Introduction of classic CD40L-CD40 signalling but not of the novel CD40L-Mac-1 interaction limits arterial neointima formation in mice

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Summary
The co-stimulatory immune molecule CD40L figures prominently in a variety of inflammatory conditions including arterial disease. Recently, we made the surprising finding that CD40L mediates atherogenesis independently of its classic receptor CD40 via a novel interaction with the leukocyte integrin Mac-1. Here, we hypothesised that selective blockade of the CD40L-Mac-1 interaction may also retard restenosis. We induced neointima formation in C57/BL6 mice by ligation of the left carotid artery. Mice were randomised to daily intraperitoneal injections of either cM7, a small peptide selectively inhibiting the CD40L-Mac-1 interaction, scM7, a scrambled control peptide, or saline for 28 days. Interestingly, cM7-treated mice developed neointima of similar size compared with mice receiving the control peptide or saline as assessed by computer-assisted analysis of histological cross sections. These data demonstrate that the CD40L-Mac-1 interaction is not required for the development of restenosis. In contrast, CD40-deficient mice subjected to carotid ligation in parallel, developed significantly reduced neointimal lesions compared with respective wild-type controls (2872 ± 843 µm² vs 35469 ± 11870 µm²). Flow cytometry in CD40-deficient mice revealed reduced formation of platelet-granulocyte and platelet-inflammatory monocyte- aggregates. In vitro, supernatants of CD40-deficient platelet-leukocyte aggregates attenuated proliferation and increased apoptosis of smooth muscle cells. Unlike in the setting of atherosclerosis, CD40L mediates neointima formation via its classic receptor CD40 rather than via its recently described novel interaction with Mac-1. Therefore, selective targeting of CD40L-Mac-1 binding does not appear to be a favorable strategy to fight restenosis.

Keywords
Inflammation, CD40L, CD40, Mac-1, restenosis, neointima formation, mice

Introduction
An extensive body of experimental and clinical evidence implicates inflammation with neointimal hyperplasia and restenosis after coronary interventions (1, 2). Following injury to the vessel wall a network of inflammatory cellular and molecular regulatory pathways are activated. Vascular inflammation involves complex heterotypic interactions between endothelial cells, platelets, and inflammatory cells including neutrophils, monocytes, lymphocytes, and mast cells. Under normal circumstances, the cellular and molecular processes governing these responses mediate repair and vascular healing. In pathological conditions, however, dysregulation of inflammatory responses results in persistent inflammation and adverse arterial remodeling contributing to the development of clinical complications such as restenosis.

The CD40/CD40L dyad represents a crucial mediator of various inflammatory diseases, such as rheumatoid arthritis, transplant rejection, multiple sclerosis, and atherosclerosis (3, 4). CD40 ligand (CD40L, CD154), a member of the tumour necrosis factor-alpha (TNFα) superfamily, is preferentially expressed on activated CD4+ T-cells and activated platelets, but is also found on other haematopoietic and non-haematopoietic cells such as monocytes/macrophages, endothelial cells (EC), and smooth muscle cells (SMC) (5, 6), cell types centrally involved in vascular disease and neointima formation. Mechanistically, CD40L induces proliferation and migration of smooth muscle cells (SMCs) by up-regulation of matrix metalloproteinase (MMP) transcript and protein levels (7) and induces expression of key inflammatory cytokines such as monocyte chemoattractant protein (MCP)-1, interleukin (IL)-1β, IL-6, and IL-8 (8, 9). In vivo, CD40L impairs the function of peripheral blood angiogenic outgrowth cells and increases...
neointima formation after arterial injury (10) while anti-CD40L antibody treatment reduces neointima formation in mice subjected to vascular injury (11). CD40L expression on platelets is associated with late restenosis after percutaneous coronary intervention (12). However, assessment of neointima formation in CD40L-deficient mice gave ambivalent results (13).

The binding partner of CD40L, CD40 has also been implicated with neointima formation: CD40-deficient mice develop less neointima after ligation and wire injury (13-15) compared with respective wild-type controls. Both, in vitro and in vivo data suggest that CD40-induced neointima formation is mediated through its adaptor protein TNF receptor-associated factor 6 (TRAF6) in vascular SMCs (14) while the role of TRAF6 in classic atherogenesis is controversial and most likely cell type-dependent (16, 17). Besides binding to CD40, CD40L also interacts with integrins such as α5β1 (18) and αMβ2 (Mac-1) (19, 20). Mac-1 itself binds to multiple ligands such as glycoprotein (GP)Ibα, ICAM-1, ICAM-2, and fibrinogen and is a crucial regulator of leukocyte recruitment by promoting firm adhesion of leukocytes to adherent platelets and fibrinogen (21). We identified the interaction of CD40L and Mac-1 as an alternative pathway for CD40L-mediated inflammation in atherogenesis (19). More recently, we identified the binding site of CD40L on Mac-1, the EQLKKSKT motif within the I-domain, and generated the selective peptide inhibitor cM7 that reduced peritoneal inflammation and inflammatory cell recruitment to the vascular bed in vivo (20). In addition, inhibition of CD40L-Mac-1 interaction abated atherosclerotic lesion development in low-density lipoprotein receptor (LDLr)-deficient mice. Mac-1 has also been associated with neointimal hyperplasia after vascular injury (22) and clinical studies showed that activation of circulating neutrophils (as determined by enhanced Mac-1 expression) coincides with late lumen loss and restenosis (23-26). Accordingly, Mac-1-deficient mice developed decreased neointima formation following vascular injury most likely due to decreased leukocyte infiltration (27).

In the light of these findings suggesting a role for both CD40L and Mac-1 in neointima formation we hypothesised that inhibition of the novel CD40L-Mac1 interaction protects from neointima formation in mice.

Methods

Mouse model of carotid artery ligation

To evaluate the effect of CD40L-Mac-1 interaction on neointima formation, male C57BL/6 mice aged eight to 10 weeks were anaesthetised on day 0 with 10% Ketamine and 2% Xylazine. The carotid artery was ligated with silk suture (6–0) close to the left bifurcation to trigger neointima formation as described previously (28). Mice then received daily intraperitoneal (i.p.) injections of the peptide inhibitor cM7 (4 µg/kg), its scrambled control scM7 (4 µg/kg, Peptide Specialty Laboratory, Heidelberg, Germany) or saline. After 28 days on normal diet all mice were euthanised and the carotid arteries were harvested. To evaluate the role of CD40 deficiency on neointima formation, CD40-deficient mice (Jackson Laboratories, Bar Harbor, ME, USA) were subjected to the same procedure and compared with male wild-type C57BL/6 mice (Jackson Laboratories). All mice were housed under specific pathogen-free conditions. All procedures were approved by the Animal Care Committee of the University of Freiburg and the local authorities (Regierungspräsidium Freiburg, Az. 35–9185.81/G-12/76).

Intravital microscopy

Male C57BL/6 mice aged eight to 10 weeks were anesthetised on day 0 with 10% Ketamine and 2% Xylazine. The carotid artery was ligated with silk suture (6–0) close to the left bifurcation as described above. Following surgery, all mice were treated i.p. with the peptides cM7 or scM7 (4 µg/kg, dissolved in sterile saline) for six days. On the sixth post-surgery day mice received i.p. injections of 200 ng of murine TNFα (R&D Systems, Minneapolis, MN, USA) 4 hours (h) before intravital microscopy. Intravital microscopy was performed as described previously (29). Mice were placed on a heating pad to maintain body temperature. Videos were taken with an intravital microscope (AxioScope Vario, Carl Zeiss, Jena, Germany) fitted with a saline immersion objective (WPlan- APOCHROMAT 20x/1,0DIC IR, Carl Zeiss) and a high sensitivity camera system (AxioCam MRm, Carl Zeiss) for 30 seconds (s) each. 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 30 s. Rolling leukocyte flux, adhering flux were quantified by blinded investigators.

Preparation and histology of ligated carotid arteries

At 28 days after carotid artery ligation, mice were euthanised and the arterial system was perfused through the left cardiac ventricle with saline and subsequently with 1% agarose. Carotid arteries were removed, fixed in 10% paraformaldehyde, and then embedded in paraffin. For histologic analysis cross-sections (5 µm) were cut at 250 µm intervals throughout the common carotid artery starting at the bifurcation. For each of the eight levels, a cross-section was stained with Movat. Movat-stained cross-sections were used for morphometric analysis of the lumen area, intimal area (the area within the internal elastic lamina [IEL] minus the lumen area), medial area (defined as the area within the external elastic lamina [EEL] minus the area within the IEL), and total vessel area (area encompassed by the EEL). Intimal volumes were determined by multiplying intimal areas with the distance analyzed. Luminal and total vessel volumes were determined by multiplying lumen and total vessel areas including eight levels.

Immunohistochemistry

In addition to the Movat staining, sections were stained with anti-mac3 (1:1000, BD Pharmingen, San Jose, CA, USA) followed by a biotinylated rabbit anti-rat secondary antibody (BA-4001, Vector Laboratories, Burlingame, CA, USA) for macrophages. For detec-
tion of smooth muscle cells, sections were stained with anti-
alpha-actin-FITC-conjugated antibody (1:1000; Sigma Diagnostics, St.
Louis, MO, USA) followed by anti-FITC-biotin conjugated anti-
body (Sigma) as described previously (30). Slides were de-
washed with AEC Substrate Chromogen (Dako, Glostrup, Denmark).

Furthermore, ICAM-1 positive cells were analysed by staining
sections with anti-ICAM-1 antibody (1:100, Abcam, Cambridge,
UK), followed by the biotinylated goat anti-rabbit secondary anti-
body (1:200, Vector). VCAM-1 positive cells were stained by using
anti-VCAM-1 antibody (1:100, Santa Cruz Biotechnologies, Santa
Cruz, CA, USA) and biotinylated rabbit anti-goat (1:200, Vector)
as the secondary antibody. Mac-3, a-actin, VCAM-1 and ICAM-1
positive cells per intimal area were determined by computer-as-
sisted image quantification (ImagePro, Media Cybernetics, Rock-
ville, MD, USA).

Flow cytometry
Flow cytometry was performed as previously described using
FACSCanntoII (Becton Dickinson) (20). Blood samples (100
µl/mouse) were collected from the retroorbital plexus after anes-
thesia with isoflurane. Cells were pre-incubated with mouse Fc-
Block (CD16/32, eBioscience, San Diego, CA, USA). Antibodies
included CD11b-FITC, CD115-PE, Ly6C/G (Gr1)-APC, CD4-Alexa488, CD8-PE, CD3-APC, and CD41-FITC (all eBios-
cience). The leukocyte population was set in a forward angle (FSC)
vs right angle scatter (SSC). Blood monocytes were quantified by
gating for CD11b and CD115 positive cell population. Inflamma-
tory monocytes were selected by additional gating for Gr1 positive
cells. For analysis of T-cells, histogram dot plots were used to select
for lymphocytes and gate for CD3-positive cells. To identify the
CD8- and CD4-positive cells, CD3 expressing cells were set in a
CD8-PE vs CD4-Alexa488 staining. Platelet-leukocyte aggregates
were detected by additional gating for CD41 in its respective cell
population (Suppl. Figures 5 and 6 for representative FACS plots,
available online at www.thrombosis-online.com). Cytometric data
was analyzed employing FlowJo software (Tree Star Inc., Ashland,
OR, USA).

For assessment of platelet activation C57BL/6 mice were treated
i.p. with cM7 or scM7 (both 4 µg/kg body weight) for four days.
Then Murine platelet preparations were obtained using a modified
protocol (31). Briefly, blood was drawn from the retroorbital plex-
us via heparinised capillaries into a tube with 300 µl ACDF buffer
(12.5 g sodium-citrate, 2 g citric acid, 10 g glucose in 500 ml Aqua
dest.; pH 4.7). To prevent platelet activation, apyrase and prostag-
landin were used. To activate platelets, isolated platelets (1x10^9)
were stimulated at room temperature with 10 µM ADP or 0.2
U/ml Thrombin. The activation of platelets was monitored by flow
cytometry using anti-mouse P-selectin antibody (Wug.E9, emfret
Analytics, Eibelstadt, Germany) and anti-mouse GPIIb/IIa anti-
body (JON/A, emfret Analytics). The staining was performed ac-
cording to the manufacturer’s recommendations and samples were
analysed with a FACS-Calibur flow cytometer (Becton-Dickinson,
Heidelberg, Germany).

Assessment of plasma cytokines and matrix metallo-
proteinases
Blood was collected by intracardial puncture from mice 28 days after
ligation of the carotid artery. Plasma concentrations of IL-6, IL-
10, IL-12p70, MCP-1 and TNFα were determined by the Cyto-
metric Bead Array (CBA, BD Biosciences) as described previously
(32). Plasma levels of CXCL4 were determined by ELISA (Mouse
CXCL4/PF4, DuoSet) according to the manufacturer’s instruc-
tions. Plasma concentrations of MMP2 and MMP9 were measured
by ELISA using the MMP2 Mouse ELISA Kit (Abcam) and Mouse
Total MMP9 DuoSet (R&D).

Cell culture
For ex vivo analysis of platelet-leukocyte interaction retroorbital
blood was collected in enoxaparin/phosphate-buffered saline
(PBS) (100 µg/ml) 1:1 and centrifuged at 100 g x 5 minutes (min)
at 37°C. Platelets were taken off and counted in a Neubauer
chamber. The red pellet was lysated with RBC Lysis Buffer on ice
and washed with cool PBS. Leukocyte were counted and incubated
with platelets in ratio of 1:5 for 20 min at 37°C followed by cen-
trifugation at 1,500 g for 3 min. Platelet-leukocyte aggregates were
quantified by flow cytometry as described above.

To study the effect of platelet-leukocyte aggregates on SMC
proliferation, murine SMCs (ATCC, CRL-2797, cell line from
C57BL/6 mice) were grown in 96-well plates to 80% confluency.
The murine smooth muscle cells were then incubated with the
supernatants of platelet-leukocyte aggregates for 12 h. Apoptosis
and proliferation of smooth muscle cells was measured by
Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, Madison,
WI, USA) and BrdU ELISA (Roche, Basel, Switzerland) according
to the instructions of the manufacturers.

Statistical analysis
Data are presented as mean ± SEM. Significance was measured
with the unpaired Student’s t-test or analysis of variance
(ANOVA), followed by Newman-Keuls Multiple Comparison Test
where appropriate. P-values < 0.05 were considered significant.

Results
Inhibition of the CD40L-Mac-1 interaction attenuates
endothelial leukocyte adhesion and rolling after
carotid ligation without affecting platelet activation
We previously showed in vitro and in vivo that leukocyte adhesion
in mice is impaired after inhibition of the CD40L-Mac1 interac-
tion with the peptide inhibitor cM7 (20, 33). We now studied the
effect of cM7 on leukocyte adhesion and rolling after carotid li-
gation in mesenterial venules. We detected a significant decrease
in leukocyte adhesion and rolling in cM7 treated mice, corroborat-
ing that inhibition of CD40L-Mac1 impairs leukocyte adhesion
(Suppl. Figure 1A, available online at www.thrombosis-online.com).
Since blockade of CD40L can increase thrombus formation (34, 35) we also measured platelet activation by quantifying the expression of activated GPIIbIIIa (clone JON-A) or P-selectin on platelets of animals treated with cM7 or scM7 control using flow cytometry (Suppl. Figure 2, available online at www.thrombosis-online.com). These data suggest that our peptide targeting the CD40L-Mac-1 interaction has no relevant effect on the tested platelet activation markers. For instance, no significant difference in platelets positive for activated GPIIbIIIa or double positive for activated GPIIbIIIa and P-selectin was detected after exposure to cM7 in comparison to scrambled control peptide. In a previous study we also showed that inhibition of the CD40L-Mac-1 interaction does not affect thrombus formation (20).

Inhibition of the CD40L-Mac-1 interaction does not attenuate neointimal hyperplasia

To determine the possible role of CD40L-Mac-1 interaction in neointima formation, C57BL/6J mice underwent ligation of the left carotid artery followed by treatment with the specific inhibitor cM7 of the CD40L-Mac-1 interaction, the scrambled control scM7, or saline for 28 days. Body weight did not differ between the groups at beginning and at the end of the study (Table 1). Analysis of consecutive Movat-stained cross-sections revealed that cM7-treated mice developed equal amounts of neointimal hyperplasia compared with both, scM7- and saline-treated mice, suggesting no role of the CD40L-Mac-1 interaction in restenosis (Figure 1). Since both CD40L and Mac-1 play a role in the attraction of macrophages and proliferation of smooth muscle cells we analysed macrophage and smooth muscle cell content in carotid cross sections. In line with our data on lesion size, immunohis-
tochemistry showed similar content of both, macrophages and SMCs, in the ligated carotid arteries of treated and untreated animals (Figure 2). Also, we did not detect a difference in endothelial cell activation as assessed by staining for ICAM-1 and VCAM-1 (Suppl. Figure 1B, available online at www.thrombosis-online.com).

cM7 does not alter T-lymphocyte, monocyte or leukocyte-platelet aggregate numbers

Since both, CD40L and Mac-1 are potentially functionally relevant for the interaction of leukocyte subsets and platelets; we analysed blood cells by flow cytometry after carotid ligation and 28 days of treatment with cM7, scM7, or saline (Figure 3). We did not detect a significant difference in the number of T-lymphocytes, inflammatory monocytes, or monocyte/granulocyte-platelet aggregates (Figure 3A-G). Plasma cytokine levels (interferone [IFN]γ, IL-10, IL-12, IL-6, MCP-1 and TNFa), MMP2 and MMP9 and CXCL4 (PF4) were not affected by CD40L-Mac-1 inhibition (Suppl. Figure 1C-E, available online at www.thrombosis-online.com).

Table 1: Baseline characteristics of mice.

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<th>cM7</th>
<th>scM7</th>
<th>NaCl</th>
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<tr>
<td>Number of mice</td>
<td>24</td>
<td>22</td>
<td>19</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
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<tr>
<td>- before ligation</td>
<td>22.4 ± 0.3</td>
<td>22.6 ± 0.2</td>
<td>22.7 ± 0.3</td>
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<tr>
<td>- after 28 days</td>
<td>25.3 ± 0.3</td>
<td>25.3 ± 0.3</td>
<td>25.4 ± 0.4</td>
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<tr>
<td>CD40</td>
<td>C57Bl/6J</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of mice</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- before ligation</td>
<td>24.0 ± 0.5</td>
<td>23.9 ± 0.2</td>
<td>26.0 ± 0.4</td>
</tr>
<tr>
<td>- after 28 days</td>
<td>25.8 ± 0.6</td>
<td>25.8 ± 0.6</td>
<td>25.8 ± 0.6</td>
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Willecke, Tiwari, et al. CD40L-Mac-1 and restenosis

Based on our finding that the novel CD40L-Mac-1 interaction did not contribute to neointima formation in our model, we tested whether CD40 as the classic CD40L receptor is important in this scenario. Therefore, we studied the effect of CD40 deficiency on neointima formation. Neointima formation in CD40-deficient mice was significantly impaired compared with respective controls (▶ Figure 4). Of note, the average vessel area was also significantly reduced in CD40-deficient mice (▶ Figure 4E). This suggests an impaired remodelling capacity in the ligated arteries of CD40-deficient mice as the medial area and the average total lumen volume were similar in both groups (▶ Figure 4B and J) (13).

CD40 deficiency decreases platelet-inflammatory monocyte and -granulocyte aggregates and plasma levels of CXCL4 (PF4)

Since CD40 is abundantly expressed by monocytes and platelets, both playing an important role in atherosclerosis and neointima formation (2), we further characterised CD40-deficient mice by flow cytometry (▶ Figure 5). T-cell numbers were similar between CD40 knockout mice and wild-type mice, although there was a tendency towards more CD8-positive T-cells in CD40-deficient mice (▶ Figure 5A-C). The number of inflammatory monocytes tended to decrease (p = 0.06) in CD40-deficient mice (▶ Figure 5D). The number of granulocytes and inflammatory monocytes attached to platelets decreased significantly in CD40-deficient mice (▶ Figure 5E-G). To test whether the decreased platelet-leukocyte aggregation is caused by a lack of CD40 on platelets or CD40 on leukocytes we co-cultured platelets and leukocytes isolated from C57BL/6 and CD40-deficient mice in vitro and measured the formation of platelet-leukocyte aggregates. Formation of platelet-leukocyte aggregates were significantly reduced when either only leukocytes or both platelets and leukocytes were CD40-deficient, suggesting that CD40 on leukocytes mediates formation of these aggregates (▶ Figure 6A). Platelet-monocyte aggregates were also significantly decreased when both platelets and leukocytes were CD40-deficient (Suppl. Figure 4A, available online at www.thrombosis-online.com). In contrast to our in vivo data platelet-granulocyte aggregates were unchanged (Suppl. Figure 4B, available online at www.thrombosis-online.com). Whereas CD40 deficiency did not change inflammatory cytokines like IL-6, IL-12, TNFa, MCP-1 and IFNy or plasma levels of MMP2 and MMP9 we detected a significant reduction in plasma levels of CXCL4 (PF4) in CD40-deficient mice after carotid ligation (Suppl. Figure 3A, available online at www.thrombosis-online.com).

![Figure 3: FACS analysis in cM7-, scM7-, and NaCl-treated mice. Relative amounts of circulating T-cells and subsets (A), CD4+ helper T-cells (B), CD8+ killer T-cells (C), inflammatory monocytes (D), platelet-monocyte aggregates (E), platelet-granulocyte aggregates (F) and platelet-inflammatory monocytes aggregates (G), are displayed. N as indicated. Error bars represent SEM.](https://www.thrombosis-online.com)
CD40-deficient platelet-leukocyte aggregates attenuate smooth muscle cell proliferation and increase apoptosis

SMC proliferation is a key event in neointima formation. To elucidate the role of platelets-leukocytes aggregates in smooth muscle cell proliferation we cultured a murine SMC cell line with supernatants of platelets-leukocytes aggregates from either C57BL/6 or CD40-deficient mice. Interestingly, SMC proliferation decreased and SMC apoptosis increased significantly when incubated with supernatants of CD40-deficient platelet-leukocyte aggregates, suggesting that CD40 can mediate SMC proliferation and apoptosis via platelet-leukocyte aggregates (▶ Figure 6B and C).

Discussion

This study elucidates the role of two important receptors of CD40L, CD40 and Mac-1, in neointima formation in response to carotid ligation. We recently discovered a new interaction between CD40L and the β2-integrin receptor Mac-1 and showed that specific inhibition of the CD40L-Mac-1 interaction by the peptide inhibitor cM7 attenuated leukocyte recruitment, inflammation, and atherogenesis in mice (20). Of note, cM7 does not interfere with CD40L’s immune and hemostatic functions (e.g. platelet activation and thrombus formation) – a side effect seen with other inhibitors of CD40L (34, 35). In accord, we detected decreased adhesion and rolling of leukocytes after carotid ligation in cM7-treated mice, whereas platelet activation was not significantly affected (Suppl. Figures 1A and 2A-C, available online at www.thrombosis-online.com).

Since a solid body of evidence suggests a role of CD40L and Mac-1 in neointima formation (7, 10, 11, 13, 22, 27) we hypothesised that specific inhibition of the CD40L-Mac-1 interaction – with preservation of the CD40L/CD40 interaction – would reduce neointima formation in a mouse model of carotid ligation. In contrast to our initial hypothesis, neointima formation in mice treated with the specific CD40L/Mac-1 peptide inhibitor cM7 did not reduce neointima formation compared with mice treated with the scrambled control peptide scM7 or saline. In addition, we also...
Figure 5: FACS analysis of peripheral blood in CD40-deficient and C57BL/6 mice: Relative amounts of circulating T-cells (A), CD4⁺ helper T-cells (B), CD8⁺ killer T-cells (C), inflammatory monocytes (D), platelet-monocyte aggregates (E), platelet-granulocyte aggregates (F) and platelet-inflammation monocytes aggregates (G), are displayed. N = 10 per group. Error bars represent SEM.

Figure 6: CD40 on leukocytes contributes to the formation of platelet-leukocyte aggregates. Platelets and leukocytes from CD40-deficient (CD40⁻⁻) and C57BL/6 mice (WT) were co-incubated as indicated and formation of platelet-leukocyte aggregates were assessed by flow cytometry (A). N = 6/group. * p < 0.05, ** p < 0.001 vs. WT/WT. Supernatants of CD40-deficient platelet-leukocyte aggregates attenuate smooth muscle cell proliferation and increase apoptosis. A murine SMC line was stimulated with supernatants of platelet-leukocyte aggregates (PLA-SN) of either C57BL/6 mice (WT) or CD40-deficient (CD40⁻⁻) mice. Proliferation of SMC was determined using a BrdU-based ELISA (B) and the rate of apoptosis was determined using Apo-ONE® Assay (C). N = 3/group. * p < 0.05, *** p < 0.001 vs. WT. Error bars represent SEM.
studied whether the inhibition of the CD40L/Mac-1 interaction had an effect on lesion composition, specifically on macrophages and SMC content in the neointima. Neointima formation is characterised by both, proliferation of SMCs and invasion of macrophages (2). Mac-1 is expressed on macrophages and in the vasculature. CD40L is also expressed on endothelial cells, macrophages, platelets, and smooth muscle cells (21). There are contradictory reports about the role of CD40L in SMC proliferation and migration in vitro. Chai et al. reported that CD40L increases SMC proliferation and migration (7), whereas Hermann et al. showed that CD40L does not stimulate SMC proliferation and migration (36).

In our study both SMC and macrophage content was not altered in anti-Mac-3- and anti-aSMC-stained cross sections of lesions of mice treated with cM7, scM7, or saline, arguing against a role of CD40L/Mac-1 interaction for SMC and macrophage proliferation and migration after carotid ligation. Treatment with the CD40L/Mac-1 inhibitor cM7 did not alter the number of T-cells, monocytes, and platelets in blood compared with treatment with scrambled control or saline.

Since the inhibition of CD40L/Mac-1 interaction does not ablate neointima formation we hypothesised that neointima is rather mediated through an interaction of CD40L with its predominant receptor CD40. Indeed, neointima proliferation was markedly reduced in CD40-deficient mice compared with wild-type mice following carotid ligation. At the same time – as seen in the study by Donners et al. (13) – the average vessel area was also markedly reduced in CD40-deficient mice, revealing an overall impaired remodelling capacity in these mice after carotid ligation. There are several studies that previously evaluated the role of CD40 and CD40L in a variety of restenosis models: Blocking CD40L with an anti-CD40L monoclonal antibody attenuated neointima formation (11). Infusion of recombinant CD40L-accelerated neointimal progression in the carotid artery after wire injury (10).

On the other hand, using flexible collars to induce neointimal thickening in carotid arteries in mice, Remskar et al. showed that CD40L ablation induced a more pronounced neointima formation. A possible explanation might be that CD40L deficiency reduces IFNγ, a cytokine that has anti-proliferative effects on SMC (37). Donners et al. reported that after wire injury, neointima formation in CD40L-deficient mice was similar compared with wild-type mice. In contrast, the same authors and another group reported that CD40-deficient mice displayed a marked reduction in neointimal proliferation and a reduction in total vessel volume (13, 14). Both studies support a key role for downstream CD40 signaling through TRAF6 in neointimal formation. As a possible explanation for the reduced neointima formation Donners et al. report a tendency towards decreased matrix-degrading protease activity (MMP9 and MMP2). In our study plasma levels of MMP9 and MMP2 were similar in CD40-deficient mice vs C57BL/6 mice after carotid ligation (Suppl. Figure 3A, available online at www.thrombosis-online.com). However, this does not rule out locally increased matrix-degrading protease activity at the side of the carotid ligation.

The fact that genetic deficiency in CD40 results in a phenotype in this model is not in contradiction to the absence of an effect for the CD40L-Mac-1 inhibition either: a) these are two very different pathways affecting distinct functions, e.g. inflammatory cell recruitment in the case of CD40L-Mac-1 vs co-stimulation dependent- and independent pro-inflammatory effects in the case of CD40, b) even CD40 may have pro- and anti-inflammatory effects depending on its contribution to classic co-stimulation, direct gene expression in non-leukocytic cells or anti-inflammatory feedback mechanisms (e.g. CD40 expression on T cells) in different in vivo models. In that respect it is also noteworthy that while we previously showed that overall genetic deficiency of CD40 in LDLR KOs does not affect atherosclerosis (19), Lutgens et al. showed that the specific deletion of the CD40-TRAF6 interaction limits atherosclerosis in the same model contributing to the complexity of pathways (17).

Since both, leukocytes and platelets are major components of neointima formation we further characterised the cellular components of the blood in these mice after carotid ligation. We observed a significant decrease of monocyte- and granulocyte-platelet aggregates in CD40-deficient mice providing a possible explanation for a decreased neointima formation in these mice. In addition, we detected a decrease of CXCL4 (PF4) in the plasma of CD40-deficient mice after ligation of the carotid artery (Suppl. Figure 3B, available online at www.thrombosis-online.com). PF4 is released in large amounts at sites of vascular injury and has been implicated to play an important role in thrombosis (38). Platelet activation and thrombus formation are the first steps following flow cessation as it takes place after carotid ligation. Activated platelets adhere to leukocytes and form circulating mixed aggregates. The latter are considered a reliable marker of a pro-thrombotic state and are associated with several cardiovascular conditions including neointima formation (39, 40). CD40/CD40L interactions play an important role in mediating platelet activation and inflammatory processes. Platelets constitutively express CD40 and CD40 ligation leads to enhanced platelet-leukocyte adhesion (41). CD40 on pla-

What is known about this topic?

- CD40L and its ligands CD40 and Mac-1 have been implicated to play a role in neointima and restenosis formation.
- We have recently developed an inhibitor of the CD40L-Mac-1 interaction to study its role in inflammatory diseases like atherosclerosis and restenosis.
- Selective inhibition of the CD40L-Mac-1 interaction limits atherosclerosis in mice.

What does this paper add?

- Deficiency of the CD40L binding partner CD40 reduces neointima formation, suggesting that the classic CD40L/CD40 interaction mediates neointima formation rather than the CD40L-Mac-1 interaction. Future studies should therefore focus on downstream targets of the CD40-CD40L interaction to prevent restenosis.
- CD40 mediates platelet-leukocyte aggregation and smooth muscle cell proliferation in vitro.
In summary, we show that inhibition of the CD40L-Mac interaction does not influence neointima formation. Deficiency of the CD40L binding partner CD40, reduces neointima formation, suggesting that the classic CD40L-CD40 interaction mediates neointima formation but not the CD40L-Mac-1 interaction.

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Conflicts of interest
None declared.

References


