

Importance of junctional adhesion molecule-C for neointimal hyperplasia and monocyte recruitment in atherosclerosis-prone mice-brief report

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# Supplement Material

#### **Material and Methods**

### H33 antibody and specificity

The anti-JAM-C antibody (clone H33, full name CRAM-13H33) was generated by Aurrand-Lions et al.<sup>1</sup>. The specificity of H33 binding selectively to JAM-C was tested in the murine squamous cell carcinoma cell line KLN205 mock-transfected or stably transfected with a JAM-C<sup>EGFP</sup> expressing fusion vector<sup>2</sup>. The molecular weight of the fusion protein containing JAM-C (30 kDa) and EGFP (40 kDa) is approximately 70 kDa. In conjunction with EGFP, flow cytometry confirmed the detection of a specific signal for JAM-C by H33 in comparison to isotype control on JAM-C<sup>EGFP</sup>-expressing cells, while an antibody to integrin  $a_V$  (RMV 7) produced a staining in both mock and JAM-C<sup>EGFP</sup>-transfected cells serving as a positive control; antibody binding was detected using an Phycoerythrin-conjugated anti-rat secondary antibody (Jackson ImmunoResearch, Ref #112-116-143) (Online Data Supplement Figure 1A). A specific band at the expected size of the fusion protein containing JAM-C (70 kDa) was in addition revealed by H33 in lysates of JAM-C<sup>EGFP</sup> but not mock-transfected KLN205 cells by Western Blotting after immunoprecipitation of JAM-C, performed as described<sup>1</sup>, while JAM-C could not be detected using anti-integrin or isotype control antibodies (Online Data Supplement Figure 1B). Furthermore, FACS analyses revealed a specific staining for JAM-C using H33 on primary lung microvascular endothelial cells (LMECs) isolated from wild type  $(Jam-C^{+/+})$  but not JAM-C-deficient  $(Jam-C^{-/-})$  mice<sup>3-5</sup> compared to control antibodies directed against L-selectin (Mel14, rat IgG2a). Staining for CD31 (GC51, rat IgG2b) on both Jam- $C^{+/+}$ 

and *Jam-C<sup>-/-</sup>* LMECs mice in comparison to isotype matched control antibodies directed against Mac-1 (M1/70, rat IgG2b) served as a positive control; primary antibody binding was detected using an Phycoerythrin-conjugated secondary anti-rat antibody (Online Data Supplement Figure 1C). Together, these data confirm binding of H33 specifically to JAM-C. In addition, H33 was shown to block JAM-B/JAM-C interactions<sup>5</sup>, angiogenesis and leukocyte homing to tumors<sup>4</sup>.

#### Mouse model of carotid artery injury

Female, 8 week old  $Apoe^{-t}$  mice (C57Bl/6 background) were fed an atherogenic diet containing 21% fat starting 1 week before transluminal carotid artery injury induced by a 0.014 inch angioplasty guide-wire in anesthetized mice (100 mg/kg ketamine/ 10 mg/ kg xylacine, *i.p.*)<sup>6</sup>. Mice received *i.p.* injections of monoclonal antibody against JAM-C (H33, 50 µg/mouse, 3/week) or isotype control antibody (rat IgG2a) starting with 2 injections peri-operatively. After 1 day or 3 weeks, arteries were harvested by *in situ* perfusion fixation with 4% paraformaldehyde and embedded in paraffin. Animal experiments were approved by local authorities and complied with the German animal protection law.

### Morphometry, immunohistochemistry and immunofluoresence stainings

Serial sections (5 $\mu$ m, 250-500 $\mu$ m from the bifurcation) stained with Movats pentachrome were analyzed by planimetry. The relative macrophage and SMC content was determined by antibody staining for Mac-3 (#550292, BD Pharmingen) detected by alkaline-phosphatase enzyme (Vector Laboratories) and counter-staining by Mayers haemalaun and  $\alpha$ -smooth muscle actin (1A4; Dako) detected by FITC-conjugated secondary antibody (Jackson ImmunoResearch). For double immunofluorescence stainings, primary affinity purified rabbit anti-JAM-C antibody (Nr. 526, provided by Prof. Imhof), anti-SM  $\alpha$ -actin ( $\alpha$ -SMA), anti-Pselectin (clone C-20, Santa-Cruz Biotechnology), anti-CD31 (M-20, Santa-Cruz Biotechnology), or appropriate isotype control antibodies were detected by secondary antirabbit Cy-3 and anti-mouse- or anti-goat FITC-antibodies (Jackson ImmunoResearch). Cell nuclei were counterstained with DAPI (Vectashield, Vector Labs). Images were visualized using a Leica DMLB microscope (Leica Microsystems, Wetzlar) and 10x0.30 numeric aperture or 40x0.75 Leica objectives. Images were captured using a JVC digital camera KY-F70B 3-CCD (Vitor, Tokyo) and processed using Diskus software.

### Cell culture and flow adhesion assay

Human coronary artery SMCs (Promocell), MonoMac6 cells and human umbilical vein endothelial cells (HUVECs) were maintained as described.<sup>6,7</sup> MonoMac6 cell adhesion to SMC was analyzed in parallel wall flow-chambers<sup>6</sup>. SMCs expressed smooth muscle specific  $\alpha$ -actin but not vWF and similarly to vWF<sup>+</sup> HUVECs displayed a strong expression of JAM-C, as analyzed by RT-PCR (see Online Data Supplement Figure 2). Confluent SMCs were activated by oxLDL (10 µg/ml, overnight) and pre-treated with/without H33 or isotype control (10 µg/ml, 30 min). MonoMac6 cells (0.5x10<sup>6</sup>/mL) were pre-treated with/without anti-Mac-1 antibody (CBRM1/29, 20 µg/ml) or isotype control (mouse IgG1) and perfused over SMC monolayers (1.5 dyn/cm<sup>2</sup>) in the continuing presence of the antibodies. The number of cells firmly adherent to SMCs was quantified by analysis of images recorded with a 3CCD video camera and recorder (JVC, Wayne, NJ).

### **Reverse-transcriptase and real time PCR**

For RNA analysis, total RNA was isolated from HUVECs and SMCs, reverse-transcribed into cDNA using Mo-MLV RT (Invitrogen). RT-PCR was performed using specific primer pairs (hJAM-C forward: GAA GCC AGT GAC CCC TGT CTG TAG AG, reverse: CAT CTG GTT TCC CTG GGT TCT TG; hcalponin forward: GCA GAT GGG CAC CAA CAA AGG AGC, reverse: CCT TGG GGG GAA AAC AGG GAA GG; hSM α-actin forward: GAT GGC TTT GGG CAG CTT GGC AG, reverse: GAG GCA GGC TAA GCG GGA TGG; hvWF forward: GTT GTG GGA GAT GTT TGC CTA CG, reverse: GAG AAC CTC ATG GTA CAC AAC AGA GC). Products were separated by agarose gel electrophoresis. The expression levels of the target genes were in addition quantified by real time PCR analysis using the QuantiTect SYBR-Green PCR kit (Opticon MJ Research) and specific primer pairs (Jam-C, Hs00230289\_m1; 18sRNA, Hs99999901\_s1, Applied biosystems).

# Platelet isolation and flow cytometric analysis

Platelet-rich-plasma was prepared from 8 week old *Apoe<sup>-/-</sup>* mice and platelets were isolated by centrifugation at 1250 g. Platelets were left untreated or incubated with thrombin (1 U/mL, 30 min, 37°C), and reacted with saturating concentrations of affinity purified anti-JAM-C antibody (rabbit anti-mouse, Nr. 526) and detected with FITC-conjugated secondary Ab and stained with anti-CD41-PE (BD Biosciences). Probes were analyzed using a BD FACSAria System (BD Biosciences) and experiments were analyzed via FlowJo Software (Tree Star).

# Ex vivo perfusion and intravital microscopy of carotid arteries

Carotid arteries of  $Apoe^{-/-}$  mice were isolated for *ex vivo* perfusion or intravital microscopy<sup>8</sup> one day after wire-injury. After transfer onto a microscope stage, arteries were perfused with

MOPS-buffered physiological salt solution (4  $\mu$ L/min), and after pre-incubation with H33 or isotype control antibodies (10  $\mu$ g/ml, 30 min), perfused with calcein-AM labeled MonoMac6 cells (10<sup>6</sup>/mL, Molecular Probes) for 8 min. For intravital microscopy, rhodamin-6G (Molecular Probes) was administered *i.v.* to *Apoe<sup>-/-</sup>* mice injected with H33 or isotype control (10  $\mu$ g/ml, 30 min before and after wire-injury) to label circulating leukocytes, and injured common carotid arteries were exposed. Leukocyte arrest to arteries was analyzed by epifluorescence microscopy (Zeiss Axiotech, x20 water immersion).

### **Statistical Analysis**

Data represent mean±SEM and were analyzed by 2-tailed Student's t-test using GraphPad

Prism (InStat software; GraphPad).

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### **Online Data Supplement Figure 1**

The murine squamous cell carcinoma cell line KLN205 was mock transfected or stably transfected with a JAM-C<sup>EGFP</sup> expressing vector (**A**,**B**). Flow cytometric analysis was performed to detect EGFP in conjunction with JAM-C by H33 or integrin  $a_V$  by RMV 7 in comparison to isotype matched control antibodies. Representative dot plots are shown (**A**). JAM-C was immunoprecipitated from cell lysates and Western Blotting was performed, revealing a specific signal at the expected size of JAM-C (70 kDa) using H33 in JAM-C<sup>EGFP</sup> but not mock-transfected KLN205, while no signal for JAM-C could be detected using integrin  $a_V$  or isotype control antibodies. The band at about 75 kDa represents unspecific binding (**B**). Flow cytometry of primary lung microvascular endothelial cells (LMECs) revealed the expression of JAM-C by H33 (black line) on *Jam-C*<sup>+/+</sup> LMECs but not *Jam-C*<sup>-/-</sup> mice, while CD31 (black line) could be detected on *Jam-C*<sup>+/+</sup> and *Jam-C*<sup>-/-</sup> LMECs mice in comparison to the respective isotype matched control antibodies (filled grey profile). Representative histograms are shown (**C**).



# **Online Data Supplement Figure 2**

HUVECs and SMCs display a robust expression of JAM-C mRNA (459 base pair product), as evaluated by RT-PCR analysis. In addition, hCASMCs show a strong mRNA expression of the smooth muscle cell markers SM  $\alpha$ -actin (358 base pairs) and calponin (471 base pairs) but not of the endothelial cell marker von Willebrand factor (vWF, 479 base pair). In contrast, HUVECs show no transcription of SM  $\alpha$ -actin or calponin but a strong expression of vWF.



# **Online Data Supplement Figure 3**

One day after injury, double-immunofluorescence staining revealed the accumulation and aggregation of P-selectin<sup>+</sup> platelets in some injured segments of  $Apoe^{-/-}$  carotid arteries (green), while no co-staining with JAM-C (red) could be detected; cell nuclei were stained by DAPI (blue, **A**). Flow cytometry of mouse platelets did not reveal any surface expression of JAM-C on unstimulated (control) or thrombin-activated mouse platelets (**B**).