TRAF-1, -2, -3, -5, and -6 Are Induced in Atherosclerotic Plaques and Differentially Mediate Proinflammatory Functions of CD40L in Endothelial Cells

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Objective—Several lines of evidence implicate CD40 ligand (CD40L, CD154) as a mediator and marker of atherosclerosis. This study investigated the involvement of tumor necrosis factor receptor-associated factors (TRAFs) in CD40 signaling in endothelial cells (ECs) and their expression in atheromata and cells involved in atherogenesis.

Methods and Results—CD40L enhanced the basal expression of TRAF-1, -2, -3, and -6, but not TRAF-5 in ECs. TRAFs associated with CD40 on ligation by CD40L. Study of ECs from TRAF-1, -2, and -5–deficient mice demonstrated functional involvement of TRAFs in proinflammatory CD40 signaling. Whereas TRAF-1 deficiency enhanced CD40L-induced IL-6 and MCP-1 expression, TRAF-2 and TRAF-5 deficiency inhibited CD40L-inducible IL-6 but not MCP-1 expression. Gene silencing in human ECs further delineated functions of TRAFs in CD40 signaling. TRAF-3 silencing in ECs showed increased CD40L-induced IL-6, MCP-1, and IL-8 expression, whereas TRAF-6 silencing increased selectively CD40L-induced MCP-1 expression. Enhanced TRAF levels in atherosclerotic lesions further supports involvement of members of this family of signaling molecules in arterial disease.

Conclusions—These results implicate endothelial TRAF-1, -2, -3, -5, and -6 in CD40 signaling in atherogenesis, identifying these molecules as potential targets for selective therapeutic intervention.

Key Words: atherosclerosis ■ CD40L ■ inflammation ■ signaling ■ TRAF

Early work on CD40/CD40L interactions focused on their pivotal role in T-cell–dependent humoral immunity.1–3 Research during the past decade, however, revealed that expression of the CD40/CD40L dyad extends beyond lymphocytes, to endothelial cells (ECs), smooth muscle cells (SMCs), and macrophages, cells resident in atherosclerotic plaques.4,5 Thus, the pathological functions of CD40/CD40L interactions transcend B lymphocyte proliferation/differentiation and immunoglobulin class switching. Stimulation of leukocytes and nonleukocytic cells with CD40L induces the expression of a plethora of proinflammatory mediators, including cytokines, chemokines, adhesion molecules, matrix degrading enzymes, and procoagulants.8–11 In vivo studies verified a crucial role for CD40/CD40L interactions in numerous chronic inflammatory diseases, including rheumatoid arthritis, complications of transplantation, cancer, and atherosclerosis.3,12–15 In accord with a pivotal role for CD40L in atherogenesis, enhanced plasma levels of soluble CD40L predict future cardiovascular events in certain patient populations16,17 and correlate with several cardiovascular risk factors in pilot studies.18,19 Surprisingly, transplantation of either wild-type or CD40L-deficient bone marrow into low-density lipoprotein receptor-deficient mice revealed that CD40L from hematopoietic cells does not contribute to atherogenesis in mice, suggesting that ECs and other resident vascular cells contribute to CD40 signaling in arterial disease.20 However, knowledge regarding underlying signaling pathways that mediate proatherogenic functions after CD40 engagement in ECs, a key cell type in vascular disease, remains limited. Identification of such pathways has considerable importance, because systemic inhibition of CD40L may have adverse consequences, given this mediator’s pivotal function in host defenses.3,12,21

Previous studies focused on CD40 signaling pathways in B lymphocytes. Kinase pathways and tumor necrosis factor (TNF) receptor-associated factors (TRAFs), cytoplasmic
adaptor proteins that mediate cytokine signaling of members of the TNF-1, Toll-like-1, and IL-1 receptor superfamilies, may participate in CD40 signaling. Therefore, this study tested the hypothesis that TRAF-1, -2, -3, -5, and -6 function in CD40L-induced proinflammatory signaling in human and murine primary ECs and whether such signaling functions vary among different CD40L-induced genes in ECs and for the same target gene even among different cell types. We also investigated TRAF expression in murine and human atherosclerotic plaques.

Materials and Methods
Human saphenous vein ECs and human macrophages were isolated, cultured, and stimulated with various cytokines. Expression of TRAF1-6 was assayed by Western blotting of whole-cell lysates and lysates of subcellular protein fractions as described previously. TRAF protein expression was also quantified in lysates from normal and diseased human carotid arteries by Western blotting and in murine aortic sections by immunohistochemistry. Murine ECs isolated from TRAF wild-type and TRAF-deficient mice, as well as human umbilical vein ECs, silenced for the respective TRAFs were stimulated with TNF-α and CD40L and assayed for IL-6, IL-8, and MCP-1 by enzyme-linked immunosorbent assay (for detailed methods please see http://atvb.ahajournals.org).

Results
CD40L Enhances the Expression of TRAF-1, -2, -3, -5, and -6 Differentially in Human Vascular ECs, SMCs, and Macrophages
All cell types expressed TRAF-1, -2, -3, -5, and -6 constitutively, as determined by Western blot analysis of protein extracts. Ligation of CD40 enhanced the total protein expression of TRAF-1, -2, -3, and -6, but not TRAF-5 in ECs. In SMCs and macrophages, stimulation with CD40L only enhanced TRAF-1 expression (Figure 1). Notably, other proinflammatory cytokines modulated expression of these TRAFs differently. Stimulation with IL-1β or TNF-α induced expression of TRAF-1, -3, and -6 in ECs and SMCs, whereas neither stimulus affected the constitutive expression of TRAF-2 or -5 (Figure 1). In contrast, stimulation with interferon-γ or transforming growth factor-β did not change the expression of any TRAF tested in these cell types (Figure 1). In macrophages TNF-α stimulation only enhanced TRAF-1 expression. Basal TRAF-5 expression was most prominent in macrophages and could be significantly enhanced by stimulation with interferon-γ. The induction of TRAF-1, -2, -3, and -6 expression by CD40L depended on time and concentration, requiring a minimum of 4 hour of stimulation with ≥0.1 μg/mL CD40L (supplemental Figure IA and IB, available online at http://atvb.ahajournals.org).

CD40 Ligation Triggers Association of TRAF-1, -2, -3, -5, and -6 With CD40 in Human Vascular ECs and Recruitment of TRAFs to the Plasma Membrane
CD40 signaling in ECs indeed utilizes TRAFs, as determined by immunoprecipitation with an anti-CD40 antibody from lysates of ECs cultures followed by immunoblot analysis of the precipitates with the respective TRAF antibody. Ligation of CD40 on ECs triggered its association with TRAF-1, -3, and -6 from 15 to 60 minutes through 16 hours (Figure 2A) in accord with the increased expression of these TRAFs after CD40L stimulation. Although CD40L also triggered the association of TRAF-2 and TRAF-5 with CD40 in ECs, this interaction peaked at 60 minutes and declined to baseline levels within 16 hours. Stimulation of ECs with CD40L also enhanced TRAF expression in the plasma membrane fractions (supplemental Figure II).

Enhanced Expression of TRAF-1, -2, -3, -5 and -6 in Atherosclerotic Compared With Nondiseased Arterial Tissue
To determine tissue expression of TRAFs, protein extracts of nondiseased, atherosclerotic (dichotomized into fibrous or atheromatous plaques as described previously), or aneurysmal arteries were analyzed by Western blotting. Nondiseased tissue had barely detectable TRAF-1, -2, -3, -5, and -6 expression with the highest amounts of TRAF-6 (Figure 2B). In contrast, diseased tissues contained all TRAFs. Compared
with fibrous plaques, atheromatous plaques contained significantly more TRAF-2 and TRAF-3 protein (supplemental Figure III). However, TRAF-5 abounded in fibrous lesions. Mouse atherosclerotic lesions also contain TRAF-1, -2, -3, -5, and -6 as shown by immunohistochemical study of longitudinal sections of aortas from low-density lipoprotein receptor-deficient mice fed a high-cholesterol diet for 16 weeks (Figure 3). Sections from aortas from mice of similar age fed a regular low-fat diet showed little or no TRAF staining (data not shown). All TRAFs colocalized with both ECs and macrophages as assessed by immunohistochemical study of Murine atheromata (supplemental Figure IV).

Distinct Roles for TRAF-1, -2, and -5 in CD40L-Induced IL-6 and MCP-1 Expression in Primary Murine ECs

To examine the functional relevance of TRAF-1 for proinflammatory CD40 signaling, we isolated ECs from TRAF-1 wild-type and TRAF-1–deficient mice and analyzed supernatants for an inflammatory response to CD40L. Exposure of CD40L enhanced the constitutive production of IL-6 and MCP-1 protein (Figure 4A and 4B). Relative levels of IL-6 and MCP-1 release in TRAF-1–deficient ECs exceeded those from wild-type ECs, indicating that TRAF-1 limits CD40 signaling.25 Stimulation with TNF-α also resulted in a relative increase of cytokine expression in TRAF-1–deficient ECs compared with wild-type cells (Figure 4A and 4B).

In contrast to TRAF-1, deficiency of either TRAF-2- or TRAF-5 diminished CD40L-inducible and TNF-α-inducible IL-6 expression (Figure 4C). TRAF-2/-5 compound deficiency did not result in a significantly greater reduction in CD40L-inducible or TNF-α–induced IL-6 expression compared with single gene deficiency (Figure 4C). TRAF-2 and/or TRAF-5 deficiency did not modulate CD40L-induced or TNF-α–induced MCP-1 expression (Figure 4D), demonstrating distinct regulation of CD40 signaling for different target genes.

Because previous reports implicated TRAF-1 particularly in the regulation of apoptosis, we investigated whether TRAF-dependent modulation of cytokine expression depends on apoptosis and cell viability.25 Caspase-3/7 expression did not differ between TRAF-1–deficient cells and wild-type controls. Supernatants from TNF-α–stimulated TRAF-5–deficient cells had even lower caspase-3/7 expression than in corresponding controls, suggesting lack of dependence of expression of these mediators of apoptosis in these cells (supplemental Figure VA). Similarly, TRAF-deficient and wild-type Murine cells had comparable cell viability as assessed by lactate dehydrogenase (LDH) release into the supernatant. Only TNF-α–stimulated TRAF-2/-5 double-deficient cells showed an increased rate of cytotoxicity (supplemental Figure VB). To ensure that the results obtained in ECs from various tissues can represent those from arterial tissue, we verified some of our findings in ECs isolated from 8 pooled aortas per group (supplemental Figure VIA to VID).
Silencing of TRAFs by siRNA Implicates TRAF-1, -2, -3, -5, and -6 in CD40 Signaling in Human ECs

Primary human umbilical vein ECs transfected with TRAF-1–directed siRNA released more IL-6 and MCP-1 but significantly less IL-8 on stimulation with CD40L than those transfected with lamin-directed siRNA, demonstrating that TRAF functions vary for different target genes (supplemental Figure VII). Transfection with TRAF-2–directed siRNA significantly reduced CD40L-stimulated expression of IL-6 and IL-8 but not MCP-1 compared with anti-lamin–transfected controls, corroborating the concept that this molecule can limit certain proinflammatory aspects of CD40 signaling in ECs. ECs treated with TRAF-5 siRNA showed decreased IL-6 but increased IL-8 expression (supplemental Figure VII). Silencing of TRAF-3 in ECs supported an inhibitory role of TRAF-3 in CD40L-induced proinflammatory gene expression, because cells treated with anti-TRAF-3 siRNA showed an increased basal and CD40L-stimulated expression of all 3 proteins. Endothelial cells silenced for TRAF-6 released similar amounts of IL-6 and IL-8 and an increased amount of MCP-1 (Figure 5).

TRAF-1, -2, and -5 Differentially Mediate Proinflammatory Functions of CD40L in Various Cell Types

To test the hypothesis that proinflammatory functions of CD40L not only show target gene selectivity but also differ for the same target gene in various cell types, we isolated peritoneal macrophages from TRAF wild-type and TRAF-deficient animals. Similar to the observations in ECs, TRAF-1-deficient macrophages expressed higher levels of IL-6 and MCP-1 on stimulation with CD40L compared with corresponding wild-type controls. Also, neither TRAF-2 nor TRAF-5 affected CD40L-inducible MCP-1 expression. However, in contrast to ECs, TRAF-5 deficiency did not affect CD40L-induced or TNF-α-induced IL-6 expression, whereas TRAF-5 deficiency combined with TRAF-2 heterozygosity effectively reduced CD40L-induced and TNF-α-induced IL-6 expression compared with wild-type controls (supplemental Figure VIII).

Discussion

This study demonstrates that CD40L and other proinflammatory cytokines differentially modulate TRAF-1, -2, -3, -5, and -6 expression in ECs, SMCs, and macrophages, key cell types in arteries and arterial disease. The results also establish the functional relevance of these TRAFs for proinflammatory signaling events in ECs, and hence in inflammatory vascular diseases such as atherosclerosis.

To date, analysis of TRAF functions after CD40 ligation focused on lymphoid cells and generated inconsistent results,3,22,25 caused in part by diverse methods and cell type-specific and target gene-specific differences in signal transduction mediated by CD40L. Indeed, CD40 signaling may even induce different pathways in the same cell type depending on the stage of differentiation.23,26 The present data demonstrate that CD40L uses TRAF-1, -2, -3, -5, and -6 differentially for IL-6, IL-8, and MCP-1 expression in ECs, supporting the concept of target gene-dependent CD40 signaling. Furthermore, TRAF-associated signaling induced by CD40L differs from activation pathways used by other proinflammatory cytokines, as indicated by the distinct modulation of TRAF expression by CD40L compared with IL-1β, TNF-α, transforming growth factor-β, and interferon-γ. In that context, our group recently described that ligation of CD40 on ECs activates Egr-1, a transcription factor not altered by TNF-α and IL-1β in this cell type.27 Our observations in macrophages, which contrast in some respects with
the findings in ECs, corroborate the notion that CD40 signaling differs not only between target genes within the same cell type but also for the same target gene in different cell types.

The inducible expression of TRAF-1 by CD40L, IL-1β, and TNF-α in ECs, SMCs, and macrophages observed here agrees with previous reports in B cells and freshly isolated monocytes. The physiological role of TRAF-1 remains controversial. Conflicting reports identify TRAF-1 as either a promoter or an inhibitor of signals triggered by CD40L and TNF-α in various cell types. Similarly, others have suggested TRAF-1 as cofactor and inhibitor of TRAF-2–dependent NF-κB and JNK activation. Our data demonstrate that TRAF-1 may indeed promote and at the same time inhibit certain proinflammatory functions of CD40L. Thus, TRAF-1 deficiency enhances both CD40L- and TNF-α–induced IL-6 and MCP-1 expression in ECs, suggesting that TRAF-1 negatively regulates these cytokines in this cell type. Our siRNA studies in human umbilical vein ECs parallel those findings for IL-6 and MCP-1, while suggesting a positive role for TRAF-1 in CD40L-induced IL-8 expression.

In contrast to TRAF-1 and TRAF-5, earlier studies have shown that overexpression of TRAF-2 in cell lines suffices to activate NF-κB and JNK. Thus, TRAF-2 likely stimulates signaling by TNF receptor family members, including CD40, in this cell type. However, several reports also described inhibitory signaling functions of TRAF-2 in lymphoid and other cells. The present report identifies TRAF-2 as an activator of CD40L-induced and TNF-α–induced IL-6 and IL-8 expression in ECs. However, TRAF-2 does not participate in MCP-1 expression initiated by the same cytokines in this cell type.

Figure 4. TRAF-1, -2, and -5 differentially mediate CD40L and TNF-α–induced IL-6 and MCP-1 expression in Murine ECs. A to D, Supernatants from ECs from TRAF-1–competent (traf1++) and TRAF-2/-5–competent (traf2−/−5−/−), as well as TRAF-1–deficient (traf1−), TRAF-5–deficient (traf5−), TRAF-2–deficient (traf2−), and TRAF-2/-5–double-deficient (traf2−/−5−) mice incubated for 24 hours with medium alone (hatched bar), recombinant murine CD40L (5 μg/mL, black bar), or recombinant murine TNF-α (10 ng/mL, gray bar) were analyzed by enzyme-linked immunosorbent assay for IL-6 (left) and MCP-1 (right) protein expression. Data are presented as means±SEM of at least 6 independent experiments per group. #P<0.05 when compared with CD40L-induced value of respective TRAF-gene competent groups. $P<0.05 when compared with TNF-α–induced value of respective TRAF-gene competent groups.

Similar to TRAF-2 deficiency, TRAF-5–deficient ECs showed significantly reduced CD40L-induced and TNF-α–induced IL-6 expression compared with wild-type controls. However, ECs treated with TRAF-5 siRNA released significantly more IL-8 protein into the supernatant, suggesting that TRAF-5 mediates and inhibits certain proinflammatory functions of these cytokines. Our data agree with previous reports in B cells, which implicated the participation of TRAF-5 in CD40 and TNF receptor signaling. In contrast to the observations in ECs, TRAF-5 does not mediate CD40L-induced or TNF-α–induced IL-6 expression in MΦ, an illustration of cell type-selective signaling by these mediators. In B cells, TRAF-2 and TRAF-5 exhibit overlapping functions in CD40 signaling.

Xu et al observed that B cells from TRAF-3–null mice show augmented CD23 and proliferate normally on stimulation with CD40L, suggesting that TRAF-3 does not require CD40 signaling. In contrast, our data demonstrate that ECs treated with siRNA targeting TRAF-3 released significantly more basal and CD40L-induced IL-6, IL-8, and MCP-1 into the supernatant than appropriate control cells, suggesting an inhibitory role for TRAF-3 in CD40 signaling. Our data agree with a previous report by Urbich et al that demonstrated that activation of TRAF-3 by shear stress abrogates CD40L–mediated endothelial activation.

Previous studies on B lymphocytes derived from TRAF-6–deficient mice suggested that TRAF-6 is instrumental in CD40L–induced proinflammatory cytokine production and B cell maturation. Similarly, Andrade et al and others implicated TRAF-6 as important mediator of CD40 signals in monocytes and macrophages. In contrast to these findings, our siRNA studies suggest that in ECs, TRAF-6, if anything, inhibits CD40L–induced proinflammatory protein expression.
highlighting once again cell-type specific differences of TRAF functions.

Previous reports implicated TRAF-1 in particular, but also other TRAFs in apoptosis. Our data suggest that TRAF-dependent protein expression does not depend on apoptosis and cell viability. We found extensive expression of TRAF-1, -2, -3, -5, and -6 in sections of murine atherosclerotic aortic arches. Co-localization studies confirmed TRAF expression by both ECs and macrophages in situ. Furthermore, lysates of human atherosclerotic and aneurysmal arteries overexpressed TRAFs compared with lysates from apparently normal arteries. These data further support a role for TRAFs in the pathogenesis of atherosclerosis. Interestingly, in contrast to TRAF-1, expression levels of TRAF-2 and -3 were significantly greater in atheromatous lesions compared with fibrous lesions or aneurysmal arteries, whereas fibrous lesions expressed significantly more TRAF-5. Atherosclerotic lesions overexpress CD40L, a mediator that can trigger mechanisms associated with plaque progression and thrombosis. Therefore, high levels of TRAF-2 expression may promote inflammatory signaling by CD40L and other cytokines, potentially linking TRAF-2 not only to atherogenesis but also to plaque complications. Because the same TRAFs may exert opposing functions for different target genes, the net effects of TRAF overexpression on inflammatory activity and atherosclerosis will require future study.

In sum, the present study provides new functional insights into signaling mechanisms initiated by the proatherogenic CD40L/CD40 dyad in vascular cells. The results demonstrate that both CD40L and TNF-α differentially use TRAF-1, -2, -3, -5, and -6 for proinflammatory signaling depending on the target gene and cell type investigated and directly implicate TRAFs in vascular disease. Manipulation of TRAFs may permit selective modulation of proatherogenic functions of CD40L and other proinflammatory cytokines of the TNF receptor-like and IL-1/Toll-like receptor superfamilies.

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Disclosure

None.

References


19. Zirlik et al TRAFs and CD40 Signaling in ECs 1107


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Expanded Materials and Methods:

Reagents – TRAF-1, -2, -3, -5, and -6 antibodies were obtained from Santa Cruz, anti-TRAF-2 also from Cell Signaling, anti-TRAF-5 also from ebioscience, and anti-Lamin antibody was purchased from Novocastra. Unless stated differently, all antibodies used for FACS were from BD Pharmingen and all ELISA antibodies from Pierce Endogen. Recombinant proteins were from R&D with exception of human CD40L from Leinco and human IL-6 and IL-8 from Pierce Endogen. All siRNA oligonucleotides were designed and purchased from Qiagen. All human experiments employed human CD40L (Leinco), all Murine experiments Murine CD40L (R&D).

Mice – TRAF-1 deficient mice were kindly provided by their originator Dr. Tsitsikov, TRAF-2 and TRAF-2/-5 deficient mice from Dr. Nakano. TRAF-2 deficient single knock out mice were also provided by Dr. Nakano with permission of their originator, Dr. T.W. Mak (Toronto, Canada). TRAF-1 deficient mice were pure C57/BL6, TRAF-2, -5 and -2/-5 deficient mice were Balb/c.

Cell isolation and culture - Human saphenous vein EC (HSVEC), human umbilical vein EC (HUVEC), and human MΦ were isolated and cultured as previously described. For isolation of murine EC, corresponding TRAF-deficient and control mice were euthanized with CO₂, and lungs, heart, brain, and liver were harvested employing sterile technique, minced with a razor blade, and digested in 0.2% collagenase type-1/1% BSA (Worthington, Lakewood, NJ and Sigma, St. Louis, MO) for 90 min at 37°C. After washing with 0.1% BSA and filtering through a 70µm nylon mesh (BD Biosciences, San Jose, CA), cells were resuspended in 0.1% BSA and incubated with an anti-mouse CD31 antibody conjugated to sheep anti-rat Dynabeads (Dynal Biotech, Oslo, Norway) for 10 min at room temperature. Cells were then separated and washed three times using a magnetic particle concentrator (Dynal Biotech) and seeded into gelatin-coated plates. After they reached confluence, a second magnetic sorting was performed with a rat anti-mouse ICAM-2 antibody (BD Pharmingen).
Cells were grown in DMEM high glucose (Cambrex) supplemented with 20% fetal bovine serum (FBS), 1% sodium pyruvate, 1% heparin, 1% bovine endothelial growth factor, 0.6% non-essential amino acids, and 1% penicillin/streptomycin. Cells were maintained in M-199 supplemented with 0.1% FBS 24h prior to experiments. Since TRAF-2- and TRAF-2/-5-deficient mice only survive 3-4 weeks, mice were genotyped at 10-14 days of age and EC were isolated subsequently.

For isolation of peritoneal MΦ, 2 ml of 4% thioglycollate (Sigma) was injected intraperitoneally in mice of desired genotype. After three days, the peritoneal cavity was flushed with 5ml of RPMI 1640. After lysing the erythrocytes with 0.155 M NH₄Cl, filtering through a 70µm nylon mesh, and washing with 0.2% BSA/PBS, cells were plated and maintained in RPMI 1640 supplemented with 5% FBS and 1% penicillin/ streptomycin. Before experiments, cells were maintained in RPMI 1640 lacking FBS for 24 h.

Preparation of protein extracts - Frozen non-atherosclerotic human carotids (n=6), atherosclerotic human carotids (n=12), dichotomized a priori into fibrous (stable; n=6) and atheromatous (n=6) plaques by morphological criteria, as well as abdominal aortic aneurysm tissue (n=6) were homogenized (IKA-Labortechnik, Ultra-turrax T 25) and lysed as described previously.

Immunoprecipitation and Western blotting- Confluent cell cultures were scraped in lysis buffer (50 mM HEPES pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 µg/ml aprotinin, leupeptin and pepstatin A), incubated 30 min at 4°C, and clarified (14,000 x g, 10 min). Lysates were precleared with Protein-G-Sepharose (30 min, 4°C, Amersham-Pharmacia), incubated (2 h, 4°C) with the specific goat or mouse anti-human CD40 antibody (10 µg/ml, Sigma; or Ancell), followed by incubation with Protein-G-Sepharose (1 h, 4°C). The beads were washed (4X) in lysis buffer and finally subjected to Western blot analysis, as described previously.

Preparation of plasma membrane fraction – Cell lysates were centrifuged at 3,500 x g for 5min. Plasma membrane fractions were pelleted from the supernatants by centrifugation at 100,000 x g for 90min.
Immunohistochemistry – Serial cryostat sections (6 µm) of Murine aortic arches from LDLR-deficient mice fed a high cholesterol diet (1.25 cholesterol, 0% cholate; Research Diets) for 16 weeks were fixed in acetone (-20°C, 5 minutes), air-dried, and stained by the avidin-biotin-peroxidase method as described previously 1.

siRNA Transfections – HUVEC were grown to 80% confluency, transfected at 250nM final concentration of specified TRAF-specific siRNA employing the Amaxa Nucleofector device (Amaxa), the HUVEC specific Amaxa Nucleofector kit, and program V-01, according to the manufacturer’s instructions. Subsequently, cells were cultured for 24h for attachment and stimulated for 24 h with the indicated concentrations of human CD40L. Cell lysates and supernatants were collected. Cells transfected with Lamin-directed or scrambled siRNA served as internal control. Transfection efficacy was >75% assessed by FACS analysis of cells transfected with FITC-labeled siRNA (Fig.I, online supplement).

Enzyme-linked immunoabsorbent assay (ELISA) - Murine and human IL-6, IL-8, and MCP-1 were quantified in the supernatant of cultures by ELISA.

Apoptosis and Cytotoxicity Assays – To assess apoptosis and cytotoxicity in Murine cells, Caspase-3/7 and LDH protein concentrations were quantified in supernatants employing commercially available assays (ApoOne Homogeneous Caspase-3/7 Assay, CytotoxOne Assay, Promega) according to the protocol of the manufacturer.

Data analysis - Western blots were analyzed densitometrically using ImageJ (NIH software). Data of at least three experiments were pooled and presented as mean ± SEM. Statistics employed the
Student’s two-tailed t test for paired or unpaired values (where appropriate). A p value <0.05 was considered statistically significant.

References


Online Figure Legends:

Fig. I: Time- and concentration-dependent induction of TRAF-1, -2, -3, and -6 by CD40L in EC. A, Lysates of EC incubated for the indicated time periods with serum-free medium alone (None) or CD40L (10µg/ml) were analyzed by Western blotting employing various TRAF antibodies. B, Lysates of EC incubated for 24 h with serum-free medium alone (None) or respective concentrations of CD40L, were analyzed by Western blotting employing respective TRAF antibodies.

Fig. II: CD40L recruits TRAF-1, -2, -3, -5, and -6 to the plasma membrane in human EC. Plasma membrane fractions of lysates of EC incubated for the respective duration with CD40L (10µg/ml) were applied to Western blot analysis with respective TRAF antibodies. For control purposes, lysates of PMA-activated Jurkat cells were applied.

Fig. III: TRAF-1, -2, -3, -5, and -6 are overexpressed in atherosclerotic and aneurysmal arterial tissue. Extracts of non-diseased (Normal), atherosclerotic (dichotomized into fibrous and atheromatous plaques), or aneurysmal (AAA) specimens were analysed by Western blots employing the indicated TRAF antibodies. Pooled densitometric values of at least six individual experiments are shown as graphs. Data represent means ± SEM. § = P≤0.05 when compared to values of the non-diseased group.

Fig. IV: TRAF-1, -2, -3, -5, and -6 colocalize with endothelial cells and macrophages in murine atherosclerotic sections. Sections of aortic arches from LDLR-deficient mice fed a high cholesterol diet for 16 weeks were double stained with the indicated TRAF antibodies and an endothelial (CD31) and macrophage marker (Mac-3) and shown at 40x magnification. Red staining signifies TRAF expression, blue staining the respective cell marker. Three independent experiments yielded similar results.

Fig. V: TRAFs, apoptosis, and cytotoxicity in murine EC. A, TRAF-1- and TRAF5-deficient cells and respective controls were stimulated with medium alone (hatched bar), CD40L (5µg/ml, black bar), and
TNFα (10ng/ml, grey bar) and Caspase 3/7 release into the supernatant was quantified employing a commercially available assay. Data are presented as means ± SEM of at least four independent experiments per group. § = P≤0.05 when compared to TNFα-induced value of respective TRAF-gene competent groups. B, Supernatants from cells treated as described under Fig. 4 A-D were assayed for LDH employing a commercially available assay. Data are presented as means ± SEM of at least four independent experiments per group. § = P≤0.05 when compared to TNFα-induced value of respective TRAF-gene competent groups.

**Fig. VI:** TRAF-1 and -5-dependent pro-inflammatory gene expression confirmed in arterial murine EC. Supernatants from endothelial cells (EC) isolated only from aortas from a pool of 8 TRAF-1 competent- (traf1\(^{++}\)) and TRAF-5-competent (traf2\(^{++}\)5\(^{++}\)) as well as TRAF-1-deficient (traf1\(-\)), TRAF-5-deficient (traf2\(^{++}\)5\(^{-}\)) mice, respectively, incubated for 24h with medium alone (hatched bar), recombinant murine CD40L (5µg/ml, black bar), or recombinant murine TNFα (10ng/ml, grey bar) were analyzed by ELISA for IL-6 (left) and MCP-1 (right) protein expression. Data are presented as means ± SD of one experiment assayed in triplicate. # = P≤0.05 when compared to CD40L-induced value of respective TRAF-gene competent groups. § = P≤0.05 when compared to TNFα-induced value of respective TRAF-gene competent groups.

**Fig. VII:** Inhibition by siRNA shows distinct roles of TRAF-1, -2, and -5 in CD40 signaling in HUVEC. HUVEC were transfected with indicated TRAF-directed siRNA (250nM final concentration), plated for 12 h in growth medium, starved for 12 h in serum-reduced medium, and incubated with either medium (hatched bar) or 10 µg/ml of recombinant human CD40L (black bar) for 24 h. Supernatants were assayed for IL-6, IL-8, and MCP-1 by ELISA. Data are presented as means ± SEM of at least four experiments per group. Representative blots are shown. Cell lysates were subjected to Western blot analysis with the antibodies indicated. Cells transfected with anti-Lamin siRNA served as internal control.
Fig. VIII: TRAF-1, -2 and -5 differentially mediate CD40L and TNFα induced IL-6 and MCP-1 expression in macrophages. A-D, IL-6 and MCP-1 was measured in the conditioned media of peritoneal macrophages isolated from TRAF-1 competent- (traf1\(^{++}\)) and TRAF-2/-5-competent (traf2\(^{++}\)traf5\(^{++}\)) as well as TRAF-1-deficient (traf1\(^{-}\)), TRAF-5-deficient (traf2\(^{++}\)traf5\(^{-}\)), TRAF-2 heterozygous /TRAF-5-deficient (traf2\(^{+}\)traf5\(^{-}\)) mice and stimulated for 24 h with media alone (Control, hatched bar), recombinant murine CD40L (5µg/ml, black bar), or recombinant murine TNFα (10ng/ml, grey bar). IL-6 and MCP-1 expression was quantified by ELISA. Data are presented as means ± SEM of at least six independent experiments per group. # = P≤0.05 when compared to CD40L-induced value of respective TRAF-gene competent groups. § = P≤0.05 when compared to TNFα-induced value of respective TRAF-gene competent groups.

Fig. IX: Transfection efficacy of siRNA in HUVEC. HUVEC transfected with FITC-labeled siRNA or non-labelled control siRNA were harvested after 24 h with trypsin-EDTA and transfection efficacy was analyzed by FACS. Representative FACS of a total of three experiments yielding similar results is shown.
Online Fig. I

A

<table>
<thead>
<tr>
<th>Time</th>
<th>TRAF-1</th>
<th>TRAF-2</th>
<th>TRAF-3</th>
<th>TRAF-6</th>
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<tbody>
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<td>5’</td>
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<td>30’</td>
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<tr>
<td>36h</td>
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<tr>
<td>CD40L (10 µg/ml)</td>
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B

<table>
<thead>
<tr>
<th>Condition</th>
<th>TRAF-1</th>
<th>TRAF-2</th>
<th>TRAF-3</th>
<th>TRAF-6</th>
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<tbody>
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<tr>
<td>IL-1β</td>
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<tr>
<td>TNFα</td>
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<tr>
<td>0.1 µg/ml</td>
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<td>0.3 µg/ml</td>
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<tr>
<td>1 µg/ml</td>
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<td>3 µg/ml</td>
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<tr>
<td>10 µg/ml</td>
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<tr>
<td>CD40L (24 h)</td>
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</table>
Plasma-membrane

TRAF-1
TRAF-2
TRAF-3
TRAF-5
TRAF-6

0 10' 30' 90' 16h

CD40L (10 µg/ml)

Online Figure II
Online Figure III
Colocalization with endothelial cells (CD31, blue staining)

| TRAF-1 | TRAF-2 | TRAF-3 | TRAF-5 | TRAF-6 |

Colocalization with macrophages (anti-Mac-3, blue staining)

Online Figure IV
Online Figure V
Online Fig. VI

A

[Bar graph showing IL-6 levels in traf1++ and traf1--]  
- Traf1++: Black bars  
- Traf1--: Grey bars

B

[Bar graph showing MCP-1 levels in traf1++ and traf1--]  
- Traf1++: Black bars  
- Traf1--: Grey bars

C

[Bar graph showing IL-6 levels in traf5++ and traf5--]  
- Traf5++: Black bars  
- Traf5--: Grey bars

D

[Bar graph showing MCP-1 levels in traf5++ and traf5--]  
- Traf5++: Black bars  
- Traf5--: Grey bars

Legend:  
- Control  
- CD40L 5µg/ml  
- TNFα 10ng/ml
Online Figure VII
Online Fig. IX

Mock transfected ECs

ECs transfected with FITC-labeled siRNA