Materials and Methods

Microbubble synthesis and labeling

Poly(n-butyl cyanoacrylate) (PBCA) MBs were synthesized as described previously\(^1,\)\(^2\). For functionalization, MBs were coated with streptavidin (12 μg in 10mM sodium acetate/2x10\(^8\) MBs) then mixed with biotinylated anti-JAM-A antibodies (clone H202-106, AbD Serotec, 10μg/2x10\(^7\) MBs). After 10 minutes incubation, conjugated MBs were separated from excess antibody by 30 minutes flotation, then re-suspended in 50 μL HEPES/Triton buffer (pH 7.0) and prepared for injection. Prior to the intravenous injection, 50 μL 0.9% saline solution was added to the MB mix.

Atherogenic murine model of endothelial dysfunction and arterial remodeling

A total number of 43 animals were included in this study. All animal experiments and study protocols were approved by local authorities and complied with the European Convention of Animal Protection law (LANUV AZ: 84.02.04.2012.A161). Flow-induced endothelial dysfunction followed by accelerated atherosclerosis was induced in a mouse model of carotid partial-ligation (PL). Eight-week-old female ApoE\(^-/-\) mice were fed an atherogenic diet (Altromin, Germany) 1 week before and 1 to 4 weeks after the partial ligation. Herein, three out of four branches of the left carotid artery, the left external and internal carotid arterial branches, and occipital artery, were surgically isolated and ligated, diverting the blood flow solely through the superior thyroid artery. The neck incision was closed by suture under aseptic conditions as described previously\(^3\). The endothelial dysfunction and plaque development was analyzed 1, 2, 3, and 4 weeks after the intervention.

In vivo molecular and functional ultrasound imaging

Mice were investigated by functional and molecular ultrasound imaging at different time points after the intervention (1, 2, 3, 4 weeks (n=3-5 / time point)). Imaging was performed under anesthesia with 1% isoflurane having the mice placed on a heated table (Vevo Mouse Handling Table)\(^2,\)\(^4\). In vivo functional ultrasound imaging was performed at first. B-, M- and PW-mode images of both the injured carotids and the contralateral arteries were recorded using a 40 MHz ultrasound probe on the Vevo770 device (Visualsonics, Toronto). Carotid arteries were imaged in the long axis, having the bifurcation in the field of view. M-mode images were used to quantify the arterial wall thickness. PW-data mode were recorded covering the whole common carotid enabling the assessment of blood flow at i) the bifurcation, ii) arteries’ origin and iii) in between. Subsequently, analysis was performed for all 3 sites and mean values are reported. In vivo molecular ultrasound measurements were performed as previously described\(^2,\)\(^4\). A dose of 2x10\(^7\) JAM-A targeted or control non-targeted MBs was injected intravenously via the cannulated tail vein under ultrasound control (4% transmitted power, mechanical index 0.036). For competitive binding studies at 2 weeks post-partial ligation, a 20-fold
higher concentration of free anti-JAM-A antibody (200 μg) was injected intravenously to each mouse 10 minutes before JAM-A targeted microbubbles (MB\textsubscript{JAM-A}) administration, to block JAM-A binding sites. In all experiments, successful MB injection was confirmed by assessing an imaging sequence of 60 seconds (10 frames/second) during the injection. Seven minutes after MB injection, molecular ultrasound imaging in contrast mode with a sequence of 60 seconds (10 frames/second) was performed (Supplemental Figure I A) to quantify the amount of bound MBs in the imaged area. To prevent MB destruction, the first third of the sequence recorded images at a low transducer output power of 4%. Subsequently, a destructive pulse (100% power) destroyed all MBs in the imaged area. The remaining imaging sequence was acquired at 4% power. Image analysis was performed using the software provided by the vendor. Region curves for the areas of interest were generated and postprocessed by subtracting the mean value of grayscale intensity for several post-destructive frames (blood and circulating microbubbles) from the mean value of several pre-destructive frames (blood, circulating and retained microbubbles)\textsuperscript{5}. The resulting grayscale intensity difference represents the amount of signal generated by retained MBs (Supplemental Figure I B). Results were then reported as % in signal decrease after MB destruction.

**Immunofluorescence**

At the end of each experiment, mice (n=4 / time point) were euthanized by intraperitoneal overdose (400 mg/kg Ketamine and 40 mg/kg Xylazine) injection of anesthetic, followed by terminal eye bleeding. Intracardial perfusion with PBS was performed to remove the remaining blood. Then, the common carotid arteries (6–7 mm) were excised, embedded in Tissue-Tek O.C.T. (4583, Sakura) and snap-frozen. Serial tissue sections (5 μm) starting with the bifurcation were obtained from isolated carotids and stained for JAM-A (CD321, clone H202-106, 1mg/ml, dilution 1:100, Serotec) and PECAM-1 (sc-1506, 200μg/ml, dilution 1:50, Santa Cruz Biotechnology) expression. Control isotype staining was performed by using normal Rat IgG (sc-2026, 400μg/ml, dilution 1:40, Santa Cruz Biotechnology), and normal Goat IgG (sc-2028, 400μg/ml, dilution 1:100, Santa Cruz Biotechnology), respectively (Supplemental Figure II). Fluorescein streptavidin (SA-5001, 1mg/ml, dilution 1:33, Vector Laboratories), and Cy3 (705-165-147, 500μg/400μl, dilution 1:300, Jackson ImmunoResearch) conjugated secondary antibodies , respectively, were used for visualization. Sections were analyzed using Diskus software (Hilgers, Germany).

**Detection of serum markers**

Quantification of cytokines IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17A, IFN-γ, TNF-α and GM-CSF in murine plasma samples (n=20, n=4 / time point) with 1:2 sample dilution was conducted using the LUNARIS Mouse 12-Plex Cytokine Kit from AYOXXA Biosystems (Cat. No. LMC-20121S). The assay was performed according to the manufacturers’ instructions.
**Quantitative Real-Time PCR**

The carotid artery tissues were collected before the intervention and 2 weeks post-partial ligation (n=3/time point) and processed for RNA extraction using RNeasy mini kit (QIAGEN). 3000 ng RNA per sample was reverse transcribed to cDNA using QuantiTect reverse transcription kit (QIAGEN), according to the manufacturer's instructions. The 3000 ng of resulting cDNA per sample was then analyzed using QuantiTect SYBR green PCR kit (QIAGEN) on DNA Engine OPTICON 2 continuous fluorescence detector, following the manufacturer's protocol. The mouse JAM-A forward primer was 5'-CAA GGC AAG GGT TCG GTG TA -3', and the reverse primer was 5'-GAA CTT CCA CTC CAC TCG GG -3', while the IL-6 forward primer 5' GCT ACC AAA CTG GAT ATA ATC AGG A -3', and the reverse primer 5'-CCA GGT AGC TAT GGT ACT CCA GAA -3' were used. Expression was assessed in relation to a housekeeping gene (HPRT1) using the comparative (2^-ΔΔCT) method.

**In vitro flow chamber**

Human aortic endothelial cells were grown and maintained in endothelial cell growth medium II, supplemented with 10% (v/v) heat inactivated fetal bovine serum, endothelial cell growth supplement, and 1% (v/v) gentamicin at 37°C in a 5% CO₂ humidified atmosphere. All reagents were purchased from Promocell.

A total of 100 µL of human aortic endothelial cells were seeded (1×10^6 cells/mL) on collagen-coated (Collagen G 4mg/ml, dilution 1:100, Merck Millipore) 35-mm Petri dishes. The cells were incubated with oxidized-LDL (10 µg/ml, Merck Millipore) for 16 hours before MB exposure. The cells were incubated with SYTO 41 (dilution 1:1000; Invitrogen) for 45 minutes and then washed with 1× PBS. The 35-mm Petri dishes were mounted into a customized parallel-wall flow chamber in a custom silicon tube closed perfusion system (standard silicon tubing, 0.76 mm inner diameter; Helixmark). Using a peristaltic pump (Gilson Inc), a continuous flow rate of 0.25 mL/min was set. A total of 1×10^7 MB_{JAM-A} or MB_{CTR} were injected into the perfusion system, and a closed loop circulation was performed for 10 minutes followed by an open loop washing step with 1× PBS for 5 minutes. For competitive binding studies, cultured human aortic endothelial cells were incubated with 20 µg free anti-JAM-A antibody for 1 hour at 37°C, followed by a 10-minute washing step using 1× PBS before MB_{JAM-A} perfusion to block JAM-A binding sites. Thirty minutes before MB flow, the cell membrane was stained using wheat germ agglutinin Alexa Fluor 488 (W11261, dilution 1:500, Thermo Fisher). After MB flow, the viable cells nuclei were stained with Hoechst (H1399, dilution 1:500, Thermo Fisher) for 5 minutes. Fluorescence images were obtained subsequently using an Axio Imager M2 fluorescence microscope (Carl Zeiss AG, Germany).
**Statistical analysis**

Differences between groups of *ex vivo* and *in vivo* MB adhesion assays were analyzed using 1-way ANOVA followed by Newman-Keuls post-hoc test. P-values of <0.05 were considered significant. Error bars shown on graphs represent the standard deviation. Statistical analysis was performed using GraphPad Prism 6.1 (GraphPad Software).

**Supplemental References**