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Full Length Article

# CCL5 deficiency reduces neointima formation following arterial injury and thrombosis in apolipoprotein E-deficient mice

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ABSTRACT

Activated platelets secrete different chemokines, among others CCL5, thereby triggering inflammatory cell recruitment into the vessel wall. Here, we investigated how CCL5 deficiency influences vascular remodeling processes. Experiments were performed in apolipoprotein E and CCL5 double deficient (ApoE$^{-/-}$ × CCL5$^{-/-}$) mice, using ApoE$^{-/-}$ × CCL5$^{+/+}$ mice as controls. The ferric chloride model was applied to induce thrombosis at the site of carotid artery injury within minutes and the formation of a smooth muscle cell-rich neointima within 3 weeks. In both groups, vascular injury resulted in thrombus formation. CCL5 deficiency did not alter thrombus resolution examined at day 7. Analysis at 21 days revealed that CCL5 absence was associated with a significant reduction in the neointima area ($p < 0.05$), neointima-to-media ratio ($p < 0.05$) and lumen stenosis ($p < 0.05$) compared to ApoE$^{-/-}$ × CCL5$^{+/+}$ mice. Immunohistochemical analysis of CCL5 receptors showed decreased CCR5 positive staining in ApoE$^{-/-}$ × CCL5$^{-/-}$ mice ($p < 0.01$), whereas the amount of CCR1 ($p = 0.053$) and Mac2-positive macrophages ($p < 0.05$) was increased. The amount of SMA-positive smooth muscle cells was lower in ApoE$^{-/-}$ mice lacking CCL5 ($p < 0.05$). Positive staining for Krüppel-like factor 4 (KLF4), an atheroprotective transcription factor, was increased in the neointima of ApoE$^{-/-}$ × CCL5$^{-/-}$ mice ($p < 0.05$) and found to co-localize with smooth muscle cells. In summary, CCL5 deficiency resulted in reduced neointima formation after carotid artery injury and thrombosis. Hemodynamic and histochemical analyses suggested that this was not due to differences in thrombus formation or resolution. Possibly, the atheroprotective effect of CCL5 deficiency is mediated by KLF4 upregulation in smooth muscle cells.

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## 1. Introduction

Atherosclerosis is a chronic inflammatory disease and its initiation and progression is controlled by different inflammatory mediators [1,2]. Chemokines play a significant role in this process [3]. The formation of an arterial, platelet-rich thrombus, e.g. after vulnerable plaque rupture, is a frequent atherosclerosis complication. Activated platelets are an important source of different chemokines [4].

The chemokine CCL5, also known as Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES), has been implicated in the progression of atherosclerotic disease [5]. Among others, activated platelets contain and release CCL5 [4,6]. Platelet-derived CCL5 is deposited on endothelial cells via interactions with specific chemokine receptors and acts as chemoattractant for monocytes, T-cells and other inflammatory cells, thereby mediating inflammatory cell transmigration into the intima [7,8]. Injection of activated platelets enhances atherosclerotic lesion formation and this was attributed to endothelial deposition of CCL5 and CXCL4 [9].

Administration of the CCL5 receptor antagonist met-RANTES [5,10] or treatment with CCL5 variant [$^{44}$AANA$^{47}$]-RANTES [11] decreased atherosclerotic lesion formation in mice. Moreover, mice lacking the CCL5 receptor CCR5 and apolipoprotein E (ApoE) exhibited a reduced development of diet-induced atherosclerosis [12]. Furthermore, CCR5 deficiency in apolipoprotein E-deficient (ApoE$^{-/-}$) mice significantly reduced neointima formation after arterial wire injury [13]. These data implicate a crucial role of the CCL5/CCR5 signaling pathway in the pathogenesis of atherosclerotic lesion formation. On the other hand, the role of CCL5 in vascular remodeling processes following vascular injury and thrombus formation has not been directly examined. In the present study, we investigated how CCL5 deficiency in ApoE$^{-/-}$ mice affects neointima formation in response to vascular injury and arterial thrombosis.

## 2. Material and methods

### 2.1. Experimental animals

ApoE$^{-/-}$ mice were crossed with CCL5$^{-/-}$ mice (both C57/BL6 background; Jackson Laboratory, Bar Harbor, ME, USA) in order to

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generate ApoE$^{-/-}$ × CCL5$^{-/-}$ mice. For all experiments, age-matched male ApoE$^{-/-}$ × CCL5$^{-/-}$ mice were examined and compared to ApoE$^{-/-}$ × CCL5$^{+/+}$ mice as controls. Mice were fed standard rodent chow throughout the study (no. V1534-0; ssniff, Soest, Germany). All animal experimental procedures were approved by local authorities (application no. 11-0398) and complied with the national and institutional guidelines for the care and use of laboratory animals. The genotype was confirmed by polymerase chain reaction of tail snip DNA.

### 2.2. Blood analyses

Blood of mice was obtained at tissue harvest by cardiac puncture. Serum cholesterol and triglyceride levels were determined at the Department of Clinical Chemistry, University Medical Center Göttingen. Differential blood cell characterization was performed with an automated cell counter (CELL-DYN Sapphire; Abbott Diagnostics, Lake Forest, IL, USA). Results were confirmed by manual counting.

### 2.3. Vascular injury model

Mice were anesthetized with isoflurane. The left carotid artery was carefully dissected and subsequently injured by placement of a 0.5 × 1.0 mm strip of filter paper soaked in 10% ferric chloride ($FeCl_3$) solution onto the adventitia for 3 min, as described [14]. At the time of vascular injury, atherosclerotic plaques were not visible at the site of injury, i.e. the common carotid artery, neither in ApoE$^{-/-}$ × CCL5$^{+/+}$ nor in ApoE$^{-/-}$ × CCL5$^{-/-}$ mice. This intervention induces the formation of an arterial, platelet-rich thrombus. In hypercholesterolemic mice, remodeling processes within 21 days at the site of carotid injury lead to the generation of a distinct smooth muscle cell-rich neointima with typical atherosclerotic plaque features, such as oxLDL-positive macrophages [15].

### 2.4. Hemodynamic measurements

Carotid blood flow was monitored using a Doppler ultrasound flow probe (0.5 VB) connected with a flow meter (model T106; Transonic Systems, Ithaca, NY, USA). Flow monitoring was started immediately before $FeCl_3$ application and continued for further 20 min after vascular injury. Occlusive thrombosis was defined as blood flow < 0.2 ml/min. The thrombotic response was classified as 'stable occlusion' (= complete flow cessation), 'unstable occlusion' (= rapid increase(s) of blood flow by >0.2 ml/min), and 'no occlusion' (= absence of complete flow cessation). The time to first thrombotic occlusion was defined as the time interval between the end of the $FeCl_3$ exposure and the first cessation of carotid artery blood flow. The flow-time product estimates the total blood volume that passed through the carotid artery after vascular injury. It was calculated by averaging the carotid artery flow over each minute of measurement and multiplying this value by the duration of flow monitoring. Data were analyzed by a computerized data acquisition program (WinDaq Lite, DATAQ Instruments, Akron, OH, USA) [16].

### 2.5. Tissue harvest and processing

Anesthetized animals were perfusion-fixed with 4% zinc formalin at 7 days (for thrombus analysis) or 21 days (for neointima analysis) following vascular injury. The left injured carotid artery segment was excised and paraffin-embedded. The contralateral uninjured artery was used as control. Paraffin blocks were sectioned at 5 μm thickness for histological studies.

### 2.6. Morphometric analyses

For thrombus analyses, paraffin sections were stained with Masson's trichrome reagent. For evaluation of neointima formation, sections were stained with Verhoeff's elastica. Three to five sections equally spaced throughout the injured arterial segment were evaluated, and the results averaged for each animal. Pictures were analyzed using an image analysis software program (Image-Pro® Plus; MediaCybernetics, Rockville, USA).

### 2.7. Immunohistochemical analyses

For immunohistochemical analyses of neointima formation, the following primary antibodies were employed: rabbit anti-mouse CCR1 (abcam, Cambridge, UK), rabbit anti-mouse CCR5 (Assay Biotechnology, Sunnyvale, CA, USA), rabbit anti-mouse Krüppel-like factor 4 (KLF4; abcam), mouse anti-mouse smooth muscle actin (SMA; abcam) and rat anti-mouse Mac2 (Cedarlane, Burlington, ON, Canada). After incubation with the primary antibody, a peroxidase-conjugated secondary antibody (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) was applied, followed by avidin-biotin-complex (Vector Laboratories, Burlingame, CA, USA) and 3-amino-9-ethylcarbazole chromogen (Invitrogen). Sections were counterstained with Gill's hematoxylin (ThermoFisher Scientific). Negative controls were generated by incubating slides with immunoglobulins of the same species at the same concentration. Stained tissue sections were preserved in ImmuMount ™ mounting medium (ThermoFisher Scientific) before inspection on an Olympus BX51 microscope (Tokyo, Japan). The (immuno)positive area was determined using computerized image analysis software (Image-Pro® Plus, MediaCyberbetics) and expressed as percentage of total tissue area. For each analysis, 3–5 randomly selected images (at 200-fold magnification) were evaluated, and the results averaged.

In order to determine which cell type primarily expressed KLF4, neointima sections were stained with a rabbit anti-mouse antibody against KLF4 (abcam), followed by a MFP488-labeled secondary antibody (MoBiTec, Göttingen, Germany), and antibodies against CD31 (SantaCruz Biotechnology, Dallas, TX, USA), SMA (abcam) or Mac2 (BIOZOL, Eching, Germany), respectively, followed by Alexa Fluor® 555-labeled secondary antibodies (abcam). Cell nuclei were counterstained using 4′,6-diamidino-2-phenylindole (DAPI; Carl Roth, Karlsruhe, Germany). Images were visualized with an Olympus BX51 microscope.

### 2.8. Statistical analysis

Continuous variables are expressed as mean ± standard error of the mean (SEM) or as median and quartiles (25th percentile; 75th percentile), if data were not normally distributed, as determined using the modified Kolmogorov-Smirnov test (Lilliefors test). According to its results, either the unpaired Student's *t*-test or the non-parametric Mann-Whitney *U* test was applied. Qualitative variables were tested by the chi-squared test. A probability (p) value < 0.05 was considered as statistically significant. Calculations were performed using GRAPHPAD PRISM 5 software (GraphPad Software, La Jolla, CA, USA).

## 3. Results

To examine the importance of CCL5 during vascular remodeling processes following arterial thrombosis, vascular injury at the common carotid artery was induced in male ApoE$^{-/-}$ × CCL5$^{+/+}$ (median age, 60 [59; 63] days) and ApoE$^{-/-}$ × CCL5$^{-/-}$ (median age, 59 [58; 62] days; p = not significant) mice fed standard rodent chow using 10% $FeCl_3$. At the time of injury, ApoE$^{-/-}$ × CCL5$^{+/+}$ and ApoE$^{-/-}$ × CCL5$^{-/-}$ mice did not differ with regard to their body weight (26.2 [24.2; 27.5] vs. 26.1 [24.1; 26.9] g). Also, we did not observe any significant differences between ApoE$^{-/-}$ × CCL5$^{+/+}$ and ApoE$^{-/-}$ × CCL5$^{-/-}$ mice concerning their serum cholesterol (331 ± 15.7 vs. 318 ± 27.0 mg/dl) and triglyceride (120 [92.3; 236] vs. 147 [110; 159] mg/dl) levels. Erythrocyte (7.9 ± 0.68 vs. 7.6 ± 0.27 × $10^6$/μl), thrombocyte (460 ± 48.4 vs.

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