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# Binding of CD40L to Mac-1's I-domain involves the EQLKKSKTL motif and mediates leukocyte recruitment and atherosclerosis – but does not affect immunity and thrombosis in mice

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## Abstract

**Rationale—**CD40L figures prominently in chronic inflammatory diseases such as atherosclerosis. However, since CD40L potently regulates immune function and haemostasis by interaction with CD40 receptor and the platelet integrin GPIIb/IIIa, its global inhibition compromises host defense and generated thromboembolic complications in clinical trials. We recently reported that CD40L mediates atherogenesis independently of CD40 and proposed Mac-1 as an alternate receptor.

**Objective—**Here, we molecularly characterized the CD40L-Mac-1 interaction and tested whether its selective inhibition by a small peptide modulates inflammation and atherogenesis *in vivo*.

**Methods and Results—**CD40L concentration-dependently bound to Mac-1 I-domain in solid phase binding assays, and a high affinity interaction was revealed by surface-plasmon-resonance analysis. We identified the motif EQLKKSKTL, an exposed loop between the α1 helix and the β-sheet B, on Mac-1 as binding site for CD40L. A linear peptide mimicking this sequence, M7, specifically inhibited the interaction of CD40L and Mac-1. cM7, a cyclisized version optimized for *in vivo* use, decreased peritoneal inflammation and inflammatory cell recruitment *in vivo*. Finally, $LDLr^{-/-}$ mice treated with intraperitoneal injections of cM7 developed smaller, less inflamed atherosclerotic lesions featuring characteristics of stability. However, cM7 did not

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interfere with CD40L-CD40 binding *in vitro* and CD40L-GPIIb/IIIa-mediated thrombus formation *in vivo*.

**Conclusions—**We present the novel finding that CD40L binds to the EQLKKSKTL motif on Mac-1 mediating leukocyte recruitment and atherogenesis. Specific inhibition of CD40L-Mac-1 binding may represent an attractive anti-inflammatory treatment strategy for atherosclerosis and other inflammatory conditions, potentially avoiding the unwanted immunologic and thrombotic effects of global inhibition of CD40L.

### Keywords

Atherosclerosis; Inflammation; CD40L; Mac-1; Peptide Inhibitor

## Introduction

Atherosclerosis is a chronic inflammatory disease governed by a network of inflammatory cytokines and immunologic phenomena[1, 2]. CD40L, a member of the tumor necrosis factor superfamily first described on T-cells, potently regulates B- and T cell function via interaction with its classic receptor CD40[3]. However, expression of CD40L is not confined to T cells but extends to a variety of cell types including those typically resident in atherosclerotic plaques such as endothelial cells (ECs), smooth muscle cells (SMCs), and macrophages. Thus, CD40L has been implicated with a variety of different inflammatory pathologies including atherosclerosis[4-7]. Functional blockade of CD40L not only reduced atherosclerotic plaque formation and progression, but also attenuated monocyte- and lipid content of these lesions while increasing numbers of collagen fibers and SMCs[6, 8, 9] — features associated with more stable plaques in humans[10]. CD40L also augments monocyte/macrophage expression of collagenases implicated in plaque rupture and of tissue factor, a trigger of thrombosis following plaque rupture. Beyond that, CD40L stabilizes thrombi through interaction with the platelet integrin GPIIbIIIa ($\alpha_{IIb}\beta_3$)[11]. While anti-CD40L antibody treatment generated promising results in early clinical trials, elevated thromboembolic complications prohibited the pursuit of this strategy[11, 12]. In addition, long-term inhibition of CD40L — as is most likely required for treatment of chronic inflammatory diseases — severely compromises host defenses, rendering generalized inhibition of CD40L an unappealing treatment strategy.

We previously reported the surprising finding that CD40L promotes atherogenesis without participation of CD40L on bone marrow–derived cells and independently of its classic receptor CD40[8, 13]. These findings point toward a role of CD40L on vascular cells such as ECs or SMCs interacting with an alternate receptor. Indeed, we suggested a novel interaction of CD40L with the leukocyte integrin Mac-1[13] promoting inflammatory cell recruitment, a crucial step in inflammation and atherogenesis. Mac-1 ($\alpha_M\beta_2$, CD11b/CD18) belongs to the $\beta_2$-family of integrins and functions as adhesive receptor mediating adhesion and transmigration of leukocytes. Mac-1 interacts with a variety of different ligands such as C3bi[14], ICAM[15], fibrinogen[16], fibronectin[17], heparin[18], GPIbα[19], and RAGE[20]. Thus, Mac-1 has been implicated with immunity, inflammation, and haemostasis[21-23]. Inhibition of Mac-1 by neutralizing antibodies attenuated atherosclerotic lesion formation in mice by impairing monocyte recruitment[13]. Here, we aimed to characterize the interaction of CD40L and Mac-1 on a molecular level, to design an inhibitory peptide specifically inhibiting this interaction, and to test whether this peptide ultimately limits inflammation and atherogenesis in mice without affecting CD40L's immunologic and thrombotic properties.

## Methods

An expanded Materials and Methods section is available in the online Data Supplement

### Recombinant protein expression

Mac-1 I-domain was produced as His-tag fusion protein by inserting the DNA-sequence coding for the Mac-1 amino acids $R^{115}$ to $S^{340}$ in pET20b (Novagen), subsequent purification by Ni-NTA immobilized metal affinity chromatography (Qiagen), and anion-exchange chromatography using Q-Sepharose (GE Healthcare). CD40L was produced as His- and c-myc-tag fusion protein by inserting the coding DNA for amino acids $E^{108}$ to $L^{261}$ in pHOG-21.

### Solid phase binding assay

Recombinant CD40L was incubated with immobilized Mac-1 I-domain in the presence or absence of blocking peptides. Binding of sCD40L was detected by addition of anti-c-myc-HRP (Invitrogen), TMB-substrate (Pierce), and colorimetric reaction. Alternatively, CD40L (Provitro) was immobilized and binding of the recombinant Mac-1 I-domain was quantified by addition of anti-His-biotin (Qiagen) and HRP-coupled streptavidin (Pierce). For binding to immobilized peptides, CD40L was biotinylated (Micro Biotinylation Kit, Sigma).

### Surface plasmon resonance

Binding was characterized on a BIAcore 3000 (BIAcore AB) by amine coupling of CD40L or Mac-1 I-domain onto a CM5 sensor chip. Experiments were performed by passing sample solutions over the receptor coated and reference flow cells at RT in PBS +0.1 % BSA at the flow rate 20 μl/min. Surfaces were regenerated by 30-s pulses of 5mM NaOH. Kd values were calculated using the kinetic rate constants using the software provided by the manufacturer.

### Dynamic and static adhesion assays

96-well plates (Nunc) were coated with sCD40L and incubated with CHO cells expressing constitutively activated Mac-1, or THP-1 cells. HEK 293-cells expressing wildtype or chimeric $\beta_2$-integrins have been previously described and have been used as indicated[24]. Briefly, cells were allowed to adhere for 20 to 50min. Cell were preincubated with blocking antibodies (10μg/ml) or peptides (50μM) as indicated. Permeabilization buffer (6mg/ml phosphatase substrate (Sigma), 1% Triton X-100, 50mM sodium acetate, pH 5.5) was added for colorimetric quantification. Alternatively, adhering cells were counted or stained for detection in fluorometer. For dynamic adhesion assays, 35-mm dishes or glass capillaries were coated with 1% BSA, CD40L, GPIbα (Abnova), fibrinogen (Sigma), or ICAM-1 (R&D systems). Adhering and rolling cells were quantified in a parallel flow chamber system (Glycotech) at the indicated shear rates and in the presence of the indicated peptides (1μM) or antibodies (10μg/ml).

### Flow cytometry

Quantification of leukocyte subsets was performed by flow cytometry. Binding of cM7 to CD40L-expressing Murine fibroblasts was determined by quantification of FITC-coupled cM7. Binding of CD40L to Mac-1 expressing CHO-cells or human leukocytes was performed by incubation with CD40L (10μg/ml) and subsequent detection with anti-penta-His antibody (Qiagen).

### Serum cytokines

Plasma concentrations of cytokines and chemokines were determined by cytometric bead array (BD Biosciences).

### Pharmacokinetics

C57BL/6J mice received intraperitoneal injections with FITC or TAMRA-labeled peptides cM7 or scM7. Fluorescence in plasma samples was measured at indicated time points. Endothelial binding was visualized by intravital microscopy.

### Murine Peritonitis

WT or CD40L$^{-/-}$ mice (Jackson Laboratories) received an injection of 3ml of 4% thioglycollate broth (Sigma). A peritoneal lavage was performed after 15h. Peritoneal exudate cells (PECs) were quantified after red cell lysis.

### Intravital microscopy

Mice received intraperitoneal injections of 200ng of Murine TNFα (R&D systems) and 100μg of peptides 5h before surgery. Mice were anesthetized by intraperitoneal injection of ketamine hydrochloride (Essex) and xylazin (Bayer). The cremaster muscle was exteriorized. Videos were taken with an intravital microscope (AxioScope Vario, Carl Zeiss) fitted with a saline immersion objective (WPlan-APOCHROMAT 20x/1,0DIC IR, Carl Zeiss). Rolling leukocyte flux was defined as the number of leukocytes moving at a velocity less than erythrocytes. Adherent leukocytes were defined as cells that remained stationary for at least 30s.

### Atherogenesis study

Eight-week-old male LDL-receptor–deficient (LDLr$^{-/-}$) mice (Jackson Laboratories) consuming a high-cholesterol diet (HCD) for 20 weeks were treated with intraperitoneal injections of the peptides cM7, scM7 (100 μg), or sterile saline three times a week. Atherosclerotic lesions were analyzed histologically as described previously[8, 13].

### *In vivo* thrombosis model

3-4 weeks old C57BL/6J mice received intraperitoneal injections of either sterile saline (100μl), the peptides cM7, scM7, or the indicated antibodies. A mesenteric arteriole was chosen and injured with ferrichloride. Platelets were stained by retroorbital injection of rhodamine 3G and visualized through an intravital microscope (AxioScope Vario, Carl Zeiss). Vessel occlusion time and thrombus embolization rate was analyzed. Tail bleeding time was determined as previously reported[11].

### Structural modeling

Mac-1 I-domain structure was visualized using Sirius visualization system 1.2 (San Diego Supercomputer Center) and a crystallographic dataset for the Mac-1 I-domain (PDB ID: 1NA5).

### Statistical analysis

Data are presented as mean±SEM. Statistical testing employed Student's unpaired t test or analysis of variance (ANOVA), followed by Newman-Keuls *post hoc* test or Mann-Whitney-U test as indicated. P values $< 0.05$ were considered significant.

## Results

### CD40L concentration-dependently binds to the Mac-1 I-domain

Since most of Mac-1's ligands — such as fibrinogen, ICAM-1, GPIbα, RAGE, C3bi, or heparin — bind to the Mac-1 I-domain[18-20, 25, 26], a stretch of ~220 amino acids within the $\alpha_M$ subunit of the integrin, we hypothesized that the I-domain also serves as binding partner for CD40L. As expected $\alpha_M\beta_2$ (Mac-1) expressing HEK cells strongly adhered to CD40L while HEK cells expressing the $\alpha_L\beta_2$ integrin (LFA-1) failed to mediate cell adhesion. However, binding of HEK cells expressing $\alpha_L\beta_2$ to CD40L could be rescued when the $\alpha_M$ I-domain replaced the $\alpha_L$ I-domain in the $\alpha_L\beta_2$ backbone, demonstrating that CD40L binds to Mac-1's I-domain (Fig. 1a). Similarly, recombinantly produced variants of the I-domain and CD40L (Supplemental Fig. I) specifically bound to each other in solid phase binding assays (Fig. 1B, Supplemental Fig. IIa). Binding was enhanced in the presence of integrin-activating $Mn^{2+}$ as observed for most $\alpha_M$ ligands[27] (Supplemental Fig. IIb). Surface plasmon resonance (SPR) analysis revealed a high affinity interaction between both molecules. $K_d$ was 214±78nM and 671±272nM for binding of chip-coupled I-domain to soluble CD40L and chip-coupled CD40L to soluble I-domain, respectively (Fig. 1C,D). Interestingly, the Mac-1 antibody clone 2LPM19c blocked adhesion of Mac-1-expressing CHO cells to fibrinogen only while both antibody clones, ICRF44 and 2LPM19c, abrogated adhesion of these cells to CD40L, suggesting that CD40L binds to the I-domain, but to a binding site distinct from that of fibrinogen (Supplemental Fig. II c, d). Anti-CD40L treatment abrogated adhesion to CD40L but not to fibrinogen, indicating specificity of the assay.

### The EQLKKSKTL motif within Mac-1's I-domain serves as binding site for CD40L

To identify the binding site used by CD40L, we employed a peptide mapping strategy using a set of linear peptides, M1-M8, originating from the hydrated surface of Mac-1's I-domain (Supplemental Table I, Fig. 2A)[19], as competitive inhibitors of the CD40L/Mac-1 dyad. In an initial solid phase binding assay evaluating the binding of the isolated Mac-1 I-domain to immobilized CD40L, the peptides M3, M4, M5, and M7 emerged as potential candidate inhibitors (data not shown). In the more physiological setting with the entire Mac-1 protein in a cell membrane environment, peptide M7, mimicking the EQLKKSKTL motif ($E^{162}$-$L^{170}$), most efficiently blocked adhesion of THP-1 cells to CD40L (61±3% inhibition, n=3, p=0.003). The extent of inhibition resembled that of a pan I-domain blocking antibody (57±24% inhibition, n=3, p=0.03, Fig. 2B). In accord, only M7 blocked binding of fluorescence-labeled CD40L to Mac-1 expressing human granulocytes and monocytes in flow cytometry (54±11% inhibition, n=3, p=0.001, Fig. 2C). Also, biotinylated CD40L specifically bound to immobilized peptide M7 (Fig. 2D).

To provide genetic proof of involvement of the EQLKKSKTL motif we tested adhesion of HEK cells expressing either wildtype $\alpha_M$ or chimeric $\alpha_M$ integin backbones substituted with the $\alpha_L$ I-domain sequences $E^{162}$-$L^{170}$ or $E^{178}$-$T^{185}$ corresponding to the sequences of M7 and M8, respectively. Consistent with our previous findings, switch of the EQLKKSKTL motif abrogated adhesion on CD40L while switch of the sequence EEFRIHFT ($E^{178}$-$T^{185}$, M8) had no effect (Fig. 2E). Interestingly, adhesion to the fibrinogen recognition peptide, P2-C, was enhanced by deletion of the CD40L-binding site (data not shown) as reported previously[28]. Finally, M7 concentration-dependently inhibited binding of CD40L to Mac-1 in a solid phase binding assay and in SPR-analysis (IC50 of 200-900nM) (Supplemental Fig. III).

### The peptide inhibitor cM7 is available in plasma and specifically interferes with CD40L-Mac-1 binding

Therapeutic application of peptides *in vivo* requires adequate plasma availability. To improve plasma stability and resistance to degradation by peptidases we modified the peptide M7 by addition of two flanking cysteine residues C- and N-terminal and subsequent cyclization by disulfide-bonds (termed cM7). A scrambled peptide, scM7, served as control for subsequent *in vivo* experiments (Supplemental Table I). In accord with previous reports using cysteine-cyclized peptides[29], cM7 coupled to fluorescein isothiocyanate (FITC) persisted in plasma between 30 minutes and 4 hours with the highest plasma availability at 30min after intraperitoneal injection (12.2±0.8μM vs. 15.8±1.6μM for 100μg and 200μg cM7, respectively, Supplemental Fig. IV a). Notably, FITC-labeled cM7 concentration-dependently bound to Murine fibroblasts over-expressing CD40L, but not to respective mock-transfected control cells (Supplemental Figure IVb). After intraperitoneal injection, fluorescent cM7, but not scM7, bound to inflamed endothelium in intravital microscopy (Supplemental Fig. IVc). To assess specificity of the peptide inhibitor cM7, we tested the adhesion of Mac-1-CHO cells to different immobilized Mac-1 ligands in the flow chamber. While cM7 concentration dependently blocked cellular adhesion to CD40L with an IC50 of 206nM (Fig. 3A,B), it did not affect adhesion to ICAM-1 and GPIbα (Fig. 3C,D). In contrast, peptide M2 blocked the interaction between Mac-1 and GPIbα as described previously, while not affecting CD40L-Mac-1 binding[19]. Finally, cM7 did not alter binding of CD40 to CD40L, whereas pre-treatment with anti-CD40 concentration-dependently blocked receptor-ligand interaction in a solid phase binding assay (Fig. 3E).

### The CD40L-Mac-1 interaction drives leukocyte recruitment *in vitro* and *in vivo*

Mac-1 potently regulates cell adhesion and migration[22, 23]. We previously demonstrated that a pan-blocking Mac-1 antibody limits thioglycollate-induced leukocyte recruitment to the peritoneal cavity while in turn injection of CD40L promoted peritoneal inflammation, suggesting that CD40L may contribute to leukocyte recruitment via Mac-1[13]. Consequently, we tested whether selective inhibition of the CD40L-Mac-1 by our peptide inhibitor would effectively limit peritoneal inflammation. Indeed, treatment with cM7 significantly decreased thioglycollate-elicited peritoneal exudate cell (PEC) accumulation in wild-type mice by 50±8% compared to scM7 (n≥6, p=0.01). Of note, treatment with cM7 had no effect in $CD40L^{-/-}$ and $Mac\text{-}1^{-/-}$ mice, suggesting that our peptide antagonist cM7 required presence of CD40L and acted specifically (Fig. 4A). Notably, total numbers of PECs were higher in saline treated $Mac\text{-}1^{-/-}$ as previously reported[30] (Supplemental Fig. V). Since Mac-1 classically functions as adhesive receptor in a variety of pathologies, we tested the functional role of the CD40L/Mac-1 interaction in inflammatory cell recruitment in intravital microscopy. Injection of cM7 but not scM7 significantly reduced rolling and adhesion by 40±11% (n≥9, p=0.03 vs. scM7) and 24±7% (n≥9, p=0.03 vs. scM7), respectively, in cremaster vessels of mice challenged with TNFα (Fig. 4B-D, Supplemental Videos 1-3). Average rolling velocity was not changed (Fig. 4E). Possible confounders, such as blood pressure, average vessel diameter, leukocyte, or platelet counts did not change between cM7- and scM7-treated mice (Supplemental Table II). Interestingly, in a second set of mice cM7 reduced rolling cell flux within minutes after intravenous delivery (Supplemental Fig. VI), suggesting direct modulation of the leukocyte adhesion cascade rather than regulating adhesion molecule expression.

In vitro, adhesion of Mac-1 expressing Chinese hamster ovarian cells (CHO) to TNFα-activated human ECs could be inhibited by selective antibodies blocking CD40L on EC or Mac-1 on CHO cells but not *vice versa* rendering direct interaction between endothelial CD40L and leukocyte Mac-1 most likely (Supplemental Fig. VII a). Anti-CD40L treatment blocked adhesion to the same extent as did treatment with anti-ICAM-1 or anti-Mac-1,

suggesting biological relevance of this interaction (Supplemental Fig. VIIb). ECs deficient for CD40L, showed significantly decreased adhering and rolling of leukocytes in the flow chamber compared with CD40L-competent EC (Supplemental Fig. VIIc,d) while expressing similar amounts of the endothelial adhesion molecules ICAM-1, -2, VCAM-1, and P-Selectin (Supplemental Fig. VII e). Platelet-leukocyte interactions critically participate in inflammation[31] and CD40L expression is up-regulated on activated platelets[32]. However, *in vitro* and *in vivo*, inhibition of either total CD40L with blocking antibodies and by cM7 did not modulate platelet-leukocyte adhesion and aggregation formation (Supplemental Fig. VIII). Also, CD40L/Mac-1 interaction did not induce inflammatory gene expression, MAP kinase- or NFκB-activation in human monocytes and murine leukocytes (Supplemental Fig. IX).

## Specific blockade of the CD40L-Mac-1 interaction attenuates atherosclerosis in mice

Inflammatory cell recruitment contributes critically to the initiation and progression of various chronic inflammatory diseases inclucing atherosclerosis[2]. Therefore, we tested whether specific inhibition of the CD40L/Mac-1 interaction mitigated atherosclerosis in mice. $LDLr^{-/-}$ mice consuming a high-cholesterol diet for 20 weeks developed significantly smaller lesions in the aortic sinus (reduction by 21±6% vs. scM7, n≥11, p=0.03) and by tendency also in abdominal aortas (reduction by 30±10 % vs. scM7, n≥9, p=0.056) when treated with cM7 compared with scM7-treated controls (Fig. 5A, B). Beyond a mere reduction in size, atherosclerotic plaques from cM7-treated animals contained significantly fewer macrophages (decrease by 37±6% vs. scM7, n≥10, p<0.001, Fig. 6B) and lower amounts of lipids (reduction by 32±5% vs. scM7, n≥11, p<0.001, Fig. 6A). In contrast, number of smooth-muscle cells (SMCs) were unchanged compared with saline treated mice but increased by 88±26% compared with scM7 treatment (n≥11, p<0.01, Fig. 6C), while infiltration of neutrophils was overall low and not changed within the groups (Supplemental Fig. X). Collagen content increased in plaques of both, the treatment and the control group (Fig. 6D). However, plaques from cM7-treated animals contained more stable collagen fibers (increase by 38±9% vs. scM7, n≥11, p=0.014, Fig. 6e, Supplemental Figure X). Thus, features of plaques from cM7-treated animals resembled those of more stable plaques in humans. Importantly, lipid levels and weights remained unchanged between cM7 and scM7-terated mice (Table 1). Presence of CD40L is critical for T-cell function, proliferation, and expression of T-cell specific cytokines[33-36]. However, we did not observe changes in cytokine profiles indicative of a switch in Th1-/Th-2 phenotype or T-cell subsets upon long-term treatment with cM7 in mice of the atherogenesis study (Supplemental Fig. XI).

## CD40L-Mac-1 interaction does not affect bleeding time and thrombus formation in mice

Haemostatic functioning of CD40L depends on interaction with either CD40 or platelet integrin GPIIb/IIIa ($\alpha_{IIb}\beta_3$)[11, 37]. The inhibition of this interaction by former therapeutic strategies employing antibodies neutralizing total CD40L provoked thromboembolic complications. Thus, confirming previous studies, treatment with an anti-CD40L blocking antibody significantly prolonged tail vein bleeding time by 74±12% (n≥4, p=0.04) in our study. Interestingly, selective blockade with cM7 did not modulate platelet activation (Supplemental Fig. VIII b) and bleeding time (Fig. 7A), suggesting that CD40L-Mac-1 interaction is specific for CD40L's inflammatory pathways. Accordingly, cM7 did not prolong vessel occlusion time in a model of arterial thrombosis (Supplemental Videos 4-10), whereas anti-CD40L and anti-CD40 treatment impaired thrombus formation in mesenterial arterioles resulting in a prolongation of the occlusion time by 113±22% (n=5, p=0.005) and 116±22% (n=4, p=0.05), respectively (Fig 7B). Furthermore, disruption of the CD40L-Mac-1 interaction by cM7 only caused a slight increase in thromboembolization rate (by 67±15%, n=5, p=0.005). However, this was a negligible effect compared with anti-CD40L and anti-CD40 treatment increasing embolization rate by 339±38% (n=6, p=0.001), and

173±40% (n=3, p=0.008), respectively. Interestingly, treatment with neutralizing anti-Mac-1 antibodies also increased the embolization rate – albeit mildly - by 131±41% (n=4, p=0.03, Fig. 7C, D).

## Discussion

We previously made the surprising observation that CD40L binds to the leukocyte integrin Mac-1 and proposed this new interaction as alternative pathway for CD40L-mediated inflammation[13]. In the present study, we characterized the CD40L-Mac-1 interaction on a molecular level and demonstrated that CD40L specifically binds to a distinct region within Mac-1's I-domain revealing a high-affinity interaction comparable with that of CD40L's interaction with $\alpha_{IIb}\beta_3$[11]. Our plasmon resonance data suggest a binding affinity in the range of 200nM of the $\alpha_M$ I-domain for CD40L, which is about 10-fold lower than the classical interaction between CD40L and CD40 as reported previously[38]. However, since it is known that integrin activity is regulated by inflammation, it is likely that the binding affinity between CD40L and Mac-1 regionally is much higher and could be further enhanced by additional interactions between CD40L and other regions of Mac-1 outside of the $\alpha_M$ I-domain. Also, binding affinity of CD40L was ~3-fold higher to the immobilized, oligomerized $\alpha_M$ I-domain, suggesting that integrin clustering might critically strengthen binding as reported previously [39].

Employing a peptide mapping strategy we identified the motif EQLKKSKTL within Mac-1's I-domain as a site involved in binding of CD40L. Our data that chimeric $\alpha_M$ integrin in which the $\alpha_L$ I-domain sequence corresponding $E^{162}$-$L^{170}$ did not bind CD40L strongly suggest that the EQLKKSKTL motif in fact represents a critical binding sequence. However, we cannot rule out that EQLKKSKTL is only part of the binding sequence or modulates binding affinity as previously observed for other Mac-1 ligands[24]. Also, other segments of the integrin within or outside of the the $\alpha_M$ I-domain could be involved. A systematic mutational analysis will have to be undertaken to address these questions in the future. We further demonstrated that small peptide inhibitors mimicking this sequence, M7 or cM7, its cyclisized version, selectively blocked binding of CD40L to Mac-1. In solid phase binding assays and cellular adhesion assays, the IC50 ranged between 200 and 900nM. However, cM7 did not affect some of the other receptor-ligand interactions described previously for both proteins. This is in accordance with Ustinov *et al*. who proposed a mosaic binding model with distinct or overlapping regions within the I-domain as potential binding sites[40].

Interestingly, the region corresponding to M7 ($E^{162}$-$L^{170}$), located on an exposed loop between the α1 helix and β-sheet B in the tertiary structure of the I-domain, has not been implicated in binding of GPIbα, NIF, C3bi, ICAM-1, or fibrinogen to Mac-1 (Fig, 2b)[19, 26, 41-43]. cM7 blocked the interaction of CD40L to Mac-1 while leaving the interactions of Mac-1 with GPIbα, ICAM-1, and fibrinogen unaffected. These data corroborate the concept that CD40L binds to a unique binding site on Mac-1. CD40L itself has at least four different receptors, including CD40, GPIIb/IIIa ($\alpha_{IIb}\beta_3$), Mac-1 ($\alpha_M\beta_2$), and $\alpha_5\beta_1$[44]. Binding of CD40 to CD40L was previously mapped to CD40L's amino residues $K^{143}$, $R^{203}$, $G^{220}$, $Y^{145}$, and $Y^{146}$ [45, 46]. Andre *et al*. showed that binding of GPIIb/IIIa to CD40L involved CD40L's $KGD^{115-117}$ sequence[11]. Whether binding of Mac-1 to CD40L also occurs at a distinct region within CD40L needs to be verified by future studies. However, our data strongly suggest this notion: We show (1) that M7 and cM7 bind to CD40L, (2) that cM7 does not affect binding of CD40L to CD40, and (3) that cM7 does not affect bleeding time and thrombus formation – CD40L's haemostatic functions, which have been shown to be dependent on interactions with both CD40 and GPIIb/IIIa. These findings

support the notion of specific binding sites for the CD40L-Mac-1 interaction on both interaction partners.

We and others previously implicated CD40L in leukocyte recruitment in inflammatory disease including atherosclerosis[6, 8]. In line with this concept, cM7 effectively limited recruitment of inflammatory cells to the peritoneal cavity and the endothelium *in vivo* demonstrating that CD40L mediates inflammatory cell recruitment by interaction with the EQLKKSKTL motif on Mac-1. Mac-1 participates in inflammation as adhesion receptor efficiently promoting both, firm adhesion and slow rolling of leukocytes[15, 47]. While this effect was thought to be solely dependent on the binding of Mac-1 to endothelial ICAM-1, Lo *et al*. proposed the existence of an alternative endothelial ligand for Mac-1[48]. In accord, Lauterbach *et al*. observed that the adhesion of neutrophils in intravital microscopy was more severely impaired in ICAM-1-Mac-1 double knock-out mice than in mice deficient in either of these factors alone[49]. CD40L could be such an alternative endothelial binding partner for Mac-1. Since platelet depositions occur at sites of inflamed EC[31] the interaction of leukocyte Mac-1 with platelets expressing CD40L could also contribute to the effects observed in our study. However, this is unlikely since we did not observe modulation of platelet activation or monocyte platelet aggregate formation by cM7. Interestingly, cM7 treatment also resulted in attenuated expression of TNFα and MCP-1 in a Murine cytokine challenge model but cM7 did not modulate inflammatory signaling and gene expression in cell culture *ex vivo*. These data suggest that cM7 does not modulate outside in signaling via Mac-1 but may rather affect inflammatory gene expression *in vivo* via secondary mechanisms, e.g. reduced leukocyte recruitment.

Since the recruitment of monocytes is considered a crucial step in the initiation and progression of atherosclerosis[2], we tested whether specific disruption of the CD40L-Mac-1 dyad would ultimately limit the development of atherosclerotic lesions *in vivo*. cM7 treatment resulted in significantly smaller, less inflamed, and potentially more stable atherosclerotic lesions. These findings agree with previous reports by us and others demonstrating that genetic deficiency or unselective inhibition of CD40L by neutralizing antibodies attenuates atherosclerotic lesion formation and favorably affects plaque morphology[5, 6, 9]. Beyond that, our data illustrate for the first time that CD40L's interaction with a distinct region on Mac-1's I-domain mediates its pro-atherogenic properties. While impaired migration of monocytes to atherosclerotic lesions may in fact be explained by the anti-inflammatory, anti-adhesive effects of cM7, the increased number of SMCs and stable collagen fibers after CD40L-Mac-1 blockade needs to be further explored. Since SMCs express CD40L[50], a cross-talk between SMCs and macrophages mediated by the CD40L/Mac-1 interaction might explain this effect.

While inflammation drives many chronic diseases including atherosclerosis few selective anti-inflammatory treatment options currently exist. In the context of atherosclerosis, statins allow a glimpse at the therapeutic potential of such strategies[51]. Also Cox-2 inhibitors exemplify the impressive therapeutic benefits but also the difficulty in developing anti-inflammatory drugs without side effects[52]. Our peptide-based strategy might overcome some of these limitations. Also, cM7 attenuated inflammation and atherogenesis rather than abrogating it completely. In fact, a more subtle inhibition of inflammatory activity may be actually the more promising and fruitful strategy in the combat of atherosclerosis where most likely long-term therapy is needed and inflammatory regenerative pathways should stay intact. Our data add a new dimension to the understanding of CD40L-mediated inflammation – for long thought to be solely dependent on the interaction with its classical receptor CD40. Our findings might revive the concept of a therapeutic blockade of CD40L. Previous concepts aimed at the global inhibition of CD40L and failed due to acute or long-term side effects. In particular, clinical data revealed thromboembolic complications most

likely due to destabilization of thrombi[11]. In accord, we show that inhibition of CD40L and CD40 prolonged bleeding time, inhibited thrombus formation, and promoted thrombus destabilization. In contrast, specific inhibition of the CD40L-Mac-1 interaction hardly affected thrombus integrity. Moreover, cM7 did not interfere with CD40-CD40L binding *in vitro* and did not induce changes in basic immunological characteristics such as alteration of Th1/Th2-phenotype as previously demonstrated for CD40L-CD40-signaling[3]. Taken together, these findings suggest the following scenario: CD40L mediates its inflammatory actions preferably via the CD40L-Mac-1 dyad, whereas its immunologic and thrombotic features rather depend on its interaction with CD40 and GPIIb/IIIa.

Indeed, the use of selective strategies to block CD40L's interaction partners as suggested in this work might enable development of tailored drugs for different CD40L-dependent conditions. cM7 effectively and specifically inhibited CD40L-Mac-1-dependent inflammatory cell recruitment and atherogenesis. Therefore, selective inhibition of the CD40L-Mac-1 interaction as achieved by cM7 treatment in this study may represent a fruitful novel strategy to combat chronic inflammatory diseases such as atherosclerosis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Non-standard Abbrevations and Acronymns

| | |
|---|---|
| **CD40L** | CD40 ligand, CD154 |
| **Mac-1** | Macrophage-1 antigen, integrin $\alpha_M\beta_2$, CD11b/CD18 |
| **EC** | Endothelial Cell |
| **SMC** | Smooth Muscle Cell |
| **GPIIb/IIIa** | Glycoprotein IIb/IIIa, integrin $\alpha_{IIb}\beta_3$ |
| **LDLr** | Low-Density Lipoprotein (LDL) Receptor |
| **GPIbα** | Glycoprotein Ib alpha |
| **RAGE** | Receptor for Advanced Glycation Endproducts |
| **ICAM** | Intercellular Adhesion Molecule |
| **C3bi** | Complement Receptor Type 3 |

## Novelty and Significance

**What is known?**

- CD40L, a member of the tumor necrosis factor superfamiliy, is a potent mediator of inflammation, immunity, and haemostasis.
- Therapeutic inhibition of CD40L by blocking antibodies was successful in attenuating inflammatory disease in clinical trials, but inhibition severely compromises immune and haemostatic function.
- Beyond its classical immune and platelet receptors CD40 and GPIIb/IIIa, CD40L also binds to the pro-inflammatory leukocyte integrin Mac-1.

**What new information does this article contribute?**

- CD40L binds to a distinct region within the Mac-1 molecule. A peptide mimicking this region, cM7, is capable of specifically blocking binding of CD40L to Mac-1.
- cM7 attenuated leukocyte recruitment, inflammation, and atherogenesis in mice, but did not interfere with CD40L's immune and haemostatic functions.
- Specific inhibition of CD40L's interaction with inflammatory partners might represent a selective, anti-inflammatory treatment strategy.

Atherosclerosis is an inflammatory disease involving a plethora of pro-inflammatory mediators. Previous reports established CD40L as marker and mediator of atherosclerotic disease. However, global inhibition of CD40L generated unfavorable side effects in clinical trials. Here, we present a strategy to selectively inhibit pro-atherogenic function of CD40L in several murine models. Given the importance of inflammatory pathways in atherosclerosis the selective inhibition of the CD40L-Mac-1 interaction as characterized in our study may be a fruitful strategy to combat chronic inflammatory diseases such as atherosclerosis.

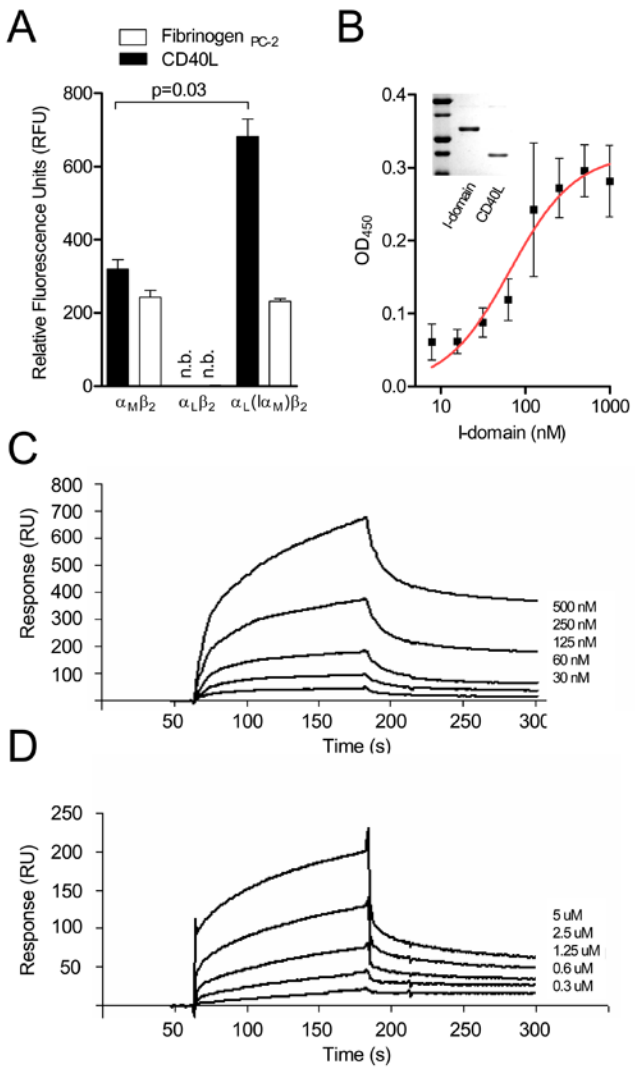


**Figure 1. CD40L concentration-dependently binds to Mac-1's I-domain**

HEK293 cells expressing either integrin $\alpha_M\beta_2$, $\alpha_L\beta_2$ or chimeric $\alpha_L\beta_2$ backbones substituted with $\alpha_M$ I-domain were allowed to adhere to immobilized CD40L or Fibrinogen recognition peptide PC-2. Cell adhesion was quantified by fluorescent staining (A). The isolated Mac-1 ($\alpha_M$) I-domain concentration-dependently bound to immobilized CD40L (B). Binding was also quantified in SPR by passing sample solutions over chip-coupled CD40L (C), Mac-1 I-domain (D), and reference flow cells in 0.1%BSA/PBS at the flow rate of 20μl/min (C, D) The insert shows recombinant, purified CD40L and I-domain on a coomassie blue-stained acrylamide gel (B). Data are presented as mean±SEM of at least three independent experiments. A representative experiment is shown (C,D). n.b. no binding.

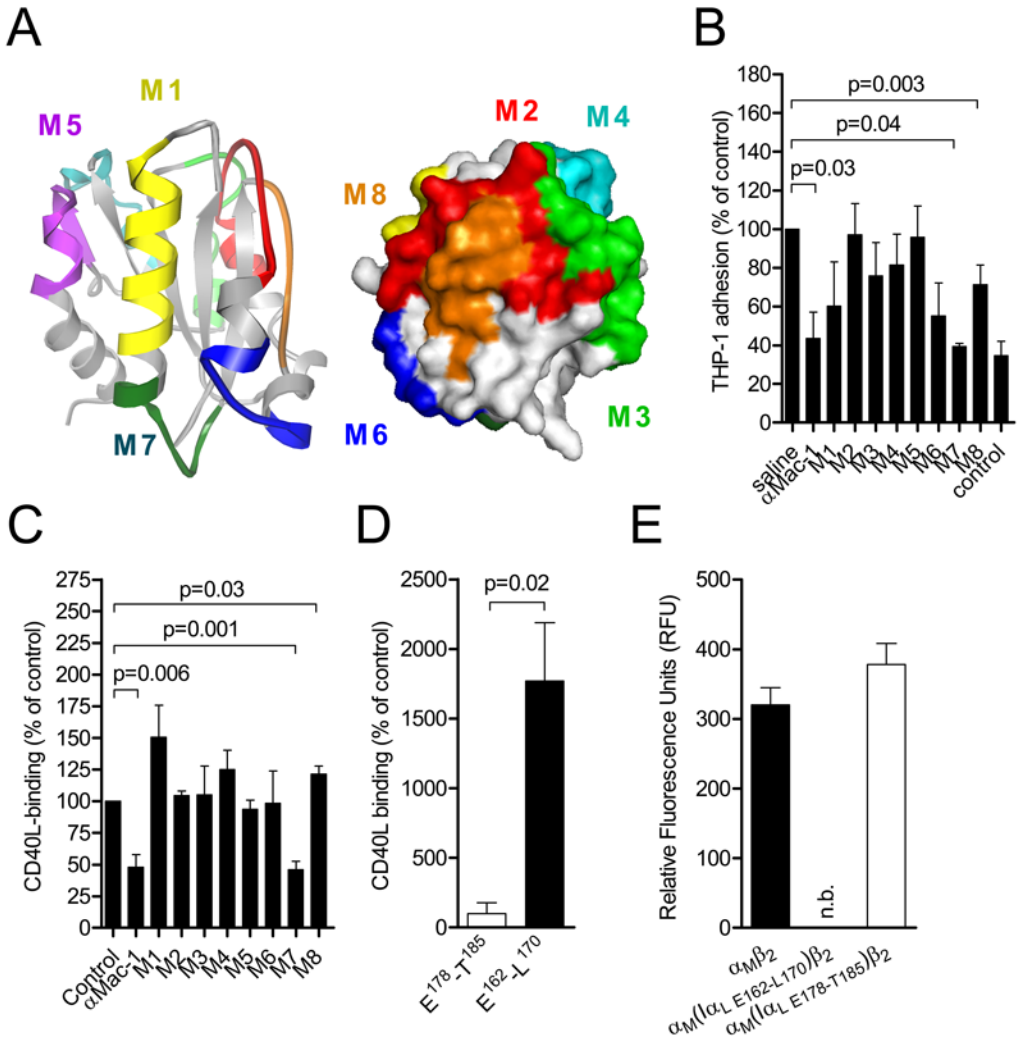


**Figure 2. CD40L binds to a distinct binding site within the Mac-1 I-domain**

Linear peptides M1 to M8 originate from the hydrated surface of the Mac-1 I-domain shown as ribbon diagram (left) and model of the hydrated surface computing the crystallographic dataset INA5 (right, A). Peptides M1-M8 (50μmol/l) were used to competitively block adhesion of Mac-1 expressing, activated human THP-1 cells to immobilized CD40L in a static adhesion assay. A pan I-domain blocking antibody (anti-Mac-1, 10μg/ml) served as control (B). Peptides were incubated with His-tagged CD40L (10μg/ml) and freshly isolated human leukocytes. Binding of CD40L to leukocytes was detected by an anti-His-FITC antibody in flow cytometry (C). Biotinylated CD40L was incubated with immobilized peptides M7 ($E^{162}$-$L^{170}$) and M8 ($E^{178}$-$T^{185}$). Direct binding was quantified by Streptavidin-HRP conjugate after removal of unbound CD40L (D). To further proof involvement of the region $E^{162}$-$L^{170}$, HEK cells expressing either wildtype $\alpha_M$ or chimeric $\alpha_M$ integin backbones substituted with $\alpha_L$ I-domain sequences $E^{162}$-$L^{170}$ or $E^{178}$-$T^{185}$ were allowed to adhere on immobilized CD40L (E). Data are presented as mean±SEM of at least three independent experiments. Three healthy male donors are included in (C). n.b. no binding.

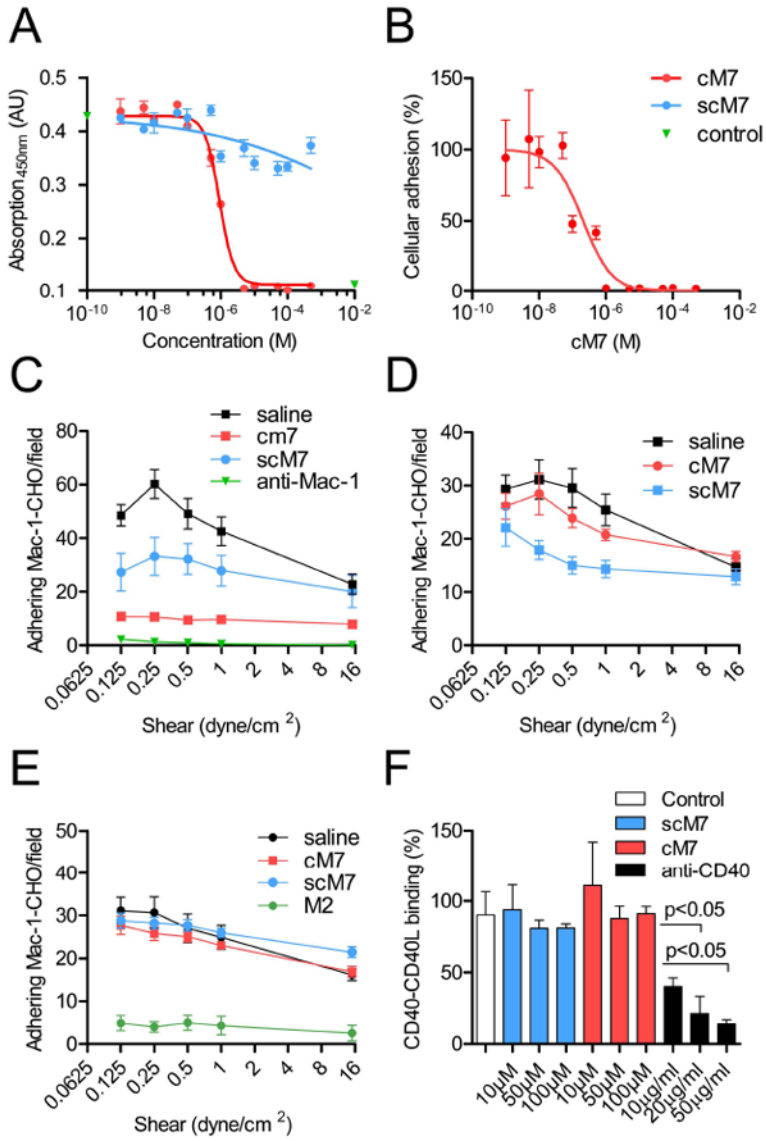


**Figure 3. The peptide inhibitor cM7 specifically interferes with CD40L-Mac-1 binding**

The Mac-1 I-domain interacted with CD40L in a solid phase binding assay in the presences of different concentrations of peptides cM7 and scM7. Anti-CD40L (100μg/ml, green arrow, right) and saline (green arrow, left) served as controls (A). Mac-1-transfected CHO cells interacted with immobilized CD40L (B, C), the alternative Mac-1 ligands ICAM-1 (D), and GPIbα (E) in flow chamber under physiological shear stress in the presence of different concentrations of cM7 (A) or at 12.5μmol/l (B-D), or a scrambled control peptide (scM7). M2 (12.5μmol/l) represents a GPIbα-specific peptide (D). CD40L (10μg/ml) was pre-incubated with different concentrations of cM7 and scM7 (10, 50, or 100μmol/l) or a blocking anti-CD40 antibody (10, 20, or 50μg/ml) and was subsequently incubated with immobilized CD40-Fc fragments (5μg/ml). Binding of CD40L to CD40 was quantified by colorimetric reaction (F). Data are presented as mean±SEM of at least three independent experiments.

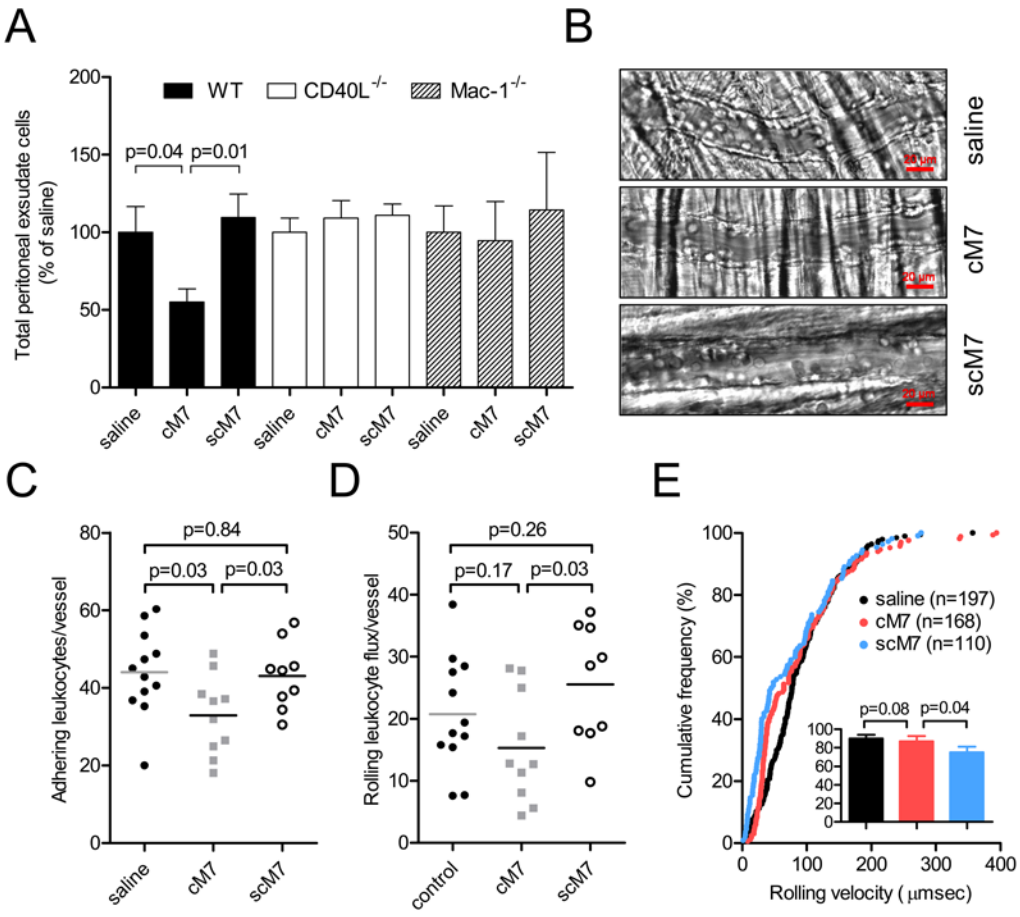


**Figure 4. CD40L-Mac-1 interaction drives leukocyte recruitment *in vivo***

C57Bl/6 wild-type, CD40L$^{-/-}$, and Mac-1$^{-/-}$ mice (n=6 per group) were injected with 3ml 4% thioglycollate broth and 100μg of the peptides cM7, scM7 or saline as indicated. After 15hrs numbers of thioglycollate-elicited peritoneal exsudate cells were determined. Numbers of PEC's were adjusted to percent of treatment with saline (A). For intravital microscopy C57Bl/6 mice were injected i.p. with TNFα (100ng), peptides cM7, scM7 (100μg) or saline. After 5 hours cremaster muscle was exteriorized, mounted under an intravital microscope (B), and leukocyte adhesion (C), rolling (D), and cumulative frequency of rolling velocity (E) were quantified. The inlay in (E) represents mean rolling velocity. Data are presented as mean±SEM. At least 9 mice per group were used for B-D. Scale bar 20μm (B).

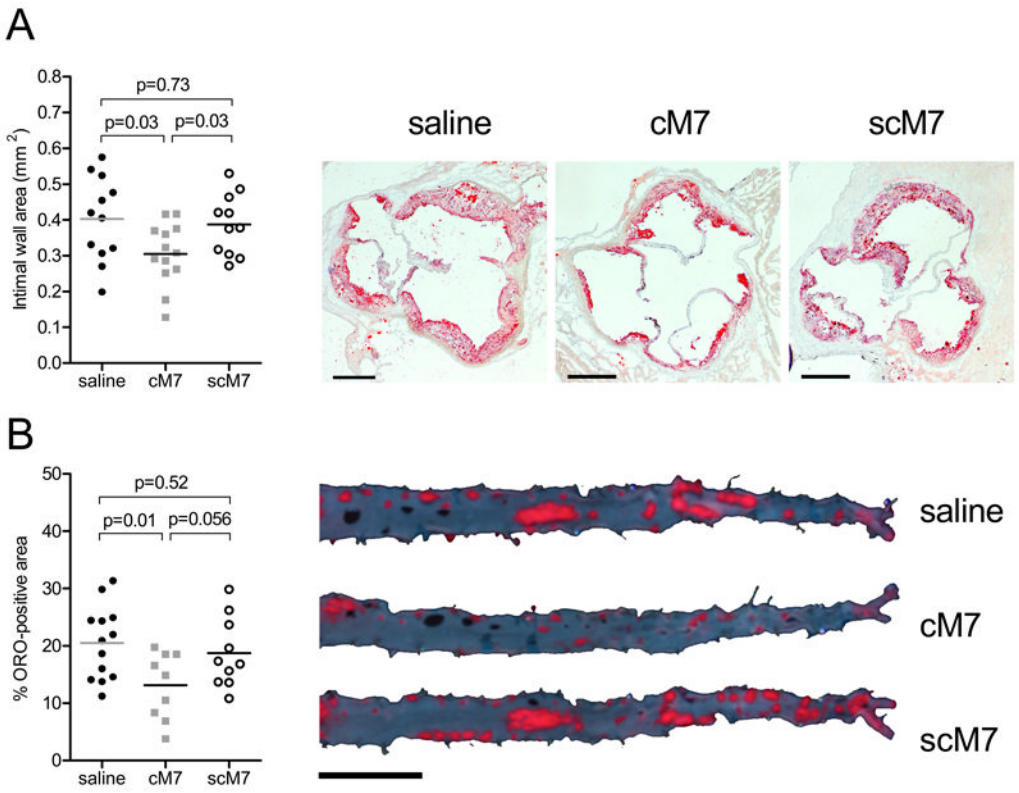


**Figure 5. Specific blockade of the CD40L-Mac-1 interaction attenuates atherosclerosis in mice**

LDLr-/- mice consumed a high-cholesterol diet for 20 weeks. Mice were injected i.p. with the specific inhibitor (100μg) of the CD40L-Mac-1 interaction, cM7 (n=13), the scrambled control peptide, scM7 (n=12), or saline (n=12), three times a week. Atherosclerotic lesion size was determined in sections of the aortic root (A) or by quantification of Oil-red-O-positive staining of *en face* preparations of the abdominal aorta (B). Data are presented as mean±SEM. Scale bar 1000μm (A, B).

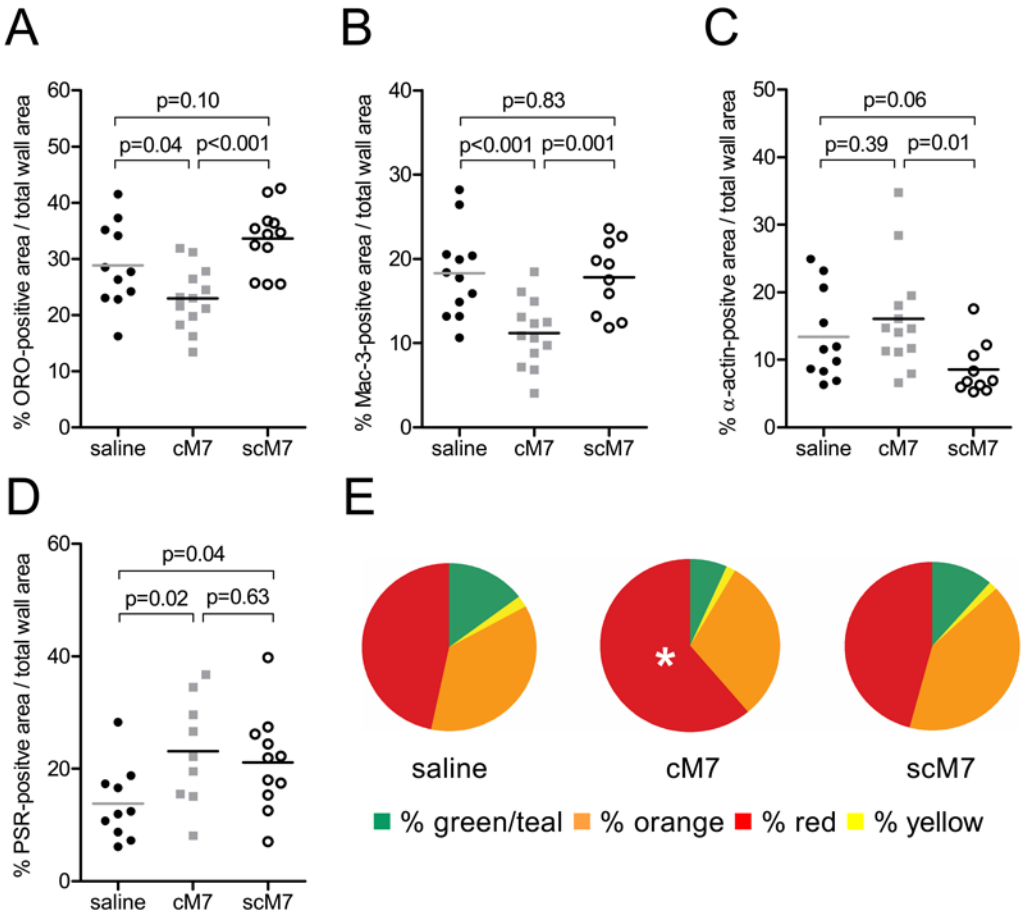


**Figure 6. Inhibition of the CD40L-Mac-1 dyad reduces macrophage and lipid accumulation in atherosclerotic lesions**

LDLr-/- mice consumed a high-cholesterol diet for 20 weeks. Mice were injected i.p. with the specific inhibitor of the CD40L-Mac-1 interaction, cM7 (n=13), the scrambled control peptide, scM7 (n=12), or saline (n=12), three times a week. Composition of atherosclerotic plaques was determined by lipid-specific Oil-red-O staining (A), or immunohistochemistry against the macrophage antigen Mac-3 (B) and smooth-muscle cell specific α-Actin (C). Collagen deposition was quantified by picrosirius-red staining as total collagen (D) and distribution of collagen fractions based on emitted color in polarizing light microscopy (E). Data are presented as mean±SEM. * indicates p-value<0.05.

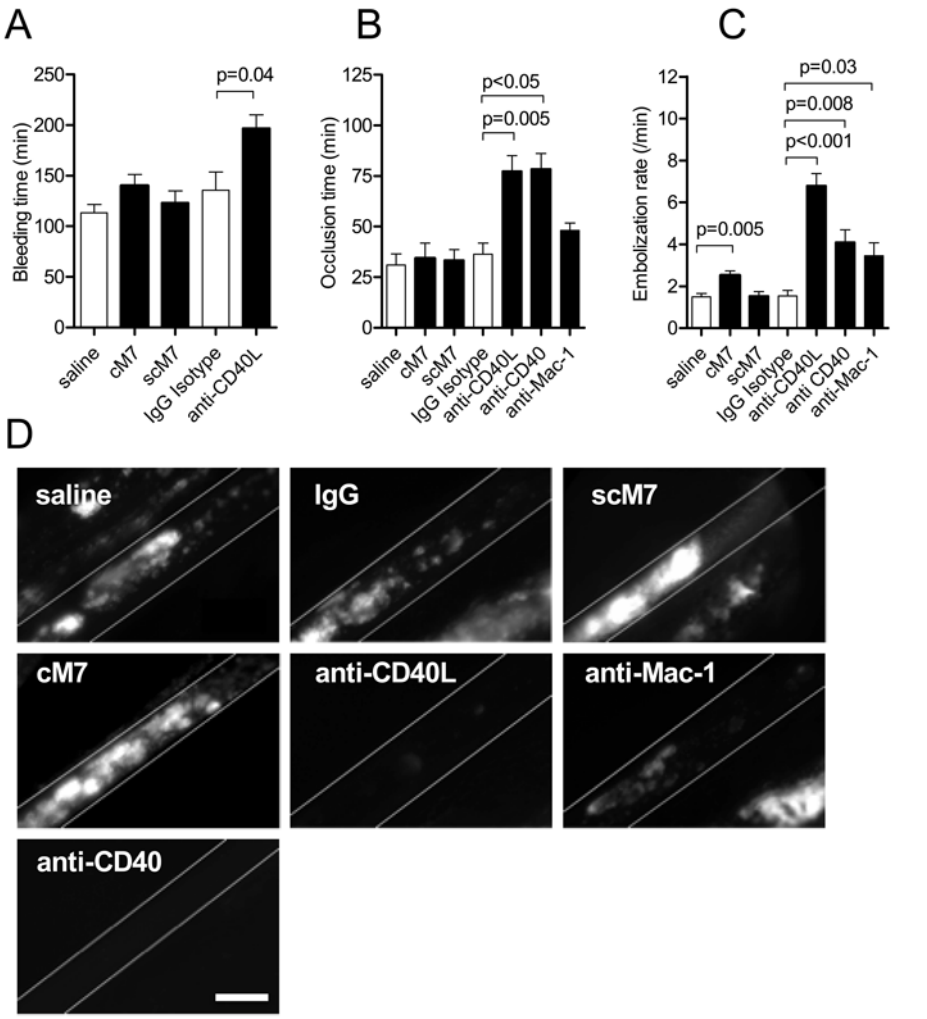


**Figure 7. CD40L-Mac-1 interaction does not mediate thrombus formation and stability in mice**

C57Bl/6 wild-type mice were injected with the peptides cM7, scM7 (100μg), blocking antibodies against Mac-1, CD40L, CD40 (100μg), IgG isotype control (100μg), or saline, before assessment of tail bleeding time (A) and *in vivo* thrombus formation (B-D) in mesenteric arterioles following injury with ferrichloride. Thromboembolization rate was defined as frequency of emboli/min (C, D). Data are presented as mean±SEM of at least 4 animals per group. Scale bar 200μm.

**Table 1**

Atherosclerosis Study Characteristics

| | | **saline** | **p*1*** | **cM7** | **p*2*** | **scM7** | **p*3*** |
|---|---|---|---|---|---|---|---|
| Weight (g) | BF | 23.8±1.7 | 0.57 | 23.4±2.3 | 0.23 | 24.2±1.2 | 0.44 |
| | AF | 36.4±3.8 | 0.65 | 35.7±3.8 | 0.74 | 35.3±2.2 | 0.37 |
| Cholesterol (mg/dl) | AF | 96.6±29.7 | 0.63 | 91.5±30.5 | 0.97 | 91.0±33.6 | 0.65 |
| Triglycerides (mg/dl) | AF | 228±97 | 0.18 | 277±107 | 0.20 | 201±190 | 0.63 |
| Visceral fat pads (g) | BF | 2.3±0.7 | 0.96 | 2.3±0.7 | 0.81 | 2.2±0.5 | 0.77 |
| Systolic blood pressure (mmHg) | AF | 103±12 | 0.23 | 98±7 | 0.79 | 97±13 | 0.25 |
| Heart rate (bpm) | AF | 655±54 | 0.44 | 638±58 | 0.29 | 660±42 | 0.80 |
| Leukocytes (×1000/μl) | BF | 12.1±2.8 | 0.41 | 11.2±3.1 | 0.13 | 13.3±3.9 | 0.35 |
| | AF | 5.23±1.31 | 0.17 | 4.54±1.28 | 0.90 | 4.62±1.68 | 0.29 |
| Platelets (×1000/μl) | BF | 557±153 | 0.51 | 529±53 | 0.25 | 562±91 | 0.93 |
| | AF | 663±138 | 0.01 | 486±198 | 0.30 | 556±135 | 0.05 |
| CD11b+ (% of leukocytes) | AF | 16.8±6.5 | 0.33 | 14.3±4.4 | 0.68 | 13.4±5.6 | 0.19 |
| Granulocytes (% of leukocytes) | AF | 13.9±4.3 | 0.60 | 13.0±3.3 | 0.93 | 13.2±4.8 | 0.70 |
| Monocytes (%of leukocytes) | AF | 9.8±3.6 | 0.06 | 7.2±2.1 | 0.50 | 6.4±3.1 | 0.03 |

Data are expressed as mean ± SD.

*1*p-value saline vs. cM7,

*2*p-value cM7 vs. scM7,

*3*p-value scM7 vs. saline,

AF: after feeding, BF: before feeding