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Author: Wezel, Anouk

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Chapter 4

Complement factor C5a induces atherosclerotic plaque disruptions

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Anouk Wezel^{1,2}

Margreet R. de Vries^{2,3}

H. Maxime Lagraauw¹

Amanda C. Foks¹

Johan Kuiper¹

Paul H.A. Quax^{2,3}

Ilze Bot^{1,2}

¹ Division of Biopharmaceutics, Gorlaeus Laboratories, Leiden Academic Center for Drug Research, Leiden University, Leiden, The Netherlands

² Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands

³ Einthoven Laboratory for Experimental Vascular Medicine, Leiden, The Netherlands

Abstract

Rationale: Complement factor C5a and its receptor C5aR are expressed in vulnerable atherosclerotic plaques; however, a causal relation between C5a and plaque rupture has not been established yet.

Methods and Results: Accelerated atherosclerosis was induced by placing vein grafts in male apoE^{-/-} mice. After 24 days, when advanced plaques had developed, C5a or PBS was applied locally at the lesion site in a pluronic gel. Three days later mice were sacrificed in order to examine the acute effect of C5a on late stage atherosclerosis. A significant increase of C5aR in the plaque was detectable in mice treated with C5a. Lesion size and plaque morphology did not differ between treatment groups, but interestingly, local treatment with C5a resulted in a striking increase in the amount of plaque disruptions with concomitant intraplaque hemorrhage. To identify the potential underlying mechanisms, smooth muscle cells and endothelial cells were treated *in vitro* with C5a. Both cell types revealed a marked increase in apoptosis after stimulation with C5a, which may contribute to lesion instability *in vivo*. Indeed, apoptosis within the plaque was seen to be significantly increased after C5a treatment.

Conclusions: We here demonstrate a causal role for C5a in atherosclerotic plaque disruptions, probably by inducing apoptosis. Therefore, intervention in complement factor C5a signalling may be a promising target in the prevention of acute atherosclerotic complications.

Introduction

Rupture of an atherosclerotic plaque is the main cause of myocardial infarction or stroke, which are still the leading causes of death worldwide^{1,2}. Features of an unstable plaque, prone to rupture, are a large lipid core covered by a thin fibrous cap depleted of smooth muscle cells. The exact mechanism of progression from an asymptomatic stable lesion to a vulnerable plaque with subsequent rupture is still incompletely understood, but apoptosis of smooth muscle cells and endothelial cells is thought to be crucial³⁻⁵. In search for new therapeutic strategies to reduce the incidence of plaque rupture, attention turned to the complement system, in particular to complement factor C5a.

The complement system, consisting of approximately 30 proteins, has been suggested to play an important role in cardiovascular diseases, amongst which atherosclerosis^{6,7}. The anaphylatoxin C5a, a split product of C5, is constantly produced during complement activation. C5a is one of the most potent inflammatory chemoattractants and promotes leukocyte recruitment to sites of inflammation⁸⁻¹¹. Furthermore, C5a has the ability to activate endothelium and induce the expression and release of numerous cytokines and chemokines such as CCL2^{12,13}. Various cell types, such as macrophages, endothelial cells, smooth muscle cells and mast cells, express receptors for C5a. Previously, we have shown that vein graft disease is aggravated by C5a as a result from attracting and activating mast cells¹⁴. Others have demonstrated that inhibition of C5aR signalling reduces atherosclerosis and neointima formation in apoE^{-/-} mice^{15,16}. Due to its pronounced effects on a number of cell types within the atherosclerotic plaque, C5a may also be one of the key components in plaque destabilization and acute plaque rupture. Previously, it has been demonstrated that elevated levels of C5a are associated with increased cardiovascular risk in patients with advanced atherosclerosis¹⁷. However, since that study was mere observational, a direct causal role for C5a could not be assigned.

A major drawback in preclinical research aiming at the identification of factors involved in acute cardiovascular syndromes is the lack of suitable atherosclerotic mouse models that resemble the complex human atherosclerotic plaque morphology with concomitant plaque rupture. Interestingly, we have recently established that lesions induced in a murine vein graft model are more complex and display both plaque disruptions and intraplaque hemorrhages¹⁸, which are important features of acute atherosclerotic plaque destabilization in humans. These findings render this an excellent model to study late stage atherosclerosis and acute complications.

In the present study we thus aimed to investigate the acute effect of complement factor C5a on late stage atherosclerosis and concomitant plaque complications in this mouse model.

Material and Methods

Mice

This study was performed in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. All animal experiments were approved by the animal welfare committee of the Leiden University Medical Center (approval reference number 10091). Male apoE^{-/-} mice (PBS/PBS treated mice: n=8; C5a/PBS treated mice: n=9; C5a/cromolyn treated mice: n=7), were obtained from the Gorlaeus Laboratory animal breeding facility (Leiden, The Netherlands) and were given water and chow *ad libitum*. Plasma total cholesterol levels were measured by enzymatic procedures using precipath standardized serum as an internal standard (Boehringer Mannheim, Germany).

Surgical intervention

Before surgery, gel placement and sacrifice, mice were anesthetized by an intra-peritoneal injection with midazolam (5 mg/kg, Roche, Woerden, The Netherlands), Domitor (0.5 mg/kg, AST Farma, Oudewater, The Netherlands) and fentanyl (0.05 mg/kg, Janssen, Beerse, Belgium). After surgery mice were antagonized with a subcutaneous injection of flumazenil (0.5 mg/kg, Fresenius Kabi, Schelle, Belgium), Antisedan (2.5 mg/kg, AST farma) and buprenorphine (0.1 mg/kg, MSD animal Health, Boxmeer, The Netherlands). Vein graft surgery was performed in order to induce advanced atherosclerotic lesions. In brief, the carotid artery of the recipient mice was cut in the middle; both ends of the artery were everted around cuffs and ligated with 8.0 sutures. The vena cava from donor littermates was harvested and sleeved over the cuffs. By placing the vein in the arterial circulation with high blood pressure, endothelial damage was induced with subsequent accelerated atherosclerosis. After 24 days when advanced lesions were present, age and weight matched mice were anesthetized. A F-127 pluronic gel (25% w/v) was placed in the surrounding tissue of the vein graft (the perivascular tissue) completely surrounding the vessel, enabling us to investigate the local effect rather than the systemic effect of our compound. The gel contained either C5a (5 µg, Hycult Biotech, Uden, The Netherlands) as has been previously described¹⁴, or PBS.

As we have previously established that C5a may exert its effects on atherosclerosis via activation of mast cells¹⁴, we have treated a subgroup of C5a challenged mice with the mast cell stabilizer cromolyn in order to identify mast cell dependent effects. Thus, from gel placement until sacrifice at day 28, mice received daily intra-peritoneal injections with a commonly used mast cell stabilizer cromolyn (50 mg/kg/day, Sigma-Aldrich, Zwijndrecht, The Netherlands)^{19,20} or PBS.

Histological and immunohistochemical analysis

Cross-sections of paraffin embedded vein grafts (5 µm thick) were stained with hematoxylin-phloxine saffron (HPS) for measurement of lesion size, fibrin content and plaque dissection analysis. Fibrin content was graded by two blinded independent investigators on a scale from 0 to 3, with 0 representing no fibrin and 3 representing severe transmural fibrin depositions. Collagen content was visualized with a picrosirius red staining. Mast cell and neutrophil staining was performed using an enzymatic chloroacetate esterase kit (Sigma-Aldrich); when granules were apparent in the vicinity of the mast cell they were scored as activated. Neutrophils were stained light pink, while the segmented nuclei were visualized by Gill's hematoxylin²¹. Composition of the atherosclerotic lesion was further evaluated by immunohistochemical stainings for macrophages (MAC3, 1:200, BD-Pharmingen, San Diego, CA, USA), smooth muscle cell actin (1:1000; Sigma-Aldrich); C5a (1:400; Hycult Biotechnology) and C5aR (1:400; SantaCruz, Dallas, Texas, USA). A TUNEL staining was performed according to manufacturer's protocol in order to detect apoptotic cells in the atherosclerotic plaque (*in situ* cell death detection kit, POD, Roche). The total amount of cells in the vessel wall area, as well as the amount of apoptotic cells, was counted manually, after which the percentage of apoptosis was calculated. All staining measurements were performed on six consecutive

cross-sections of the vein grafts, approximately 150 μm spaced, in a blinded manner by a single observer. Plaque dissection analysis was determined over a total vein graft length of 1800 mm. The disruptions were defined as a connection or fissure between the lumen and part of the vessel wall underneath the adventitia, filled with fibrin and erythrocytes. Quantification of the lesion area and immunostained positive area were performed using computer assisted software (Qwin, Leica, Cambridge, UK). In brief, the total intimal area was measured, as well as the stained area. The stained area was then calculated as a percentage of the total intimal area.

Cell culture

To generate bone marrow derived macrophages (BMDMs), cells were isolated from bone marrow of C57Bl/6 mice and cultured for 7 days in RPMI medium supplemented with 20% fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin (all from PAA, Colbe, Germany) and 30% L929 cell-conditioned medium (as the source of macrophage colony-stimulating factor (M-CSF)) as has been described previously²²⁻²⁴. Primary cultured murine smooth muscle cells (vSMC)²⁵ and a murine cell line for endothelial cells H5V²⁶ were cultured in DMEM medium supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin.

Collagen synthesis assay

To measure collagen production by vSMC, cells were seeded at a density of 0.2×10^6 cells per well. Control medium or medium containing 0.2 nM, 2 nM or 20 nM C5a was added after attachment of the cells. Also, 1 μCi [³H]Proline (Perkin Elmer, Groningen, The Netherlands) together with 50 $\mu\text{g}/\text{mL}$ ascorbic acid was added and incubated overnight at 37°C. Cells were taken up in 20 mM Tris HCl/0.36 mM CaCl₂ (pH=7.6) and sonicated for 2 minutes. Collagen was degraded by incubation with 100 U/mL collagenase for 2 hours at 37°C, after which samples were centrifuged for 15 minutes at maximum speed. Proteins were precipitated for 30 minutes on ice using 50% trichloroacetic acid, after which [³H]Proline content in the supernatant as a measure for collagen production was quantified in a liquid scintillation analyzer (Packard 1500 Tricarb, Downers Grove, IL, USA). Protein content was measured using a standard BCA protein assay.

Macrophage activation

BMDMs were plated in triplicate at a density of 0.5×10^6 cells/mL. C5a was added in a concentration range of 0.2 nM, 2 nM, 20 nM or 200 nM and incubated overnight at 37°C. In order to investigate the effect of C5a on cytokine release, IL-6 and MCP-1 were measured by means of ELISA (BD Bioscience, San Diego, CA, USA). Cells were lysed for RNA isolation.

RNA isolation, cDNA synthesis and qPCR

Guanidine thiocyanate (GTC) was used to extract total RNA from BMDMs²⁷. RNA was reverse transcribed by M-MuLV reverse transcriptase (RevertAid, MBI Fermentas, Leon-Roth) and used for quantitative analysis of mouse genes (Supplemental Table 1) with an ABI PRISM 7700 Taqman apparatus (Applied Biosystems, Foster City, CA, USA). Murine hypoxanthine phosphoribosyltransferase (HPRT) and murine ribosomal protein 27 (RPL27) were used as standard housekeeping genes.

Annexin V staining

Annexin V staining was performed to detect apoptosis. vSMC and H5V were plated in quadruplicate at 0.4×10^6 cells/mL in medium. After attachment of the cells, C5a was added overnight in a concentration range of 2 nM, 20 nM or 200 nM, which is based on previous literature²⁸ or H₂O₂ as a positive control. Cells were washed and stained with fluorochrome-conjugated Annexin V. To distinguish late stage apoptosis

from early apoptosis, cells were subsequently stained with Propidium Iodide Staining Solution. In late stage apoptosis the cell membrane loses its integrity, these cells then become double-positive for Annexin V and Propidium Iodide. Analysis was performed by flow cytometry (FACS CantoII, BD Bioscience, Breda, The Netherlands).

Statistical analysis

Data are expressed as mean \pm SEM. A Fisher's exact test was used to measure the statistical significance between the amounts of plaque dissections. A 2-tailed Student's t-test was used to compare individual groups. Multiple group comparisons were analysed by 2 way ANOVA. Non-parametric data were analyzed using a Mann-Whitney U test. A value of $P < 0.05$ was considered significant.

Results

Lesion size, C5a and C5aR expression

In order to investigate the effect of C5a on the composition of advanced plaques, age- and weight-matched apoE^{-/-} mice with advanced lesions were treated locally with C5a or PBS. No differences were found in plasma cholesterol levels; also, plasma IL-6 concentration did not differ between groups (Supplemental Figure 1). Analysis of atherosclerotic plaques revealed no difference between groups in lesion size, measured as vessel wall area (PBS: $3.9 \pm 0.4 \cdot 10^5 \mu\text{m}^2$; C5a: $3.9 \pm 0.3 \cdot 10^5 \mu\text{m}^2$; C5a/cromolyn: $4.4 \pm 0.4 \cdot 10^5 \mu\text{m}^2$; Figure 1A).

The presence of C5a and C5aR in the atherosclerotic lesions was determined by immunohistochemical stainings. No differences in C5a expression were found between the three groups (PBS: $2.1\% \pm 0.6\%$; C5a: $1.4\% \pm 0.4\%$ $P=0.4$ compared to PBS; C5a/cromolyn: $1.0\% \pm 0.4\%$ $P=0.2$ compared to PBS; Figure 1B). Interestingly, treatment with C5a resulted in a significant increase of C5aR expression in the lesions (PBS: $0.6\% \pm 0.1\%$; C5a: $1.6\% \pm 0.3\%$ $P < 0.01$ compared to PBS; C5a/cromolyn: $1.8\% \pm 0.4\%$ $P < 0.05$ compared to PBS; Figure 1C). Both C5a and C5a receptor staining was distributed heterogeneously within the lesions, suggesting expression by multiple cell types in the intimal layer. In line with literature^{7,8}, we found C5a and C5aR to be expressed by macrophages, endothelial cells and vascular smooth muscle cells (Supplemental Figure 2).

Plaque morphology

Macrophage, smooth muscle cell and collagen content were measured to establish plaque phenotype. C5a treatment did not affect macrophage content as measured by MAC3 staining (PBS: $13.2\% \pm 1.2\%$; C5a: $13.3\% \pm 3.9\%$; C5a/cromolyn: $9.2\% \pm 2.2\%$; Figure 2A). Also, the collagen content (PBS: $21.7\% \pm 3.1\%$; C5a: $19.9\% \pm 2.4\%$; C5a/cromolyn: $18.3\% \pm 2.1\%$; Figure 2B) and the amount of smooth muscle cells (PBS: $29.0\% \pm 3.9\%$; C5a: $36.9\% \pm 2.3\%$; C5a/cromolyn: $31.7\% \pm 4.6\%$; Figure 2C) did not differ between groups.

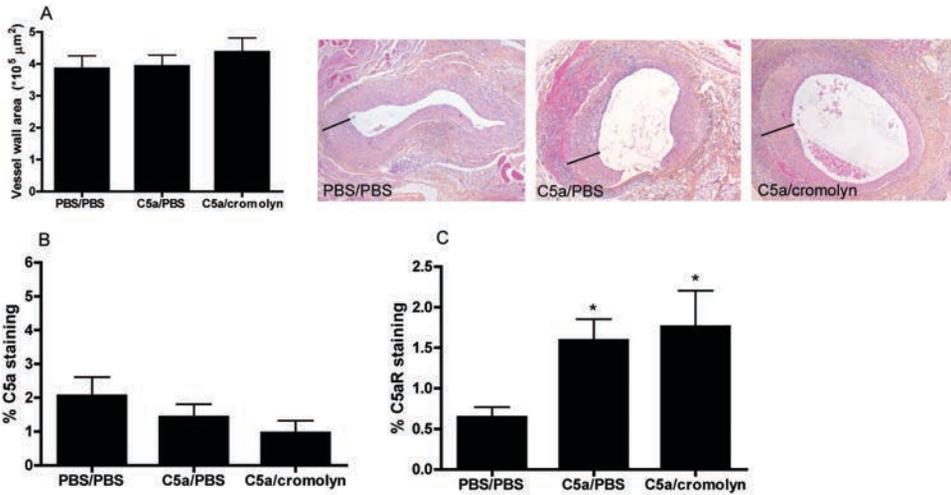


Figure 1. Local application of C5a in late stage atherosclerosis did not result in a change of lesion size (A). Three representative pictures (40x magnifications) show HPS (hematoxylin-phloxine saffron) stained vessel wall segments of all treatment groups, used for measurement of lesion size and plaque rupture analysis. Lines indicate vessel wall thickness. Immunohistochemical staining revealed no changes in C5a expression between treatment groups (B). However, C5a receptor (C5aR) expression was significantly upregulated in mice treated with C5a (C). (PBS/PBS treated mice: $n=8$; C5a/PBS treated mice: $n=9$; C5a/cromolyn treated mice: $n=7$; $*P < 0.05$ compared to PBS/PBS)

Local application of C5a resulted in a trend towards an increased amount of activated mast cells ($P=0.07$; Figure 2E), which was decreased in mice treated with cromolyn. Moreover, the total amount of perivascular mast cells was significantly increased after C5a treatment, which could partly be inhibited by cromolyn (PBS: 0.3 ± 0.1 mast cells/ mm^2 ; C5a: 0.9 ± 0.2 mast cells/ mm^2 ; C5a/cromolyn: 0.6 ± 0.1 mast cells/ mm^2 ; Figure 2D). These findings are in line with our previous data, where we established that local treatment with a specific mast cell activator results in activation of perivascular mast cells and subsequent recruitment of newly infiltrating mast cells into the vessel wall¹⁹.

Neutrophil count in the intima did not differ between the three treatment groups (Figure 2F). Also, no signs of leaky vessels were detected, defined as the presence of erythrocytes outside vascular-like structures in the intima; excluding areas in which erythrocytes were present due to plaque disruptions¹⁸.

Plaque disruptions and fibrin depositions in C5a treated groups

HPS staining was used for further analysis of lesion morphology. Interestingly, local treatment with C5a showed an increase in the amount of plaque disruptions from 13% in the PBS treated group up to 56% in the C5a treated group. Plaque disruptions were defined as the presence of erythrocytes in the lesions with concomitant fibrin depositions. Moreover, additional analysis revealed a significant increase in the length of disruptions after C5a application ($P < 0.05$). Cromolyn treatment did not reduce the number (71%) or length of the disruptions (Figure

3A and 3B). Analysis of fibrin content in the plaque revealed a significant increase after treatment with C5a (Figure 3C; $P < 0.05$). Again, cromolyn application did not reduce this effect, indicating a mast cell independent role of C5a in plaque rupture. To explain these findings, we next performed *in vitro* studies to evaluate the direct effect of C5a on macrophages, smooth muscle cells and endothelial cells.

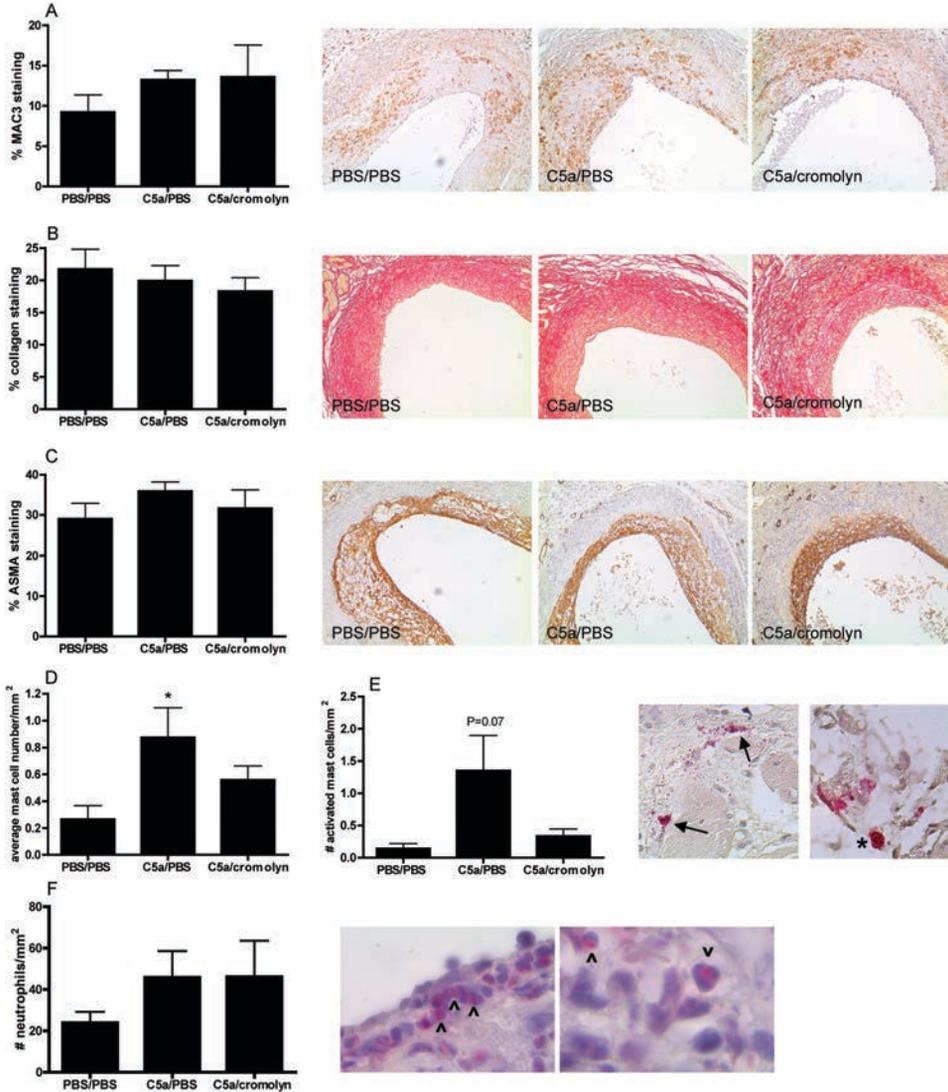


Figure 2. Plaque composition was investigated by staining for macrophages (MAC3, A), collagen (Sirius Red, B) and smooth muscle cells (ASMA, C). Representative pictures (100x magnifications) show immunohistochemical stainings of all treatment groups. Also, the total amount of mast cells in the perivascular tissue was determined (D) as well as the amount of activated mast cells (E). Pictures show activated mast cells (arrows) with granules present in the vicinity of the cell. Also, a resting mast cell (*) is shown with all granules contained inside the cell. Neutrophils in the intima were stained with a naphtol CAE staining (F). Pictures show neutrophils stained light pink with clearly visible segmented nuclei (^). (PBS/PBS treated mice: n=8; C5a/PBS treated mice: n=9; C5a/cromolyn treated mice: n=7; * $P < 0.05$ compared to PBS/PBS).

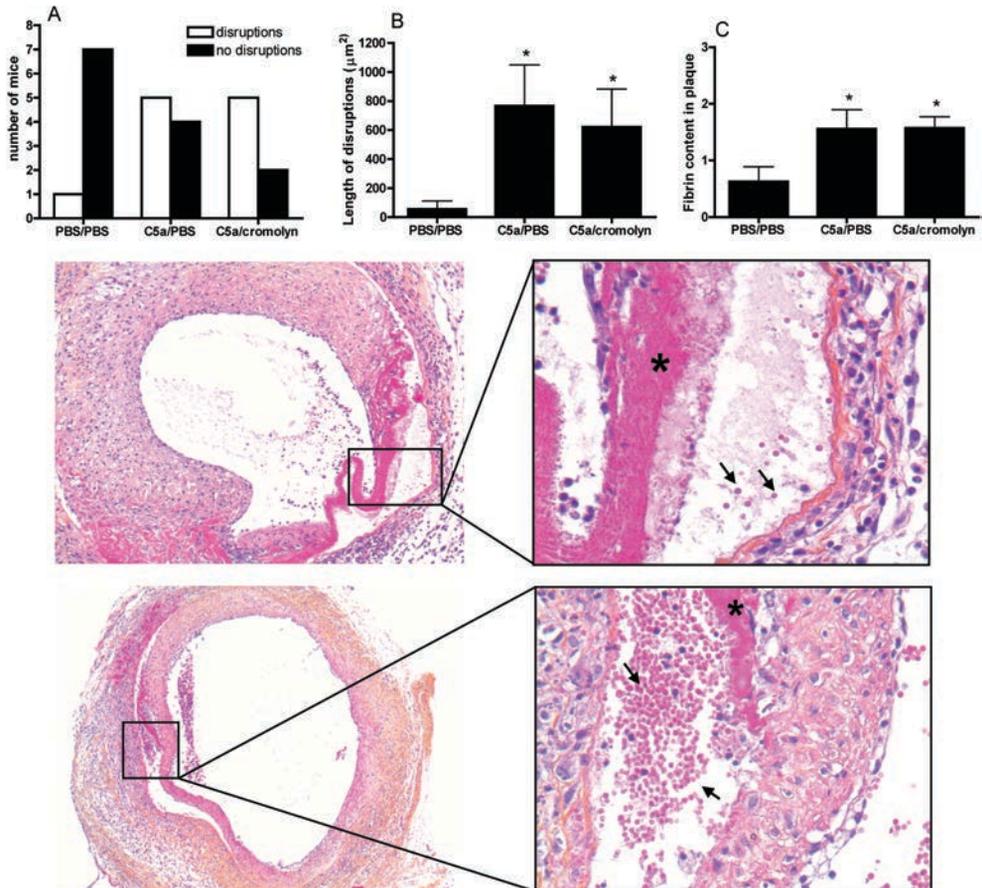


Figure 3. Three days after local application of C5a in late stage atherosclerosis, increased amount of plaque disruptions, as defined by the presence of erythrocytes in the lesions (arrows) with concomitant fibrin depositions, were present in the treated groups (A), while also the length of the disruptions was increased (B). Fibrin content (*) was significantly increased in mice treated with C5a (B). Cromolyn, a common mast cell stabilizer, did not reduce this effect. (PBS/PBS treated mice: $n=8$; C5a/PBS treated mice: $n=9$; C5a/cromolyn treated mice: $n=7$; $*P<0.05$ compared to PBS/PBS).

In vitro activation by C5a

Activation of BMDMs by C5a resulted in an increase of MCP-1 secretion of almost seven fold (200 nM C5a: 888.0 pg/ml; control: 128.8 pg/ml; $P<0.001$). No changes were detected in IL-6 secretion by C5a activated macrophages. Collagen synthesis rate of smooth muscle cells remained unchanged after treatment with C5a (data not shown). Furthermore, the TIMP1/MMP9 ratio, as a marker of the proteolytic status of C5a stimulated macrophages, did not significantly change after C5a stimulation (Figure 4).

Treatment of endothelial cells with a concentration range of C5a (2 nM -200 nM) did not induce changes in the expression of the adhesion molecules V-CAM, I-CAM or PECAM (Supplemental Figure 3).

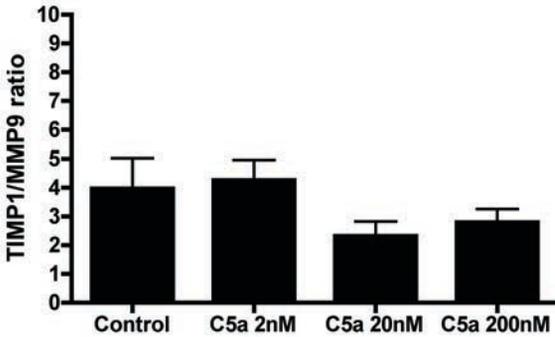


Figure 4. Expression levels of TIMP1/MMP9 ratio in BM derived macrophages showed no significant differences after activation with increasing concentrations of C5a.

In vitro apoptosis

One of the features of plaque instability is smooth muscle cell apoptosis^{3,4}. To investigate the effect of C5a on smooth muscle cells viability, we added C5a in concentrations ranging from 2 nM, 20 nM to 200 nM to cultured smooth muscle cells. Interestingly, we found a clear dose-dependent increase in smooth muscle cell apoptosis after exposure to C5a. Both the percentage of early apoptosis (200 nM C5a: 37.8% ± 0.9%; control: 13.2% ± 3.9%; $P < 0.001$; Figure 5B), as the percentage of late stage apoptosis (200 nM C5a: 34.6% ± 2.3%; control: 3.8% ± 0.8%; $P < 0.0001$; Figure 5D), were significantly increased compared to the negative control. Consequently, the amount of viable cells was drastically decreased after C5a treatment (200 nM C5a: 20.0% ± 2.2%; control: 77.4% ± 5.2%; $P < 0.0001$; Figure 5F).

Since disturbed endothelial lining along the luminal site of the vessel predisposes to plaque erosions and instability as well, we stimulated endothelial cells with the same C5a concentration range. Early apoptosis was significantly increased compared to the negative control (200 nM C5a: 24.3% ± 2.4%; control: 10.3% ± 0.9%; $P < 0.005$; Figure 5A) in a dose-dependent manner. Also, a concomitant trend towards increased late stage apoptosis was detected (200 nM C5a: 24.9% ± 3.9%; control: 15.9% ± 1.6%; $P = 0.07$; Figure 5C). The percentage of viable cells was significantly decreased after treatment with C5a (200 nM C5a: 35.3% ± 4.1%; control: 57.2% ± 3.1%; $P < 0.01$; Figure 5E). Cromolyn did not affect C5a-induced apoptosis of either endothelial cells or smooth muscle cells (data not shown).

To investigate whether apoptosis is induced via apoptotic caspases, we measured mRNA levels of caspase-1, caspase-3 and Bax in endothelial cells after stimulation with 2 nM, 20 nM or 200 nM C5a. We did not detect any changes between caspase-1 or Bax expression, however, we observed a significant dose-dependent increase of caspase-3 expression after C5a stimulation (Supplemental Figure 4), suggesting that C5a mediated apoptosis is mediated via the caspase cascade.

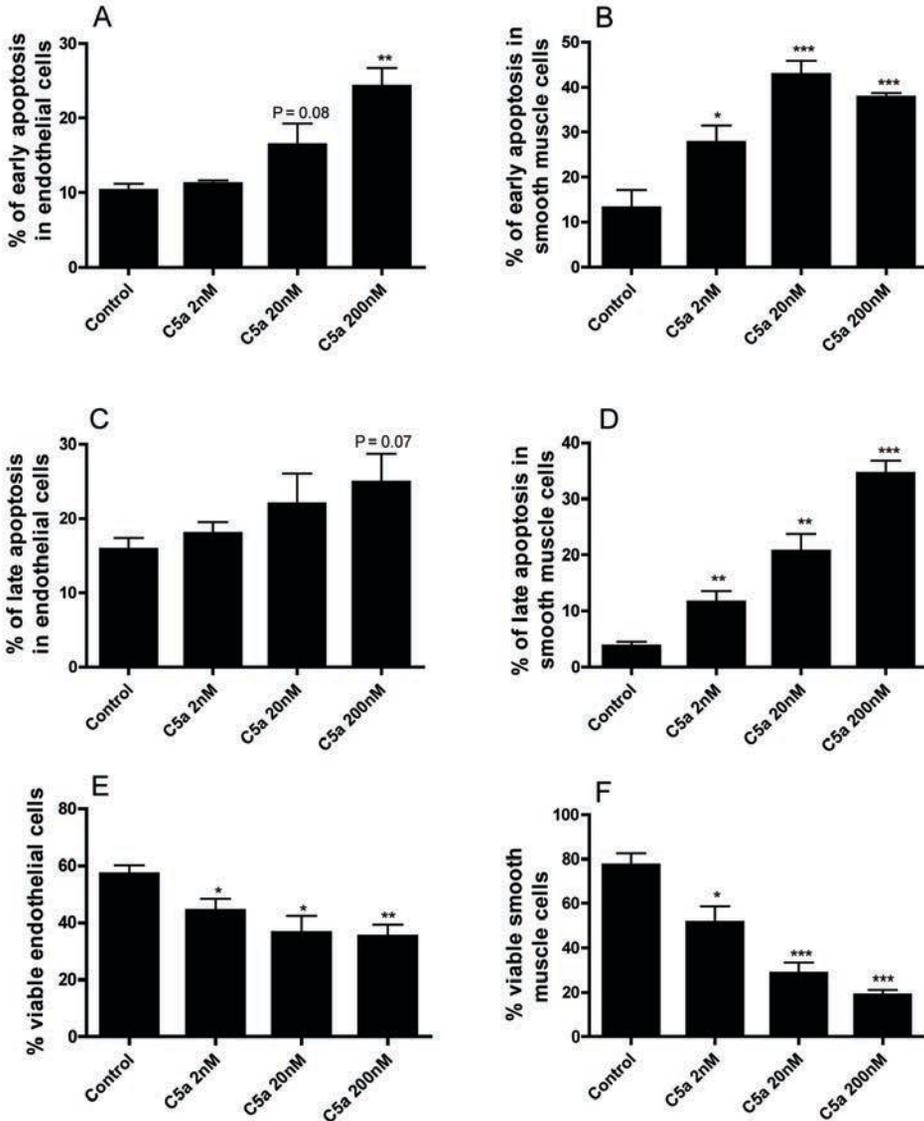


Figure 5. Treatment of smooth muscle cells *in vitro* by C5a 2 nM, C5a 20 nM and C5a 200 nM showed a marked dose-dependent increase of both early (B) and late apoptosis (D). Consequently, the amount of viable smooth muscle cells after C5a stimulation was significantly decreased (F). Also, early apoptosis (A) and late apoptosis (C) of endothelial cells were increased after C5a stimulation. The amount of viable endothelial cells showed a dose-dependent decrease (E). Both endothelial cell and smooth muscle cell apoptosis can contribute to plaque destabilization. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

In vivo apoptosis

In order to confirm our *in vitro* results, we performed a TUNEL staining to detect apoptosis within the atherosclerotic plaque. Local application of C5a resulted in a significant increase in the percentage of apoptotic cells in the vessel wall, thereby

validating our *in vitro* findings (PBS: $2.4\% \pm 0.6\%$ of the total cell population; C5a: $5.9\% \pm 1.4\%$; $P < 0.05$; Figure 6). Cromolyn treatment slightly reduced this effect, however there was still an increase in apoptotic cells apparent (PBS: $2.4\% \pm 0.6\%$; C5a/cromolyn: $4.1\% \pm 0.6\%$; $P = 0.075$ compared to PBS; Figure 6). Apoptotic cells were apparent along the endothelial lining of luminal site of the vessel, as well as in smooth muscle cell rich areas. These data identify the induction of apoptosis as a potential mechanism via which C5a can induce plaque disruptions.

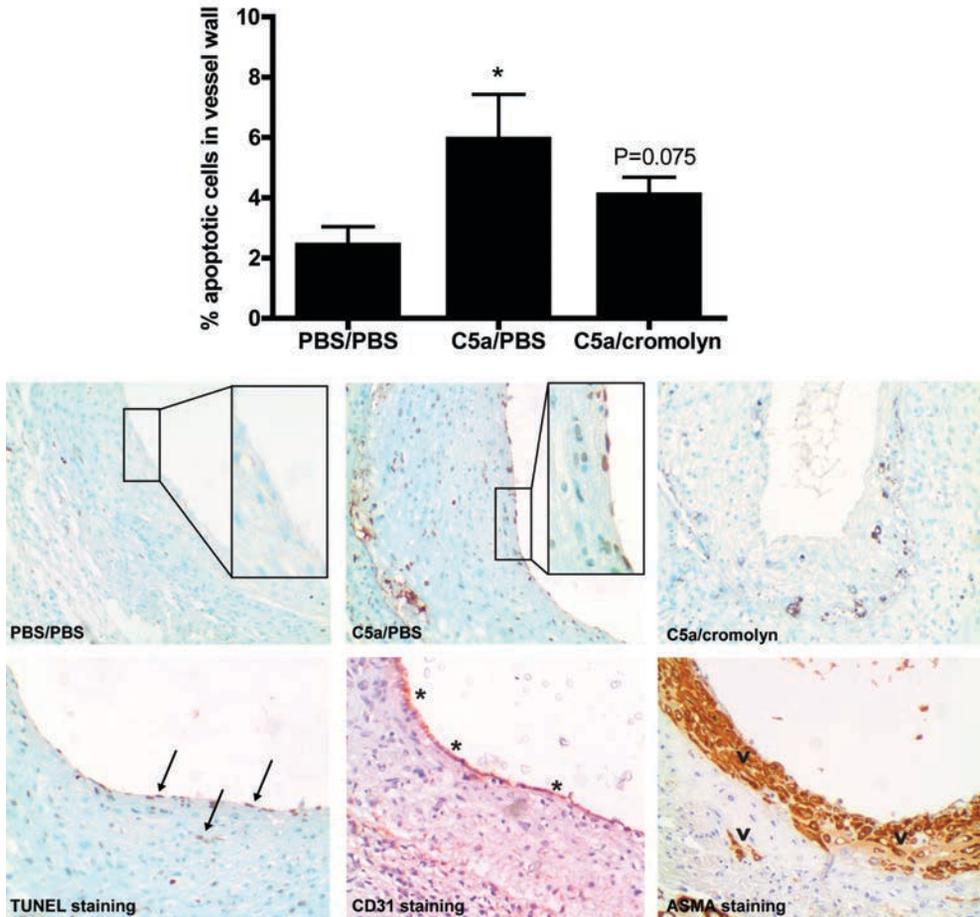


Figure 6. Apoptosis measurement is depicted as the percentage of apoptotic cells of the total number of cells within the vessel wall. Perivascular treatment with C5a resulted in an increase of apoptosis ($*P < 0.05$), which was not affected by cromolyn treatment. Top pictures show representative TUNEL stained sections of all three treatment groups (200x magnifications). Enlargements demonstrate endothelial cell apoptosis in C5a treated groups, which is mostly absent in control mice (400x magnifications). Pictures in the lower panel show representative micrographs of consecutive slides (200x magnifications). From left to right are shown: TUNEL positive staining for apoptotic cells (arrows); CD31 positive staining for endothelial cells (*) and alpha smooth muscle cell actin positive staining (^). (PBS/PBS treated mice: $n = 8$; C5a/PBS treated mice: $n = 9$; C5a/cromolyn treated mice: $n = 7$; $*P < 0.05$ compared to PBS/PBS).

Discussion

The current study describes a causal role for complement factor C5a in acute atherosclerotic plaque disruptions, which was studied by the perivascular application of C5a in advanced atherosclerotic lesions. Acute activation by C5a resulted in increased amount of plaque disruptions with concomitant intraplaque hemorrhage and fibrin depositions. *In vitro* stimulation with C5a led to a striking increase of smooth muscle cell and endothelial cell apoptosis. Moreover, increased apoptosis was confirmed in the vessel wall after C5a treatment, which may contribute to the plaque disruptions observed in this study.

Previously, we have demonstrated that local application of C5a at time of graft placement aggravates vein graft disease via activation of perivascular mast cells¹⁴. In that previous study, mice received C5a treatment only during the first days after surgery; therefore C5a had the most prominent effects on the initiation of the lesion. In this initial process both attraction and activation of mast cells have shown to be important. Interestingly, in the current study, where effects of C5a on advanced lesions were investigated, results are indicative of a mast cell independent mechanism as the mast cell stabilizer cromolyn did not prevent the C5a induced effects on plaque disruptions. These effects can be explained by differences in relative cellular content of early versus advanced lesions. In early lesions recruitment of inflammatory cells, possibly via mast cell derived chemokines such as MCP-1, plays a prominent role, while in advanced lesions a loss of matrix deposition and increased apoptosis are detrimental for plaque stability. The mechanism behind C5a activation may therefore differ between early as compared to advanced stages of atherosclerosis. Indeed, in our previous study, we have established that C5a attracts and activates mast cells, resulting in a more profound influx of inflammatory cells and accelerated vein graft disease. In mature lesions however, the amount of immune cells present in the vessel wall is already strongly increased. Although we again observe an increased number of mast cells in this study, and mast cell proteases have been implicated in plaque stability and apoptosis^{19,29,30}, C5a itself may have more direct and pronounced effects on other cell types involved in maintaining plaque stability, such as the smooth muscle cell. Features of an unstable atherosclerotic plaque, prone to rupture, have been described extensively³¹. A thin fibrous cap devoid of collagen and smooth muscle cells, a large acellular necrotic core and increased inflammation and neovascularization are detrimental for plaque stability. Disruptions described in the current experiment are not equal to plaque ruptures in the classical definition, in which a thin cap covering a lipid core ruptures, resulting in atherothrombosis³². This may be due to the fact that the vein grafts described in the current study do not contain large necrotic cores similar to human plaques, but smaller necrotic cores distributed heterogeneous throughout the intima. However, we would like to

emphasize that, as of yet, there have not been any mouse models described that completely resemble human plaque rupture, and its complex lesion morphology³³⁻³⁵. In contrast to many other mouse models, this vein graft model does exhibit a complex morphology including neovascularization, calcifications and disruptions consisting of fibrin depositions and intraplaque hemorrhage, which has extensively been described by De Vries *et al.*¹⁸. We therefore believe that this model can make a valuable contribution to research regarding the underlying mechanisms behind plaque disruptions.

To elucidate the mechanism by which C5a causes plaque disruptions, we investigated the effect of C5a *in vitro* on various cell types. Speidl *et al.* have previously demonstrated an increase of MMP expression after C5a administration to human macrophages³⁶. Because collagen turnover is important for the integrity of the fibrous cap, we determined the ratio of TIMP1/MMP9 expression after treating murine macrophages with C5a. In line with our *in vivo* data, where we did not observe any effects on lesional collagen content, we did not observe any changes in the TIMP1/MMP9 ratio, while we also did not observe any effects on smooth muscle cell dependent collagen production. Taken together, these data suggest that C5a does not acutely affect plaque stability via interference in matrix homeostasis.

Next, we investigated the effect of C5a on cellular apoptosis, a pathway which has also been suggested to contribute to plaque instability^{37,38}. Intriguingly, a marked dose-dependent increase of both early and late apoptosis was detected when administering C5a to smooth muscle cells as well as to endothelial cells. These results are in line with previous findings that C5a can induce apoptosis of adrenomedullary cells and neuronal cells^{39,40}. We here demonstrate that C5a causes a striking increase in apoptosis of smooth muscle cells. In late stage atherosclerosis, smooth muscle cell apoptosis is responsible for a potent inflammatory response, loss of collagen and thinning of the fibrous cap, whereas it causes no inflammation or thrombosis in normal arteries⁴¹. Specific apoptosis of smooth muscle cells in established atherosclerotic lesions resulted in plaques with unstable features⁴². Also, it has been shown that induction of smooth muscle cell apoptosis by specific SIRT1 deletion results in reduced cap/intima ratio⁴³. Together, these data highlight the importance of viable smooth muscle cells to the fibrous cap. Furthermore, we detected a remarkable increase of endothelial cell apoptosis after addition of C5a. Previously it has been described that C5a induces apoptosis of endothelial cells⁴⁴. Induction of endothelial cell apoptosis may result in erosions at the luminal site of atherosclerotic lesions, making the surface prone to thrombus formation and subsequent acute cardiovascular syndromes⁴⁵. Indeed, local C5a treatment resulted in an increased percentage of apoptotic cells within the atherosclerotic plaque. However, it should be noted that most of the cells in the lesion are C5aR positive; therefore we cannot completely exclude the

possibility of effects of C5a on other C5aR positive cells besides endothelial and smooth muscle cells.

Although the local concentration of C5a *in vivo* was artificially created in this study, it should be realized that previous reports described an increase in serum C5a in patients suffering from cardiovascular disease, furthermore, C5a levels did correlate with cardiovascular events and late lumen loss in drug eluting stents^{17,46}. C5a in the plaque and adventitia may thus derive via diffusion through perivessels; also, it may be locally derived from various cell types in the vessel wall, such as smooth muscle cells, macrophages and endothelial cells.

In conclusion, we here demonstrate a causal role for C5a in the induction of atherosclerotic plaque disruptions. C5a exposure resulted in a marked increase of endothelial cell and smooth muscle cell apoptosis *in vitro*. Moreover, increased apoptosis *in vivo* was observed, which may contribute to plaque destabilization. With respect to rheumatoid arthritis and psoriasis, phase Ib/IIa clinical trials for a C5a inhibitor have already been completed⁴⁷, and a similar therapeutic strategy using complement factor C5a as a target may be promising in the prevention of acute cardiovascular events.

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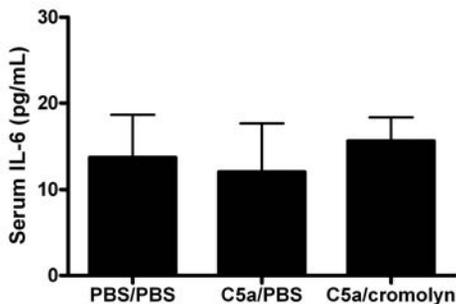
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Supplemental Figures

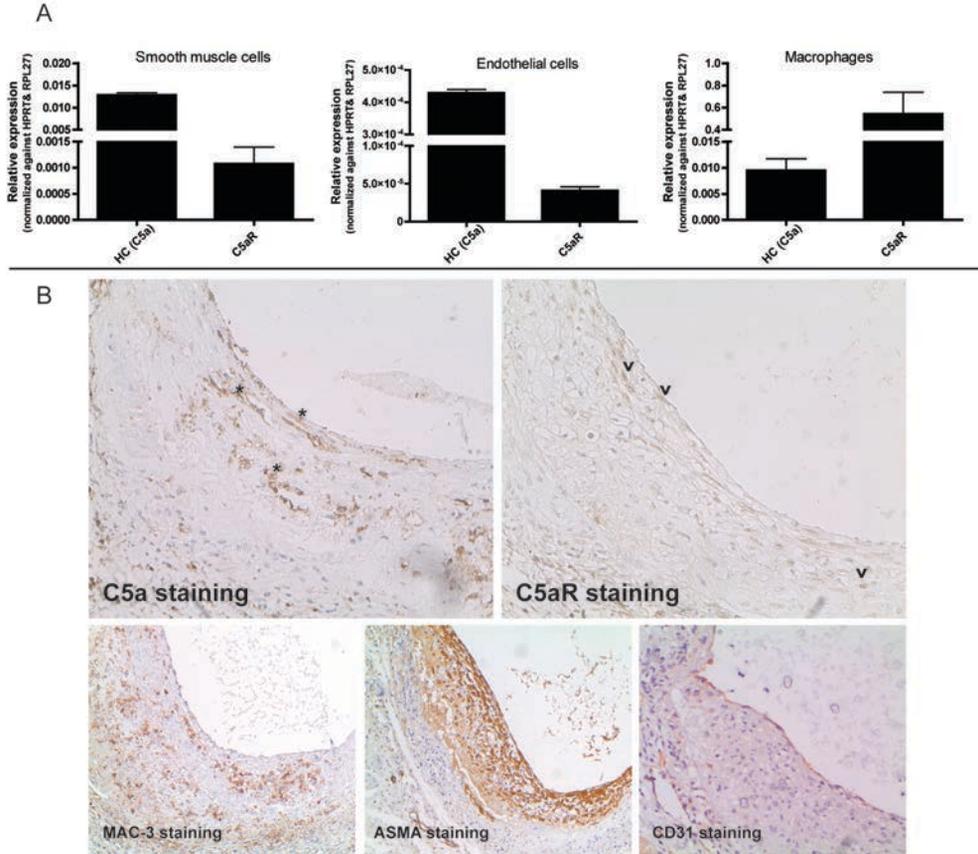
Supplemental Table 1. QPCR primers used for the *in vitro* experiments.

Gene	Forward primer	Reversed primer
TIMP1	ACACCCCAGTCATGGAAAGC	CTTAGGCGGCCCGTGAT
MMP9	CCCTGGAAGTCACACGACATCTTC	CTCATTTTGGAACTCACACGCCAG
I-CAM-1	GTCCGCTTCCGCTACCATCAC	GGTCCTTGCCACTTGTCTGCC
V-CAM-1	AGACTGAAGTTGGCTCACAATTAAGAAG	AGTAGAGTGC AAGGAGTTCGGG
PECAM-1	GTCTTGTGCGAGTATCAGAATTTGAG	TACCAGGCCGCTTCTCTTGA
HC (C5a)	ACACTGCGACTCTTCTGGTCACT	CCAGGTTGGCATTGGTACAGCTC
C5aR	GACCCCATAGATAACAGCA	CAGAGGCAACACAAAACCCA
Caspase-1	GGCATTAAAGAAGGCCCATATAGAGA	TGAGCCCTGACAGGATGTC
Caspase-3	AACCTCCATAAGAGCACTGGAATGTC	ACTTGGTATTTTCAGGCCCATGA
Bax	CGTGGTTGCCCTCTTCTACTTT	TGATCAGCTCGGGCACTTTA
TNFα	GCCTCTTCTCATTCTGCTTGTG	ATGATCTGAGTGTGAGGGTCTGG
HPRT	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAGAAGCTTATAG
RPL27	TGAAAGTTAGCGGAAGTGC	TTTCATGAACTTGCCCATCTC

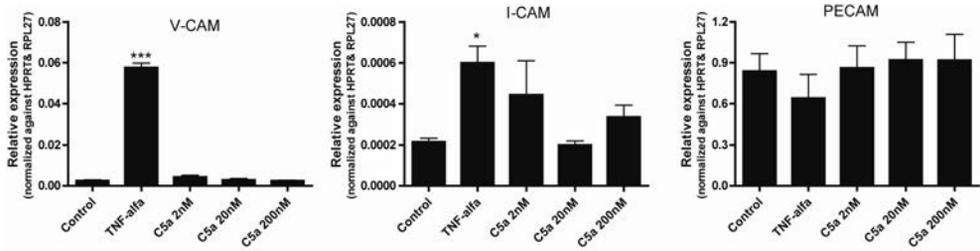
Supplemental Figure 1. Plasma IL-6 concentration did not differ between the three treatment groups. (PBS/PBS treated mice: n=8; C5a/PBS treated mice: n=9; C5a/cromolyn treated mice: n=7).



Supplemental Figure 2. Q-PCR analysis of mRNA, isolated from smooth muscle cells, macrophages and endothelial cells, show the expression of both HC (C5a) and C5aR (A). (B) Representative micrographs (C5a(*), C5aR(^); CD31: 200x magnification. MAC-3, ASMA: 100x magnification) indicate that cells expressing C5a and C5aR in the vessel wall are mostly smooth muscle cells, endothelial cells and macrophages.



Supplemental Figure 3. Mouse endothelial cells (H5V) were stimulated with increasing concentrations of C5a *in vitro*. TNF α stimulation was used as a positive control and resulted in a significant upregulation of both V-CAM and I-CAM. Stimulation with C5a however did not result in a changed expression of V-CAM, I-CAM or PECAM. (*P<0.05; *** P<0.001).



Supplemental Figure 4. Mouse endothelial cells (H5V) were stimulated with 2 nM, 20 nM, or 200 nM C5a *in vitro*. No changes were observed in the gene expression levels of caspase-1 or Bax, however the expression of caspase-3 was significantly and dose-dependently increased after C5a treatment. The expression level of the pro-apoptotic cytokine TNF α did not show any differences after C5a stimulation. (*P<0.05; ** P<0.01).

