

CD40L Deficiency Protects Against Aneurysm Formation

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Objective—The mechanisms underlying formation of arterial aneurysms remain incompletely understood. Because inflammation is a common feature during the progressive degeneration of the aortic wall, we studied the role of the costimulatory molecule CD40L, a major driver of inflammation, in aneurysm formation.

Approach and Results—Transcriptomics data obtained from human abdominal aortic aneurysms and normal aortas revealed increased abundance of both CD40L and CD40 in media of thrombus-free and thrombus-covered human abdominal aortic aneurysms samples. To further unravel the role of CD40L in aneurysm formation, apolipoprotein E-deficient (*Apoe*^{−/−}) and *Cd40l*^{−/−}*Apoe*^{−/−} mice were infused with angiotensin II for 7 and 28 days. Only a minority of *Cd40l*^{−/−}*Apoe*^{−/−} mice (33% and 17%) developed (dissecting) aneurysms compared with 75% and 67% of *Apoe*^{−/−} littermates after 7 and 28 days of infusion, respectively. Total vessel area of the aorta at the suprarenal level was 52% smaller in angiotensin II-infused *Cd40l*^{−/−}*Apoe*^{−/−} mice compared with that in angiotensin II-infused *Apoe*^{−/−} mice. Chimeric *Apoe*^{−/−} mice repopulated with *Cd40l*^{−/−}*Apoe*^{−/−} bone marrow afforded a similar protection against dissecting aneurysm formation. Moreover, lack of CD40L protected mice from fatal aneurysm rupture. T helper cell and macrophage accumulation in aneurysmal tissue was reduced in *Cd40l*^{−/−}*Apoe*^{−/−} mice with a concomitant decrease in expression of proinflammatory chemo- and cytokines. In addition, aneurysms of *Cd40l*^{−/−}*Apoe*^{−/−} mice displayed reduced abundance of matrix metalloproteinase-13 and an increase in tissue inhibitor of metalloproteinase-3 while activity of matrix metalloproteinase-2 and matrix metalloproteinase-9 was diminished.

Conclusions—Deficiency of (hematopoietic) CD40L protects against dissecting aneurysm formation and reduces the incidence of fatal rupture. This is associated with a decreased accumulation and activation of inflammatory cells and a dampened protease activity in the arterial wall.



Visual Overview—An online [visual overview](#) is available for this article. (*Arterioscler Thromb Vasc Biol.* 2018;38:1076-1085. DOI: 10.1161/ATVBAHA.117.310640.)

Key Words: aneurysms ■ angiotensin II ■ cytokines ■ inflammation ■ matrix metalloproteinase 9

An abdominal aortic aneurysm (AAA) is defined as a permanent dilation of the abdominal aorta by >50% or >3 cm and occurs in up to 8.00% of men over the age of 65 years and between 0.37% and 1.53% of women over the age of 60 years.^{1,2} Complications of AAAs, such as rupture or dissection, have an associated mortality of >80%.³ Inflammation is of major importance in aneurysm formation and progression.^{1,4,5} Patients with an AAA display increased serum concentrations of interleukin-1 β , TNF (tumor necrosis factor) α , CCL2 (chemokine [C-C motif] ligand 2), interleukin-6, and interferon γ (IFN γ) while several inflammatory mediators affect AAA development

in mice.^{6–9} Similarly, expression levels of matrix metalloproteinase (MMP) 9 are increased in human AAA tissue, and levels of tissue inhibitor of metalloproteinase-2 are decreased.¹⁰

A TNF-related signaling pathway implicated in inflammation and matrix turnover is the CD40-CD40L dyad.¹¹ In atherosclerosis, inhibition of CD40-CD40L signaling decreases plaque size and results in a plaque phenotype that contains high amounts of fibrosis and low amounts of inflammation, reminiscent of a stable plaque.^{12–15} Here, we investigate the role of CD40L in AAA development in humans and mice.

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Nonstandard Abbreviations and Acronyms

AAA	abdominal aortic aneurysm
AngII	angiotensin II
<i>Apoe</i> ^{-/-}	apolipoprotein E-deficient
CCL	chemokine (C-C motif) ligand
IFN γ	interferon γ
MMP	matrix metalloproteinase
TGF	transforming growth factor
Th	T helper
TNF	tumor necrosis factor

Materials and Methods

Microarray

Patient samples were obtained from elective open surgery for AAA treatment and control samples from the abdominal aorta of solid organ transplant donors. A total of 76 samples were obtained from AAA with thrombus-covered vessel wall, 34 from AAA with thrombus-free vessel wall, and 13 samples from the abdominal aortas of solid organ transplant donors. The elective open aortic repair surgeries were performed between 2009 and 2016, the solid organ transplant surgeries in 2014, both at the Karolinska University Hospital in Stockholm, Sweden. The samples were immediately immersed in RNA stabilization solution (RNAlater, Thermo Fisher Scientific, Waltham, MA), separated into intima/medial and adventitial wall layers, and then stored in -80°C . RNA was isolated using Qiazol Lysis Reagent (Qiagen) and purified using the RNeasy minikit (Qiagen). The concentration of RNA was measured using Nanodrop ND 1000 (Thermo Fisher Scientific). Gene expression was analyzed with Affymetrix HTA 2.0 Genechip arrays (Thermo Fisher Scientific). Raw CEL intensity files were normalized through Guanine Cytosine Count Normalization, Signal Space Transformation, and Robust Multi-array Average by use of the Affymetrix Expression Console software (Thermo Fisher Scientific). The Bioconductor tool limma¹⁶ was used in R¹⁷ to determine differential gene expression, with the empirical Bayes moderation of standard errors for the microarray linear model. The study was approved by the Regional Ethical Review Board in Stockholm (Dnr 2009/9–31/4, Dnr 2013/615–31/4).

Mice

Cd40l^{-/-} apolipoprotein E-deficient (*Apoe*^{-/-}) and *Apoe*^{-/-} mice on a C57BL/6 background were bred in our animal facility. More than 10 backcrosses with *Apoe*^{-/-} mice were performed to obtain double knockout mice. Mice were fed a normal laboratory diet throughout the experiment. At 10 weeks of age, Alzet osmotic minipumps (Model 2004; Durect Corporation, Cupertino, CA), containing angiotensin II (AngII; Sigma A9525), were implanted subcutaneously into both *Apoe*^{-/-} (n=27) and *Cd40l*^{-/-}*Apoe*^{-/-} mice (n=26). AngII was infused at a rate of 1000 ng/kg per minute for 28 days or 7 days in *Apoe*^{-/-} (n=7) or *Cd40l*^{-/-}*Apoe*^{-/-} (n=15).¹⁸ At the end of the experimental period, mice were euthanized after 4 hours of fasting. Blood was collected from the right ventricle for plasma cholesterol concentrations. The presence of macroscopic dissecting aneurysm and rupture was noted.

Mice were randomly divided into 2 groups. One group was used for histological and morphometric analysis (28 days: *Apoe*^{-/-}: n=12 and *Cd40l*^{-/-}*Apoe*^{-/-}: n=10, 7 days: *Apoe*^{-/-}: n=5 and *Cd40l*^{-/-}*Apoe*^{-/-}: n=6) and a second group for mRNA and protein analyses (*Apoe*^{-/-}: n=16 and *Cd40l*^{-/-}*Apoe*^{-/-}: n=15, 7 days: *Apoe*^{-/-}: n=6 and *Cd40l*^{-/-}*Apoe*^{-/-}: n=13). All animal experiments were performed under approved institutional animal care of the respective universities.

Bone Marrow Transplantation

Apoe^{-/-} mice (n=20) were lethally irradiated (10 Gy, 0.5 Gy/min; MU 15F/225 kV; Philips) and injected intravenously with 5×10^6 bone marrow-derived cells from *Apoe*^{-/-} and *Cd40l*^{-/-}*Apoe*^{-/-} mice.¹³ Mice

were maintained in filter-top cages and given water containing 60 000 U/L polymyxin B sulfate and 100 mg/L neomycin (both Invitrogen) from 1 week before bone marrow transplantation until 4 weeks afterward. Six weeks after transplantation, osmotic minipumps were inserted subcutaneously into both the *Apoe*^{-/-} and *Cd40l*^{-/-}*Apoe*^{-/-} mice. AngII was infused at a rate of 1000 ng/kg per minute. Mice were euthanized after 28 days of infusion.

Histological Analyses

For morphometric analyses, the entire arterial tree was perfused with PBS containing 0.1 mg/mL sodium nitroprusside dihydrate (Sigma), followed by 1% paraformaldehyde via a catheter inserted into the left ventricle. The arterial tree was excised and fixed overnight in 1% paraformaldehyde and embedded in paraffin. Cross-sections (4 μm) of the suprarenal area of the abdominal aorta (with or without dissecting aneurysms) were obtained at 12 different levels at 200 μm intervals. Cross-sections from each level were stained with hematoxylin and eosin and Elastica von Gieson. Total vessel area on all 12 levels was measured on a Leica DM6000 Light microscope (Leica Microsystems) coupled to a computerized morphometry system (Leica Qwin 3.5.1).

Immunohistochemistry

To characterize accumulation of inflammatory cells in aneurysmal tissue and the surrounding tissue, cross-sections were immunostained with anti-MAC-3 (BD Lifescience) for presence of macrophages, anti-CD45 (Becton & Dickinson) for leukocyte infiltration, and anti-CD3 (Dako) for T cell infiltration, anti-B220(1D3) for B cell infiltration, anti-CD4(RM4-5), or anti-CD8(5H10), kindly provided by Dr Duijvestijn. MAC-3, CD45, and CD3 immunostainings were quantified using a scoring system where no chromogen yielded a score of 0 and maximum color corresponded to a score of 5. Scoring was performed by 1 investigator blinded to the identity of the sections. The intraobserver variability was <10%.

RNA and Protein Analyses

Abdominal aortas of mice were excised for RNA or protein analyses and immediately snap frozen in liquid nitrogen and stored at -80°C until further use. The presence of a macroscopic visible aneurysm was documented.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

Aortic tissues were homogenized, and RNA was isolated using the Qiagen RNeasy mini kit (Qiagen). For the preparation of cDNA, the iScript cDNA Synthesis Kit (Bio-Rad) was used according to manufacturer's protocols. Quantitative polymerase chain reaction was performed using the SYBR Green method. A total of 10 ng of cDNA was used with the 2 \times quantitative polymerase chain reaction master mix (Bio-Rad). Expression of matrix metalloproteinases and their inhibitor genes pro- and anti-inflammatory genes and the regulatory T cell-specific gene *Foxp3* were analyzed. Finally, CD40 and CD40L expression were analyzed. The reference genes *cyclophilin A* and β -actin were used to normalize RNA abundance.

Gelatin Zymography

MMP-2 and MMP-9 activities were determined in aortic/aneurysm tissues of *Apoe*^{-/-} and *Cd40l*^{-/-}*Apoe*^{-/-} mice. Tissue samples were extracted in 150 μL sodium dodecyl sulfate lysis (10% glycerol, 20% sodium dodecyl sulfate, 10% 1.5 mol/L Tris pH 6.8) buffer by cutting the tissue into small pieces after which total protein concentration was adjusted to 75 μg . Murine vascular smooth muscle cells incubated with TNF- α +platelet-derived growth factor was used as a control. MMP-2 and MMP-9 activities in extracts were detected as described previously.¹⁹ Briefly, 4 μL of tissue extract was diluted (1:5) with distilled water. Samples were electrophoresed in the presence of nonreducing buffer at 4°C in 7.5% sodium dodecyl sulfate polyacrylamide gels containing 2 mg/mL gelatin. After the removal of sodium dodecyl sulfate, gelatinase activity was revealed by overnight incubation at 37°C and subsequent staining with 0.1% Coomassie brilliant

blue. Zymograms were quantified in the linear range by densitometry with Quantity One 1-D Image Analysis software system (Bio-Rad).

Lipid Analyses

Plasma cholesterol concentrations were measured using an enzymatic assay (Roche).

Statistical Analyses

Data are expressed as mean±SEM. The Mann–Whitney nonparametric test was used to compare the *Cd40l*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} groups. When analyzing incidence of aneurysm formation and incidence of death before end of the experiment, the Fisher exact test was used. Kaplan–Meier survival curves were analyzed using log-rank (Mantel–Cox) test. Flow cytometry data were analyzed using Student *t* test. Data with probability values <0.05 were considered statistically significant.

Results

Increased Expression of CD40L and CD40 in Human AAA

Microarray data from the Stockholm AAA Biobank were used to study the expression levels of CD40L and CD40 mRNA in human AAA disease. Tissue samples of thrombus-free (n=34) and thrombus-covered (n=76) AAA wall segments, collected from open aortic repair conducted between the years 2009 and 2016, as well as tissue samples of nonaneurysmal abdominal aorta (n=13), obtained from solid organ transplant surgeries performed in 2014, were immersed immediately in RNA stabilization solution and then separated into intima/medial and adventitial layers. Microarray-based gene expression analyses of these samples revealed overexpression of both CD40 and CD40L in the thrombus-free and thrombus-covered AAA aortic media compared with control aortic media (Figure 1). In mice, CD40L expression was also increased in the abdominal aorta when comparing *Apoe*^{-/-} mice who did not receive AngII infusion to mice receiving AngII for 7 days (relative

expression±SEM 0 versus 7 versus 28 days Ang II=0 days: 1.00±0.37 versus 7 days: 2.12±0.15 versus 28 days: 1.04±0.60).

CD40L Deficiency Protects Against AngII-Induced Dissecting Aneurysm Formation in *Apoe*^{-/-} Mice

After 28 days of AngII infusion, the incidence of mature aneurysms (characterized by extensive dilatation±dissection and/or hemorrhage) was only 17% in *Cd40l*^{-/-}*Apoe*^{-/-} mice compared with 67% in *Apoe*^{-/-} mice (Figure 2A). Overall survival was not significantly different between groups (Figure 2B). The protective effect of CD40L deficiency was reflected in a 52% smaller total vessel area in *Cd40l*^{-/-}*Apoe*^{-/-} mice (Figure 2C and 2D). However, once a dissecting aneurysm had developed in *Cd40l*^{-/-}*Apoe*^{-/-} mice, no differences in aneurysm area were found compared with *Apoe*^{-/-} mice that had developed a dissecting aneurysm (Figure 2E). No changes in body weight or plasma cholesterol concentrations were observed (Figure I in the [online-only Data Supplement](#)).

After 28 days of AngII infusion, lack of CD40L led to significantly lower abundance of CD45⁺, CD4⁺, and Mac3⁺ leukocytes in the abdominal vascular wall, whereas the number of B220⁺ B cells and CD8⁺ T cells was not significantly affected (Figure 2F–2L). Moreover, abdominal aortas of AngII-treated *Cd40l*^{-/-}*Apoe*^{-/-} mice exhibited reduced transcription of the chemokines CCL2 and CCL3 (Figure 3A) and the cytokine interleukin-12 with a trend toward lower IFN γ (*P*=0.05; Figure 3B) when compared with AngII-treated *Apoe*^{-/-} mice. CD40 transcript levels did not change while CD40L levels could barely be detected in abdominal aortas of *Cd40l*^{-/-}*Apoe*^{-/-} mice (Figure 3C and 3D).

Furthermore, when *Cd40l*^{-/-}*Apoe*^{-/-} bone marrow was transplanted into lethally irradiated *Apoe*^{-/-} mice, the incidence of dissecting aneurysm formation in *Cd40l*^{-/-}*Apoe*^{-/-} chimeras was significantly reduced (30% in *Cd40l*^{-/-}*Apoe*^{-/-} chimeras versus 63% in *Apoe*^{-/-} chimeras). In addition,

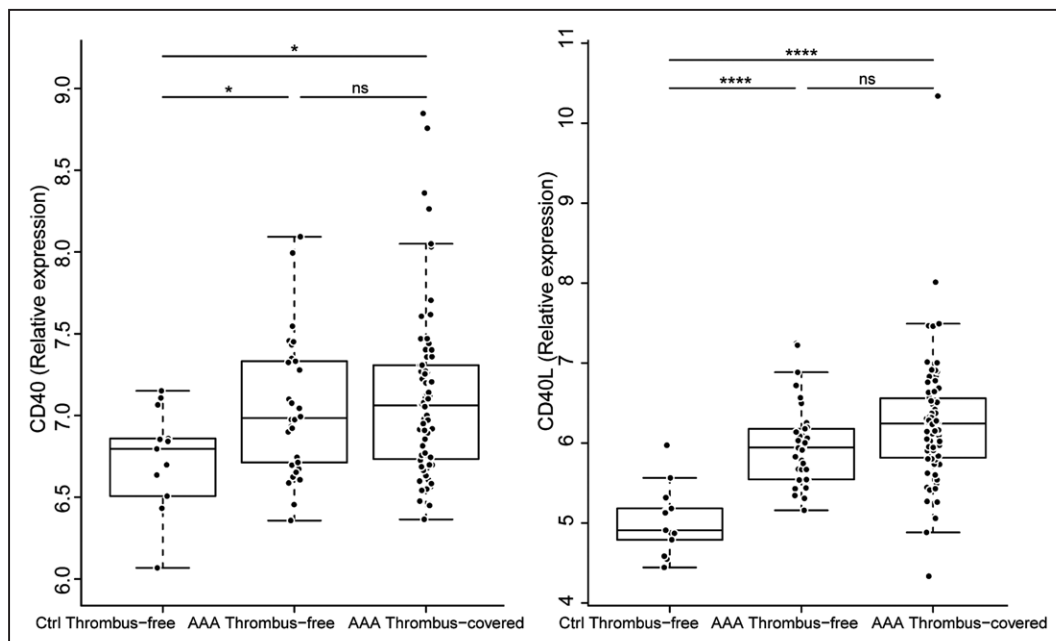


Figure 1. CD40L and CD40 are overexpressed in human aortic abdominal aneurysm (AAA) media. Microarray on CD40 and CD40L, thrombus-free (n=34) and thrombus-covered (n=76) human AAA samples obtained from elective surgery were compared with control thrombus-free samples (n=13). ns indicates nonsignificance. **P*<0.05, *****P*<0.0001.

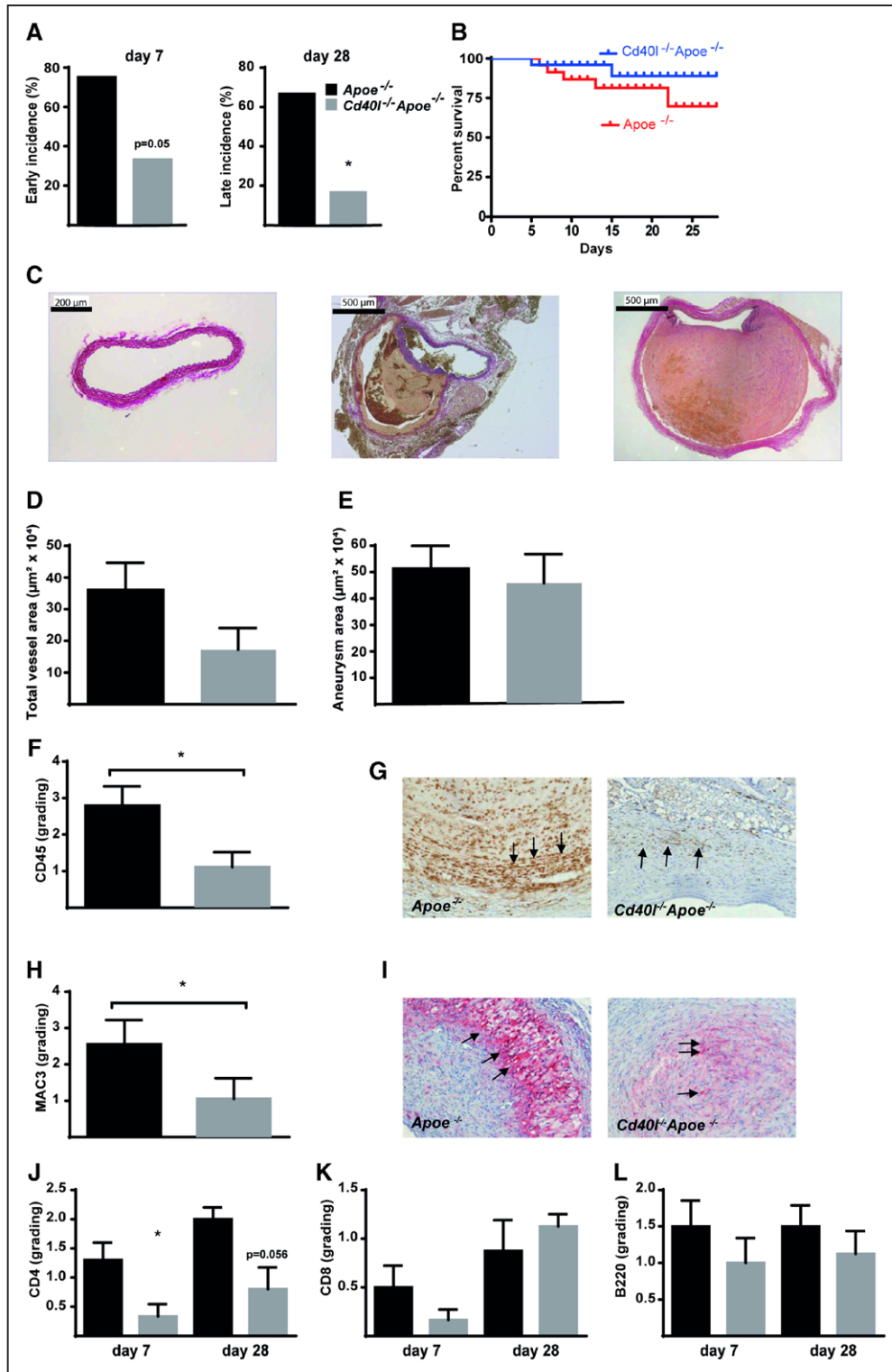


Figure 2. CD40L deficiency reduces dissecting aneurysm formation. Male *Cd40l*^{-/-} apolipoprotein E-deficient (*Apoe*^{-/-}) and *Apoe*^{-/-} mice were infused with angiotensin II (AngII) via minipumps and analyzed after 7 or 28 d. **A**, Incidence of aneurysms at 7 d and at 28 d. **B**, Survival curves. **C**, Representative photomicrographs of Elastin-van-Gieson staining of a normal aorta (left) in a nonresponding *Cd40l*^{-/-}*Apoe*^{-/-} mouse and advanced dissecting aneurysm in an *Apoe*^{-/-} (middle) and *Cd40l*^{-/-}*Apoe*^{-/-} mouse (right) after 28 d of AngII infusion. **D**, Total vessel area and **(E)** aneurysmal area of the abdominal aorta of *Apoe*^{-/-} and *Cd40l*^{-/-}*Apoe*^{-/-} mice. **F–L**, Immunohistochemical analysis of immune cell infiltrate in aneurysmal tissue of *Apoe*^{-/-} and *Cd40l*^{-/-}*Apoe*^{-/-} mice. Semiquantitative analysis of **(F)** CD45⁺ cells with **(G)** representative photomicrographs and **(H)** Mac3⁺ cells with **(I)** representative photomicrographs after 28 d of infusion (arrows indicate areas of positive staining). Semiquantitative analysis of **(J)** CD4⁺, **(K)** CD8⁺, and **(L)** B220⁺ cells after 7 and 28 d of infusion. **P*<0.05; scale bar depicted in μm.

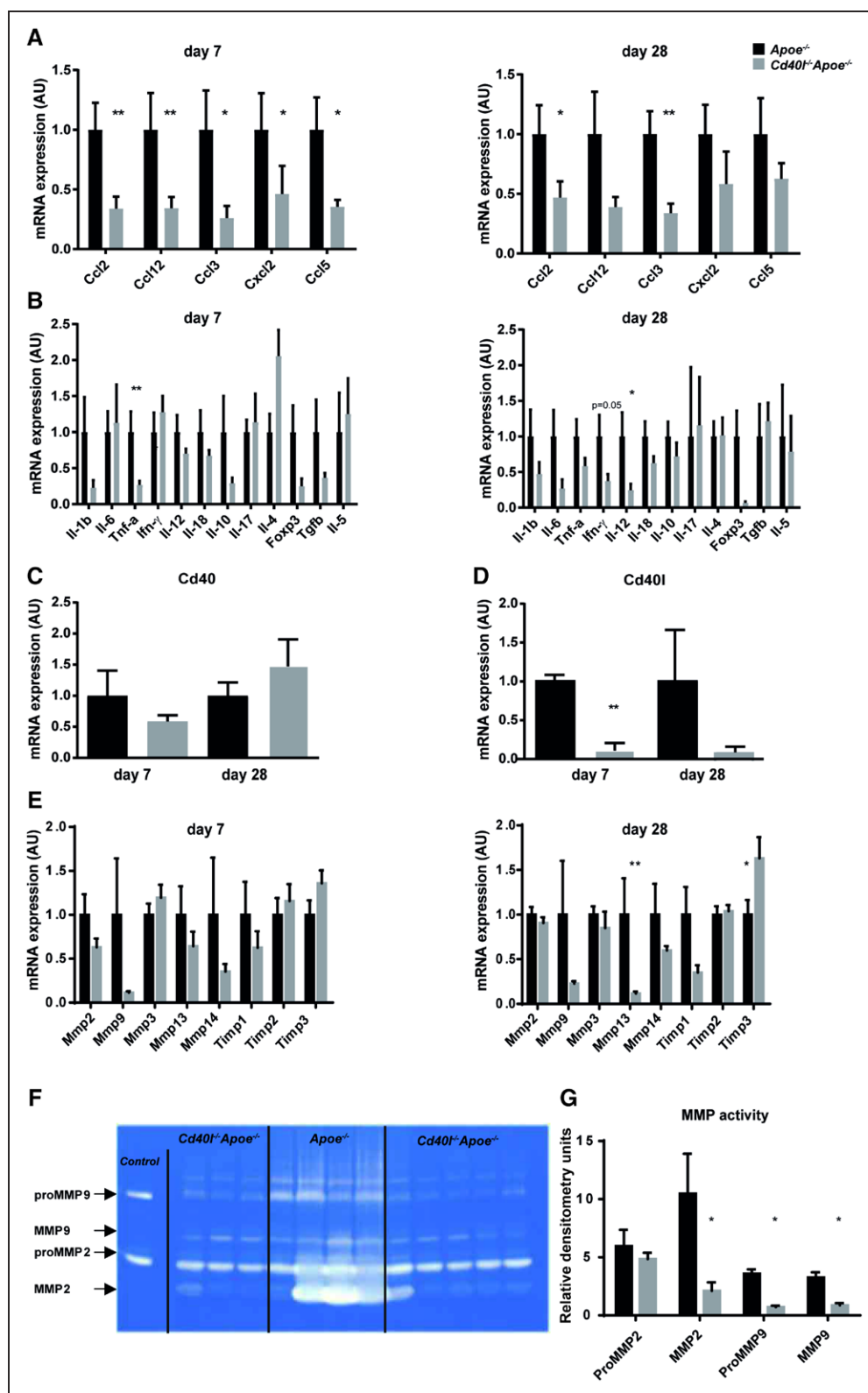


Figure 3. CD40L deficiency limits inflammation and proteolytic activity in dissecting aneurysms. Male *Cd40l*^{-/-} apolipoprotein E-deficient (*Apoe*^{-/-}) and *Apoe*^{-/-} mice were infused with angiotensin II-releasing minipumps and analyzed after 7 or 28 d. Quantitative polymerase chain reaction (Q-PCR) of chemokines after (A) 7 and 28 d of infusion, (B) cytokines after 7 and 28 d of infusion. C and D, Q-PCR of CD40 and CD40L levels. E, Q-PCR of matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) after 7 or 28 d. F and G, Quantification of MMP activity by gelatin zymography. Vascular smooth muscle cells, incubated with TNF (tumor necrosis factor) α and platelet-derived growth factor, were used as positive control. Samples were abdominal aortic tissue from *Apoe*^{-/-} and *Cd40l*^{-/-}*Apoe*^{-/-} mice (d28). F, Representative zymogram and (G) quantification of MMP activity of *Apoe*^{-/-} and *Cd40l*^{-/-}*Apoe*^{-/-} mice (**P*<0.05; ***P*<0.01). AU indicates arbitrary units.

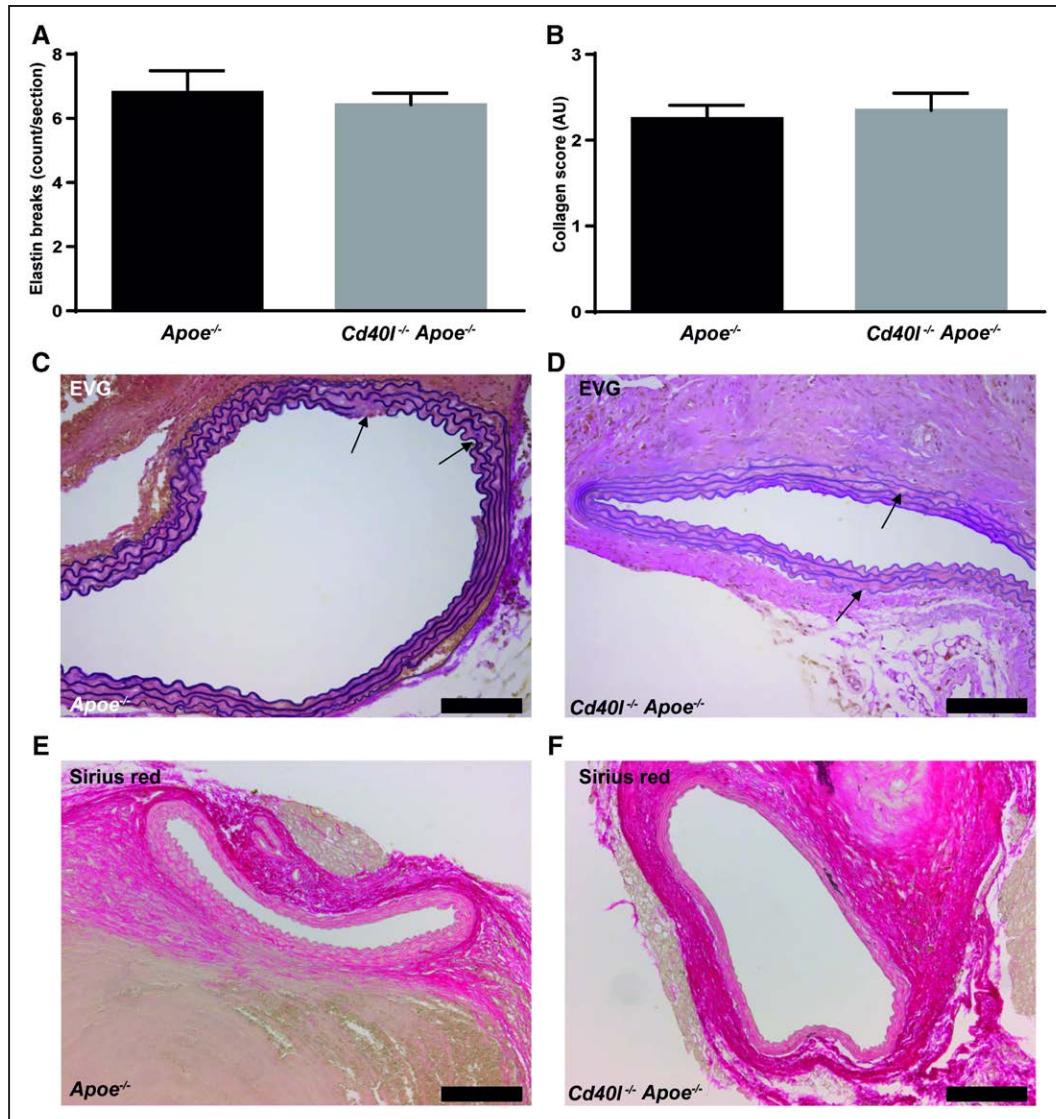


Figure 4. CD40L deficiency does not affect incidence of elastin breaks or collagen abundance. Male *Cd40l*^{-/-} apolipoprotein E-deficient (*Apoe*^{-/-}) and *Apoe*^{-/-} mice were infused with angiotensin II via minipumps and analyzed after 28 d. **A**, Mean incidence of elastin breaks on Elastica von Gieson (EVG) staining per section in aneurysms. **B**, Semiquantitative analysis of collagen content on Sirius red staining in *Apoe*^{-/-} and *Cd40l*^{-/-} *Apoe*^{-/-} mice after 28 d. Representative images of aneurysms from **(C)** *Apoe*^{-/-} mice and **(D)** *Cd40l*^{-/-} *Apoe*^{-/-} mice depicting elastin breaks (indicated by arrows; scale bar=100 μm) and from Sirius red staining in **(E)** *Apoe*^{-/-} and **(F)** *Cd40l*^{-/-} *Apoe*^{-/-} mice (scale bar=200 μm).

Cd40l^{-/-} *Apoe*^{-/-} chimeras were protected from death by aneurysm rupture indicating a strong contribution of hematopoietic CD40L in the pathogenesis of aneurysm formation (Figure IIIA and IIIB in the [online-only Data Supplement](#)).

CD40L Deficiency Reduces Proteolytic Activity

Paralleling the decrease in inflammation, absence of CD40L significantly reduced indicators of proteolytic activity. After 28 days of AngII infusion, MMP-13 mRNA levels were decreased, whereas tissue inhibitor of metalloproteinase-3 mRNA abundance increased in the abdominal aorta of *Cd40l*^{-/-} *Apoe*^{-/-} mice compared with *Apoe*^{-/-} controls (Figure 3E). Moreover, gelatin zymography revealed a significant decrease in both MMP-2 and MMP-9 activity, as well as pro-MMP-9

accumulation in abdominal aortas of *Cd40l*^{-/-} *Apoe*^{-/-} mice, suggesting an intrinsic protection against dissecting aneurysm formation in the arterial wall of *Cd40l*^{-/-} *Apoe*^{-/-} mice (Figure 3F and 3G). No differences in the incidence of elastin breaks or the amount of collagen in the media of the abdominal aorta were observed between groups (Figure 4A–4F).

CD40L Deficiency Is Protective in Early Aneurysm Development

To identify processes preceding mature aneurysm formation, mice were analyzed after only 1 week of AngII infusion. Initial signs of aneurysm formation (visible dilatation, vascular hematomas) were present in a large portion of *Apoe*^{-/-} mice after 7 days of infusions, with a lower incidence in the

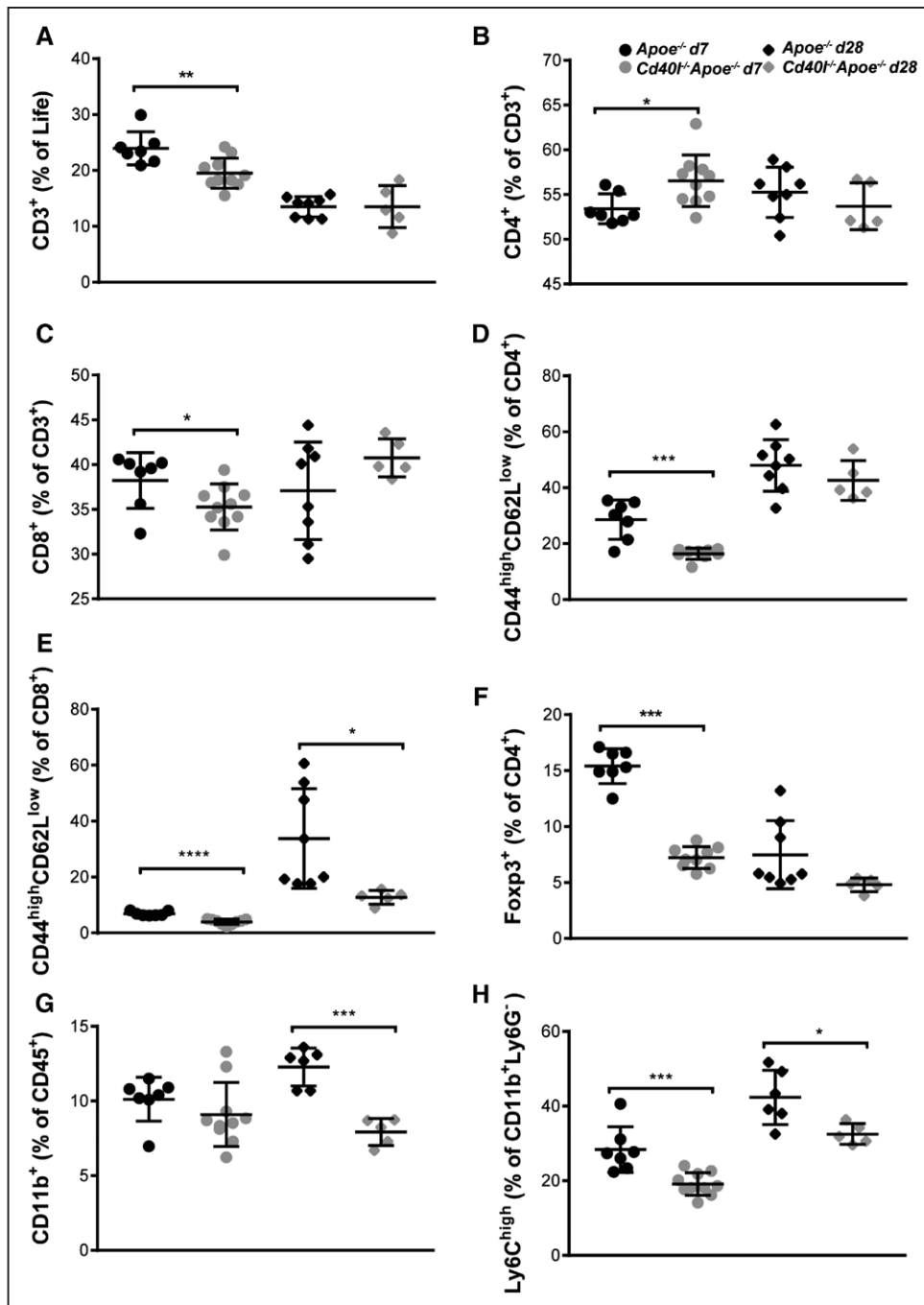


Figure 5. Reduced effector T cells and Foxp3⁺ regulatory T cells after 7 d. Flow cytometric analysis of spleens from apolipoprotein E-deficient (*Apoe*^{-/-}) and *Cd40l*^{-/-}*Apoe*^{-/-} mice after 7 or 28 d of infusion. **A**, CD3⁺ cells, **B**) CD4⁺ T helper cells, **C**) CD8⁺ cytotoxic T cells, **D**) effector CD4⁺ T cells, **E**) effector CD8⁺ cells, **F**) Foxp3⁺ regulatory T cells, **G**) CD11b⁺ cells, and **H**) Ly6C^{high} monocytes. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

Cd40l^{-/-}*Apoe*^{-/-} group (*P*=0.05; Figure 2A). Furthermore, CD40L deficiency reduced the abundance of inflammatory cells, in particular of CD4⁺ T cells, in the abdominal aorta, whereas the accumulation of B220⁺ B cells was not affected (Figure 2J–2L). Moreover, in absence of CD40L, abdominal aortas showed a decrease in CCL2, CCL12, CCL3, CXCL2 (chemokine [C-X-C motif] ligand 2), and CCL5, and TNF α transcript abundance suggesting that absence of CD40L ameliorates inflammatory responses in the initial stages of aneurysm formation (Figure 3A and 3B). Accordingly, flow

cytometric analysis of spleen and blood revealed a decrease in CD11b⁺, Ly6G⁺, Ly6C^{high} cells as well as CD4⁺ and CD8⁺ effector cells in *Cd40l*^{-/-}*Apoe*^{-/-} mice (Figure 5; Figure III in the [online-only Data Supplement](#)).

The fraction of CD4⁺FoxP3⁺ regulatory T cells was also decreased (Figure 5F). However, this can be explained by a negative effect of inhibiting CD40-CD40L on regulatory T cells via interleukin-2-mediated decreased regulatory T cell expansion and survival after dendritic cell encounter as described previously.²⁰

Discussion

Here, we show that CD40L deficiency strongly reduces the incidence of dissecting aneurysms by limiting inflammation and extracellular matrix degradation in the arterial wall. However, once a dissecting aneurysm develops, CD40L deficiency cannot prevent the progression as shown by similar sizes of developed aneurysms.

We found that CD40L deficiency especially dampened Th1 (T helper 1)-related cytokines in aneurysms and that production of these cytokines via CD40-CD40L signaling is imperative in dissecting aneurysm formation. This observation is in agreement with an AAA-eliciting role of the hallmark Th1 cytokines IFN γ and TNF α as reported before.^{21,22} However, there is still debate and contradiction whether AAA is promoted by Th1-, Th2-, or Th17-dominated cytokine responses, and multiple reports have conflicting outcomes.^{4,6,23} Xiong et al²² showed a pivotal role for TNF α in calcium chloride-induced AAAs, whereas Shimizu et al²⁴ showed that allograft AAAs were increased in IFN γ -deficient mice and harbored a Th2-skewed cytokine environment. Zhou et al²⁵ found a pathogenic role for IFN γ -producing CD8⁺ T cells and found that IFN γ antagonism prevents disease in wild-type mice in an elastase-infusion model. However, King et al²⁶ observed greatly increased aneurysm severity in AngII-induced dissecting aneurysms in IFN γ ^{-/-} mice. Yet, in mice lacking CD40L, we observed a decrease in TNF α at day 7, as well as IFN γ , and interleukin-12 28 days postaneurysm initiation, respectively. These findings are in accordance with previous studies underlining the importance of Th1 cytokines and reflect the complex role of cytokines in aneurysm pathophysiology. Indeed, different cytokines, chemokines, and proteases seem affected by CD40L at different time points in the disease process. The significant decrease in the inflammatory subsets of T cells and monocytes in *Cd40l*^{-/-}*Apoe*^{-/-} mice, combined with the aforementioned decrease in proinflammatory cytokines and chemokines, points toward a generalized reduction in the inflammatory status of these mice. This is in accordance with the strong proinflammatory effects of CD40-CD40L signaling in other disease models.^{11,17}

Another cytokine/growth factor imperative for AAA development is TGF β (transforming growth factor beta).²⁷ However, the specific role is controversial. Notably, TGF β abundance is increased in AAA vascular tissue, both in syndromic diseases, such as Marfan, as well as nonsyndromic disease.²⁸ TGF β activity was recently ascribed a protective function against AAA formation, in particular via a key component of the canonical TGF β signaling pathway, SMAD3.^{7,29} We observed a modest decrease in TGF β levels after 7 days but no change after 28 days in CD40L deficiency, suggesting that CD40L mostly drives inflammation during disease initiation. Discrepancies in study results may derive from different models used to induce AAA with diverging pathophysiological mechanisms.³⁰

Modulation of MMP activity and other proteolytic mediators is an important mechanism in reducing the incidence of AAA.³¹ Mice deficient in MMP-2, MMP-3, and -9 displayed fewer AAAs.^{32,33} CD40L is a potent inducer of MMP activation.¹¹ Accordingly, CD40L deficiency resulted in decreased MMP-2 and -9 activity and MMP-13 mRNA abundance in AAA tissue. Interestingly, we found that the effects on

inflammation preceded effects on proteolysis as shown in the difference between findings after 7 and 28 days. This indicates an early effect of CD40L on inflammation in the formation of dissecting aneurysms while at later time points, proteolysis and extracellular matrix degradation seem affected by CD40L in AngII-induced dissecting aneurysms.

Although this study is the first to examine the effects of CD40-CD40L signaling on aneurysm formation in a gene-deficient model, previous studies have investigated CD40-CD40L as a potential biomarker or therapeutic target. The antiplatelet drug ticagrelor, which inhibits CD40-CD40L, among other functions, when used on tissue from human AAA patients, inhibits MMP-2 but not MMP-9.³⁴ Interestingly, an anti-CD40L monoclonal antibody also only selectively blocked MMP-2.³⁴ In the present study, we found a significant decrease of only MMP-13 mRNA levels although we noticed decreased activity of MMP-2 and (pro)MMP-9 by gelatin zymography.

Thus far, no correlation of (s)CD40L levels with AAA incidence have been reported.^{35,36} However, concentrations of sCD40L, mainly secreted by platelets, are not necessarily related to local CD40-CD40L activity nor rule out a function of CD40-CD40L signaling in disease pathology, as also seen by conflicting data on sCD40L as a biomarker for cardiovascular disease, despite a proven role of CD40-CD40L in atherosclerosis.¹⁷ Furthermore, when measured inside the luminal thrombus layer from patients with AAA in a separate study, sCD40L levels were increased compared with other layers.³⁷ In addition, plasma levels of sCD40L were increased in patients compared with controls underlining a potential role for CD40L in human disease and reflecting the results of our study.³⁷ We now show that indeed, transcript levels of CD40 and CD40L are increased in the arterial media of human thrombus-free or thrombus-covered AAA compared with nonaneurysmal control vessels.

Lack of CD40L mitigated dissecting aneurysmal disease both in a full gene-deficient as well as in a bone marrow transplant model, suggesting that CD40L on hematopoietic cells is a major contributor to the pathogenesis of dissecting aneurysm formation. However, a role of CD40L on nonhematopoietic cells cannot be ruled out. Moreover, as of now, it is unclear on which cell types CD40L exerts its effects or which signaling pathways are responsible for these effects of CD40L. Further studies using cell-specific gene-deficient models and inhibition of specific parts of the CD40-CD40L signaling cascade will further elucidate the function of this potent costimulatory molecule pair in vascular disease, including AAA, and potentially direct new therapeutic strategies.

To date, therapeutic options preventing growth or rupture of AAA are limited, and surgical interventions remain challenging, especially in the elderly. Therefore, novel pharmacological strategies are an unmet medical need in patients. Our data reveal that blocking CD40L protects the arterial wall from dissecting aneurysm formation by reducing the proteolytic profile, preceded by reduced inflammation. This protection is associated with lower risk of rupture. Thus, therapies inhibiting CD40L or downstream targets in the CD40-CD40L pathway may pose an attractive option for the stabilization of AAAs.

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Disclosures

None.

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Highlights

- The high morbidity and mortality associated with abdominal aortic aneurysm and the lack of efficient pharmacological intervention strategies emphasize the need to invest in novel therapies.
- CD40L is a key component in dissecting aneurysm pathogenesis by promoting (Th1 driven) inflammation and extracellular matrix degradation.
- Inhibition of CD40L is an attractive novel drug target for the stabilization of abdominal aortic aneurysm.