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## **Innate immune modulation in atherosclerosis and vascular**

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### **Citation**

Wezel, A. (2014, December 11). *Innate immune modulation in atherosclerosis and vascular*. Retrieved from <https://hdl.handle.net/1887/29988>

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**Title:** Innate immune modulation in atherosclerosis and vascular remodelling

**Issue Date:** 2014-12-11

## Chapter 3

### Complement factor C5a as mast cell activator mediates vascular remodelling in vein graft disease

*Cardiovasc Res. 2013; 97: 311-20*

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## Abstract

**Aims:** Failure of vein graft conduits due to vein graft thickening, accelerated atherosclerosis, and subsequent plaque rupture is applicable to 50% of all vein grafts within 10 years. New potential therapeutic targets to treat vein graft disease may be found in components of the innate immune system, such as mast cells and complement factors, which are known to be involved in atherosclerosis and plaque destabilization. Interestingly, mast cells can be activated by complement factor C5a and, therefore, a direct role for C5a-mediated mast cell activation in vein graft disease is anticipated. We hypothesize that C5a-mediated mast cell activation is involved in the development and destabilization of vein graft lesions.

**Methods and results:** Mast cells accumulated in time in murine vein graft lesions, and C5a and C5a-receptor (CD88) expression was up-regulated during vein graft disease in apolipoprotein E-deficient mice. Mast cell activation with dinitrophenyl resulted in a profound increase in vein graft thickening and in the number of plaque disruptions. C5a application enhanced vein graft lesion formation, while treatment with a C5a-receptor antagonist resulted in decreased vein graft disease. C5a most likely exerts its function via mast cell activation since the mast cell inhibitor cromolyn totally blocked C5a-enhanced vein graft disease.

**Conclusion:** These data provide evidence that complement factor C5a-induced mast cell activation is highly involved in vein graft disease, which identifies new targets to prevent vein graft disease.

## Introduction

Venous bypass grafting, frequently used in cardiac and peripheral vascular surgery, often fails acutely due to thrombosis and on the long term through vein graft thickening, accelerated atherosclerosis, and plaque rupture<sup>1,2</sup>. Recently, our group and others have conclusively demonstrated that perivascular mast cells contribute to atherosclerotic plaque progression and destabilization in mice<sup>3-5</sup>. Although many similarities exist between atherosclerosis and vein graft disease (VGD), it is still unknown whether mast cells play a causal role in the development of VGD. Furthermore, the triggers that lead to mast cell activation in atherosclerosis or VGD are still unresolved. A potential mechanism for mast cell activation in patients is via the complement system<sup>6</sup>. Complement factors are expressed during the development of atherosclerosis<sup>7</sup>. In particular, C3a and C5a have been detected in advanced atherosclerotic plaques<sup>8</sup> and we have previously demonstrated that complement factor C3 and C1q are involved in VGD in mice<sup>9,10</sup>.

C5a is one of the major biologically active components of the complement cascade downstream of C3 and exerts its functions mainly via the canonical C5a receptor (C5aR, CD88). C5a induces chemotaxis of numerous cell types including mast cells and monocytes<sup>11</sup>. It has been demonstrated that plasma C5a levels correlate with an adverse outcome in patients with severe atherosclerosis<sup>12</sup>. Moreover, in a phase III trial with patients undergoing coronary artery bypass surgery or aortic valve replacement, the administration of an antibody against C5, which is the precursor to C5a, showed decreased mortality<sup>13</sup>.

In the current study, we aimed to investigate the role of mast cells and complement factor C5a in a mouse model of VGD. We show that either omission or stimulation of mast cells or C5a results in modulation of vein graft thickening (VGT). Furthermore, we demonstrate that C5a mediated the activation of mast cells is strongly involved in the processes implicated in VGD. These data strongly suggest that mast cells and the C5a-C5aR axis play a central role in the development of VGD and show that C5a is a potent mast cell activator during cardiovascular disease processes.

## Methods

A detailed description of the Methods is available in the Supplementary material online.

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 numbers 09148 and 10091). Vein graft surgery was performed by donor caval vein interpositioning (caval vein of  $\pm$  2mm length) in the carotid artery of recipient mice. These were either C57BL/6 control mice, apolipoprotein E-deficient (apoE<sup>-/-</sup>) or mast cell-deficient Kit(W<sup>sh</sup>/W<sup>sh</sup>) male mice (10–20 weeks old). Before surgery, mice were anaesthetized with midazolam (5 mg/kg, Roche Diagnostics), medetomidine (0.5 mg/kg, Orion), and fentanyl (0.05

mg/kg, Janssen Pharmaceutical). The adequacy of the anaesthesia was monitored by keeping track of the breathing frequency and the response to toe pinching of the mice. After the procedure, the mice were antagonized with atipamezol (2.5 mg/kg, Orion) and fluminasenil (0.5 mg/kg Fresenius Kabi). Buprenorphine (0.1 mg/kg, MSD Animal Health) was given after surgery to relieve pain.

A detailed flow chart displaying the *in vivo* experimental set-up is shown in the Supplementary material online, Figure S1. In all experiments vein grafts were left *in situ* for 28 days (with exception of the time courses). Formalin fixed, paraffin embedded, and vein grafts were histological and morphometrically analysed as previously described<sup>14,15</sup>. Cholesterol levels were determined before surgery and at sacrifice. Furthermore, sysmex analysis of blood cells was performed for all experiments and no significant differences were detected in the % of WBC populations between the treatment groups and their appropriate controls. In Supplementary material online, Table S1, white blood cell analysis of the C5a and cromolyn (Cro) experiment is shown.

First, we aimed to demonstrate the presence of mast cells, C5a and C5aR in vein grafts of hypercholesterolaemic apoE<sup>-/-</sup> mice. For this, we used immunohistochemistry and RT-PCR analysis on time courses of paraffin and RNA material of three to four mice per time point. Analyses were performed as described in the Supplementary material online.

Secondly, the involvement of mast cells in VGD was determined by the analysis of vein graft lesions in either mast cell-deficient Kit(W<sup>sh</sup>/W<sup>sh</sup>) mice ( $n = 8$  mice/group) or by investigating the effect of local mast cell activation with dinitrophenyl hapten (DNP) on vein graft remodelling in apoE<sup>-/-</sup> mice. After skin-sensitizing, apoE<sup>-/-</sup> mice ( $n = 11$ /group) were subjected to vein graft surgery and subsequently the vein grafts were treated locally with DNP in pluronic gel<sup>16</sup>.

Thirdly, the effect of interfering in C5a signalling was investigated. ApoE<sup>-/-</sup> mice ( $n = 7$ /group) were challenged perivascularly with either 0.5 or 5 µg of recombinant mouse C5a (HyCult Biotechnology) or vehicle in pluronic gel. The effects of inhibiting C5a function were also studied. ApoE<sup>-/-</sup> mice ( $n = 7$ /group) were treated daily with subcutaneous injections of the C5a-receptor antagonist hydrocinnamate-[OP-(D-Cha)WR] (PMX205) (0.3 mg/kg)<sup>17</sup> or vehicle solution, starting 1 day prior to surgery.

To determine whether C5a effects were mast cell dependent, a group of C5a stimulated apoE<sup>-/-</sup> mice (5 µg/mouse in pluronic gel) or PBS gel controls were treated twice weekly with the mast cell stabilizer Cro (50 mg/kg, Sigma) and compared with mice treated with PBS ( $n = 9$ /group).

Finally, to confirm whether C5a activation of mast cells could be a functional pathway in human atherosclerotic lesions, the presence of mast cells, C5a and C5aR in human specimens were analysed. Human vein graft ( $n = 8$ ) and carotid endarterectomy ( $n = 25$ ) tissues were obtained in accordance with guidelines set out by the 'Code for Proper Secondary Use of Human Tissue' of the Dutch Federation of Biomedical Scientific Societies (Federa) and conform with the principles outlined in the Declaration of Helsinki.

All data are presented as mean ± SD. For the time courses, statistical analysis was performed using a repeated measures ANOVA with a Bonferroni *post hoc* test. *In vivo* experiments were compared with a Krukall-Wallis test followed by a non-parametric Mann-Whitney test to compare individual groups. For *in vitro* studies, a two-tailed Student's *t*-test was used. *P*-values <0.05 were regarded significant.

## Results

### *Mast cells, C5a and C5aR in murine vein grafts*

The presence of mast cells and expression levels of C5a and C5aR were assessed by immunohistochemistry and RT-PCR (primer sequences; Supplementary material online, Table S2) in vein grafts interpositioned in carotid arteries of hypercholesterolemic apolipoprotein E-deficient mice. The vein grafts were harvested at several time points after surgery ( $n = 3-4$  per time point). Mast cells were found in small quantities in the adventitia of the ungrafted caval veins. Directly after engraftment the number of perivascular mast cells decreased and from 3 days on the number of mast cells increased profoundly ( $t = 6$  h:  $0.4 \pm 0.1$  mast cells/mm<sup>2</sup> vein graft vs. 28 days:  $4.2 \pm 0.6$  mast cells/mm<sup>2</sup> vein graft,  $P = 0.045$ , Figure 1A). Mast cells in the atherosclerotic lesion itself were very rare. Both resting and activated mast cells were found in the perivascular tissue (Figure 1B). No differences in the activation status of the mast cells were found between the different time points (data not shown). An increase in C5 mRNA expression was seen at 6 h ( $P = 0.015$ ) after surgery (Figure 1C), which is in agreement with the finding of increased C5a protein expression at this time point (Figure 1D). C5a was detected in leukocytes adhering to the lumen and in the adventitia, where mast cells particularly reside. C5a was also detected in the regenerating endothelium from 7 days on. At later time points (14 and 28 days), the expression of C5a was also seen in macrophages and some smooth muscle cells (SMCs) associated with thickening of the graft. C5aR mRNA was maximally expressed (nine-fold increase vs. ungrafted vein  $P = 0.043$ ) at 1 day after surgery and declined after 3 days to a three-fold increase in relative expression ( $P = 0.073$ ) (Figure 1E). In the first day after surgery, C5aR protein expression was seen in invading inflammatory cells and at later time points also in SMCs and macrophages (Figure 1F).

### *Effect of mast cell deficiency and mast cell stimulation on vein graft morphology*

To investigate whether there is a causal relation between mast cell accumulation and the development of VGD, mast cell-deficient Kit<sup>(W-sh/W-sh)</sup> and control C57BL/6 mice underwent vein graft surgery. At 28 days after surgery, a decrease in VGT of 36% was seen in the Kit<sup>(W-sh/W-sh)</sup> mice when compared with control mice ( $0.52 \pm 0.18$  vs.  $0.33 \pm 0.13$  mm<sup>2</sup>, respectively,  $P = 0.036$ , Figure 2). The total vessel area (lesion area + lumen area) ( $0.89 \pm 0.22$  vs.  $0.71 \pm 0.22$  mm<sup>2</sup>,  $P = 0.141$ ) and the lumen area ( $0.38 \pm 0.09$  vs.  $0.38 \pm 0.10$  mm<sup>2</sup>,  $P = 0.753$ ) were not significantly different. Consequently, no effect on outward remodelling could be detected.

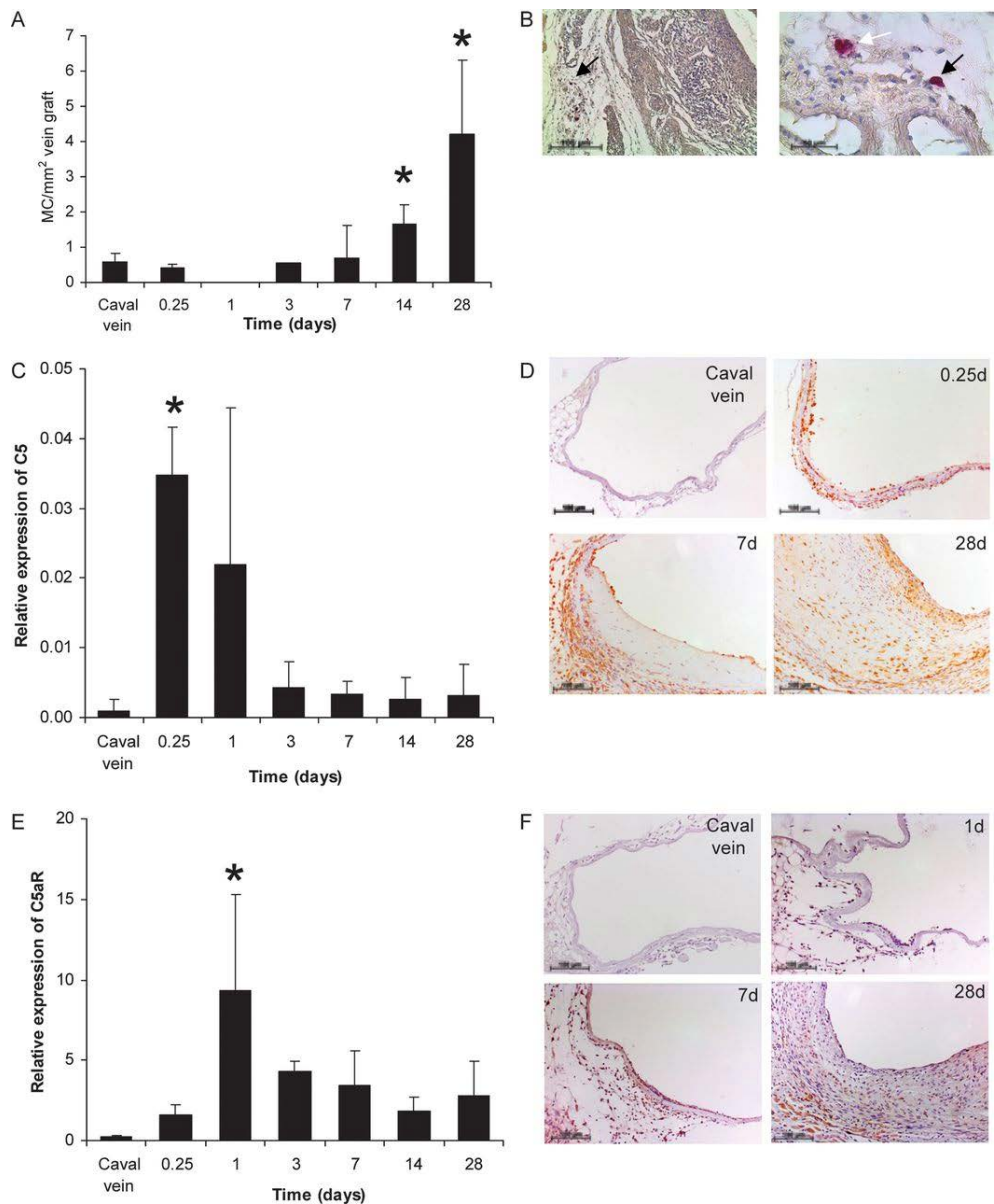


Figure 1. (A) Perivascular mast cells were scored in caval veins (0) and vein grafts at 6 h (0.25 day), 1, 3, 7, 14, and 28 days after surgery (three to four vein grafts/time point). Mast cell numbers increased from 3 days on to a significant increase (compared with vein graft at 0.25 day) at day 14 and 28 (B). Both resting (black arrow) and activated (white arrow) mast cells were found in the adventitia of the vein grafts. C5a and C5a-receptor expression on mRNA (C and E) and protein level (D and F) were assessed in time in vein grafts. Relative expression of both C5a and C5aR increased rapidly immediately after surgery due to the influx of positive inflammatory cells. At later time points expression decreased due to the influx in the vein graft of C5a- and C5aR-negative cells and extracellular matrix. \* $P < 0.05$ , representative scale bars are added to the photographs.



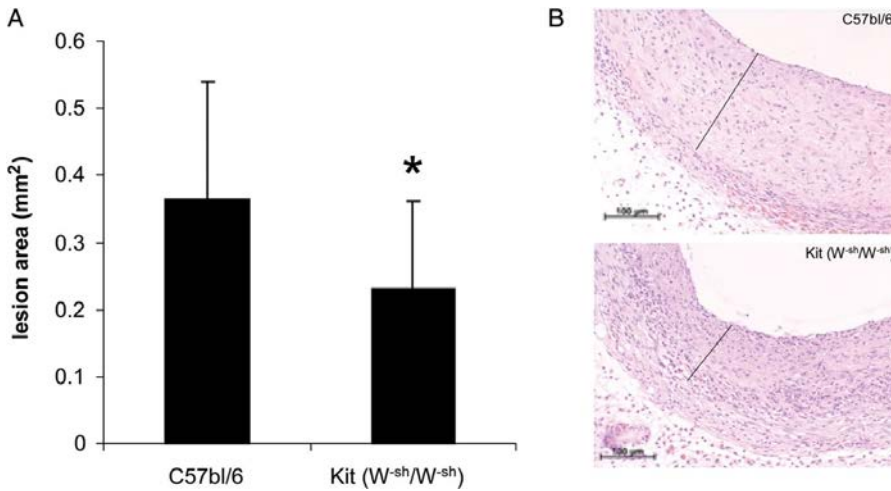


Figure 2. (A) A decrease in the  $\text{Kit}^{(W-sh/W-sh)}$  lesion area was seen when compared with control C57BL/6 lesions 28 days after surgery (\* $P < 0.05$ ). (B) Representative cross-sections of vein grafts in C57BL/6 and  $\text{Kit}^{(W-sh/W-sh)}$  mice (HPS staining, magnification  $\times 20$ ). Black line indicates the thickness of the lesion.

Next, skin-sensitized  $\text{apoE}^{-/-}$  mice were challenged locally at the vein graft with DNP, which results in acute mast cell activation, or vehicle control to study the effects of mast cell activation on VGD. Mast cell activation resulted in a 46% increase in the lesion area compared with the vehicle control group ( $0.36 \pm 0.08$  vs.  $0.52 \pm 0.20$  mm<sup>2</sup>  $P = 0.011$ , Figure 3A and B). No significant differences were found in the total vessel area and the lumen area (data not shown). Plaque phenotype analysis revealed that the DNP-challenged group showed a 50% reduction in lesional SMCs (DNP:  $12 \pm 6\%$ , vehicle:  $25 \pm 5\%$ ,  $P = 0.001$ , Figure 3C), especially in the cap region. The relative collagen content (DNP:  $27 \pm 6\%$ , vehicle:  $25 \pm 10\%$ ,  $P = 0.457$ , Figure 3D) and the macrophage content (DNP:  $18 \pm 3\%$ , vehicle:  $17 \pm 7\%$ ,  $P = 0.341$ , Figure 3E) did not differ significantly between the groups. At 28 days after DNP challenge, no differences in the number of mast cells were seen, nor did we detect a difference in the activation status (data not shown). Strikingly, vein grafts in the DNP group did show severe signs of plaque rupture complications<sup>15</sup>. Half of the vein grafts treated with DNP ( $n = 12$ ) showed lesions with de-endothelialized areas and intramural thrombus (erosions) and two vein grafts showed a dissection, in which a tear starting at the lumen and up to the outer vein graft wall, filled with erythrocytes was seen. In contrast, only three vein grafts of the vehicle-treated group ( $n = 12$ ) showed lesions with erosion ( $P = 0.05$ , Figure 3B). Plaque erosion is characterized by a loss of endothelial cells and therefore we scored the coverage of the lumen with CD31 positive cells. DNP treatment resulted in a 40% reduction in coverage compared with controls ( $P = 0.001$ , Figure 3G and H). Not only sections with intramural thrombi were devoid of endothelial cells, but also apparently asymptomatic lesions in the DNP group

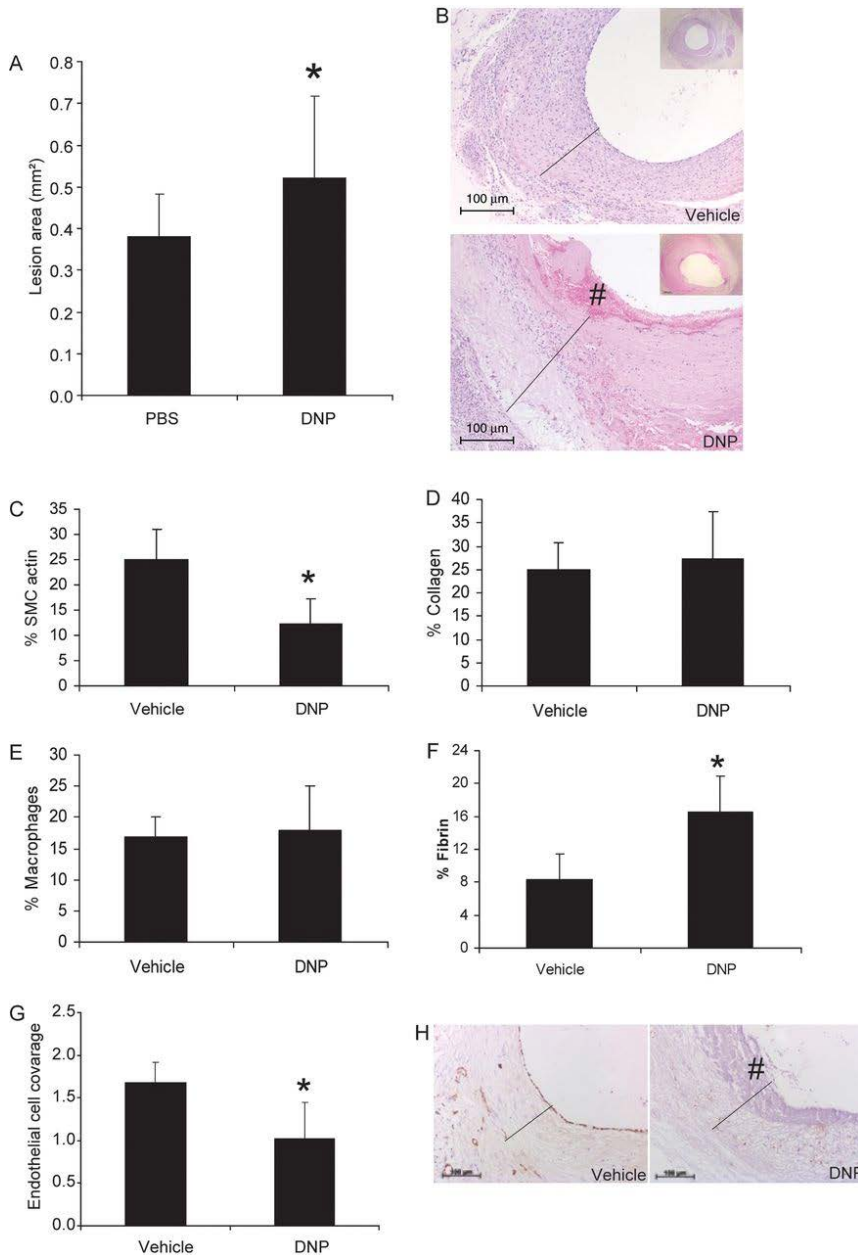


Figure 3. (A) Local treatment of apoE<sup>-/-</sup> mice with dinitrofluorobenzene (DNP) in pluronic gel resulted in increased vein graft lesion areas at 28 days after surgery compared with the vehicle-treated group. (B) Typical cross-sections of vein grafts after treatment with vehicle or DNP (HPS staining, magnification  $\times 20$ , insets magnification  $\times 5$ ). Note the erosion in the DNP-treated group (#). DNP treatment resulted in more disruptions of the vein grafts [8/12 (DNP) vs. 3/12 (PBS)] (C) Percentage of positive smooth muscle cells (SMC) was significantly decreased after DNP challenge. (D) The relative collagen content did not differ between the groups. (E) Percentage of the macrophage content was not affected by DNP challenge, whereas DNP treatment affected endothelium coverage significantly (F), which resulted in a significantly increased fibrin content in the DNP group (G and H). \* $P < 0.05$ ; black lines in the photographs indicate the thickness of the lesions.

showed less endothelial coverage. De-endothelialization can result in enhanced fibrin deposition and indeed, the DNP group demonstrated a two-fold increase in the fibrin content (DNP;  $17 \pm 3\%$ , vehicle;  $8 \pm 4\%$   $P = 0.001$ , Figure 3F).

*Effect of C5a application and C5aR antagonist PMX205 on vein graft morphology*

C5a-induced mast cell activation was demonstrated by the release of tryptase and a concentration-dependent release of CCL2 from cultured bone marrow-derived mast cells (Supplementary material online, Table S3). To study the involvement of C5a in the development of VGT, recombinant C5a was applied in increasing concentrations (0, 0.5, and 5  $\mu\text{g}$ ) directly to the vein graft at the time of surgery. Topical application of C5a resulted in a dose-dependent increase in the lesion area (control:  $0.24 \pm 0.05 \text{ mm}^2$ , 0.5  $\mu\text{g}$  C5a:  $0.29 \pm 0.1 \text{ mm}^2$ , 5  $\mu\text{g}$  C5a:  $0.41 \pm 0.1 \text{ mm}^2$ , Figure 4A and B). The total vessel area and the luminal area were, however, comparable between the three groups (data not shown). Remarkably, at 28 days after surgery perivascular mast cell numbers show a trend towards an increase after C5a application (control:  $2.77 \pm 1.7 \text{ cells/mm}^2$  vein graft, 0.5  $\mu\text{g}$  C5a:  $4.36 \pm 1.6 \text{ cells/mm}^2$  vein graft,  $P = 0.100$ , 5  $\mu\text{g}$  C5a:  $5.47 \pm 3.2 \text{ cells/mm}^2$  vein graft,  $P = 0.086$  compared with control, Figure 4C). Since C5a is a potent chemotactic factor for monocytes/macrophages, the macrophage content was studied. A dose-dependent increase in macrophage contribution to the VGT was seen in C5a-treated vein grafts (Figure 4D). The amount of macrophages increased from  $20 \pm 5\%$  in the controls to  $22\% \pm 9\%$  ( $P = 0.110$  compared with control) in mice treated with 0.5  $\mu\text{g}$  C5a, and even up to  $33 \pm 7\%$  in mice which received 5  $\mu\text{g}$  C5a ( $P < 0.05$  compared with both groups, Figure 4D). Next, the effect of the C5aR blockade by systemic treatment with the C5aR antagonist, PMX205 was delineated. Analysis of the thickened vein graft after 28 days revealed that treatment with PMX205 resulted in a 41% decrease in the lesion area, when compared with control mice (control:  $0.39 \pm 0.16 \text{ mm}^2$ , PMX205  $0.23 \pm 0.07 \text{ mm}^2$ ,  $P = 0.035$ , Figure 4E and F). The VGT consisted for  $24 \pm 8\%$  of macrophages. In PMX205-treated vein grafts, the macrophage contribution was  $16 \pm 8\%$  ( $P = 0.012$ , Figure 4G). Strikingly, adventitial mast cells were found to be reduced after PMX205 treatment (PMX205:  $2.32 \pm 0.6 \text{ cells/mm}^2$  vein graft, control:  $4.14 \pm 2.0 \text{ cells/mm}^2$  vein graft,  $P = 0.042$ , Figure 4H). No differences in rupture complications were seen between the groups.

#### *C5a-induced vein graft thickening is abolished by mast cell stabilization*

To elucidate the mast cell-dependent effects of C5a in VGD, mice were locally treated with either a PBS or C5a (5  $\mu\text{g}$ ) loaded pluronic gel ( $n = 20/\text{group}$ ). Of each group, 10 mice were treated twice weekly with the mast cell inhibitor Cro or PBS control (ip). Cro treatment resulted in a decrease in the lesion area of 22% (PBS/PBS  $0.38 \pm 0.1 \text{ mm}^2$ , PBS/Cro  $0.29 \pm 0.06 \text{ mm}^2$ ;  $P = 0.044$ , Figure 5A and B) and

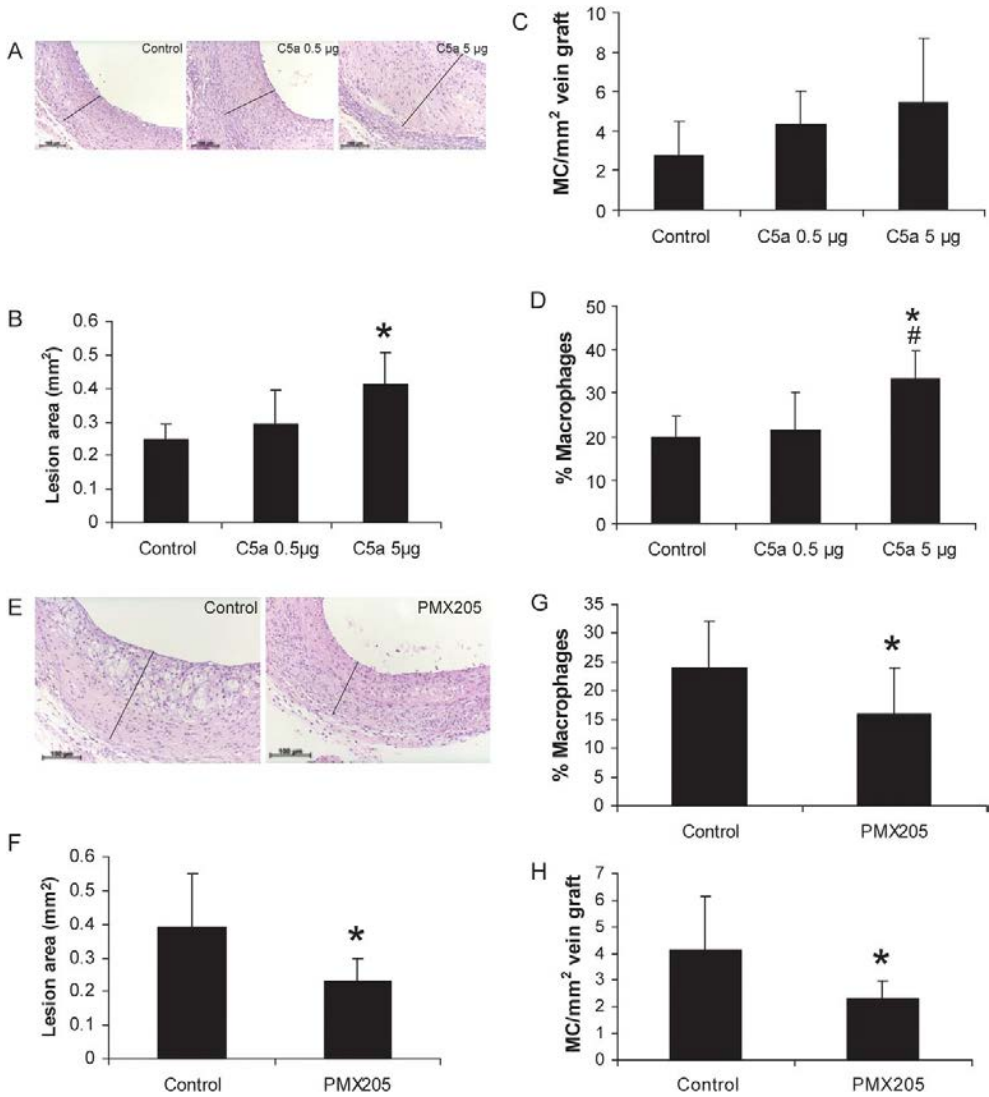


Figure 4. (A) Representative cross-sections of vein grafts, 28 days after surgery, treated with PBS or 0.5 or 5  $\mu$ g C5a (HPS staining, magnification 20 $\times$ ). (B) The quantification of vein graft lesion areas reveals a dose-dependent increase in the lesion size. (C) Perivascular mast cell numbers tend to increase after C5a application, although this did not reach significance. (D) The relative macrophage content of the vein graft wall tend to increase after 0.5  $\mu$ g C5a application. Application of 5  $\mu$ g C5a to the grafts significantly increased macrophage content (\* $P$  = 0.001 compared with control grafts; # $P$  = 0.008 compared with grafts treated with 0.5  $\mu$ g C5a). (E) Direct effects of the C5aR blockade on vein graft disease was assessed by systemic treatment with a C5aR antagonist, hydrocinnamate-[OP-(D-Cha)WR] (PMX205). Representative cross-sections of vein grafts, 28 days after surgery (HPS staining, magnification 20 $\times$ ). (F) PMX205 treatment resulted in a significant decrease in the lesion area compared with control mice. (G) A significant reduction in relative macrophage contribution can be seen in the PMX205-treated vein grafts. (H) The number of adventitial mast cells was significantly reduced after PMX205 treatment. \* $P$  < 0.05; black lines in the photographs indicate the thickness of the lesions.

a decrease in the total vessel area of 19% ( $P = 0.037$ ), whereas the lumen area was not affected. As shown previously, local C5a treatment resulted in an increase in the lesion area of 79% (C5a/PBS  $0.68 \pm 0.11 \text{ mm}^2$   $P = 0.001$ ) compared with the control group (Figure 5A and B). Strikingly, treatment with both C5a and Cro (C5a/Cro  $0.32 \pm 0.12 \text{ mm}^2$ ) resulted in a major decrease in the lesion area (compared with the C5a/PBS group,  $P = 0.001$ ) to the level of the group treated with Cro only. A decrease in the total vessel area of 30% ( $P = 0.003$ ) was seen, whereas no differences were observed in the lumen area when comparing C5a/Cro vs. C5a/PBS treatment. The relative SMC content was demonstrated to be reduced in the C5a/PBS group by 41% when compared with the PBS/PBS group ( $P = 0.002$ ) and a decrease of 31% compared with the PBS/Cro group was found ( $P = 0.021$ , Figure 5C). The collagen content did not differ between the groups (PBS/PBS  $26 \pm 6\%$ , PBS/Cro  $24 \pm 12\%$ , C5a/PBS  $26 \pm 14\%$ , C5a/Cro  $27 \pm 11$ ,  $P = 0.499$ , Figure 5D). In contrast, Cro treatment alone resulted in a 25% reduction in the macrophage content when compared with controls ( $P = 0.038$ ), which could be caused by inhibition of endogenous mast cell activation during the development of VGD (Figure 5E). The C5a/PBS group showed a 44% increase in the macrophage content when compared with the PBS/PBS group ( $P = 0.001$ ). When compared with the C5a/PBS-treated mice, the C5a/Cro group displayed a decrease of 61% in the macrophage content ( $P = 0.001$ ) as a result of the Cro treatment. Although local C5a treatment resulted in a more unstable morphology, no differences were found in the occurrence of plaque rupture complications (PBS/PBS 3/10, PBS/Cro 3/10, C5a/PBS 1/10, and C5a/Cro 2/10).

#### *Expression of mast cells and C5a(R) in human vein graft and carotid endarterectomy specimens*

To confirm whether C5a activation of mast cells is an endogenous and functional pathway in human atherosclerotic lesions, we analysed the presence of mast cells, C5a and C5aR in human saphenous vein grafts and carotid endarterectomy specimens. Mast cells were detected throughout the entire lesion in both types of tissue and were especially found in regions of neovascularization, either in the adventitia or in the intima (Figure 6). C5aR staining was abundantly present in all layers in both types of specimen. In SMCs, endothelial cells and regions of inflammatory cells, including mast cells, C5aR expression was detectable. C5a staining was less pronounced and mostly limited to regions of intimal SMCs, inflammatory cells, near necrotic cores, and neovascularization.

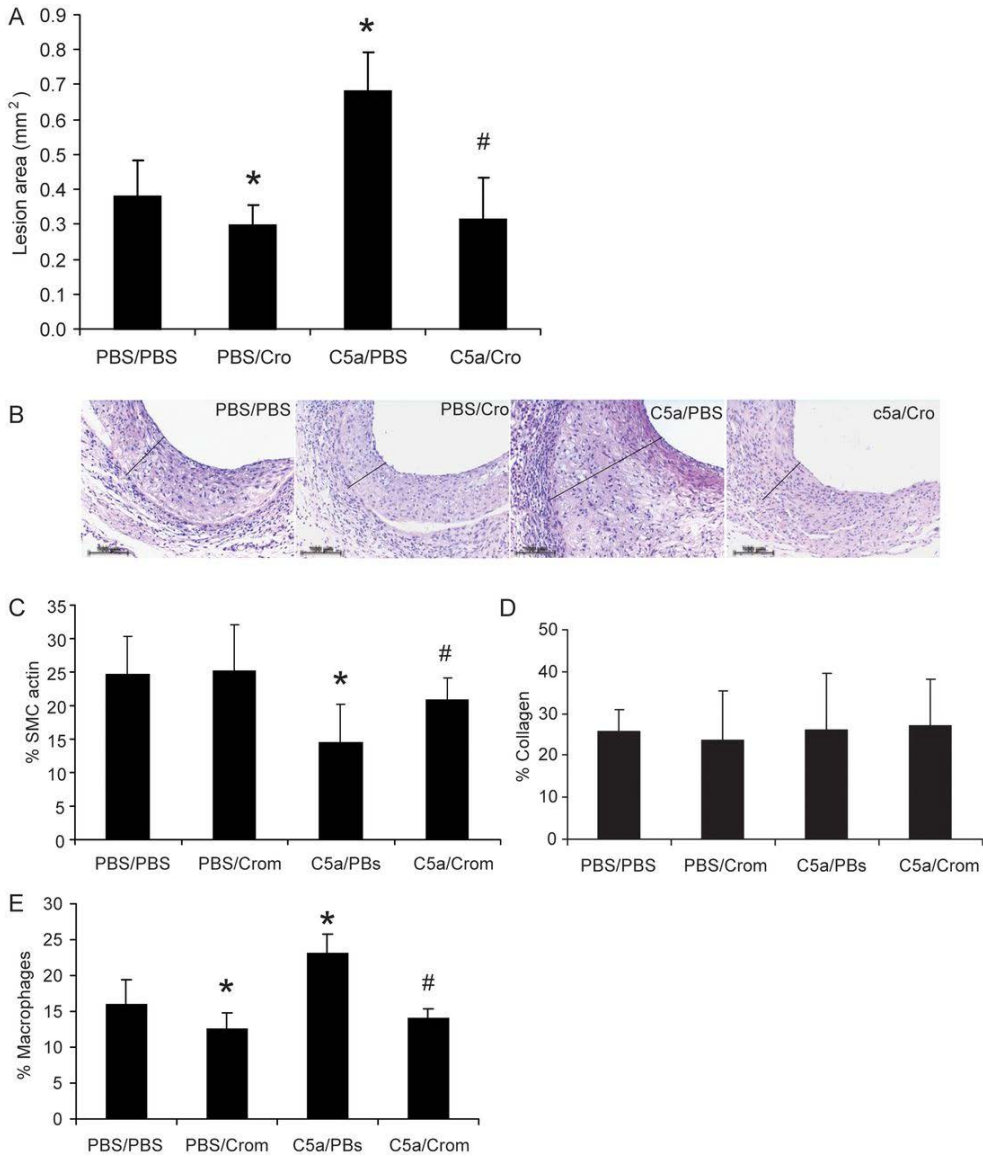


Figure 5. (A) Quantification of the vein graft wall area, 28 days after vein graft surgery, in *apoE*<sup>-/-</sup> mice treated with combination of PBS or with 5  $\mu$ g C5a locally in pluronic gel and twice weekly injections with either PBS or cromolyn (Cro). Cro treatment resulted in a decrease in the lesion area (\* $P < 0.05$  compared with the control group) C5a treatment results in increased lesion areas (\* $P < 0.05$  compared with the control group). Treatment with both C5a and cromolyn resulted in lesion areas comparable with that of the cromolyn-alone-treated group (# $P < 0.05$  compared with the c5a-treated group) (B) Representative cross-sections of vein grafts (HPS staining, magnification  $\times 20$ , black lines indicate the thickness of the lesions). (C) Percentage of SMCs was significantly decreased after C5a application. (D) The quantification of the relative collagen content did not show significant differences between all groups. (E) The relative macrophage content was significantly decreased after Cro treatment and C5a application resulted in a significant increase in the macrophage content which could be abolished by Cro, \* $P < 0.05$ .



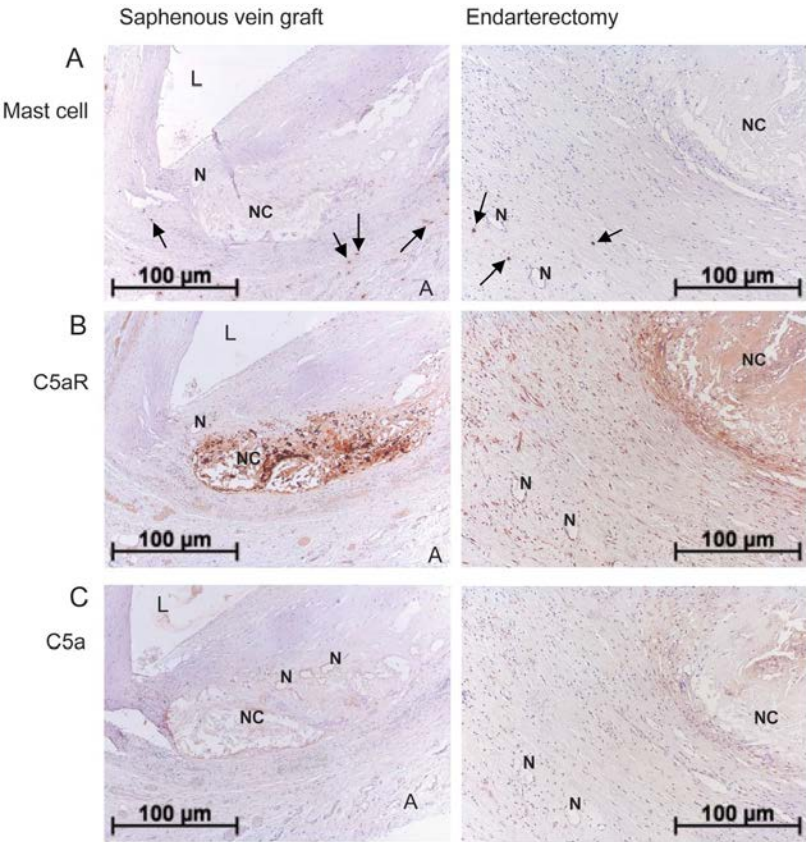


Figure 6. The presence and localization of mast cells, C5a and C5a-receptor in human saphenous vein grafts, and carotid endarterectomy material was assessed by immunohistochemistry. (A) Mast cells (black arrows) were found throughout all layers of the lesion but primarily in the adventitial and intimal regions near neovessels. (B) Abundant staining of C5aR was seen in all major cell types present in the lesions; inflammatory cells, smooth muscle cells, and endothelial cells. (C) C5a presence was seen near necrotic cores and neovessels. Furthermore colocalization of C5a with smooth muscle cells and inflammatory cells was seen frequently (NC, necrotic core; N, Neovessels; L, Lumen; and A, Adventitia).

### Discussion

We have demonstrated that mast cells and complement factor C5a, both innate immunity members, are inextricably linked in the contribution to VGD. Mast cells, C5a and C5aR were demonstrated to be present in diseased human vein grafts as well as in carotid endarterectomy specimen. This is in accordance with colocalization of C5a and the C5aR with mast cells in other types of atherosclerotic lesions as shown previously<sup>8,18</sup>. The expression of C5aR was shown in neointimal lesions and brachiocephalic artery plaques<sup>19,20</sup>, and we now demonstrate co-expression and colocalization of mast cells, C5a and C5a receptor in murine vein grafts. This identifies the C5a-C5aR axis as a potential endogenous mast cell activation route.

Local mast cell challenge with C5a resulted in an increase in the number of perivascular mast cells together with a dose-dependent increase in VGD. Interestingly, treatment of vein grafts with a specific C5aR antagonist (PMX205) resulted in decreased VGT, which coincides with recent findings of decreased neointimal lesion development after C5aR targeting using C5aR-specific antibodies or the related C5aR antagonist PMX53<sup>19,20</sup>. Morphologic analysis also revealed a strong reduction in the macrophage content. The recruitment of monocytes/macrophages into atherosclerotic plaques requires up-regulation of adhesion molecules and CCL2<sup>21</sup>. Shagdarsuren *et al*<sup>19</sup> found that treatment with PMX53 resulted in decreased macrophage content and reduced VCAM-1 expression. In addition, C5aR antagonism in a renal allograft model resulted in attenuated macrophage infiltration due to reduced levels of CCL2 and ICAM-1<sup>22</sup>. Interestingly, adhesion molecules and the CCR2/CCL2 pathway have previously been shown to be involved in the recruitment of mast cells<sup>23</sup>, which suggests that a reduction in CCL2 may have accounted for the reduced number of mast cells we found after C5aR treatment. *In vitro* studies demonstrated that C5a-mediated mast cell activation not only led to the release of the mast cell protease tryptase, but also to a dose-dependent release of chemokine CCL2, which may have, at least in part, caused the increased levels of lesional macrophages and mast cells that were seen in the C5a-treated mice.

To demonstrate a causal role for C5a activation of mast cells in VGD, vein grafts were treated locally with C5a while simultaneously mast cell activation was blocked by the use of Cro. Cro was shown to completely reverse the C5a-induced adverse effects on vein graft atherosclerosis, by decreasing the vein graft lesion size comparable with that of mice treated with Cro only. We have previously shown that Cro is a specific inhibitor of mast cells with no effects on macrophages or other cell types present in atherosclerotic plaques<sup>4</sup>. This demonstrates that C5a mainly acts on vein graft atherosclerosis through mast cell activation.

Mast cells do not only play a role in atherosclerotic lesion growth, but are also involved in plaque destabilization and plaque complications as rupture and erosions<sup>24</sup>. We investigated the effect on plaque stability after modulation of mast cell activation or C5a intervention. Interestingly, challenge with DNP resulted in a major increase in plaque complications and in particular plaque erosions. Plaque erosion, a frequent cause of acute coronary death, is characterized by a loss of endothelial cells<sup>25</sup>. Moreover, subendothelial mast cells have been demonstrated to colocalize with sites of atheromatous erosions<sup>26</sup>. In this study, mast cell activation by DNP challenge resulted in diminished endothelial cell coverage, which may be a consequence of endothelial cell apoptosis due to mast cell activation<sup>4</sup>. Mast cell mediators, such as chymase, TNF- $\alpha$ , histamine, and tryptase, have separately been reported to induce increased endothelial cell permeability and apoptosis<sup>27-29</sup>,



suggesting that mast cells can actively induce plaque erosion. Furthermore, mast cells can also induce SMC<sup>30,31</sup> and macrophage apoptosis<sup>4</sup> and secrete and activate matrix metalloproteinases<sup>32,33</sup>, all important promoters of plaque destabilization and rupture. Interestingly, factors involved in intramural thrombus formation (plasmin, thrombin) can cleave C5 into C5a<sup>34</sup>. This C5a could in turn attract and activate mast cells in the vicinity of the plaque which can result in an on-going loop of atherothrombosis. We have previously shown that mast cell activation by means of a DNP challenge induced acute intraplaque haemorrhage in collar-induced atherosclerosis<sup>4</sup> and we now show that a single DNP challenge directly after surgery resulted in vein graft destabilization and plaque disruptions. Together, these data provide clear evidence that mast cell degranulation can result in atherothrombotic events.

The increase in vein graft atherosclerosis after C5a application was accompanied by a decrease in plaque stability due to a reduction in relative SMC content and an increase in the macrophage content compared with the control group. In contrast, the C5a/Cro-treated group revealed a more stable composition (more SMC, less macrophages) than the C5a-treated group. Although there is evidence that C5a can induce rupture<sup>8,18,35</sup>, we did not observe more rupture-associated complications in the lesions after C5a application when compared with control or Cro treatment. This may be caused by the difference in the activation route of C5a vs. DNP, which results in different mast cell releasate compositions<sup>14</sup>. This may thus affect endothelial survival differently. It is also described that C5a can inhibit angiogenesis<sup>36</sup>, an important factor affecting plaque instability.

In this study, we conclusively demonstrate that mast cells actively contribute to the progression and destabilization of VGD. Furthermore, the complement factor C5a promotes mast cell-dependent VGT, suggesting that complement activation is an important endogenous route of mast cell activation in VGD. Previously, C5 inhibition was demonstrated to be effective in a phase III cardiovascular disease trial<sup>13</sup>. Our finding that mast cells and C5a colocalize in human endarterectomy and vein graft specimens is of importance for the working mechanism of C5 in cardiovascular disease. Although the mouse vein graft model used in the study shows strong homology with human VGD, it should be realized that differences exist between murine and human vein grafts. Despite these differences, we here conclude that C5a-mediated mast cell activation can be a promising therapeutic route of intervention to the prevention of VGD that definitely deserves further investigation.

## Funding

This work was supported by the Netherlands Organization for Scientific Research (916.86.046 to I.B.) and the Netherlands Heart Foundation (2010B029 to A.W.).

## Acknowledgments

The kind gift of PMX205 and useful suggestions for the experimental set-up by Professor Steve Taylor is greatly appreciated.

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## Online data supplement

### Detailed Methods

#### *Animals*

All animal work was performed in compliance with Dutch government guidelines. Male mast cell deficient Kit(W<sup>sh</sup>/W<sup>sh</sup>) mice and apolipoprotein E-deficient (apoE<sup>-/-</sup>) mice (cross bred for more than eighteen generations on a C57BL/6 background) and bred in the local animal facility and male C57BL/6 mice (Charles River Laboratories), were used for all experiments. All mice were between 10-20 weeks old at time of surgery. Before surgery apoE<sup>-/-</sup> mice were randomized to the different groups on basis of plasma cholesterol levels (Roche Diagnostics) and body weight. At sacrifice (28 days after surgery, with exception of the time courses) cholesterol levels and body weight were not significantly different between the treatment groups and their controls. Furthermore, sysmex analyses of blood cells was performed for all experiments and no significant differences were detected in the % of WBC populations between the treatment groups and their appropriate controls. In Supplemental table I white blood cell analysis of the C5a and cromolyn experiment is shown.

#### *In vivo experimental setup*

A detailed flow-chart displaying the *in vivo* experimental setup is shown in Supplemental Figure 1. The presence of mast cells, C5a and C5aR was determined in vein grafts of hypercholesterolemic apoE<sup>-/-</sup> mice. For this, immunohistochemistry and RT-PCR analysis were performed on time courses of paraffin and RNA material of 3-4 mice per time point. To demonstrate the contribution of mast cells to vein graft disease, autologous vein grafts were placed in mast cell deficient Kit(W<sup>sh</sup>/W<sup>sh</sup>) mice and control C57BL/6 mice (n=8/group). To determine the effect of local mast cell activation apoE<sup>-/-</sup> mice were skin-sensitized for 2 consecutive days with dinitrofluorobenzene (DNFB, 0.5% v/v, Janssen Chimica) or vehicle control solution (acetone: olive oil 4:1, n=12 per group) as previous described<sup>1</sup>. One week after skin-sensitization and directly after surgery 50 µg dinitrophenyl hapten (DNP) in pluronic gel (25% wt/vol, Sigma)<sup>2</sup> or pluronic gel alone was applied around the vein grafts.

The effect of modulation of C5a signalling was investigated by challenging apoE<sup>-/-</sup> mice perivascularly with either 0.5 µg or 5 µg of recombinant mouse C5a (HyCult Biotechnology) or vehicle control, which was applied around the vein grafts in pluronic gel (25% wt/vol) directly after surgery (n=7/group). In order to inhibit C5a function, mice were treated daily, starting 1 day prior to surgery, with subcutaneous injections of 0.3mg/kg hydrocinnamate-[OP-(D-Cha)WR] (PMX205)<sup>3</sup> or vehicle solution (propylene glycol: sterile water 3:7, n=7/group). PMX205 displays potent antagonizing activity for the C5a receptor and was synthesized as described previously<sup>4</sup>. To determine the mast cell dependent C5a effects, a group of C5a (5 µg) stimulated mice were treated twice weekly with intraperitoneal injection of the specific mast cell stabilizer, cromolyn (50 mg/kg, Sigma) versus C5a (5µg), cromolyn and PBS treated mice (n=9/group).

#### *Vein graft surgery*

Before surgery and at sacrifice, mice were anesthetized by an intra-peritoneal injection with midazolam (5 mg/kg, Roche), medetomidine (0.5 mg/kg, Orion) and fentanyl (0.05 mg/kg, Janssen). After the surgery mice were antagonized with subcutaneous injection of atipamezol (2.5 mg/kg, Orion), fluminasemid (0.5 mg/kg, Braun) and buprenorphine (0.05 mg/kg, Schering-Plough). The adequacy of the anaesthesia was monitored by keeping track of the breathing frequency and the response to toe pinching of the mice. Vein graft surgery was performed as described previously<sup>5</sup>. In brief, thoracic caval veins from donor littermates were harvested. In recipients, the right carotid artery was dissected and cut in the middle. The artery was everted around the cuffs that were placed at both ends of the artery and ligated with 8.0 sutures. The

caval vein was sleeved over the two cuffs, and ligated. At sacrifice, after 3 minutes of *in vivo* perfusion-fixation, vein grafts were harvested and fixed in 4% formaldehyde, dehydrated and paraffin-embedded for histology or snap frozen for RNA analysis.

#### *RNA isolation, cDNA synthesis and RT-PCR*

Total RNA was isolated from vein grafts harvested on several time points (3-4 vein grafts/time point, 6h, 24h, 3d; 7d; 14d and 28d after surgery). Also, caval veins of donor mice were included. RNA was isolated and cDNA was synthesized as described previously<sup>6</sup>. Intron-spanning polymerase chain reaction (PCR) primer sets were designed using Primer Express 1.5 software (Supplemental table II). Relative mRNA expression levels ( $\Delta C_t$ ) were calculated by subtracting the average cycle threshold ( $C_t$ ) per time point from  $C_t$  value of the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) ( $\Delta C_t = C_{t \text{ target gene}} - C_{t \text{ average housekeeping genes}}$ ). The  $\Delta C_t$  levels at each time point were compared with the mRNA expression levels of C5a and C5aR in the non-transplanted caval veins ( $\Delta \Delta C_t$ ) and a mean fold induction was calculated as  $2^{-\Delta \Delta C_t}$ .

#### *Histological and immunohistochemical assessment of vein grafts*

Six consecutive sections, with 150  $\mu\text{m}$  interspace (30 sections of 5  $\mu\text{m}$  thick), per vessel segment were routinely stained with hematoxylin-phloxine-saffron (HPS). Picrosirius red staining was used to visualize collagen content. Mast cells were visualized with aqueous toluidine blue (Sigma), whilst mast cell activation status was detected with an enzymatic kit (Naphtol-CAE, Sigma). Mast cells were marked as activated when granules could be detected near the mast cells. The following antibodies were used for immunohistochemical stainings; C5a (HyCult Biotechnology) C5aR (CD188, SantaCruz), MAC3 (macrophages, BD-Pharmingen), smooth muscle cell actin (Sigma), fibrin (Quickzyme), CD31 (Endothelial cells, Abcam) and neutrophil (Serotec) as described previously<sup>2,7</sup>. For each antibody, isotype-matched antibodies were used as negative controls and staining was absent in sections incubated with these antibodies (data not shown).

#### *Morphometric analysis of vein grafts*

Vein grafting in normocholesterolemic mice results in vessel wall thickening primarily caused by smooth muscle cell accumulation. Whereas under hypercholesterolemic conditions foam cell accumulation and accelerated atherosclerosis are observed. Since elastic laminae do not exist in these grafts of venous origin, we analyzed the putative vessel wall area (or lesion area) by measuring total vessel area (the area of the vessel within the adventitia) and the lumen area. Next the lesion area was calculated (total vessel area – lumen area). Plaque rupture complications were categorized in three categories<sup>7</sup>. Plaque hemorrhage; when extravasated erythrocytes were found adjacent to neovessels. A dissection was defined as a connection between the lumen and the part of the vessel wall underneath the adventitia filled with fibrin and erythrocytes. In case fibrin was found at the luminal side underneath a denudated endothelial layer, coinciding with erythrocytes and neutrophils, this was defined as erosion with intramural thrombosis. In this model occlusive thrombosis is rarely seen.

Quantitative morphometric analysis (Qwin, Leica) of vein grafts was performed on six HPS stained, equally spaced, sections. (Immuno) histochemical stainings were quantified by computer assisted analysis (Qwin, Leica)<sup>8</sup>. The immuno-positive area measured is expressed as a percentage of the lesion area. Mast cell presence was counted manually in four sections of each vein graft and expressed as number of mast cell per  $\text{mm}^2$  vein graft. Endothelial coverage of the lumen was analyzed in 6 sections per vein graft. For this, sections were attributed to 3 grades; 0 for no endothelial cell coverage, 1 when partially coverage was detected and 2 for full coverage.

### Cell culture

Bone marrow derived mast cells (BMMCs) were cultured as described before<sup>9</sup>. After 4 weeks in culture, BMMCs were degranulated in HEPES-tyrode supplemented with 0.1% fatty acid free BSA (Sigma) with either recombinant mouse C5a (Hycult) or 0.5 mg/mL compound 48/80 (Sigma-Aldrich) for 30 minutes at 37°C. Cells were centrifuged (1,500 rpm, 5 minutes) and the releasate was used for further experiments. Tryptase release was determined as shown previously (DAKO)<sup>2</sup>. To determine release of CCL2, a mouse CCL2 ELISA kit (Biosource Diagnostics) was used according to manufacturer's protocol. Results were plotted as percentage of total release, which was measured in mast cells lysed with 10% Triton-X100 (Sigma), while supernatant of non-degranulated mast cell was used as control (0%).

### Human Vascular Material Analysis

Vein graft (n=8) and endarterectomy (n=25) tissues were obtained in accordance with guidelines set out by the 'Code for Proper Secondary Use of Human Tissue' of the Dutch Federation of Biomedical Scientific Societies (Federa). Samples were collected, paraffin embedded and 5 µm sections were prepared. Antibodies against Tryptase (Dako) C5aR (SantaCruz) and C5a (Abcam) were used to detect mast cells, C5a and C5a Receptor in consecutive sections.

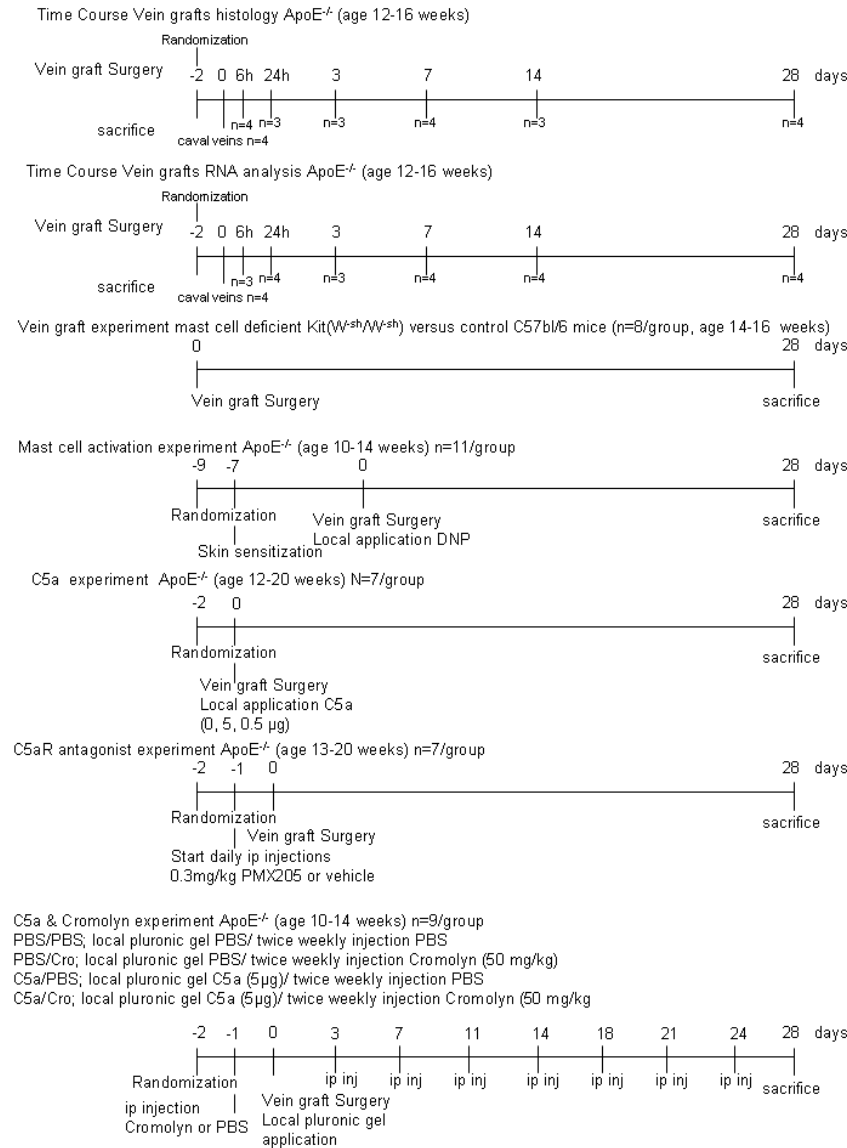
### Statistical Analysis

All data are presented as mean ± SD. Statistical analysis was performed using SPSS 17.0 for Windows. For the time courses, statistical analysis was performed using a repeated measures ANOVA with a Bonferroni Post Hoc test. To determine statistical significance for the *in vivo* experiments, comparisons were made using the non-parametric Kruskal-Wallis test. In case of significance, each group was separately compared to the control group using the Mann-Whitney test. For *in vitro* studies a 2-tailed Student's t-test was used to compare individual groups. Probability-values <0.05 were regarded significant.

**Supplemental Table I.** White Blood Cell analysis (sysmex) of the C5a and cromolyn experiment

Group	Neutrophils (%)	Monocytes (%)	Lymphocytes (%)	Eosinophils (%)
PBS/PBS	22.0 ± 2.3	6.0 ± 0.5	69.7 ± 1.8	2.3 ± 0.2
C5a/PBS	18.6 ± 0.9	5.5 ± 0.3	73.2 ± 0.9	2.7 ± 0.5
PBS/Cro	27.6 ± 2.7	5.7 ± 0.3	64.0 ± 2.6	2.6 ± 0.5
C5a/Cro	24.4 ± 2.4	5.9 ± 0.7	66.9 ± 2.9	2.9 ± 0.8

**Supplemental Figure I.** Detailed flow-chart of the *in vivo* experimental setup



**Supplemental Table II.** Primer sequences of housekeeping gene HPRT, Complement factor C5a, and its receptor, C5aR.

Gene	Forward primer	Reverse primer
HPRT	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
C5a	GGATTCAAGCGCATAATAGCA	ACCCGGATGTTGACTCCTC
C5aR	GCATCCGTCGCTGGTTAC	TGCTGTTATCTATGGGGTCCA

**Supplemental Table III.** Tryptase and CCL2 content of bone marrow derived mast cells (BMMC) releasate after C5a and compound 48/80 stimulation indicated as % of total release. Stimulation of C5a resulted in a release of tryptase comparable to that of the positive control, compound 48/80. Furthermore, C5a stimulation of BMMC led to a dose-dependent release of CCL2.

Compound	Tryptase	CCL2
10 nM C5a	16.4 ± 0.5	1.5 ± 0.9
100 nM C5a	14.4 ± 2.8	9.4 ± 3.2
0.5 µg/mL compound 48/80	16.4 ± 1.5	10.3 ± 3.9

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