Supplementary information

The anti-inflammatory peptide Catestatin blocks chemotaxis

Elke M. Muntjewerff[1,2], Kristel Parv[3], Sushil K. Mahata[4,5], N. Koen van Riessen[1], Mia Phillipson[3,6], Gustaf Christoffersson[3,6*], Geert van den Bogaart[1,2*]

[1] Department of Tumor Immunology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, the Netherlands

[2] Department of Molecular Immunology and Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, the Netherlands.

[3] Department of Medical Cell biology, Uppsala University, Uppsala, Sweden

[4] VA San Diego Healthcare System and

[5] Department of Medicine, University of California San Diego, La Jolla, California, USA

[6] Science for Life Laboratory, Uppsala University, Uppsala, Sweden

*** Correspondence:**
Geert van den Bogaart: g.van.den.bogaart@rug.nl

Gustaf Christoffersson: gustaf.christoffersson@scilifelab.uu.se

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1. Material and Methods

1.1 Animals and human bloods samples

Male and female C57BL/6J (Taconic, Denmark) and *Cx₃cr1*^{GFP} (1) mice weighing 20-26 g were used. All animal experiments were approved by the Regional Animal Ethics committee in Uppsala, Sweden. For the mouse transwell assays, female C57BL/6J mice (Charles River, USA) were used and approved by the Regional Animal Ethics committee in Nijmegen, the Netherlands (protocol: 2015-0019TIL-067).

1.2 Cells and culture conditions

The research with human blood samples at the Department of Tumor Immunology complies with all institutional and national ethics regulations and has been approved by the ethics committee of Sanquin blood bank. All blood donors were informed of the research and have granted their consent. Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) obtained from buffy coats of healthy individuals as described (2). Briefly, buffy coats were centrifuged at 2,100 rpm for 20 minutes at room temperature (RT) in tubes containing density grading media (Ficoll). The peripheral blood mononuclear cells (PMBCs) present in the supernatant were removed by a series of washing steps (cold PBS + 1 mM EDTA + 10% BSA). This was followed by LS column magnetic antibody cell sorting (MACS) of the $CD14^+$ population using Miltenyi Biotech CD14 human microbeads. After the isolation, the $CD14^+$ monocytes were centrifuged (5 minutes at 2,100 rpm) and resuspended in RPMI-medium (Thermo Fisher Scientific). Cells were used immediately or frozen and stored in liquid nitrogen.

1.3 Gradientech assay

A CellDirector 2D device (Gradientech) was coated with bovine serum overnight at 37°C. Monocytes were activated with LPS for 1 h, washed with PBS, and seeded in the device in 200 µl RPMI-1640 medium. After one hour at 37°C, the two supplied syringes with 1 ml of RPMI-1640 medium, with one containing 5 µM CST and/or 0.5 nM CCL2 (300-04, PeproTech) were attached to the CellDirector and a flow rate of 5 µl/min was applied. Monocyte movement was visualized with an Axiovert 200 M microscope with a 5× objective (Zeiss, Jena, Germany). Movies were recorded at 2 frames/min for 3 hours. Cell movement was analysed using the Tracking Tool PRO software (Gradientech).

1.4 Human transwell migration assay

Human monocytes were pre-incubated with medium, 5 µM CST or 100 ng/ml LPS for 30 min. followed by PBS washes. Afterwards 250,000 cells were seeded in the top compartment of a 6.5 mm Transwell with 5.0 µm pore Polycarbonate Membrane Insert (3421, Corning). The lower compartment contained 550 µl complete RPMI-1640 medium with 50 ng/ml CCL2 (300-04, PeproTech), 50 ng/ml IL-8 (200-08M, PeproTech), 50 ng/ml N-Formylmethionyl-leucyl-phenylalanine (fMLP; F3506, Sigma-Aldrich) and/or 5 µM CST (previously titrated, Fig. S1). After three hours, the cells in the lower compartment were collected and washed with PBS. The upper compartment was washed with PBS, followed by trypsin treatment for 5 min at 37°C to release cells that attached to the transwell insert (Fig. S6A). After neutralizing the trypsin with complete RPMI-1640 medium, the cells were washed with PBS. Cells were counted with the flow cytometer (MACSQuant).

1.5 *Mouse transwell migration assay*

Mouse legs were harvested and bone marrow cells were isolated. Briefly, legs were cleaned and were flushed to obtain bone marrow cells. Afterwards, bone marrow cells were treated with ACK lysis buffer to remove red blood cells. After PBS washes, the cells were layered on a density grading media gradient (from bottom to top: 81%, 68%, 52% ficol) and spun down for 25 min. (RT, 700 rcf, no brake). Monocytes and granulocytes were collected together in the layer between 81% and 68%. Cells were washed with media and 100,000 cells were added to the top compartment of a 6.5 mm Transwell with 3.0 µm pore Polycarbonate Membrane Insert (3415, Corning). The lower compartment contained a microscopy glass and 550 µl complete RPMI-1640 medium with 50 ng/ml CCL2, IL-8, fMLP and/or 5 µM CST. After three hours, the upper wells were removed and the plate was spin down (1,000 rcf, 3 min.) to ensure cell attachment to the glass slide. Cells were washed with PBS, fixed in ethanol and stained for hematoxylin and eosin (HE). Total migrated cells were counted and the HE stain was used to discriminate granulocytes and monocytes based on nuclear morphology.

1.6 Cremaster muscle imaging

Monocyte (CD115-mAb, 135520, Biolegend) and granulocyte (Ly6G-mAb, 127626, Biolegend) migration was imaged in the cremaster muscle of mice superfused with pre-warmed (37°C) bicarbonate-buffered saline solution (pH 7.4) (3) containing CST (5 µM) and/or CXCL2/MIP-2 (0.5 nM) (250-15, PeproTech). A bright-field intravital microscope (Leica DM5000B) with a 25×/0.6W (Leica) objective and connected to an Orca R2 camera (Hamamatsu; Volocity acquisition software) was used to record movies of five minutes at 0, 30, 60, 90 min after chemokine addition. Venules with diameter range of 20–30 µm were imaged. Movies were analysed using ImageJ and corrected using the Hyperstackreg ImageJ macro. For rolling flux, all cells rolling in the vessel were counted. For rolling speed, velocity over a 100 µm section of the vessel was analysed. In the same 100 µm section, cells were considered adherent if they remained stationary for at least 3 min.

1.7 Pancreatic islet isolation and culture

Islets were isolated from C57BL/6 mice as described previously (4). Briefly, the mice were terminated using cervical dislocation, followed by injection of ice-cold collagenase A from *Clostridium histolyticum* (2.5 mg/ml) (Roche) in Hank's balanced salt solution (HBSS) into the pancreas via the common bile duct. Following pancreas removal, it was incubated at 37°C in a water bath for 18 minutes. Islets were separated from the exocrine fraction using density-gradient centrifugation (Histopaque 1077 and GlutaMAX RPMI1640 (Gibco)). Thereafter islets were handpicked and maintained in the islet culture medium (GlutaMAX RPMI1640 (Gibco) with added D-glucose (11.1 mM), FBS (10%) and penicillin-streptomycin solution (#L0022, Biowest)) overnight at 37°C and 5% CO_2.

1.8 Aortic ring assay with pancreatic islet culture

Aortic ring isolation was carried out as previously described (5). Briefly, 13-16-week-old *Cx3cr1*^{GFP} mice were euthanized, followed by dissection of the thoracic aorta. Under a stereo-microscope, extraneous fat, tissue, and branching vessels were carefully removed, and perfused with serum-free OptiMEM medium (Thermo Fisher) with penicillin-streptomycin solution. The aorta was sectioned into 1 mm thick rings. After overnight starvation in serum-free Opti-MEM medium, rings were embedded in 1 mg/ml rat tail collagen I (#ALX-522-435-0100,

Enzo Life sciences) adjacent to pancreatic islets (2-5 islets per ring), which were isolated from C57BL/6 mice as described in section 1.7, in 8 well Nunc Lab-Tek II microscope chambers (Thermo Fisher). After 1 h, embedded rings were cultured with 300 µl of OptiMEM with 2.5% FBS, 11.1 mM glucose, penicillin-streptomycin, M-CSF (40 ng/ml) to stimulate CX_3CR1^{GFP+} macrophage survival and 5 µM CST for six days. On day six, rings were imaged using a Zeiss LSM700 (Carl Zeiss) confocal microscope.

1.9 Aortic ring assay quantification

The numbers of CX_3CR1^{GFP+} cells were quantified using the image analysis software Imaris (Bitplane). The location of the CX_3CR1^{GFP+} cells was determined using the Surface Center of Mass Position to Spots object plugin after manually defining the aorta. Afterwards, the fraction of CX_3CR1^{+} cells on either side of the center of mass was determined. For analyzing angiogenesis, staining with anti-CD31 antibody conjugated to Alexa Fluor 647 (#102515, Biolegend) was carried out prior to imaging. Aortic rings that did not show any sprouting were excluded from further analysis. Vessels were analyzed using Fiji image analysis software (6). Sprouts that originated directly from the ring endothelium were considered main sprouts, and branches as divarications from main sprouts.

1.10 Statistical data analysis

Data are expressed as mean ± SEM. One-way ANOVA with Bonferroni post-hoc tests or non-parametric Mann-Whitney test were applied for multiple comparisons. Outliers were identified using ROUT test (Q=1%). A p value < 0.05 was considered statistically significant.

References material and methods

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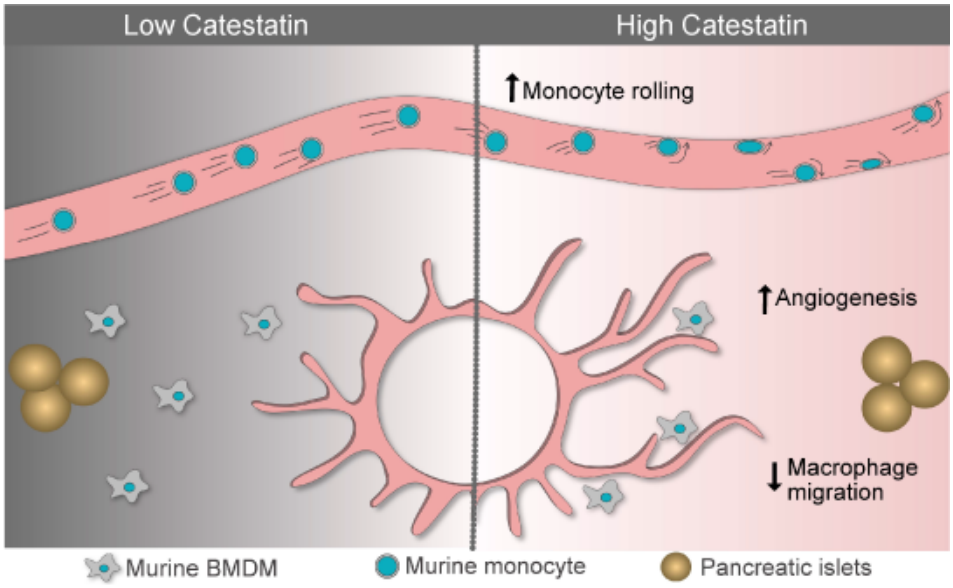
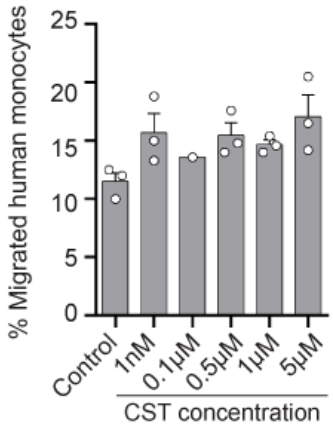
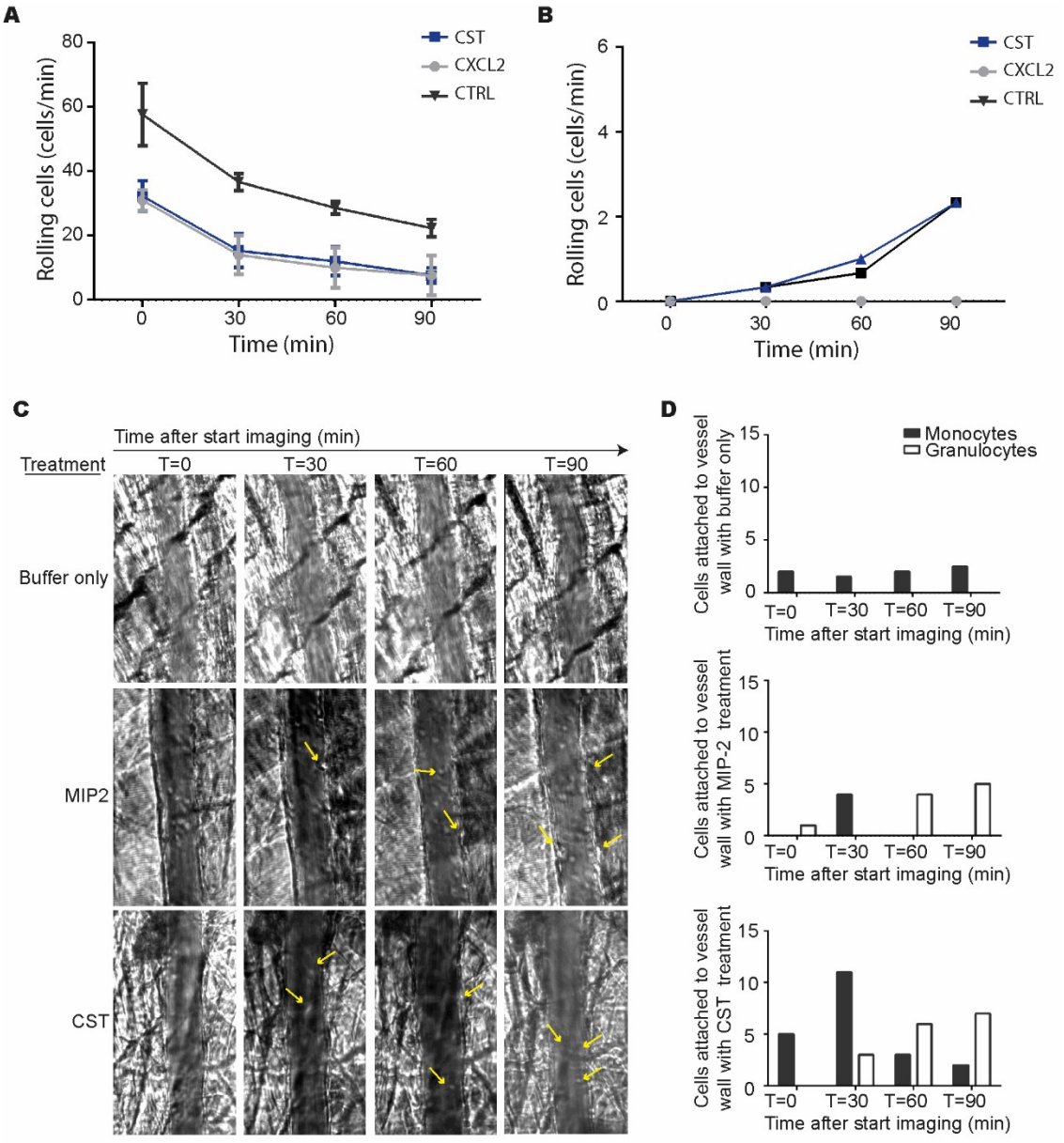


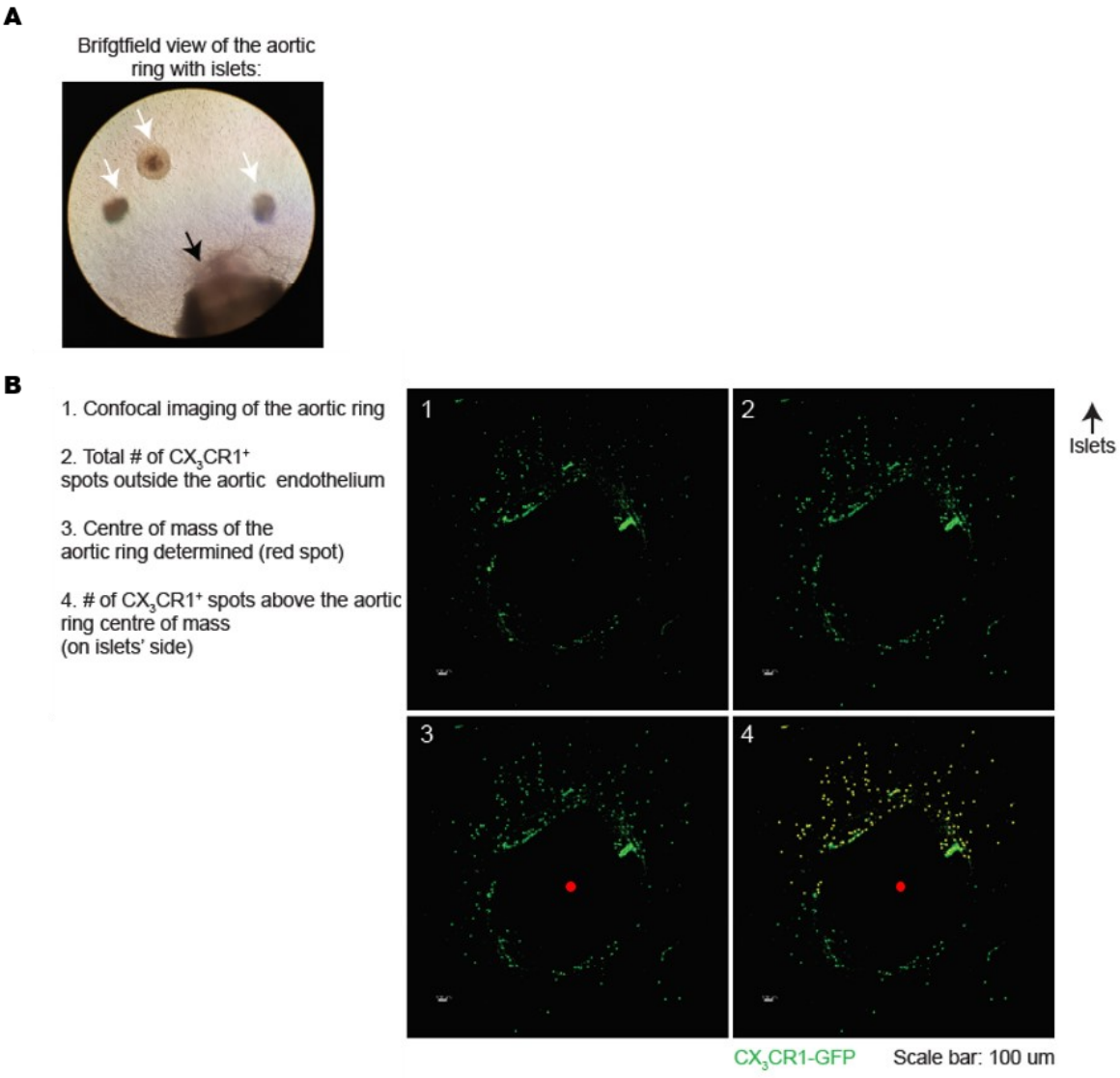
Graphical abstract: Catestatin blocks monocyte and granulocyte migration towards inflammatory chemokines.



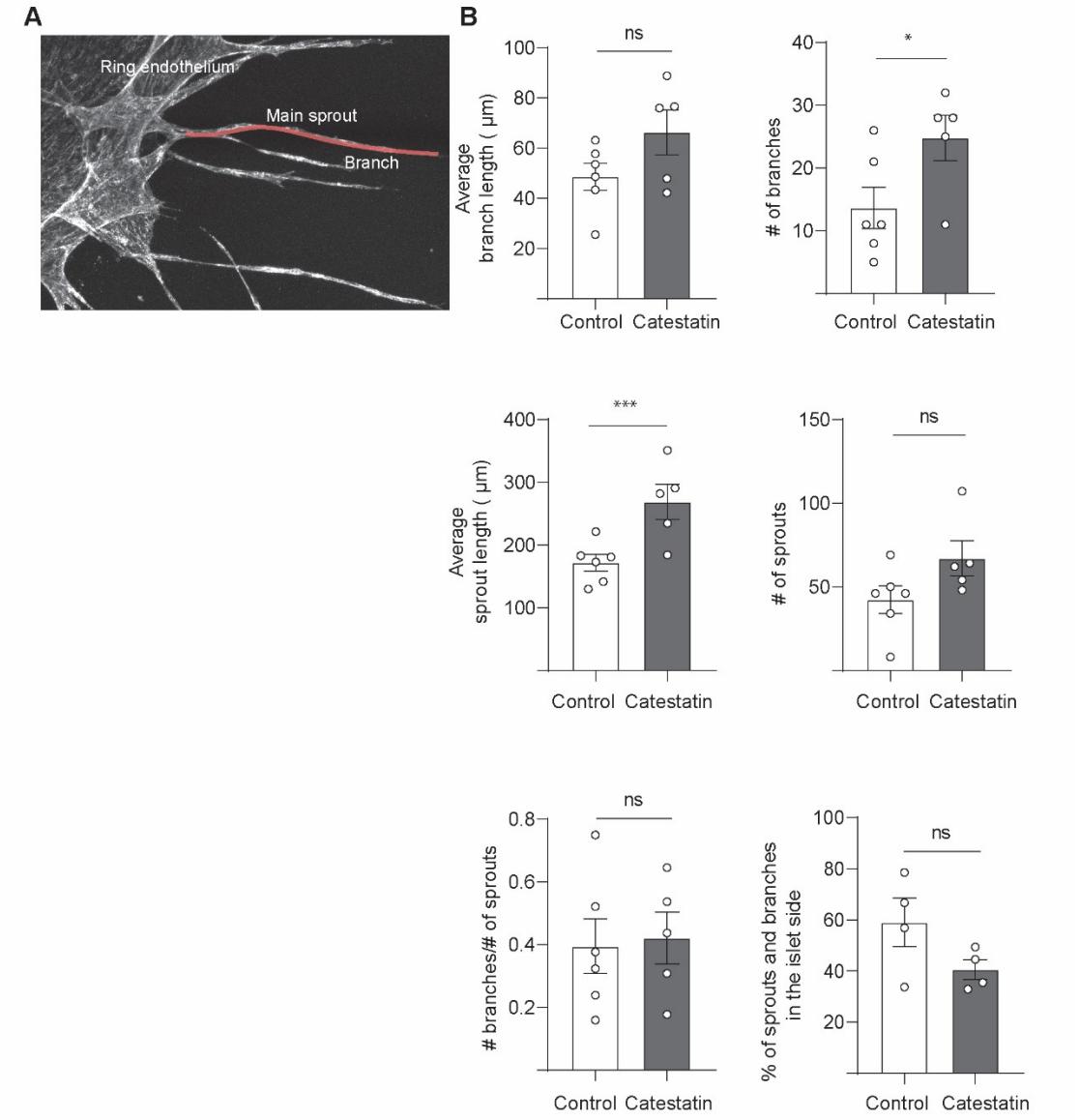
Sup. Fig. 1: Migration of human monocytes towards various CST concentrations. Percentages of migrated human monocytes towards lower compartment of the transwell assay filled with only medium or medium containing the indicated CST concentrations. Datapoints show individual donors.



Sup. Fig. 2: Attachment of granulocytes and monocytes to vessel wall. (A) Venules of the cremaster muscle were overflown with bicarbonate-buffered saline buffer (buffer only control), the chemoattractant MIP-2, or CST as shown in main Fig. 1D-F. Graph shows quantification of rolling cells (cells/min). **(B)** Quantification of cell in tissue. **(C)** Representative brightfield snapshots of *in vivo* cremaster muscle imaging as in main Fig. 1D-F. **(D)** Quantification of adherent granulocytes (visualized by Ly6G-mAb, main Fig. 1F) and monocytes (brightfield, panel C) after 0, 30, 60 and 90 minutes (N=1-2).

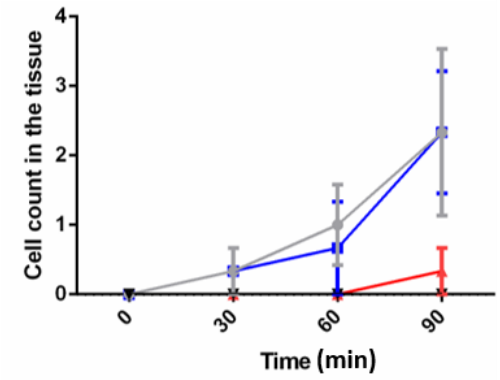


Sup. Fig. 3: Quantification of CX3XR1^{+} cell movement in the aortic ring model. (A) Brightfield image of the aortic ring with islets. **(B)** Description of CX3CR1-cell movement quantification by determination of total amount outside the aortic ring (endothelium), center of mass (red spot) and the islet side (black arrow).



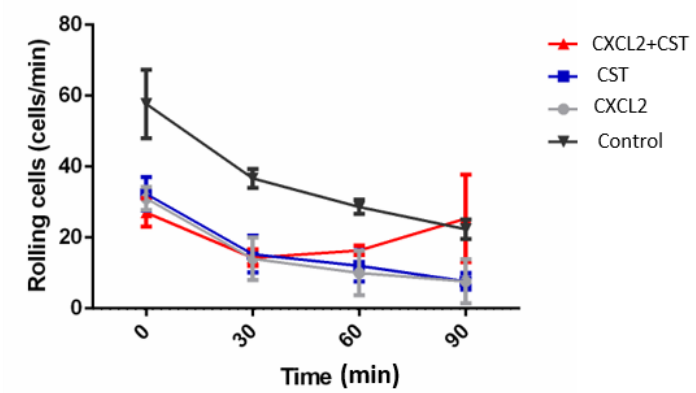
Sup. Fig. 4: Branches and sprouts in the aortic ring assay. (A) Representative images of angiogenesis quantification of main figure 2C-D. The images show the ring endothelium, main sprout (red), and branch (gray). **(B)** Quantification of total number of sprouts and branches separately and their lengths (N=5-6). Mann-Whitney test *: P<0.05; ***P<0.001; ns: not significant.

A



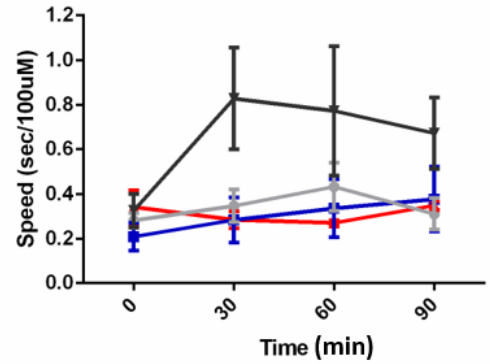
CTRL vs.	CXCL2+CST	CST	CXCL2
T=0	Ns	ns	Ns
T=30	Ns	ns	ns
T=60	Ns	ns	ns
T=90	Ns	**	**

B



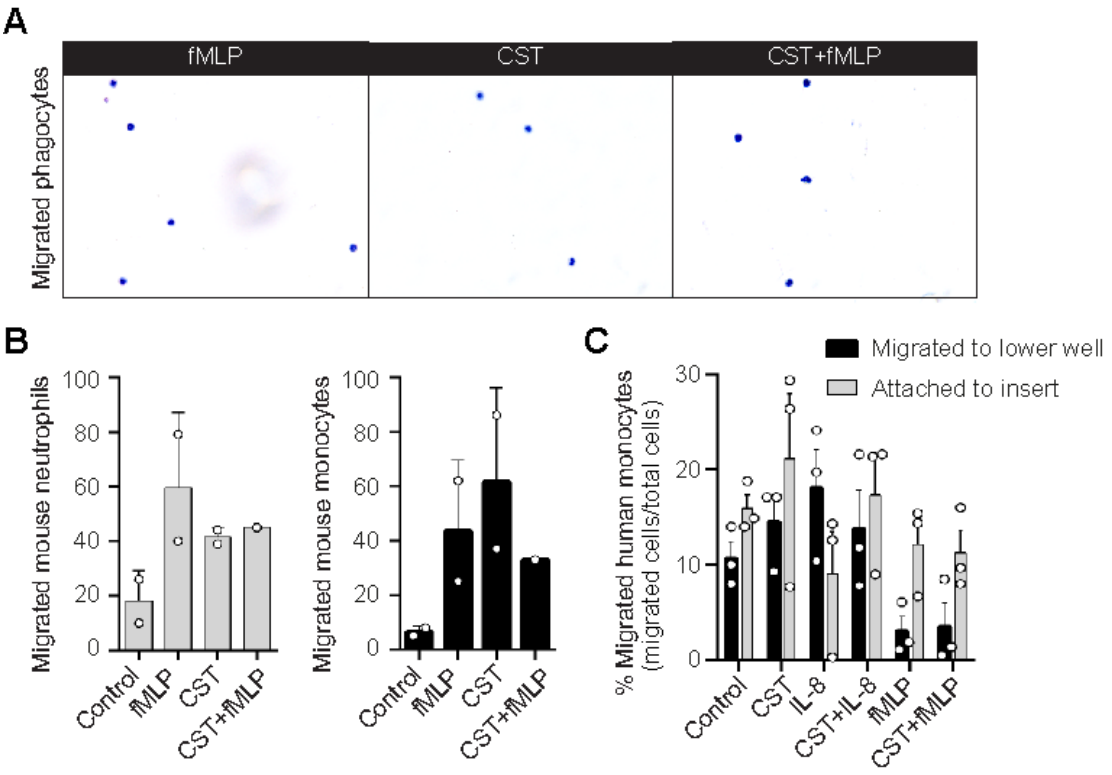
CTRL vs.	CXCL2+CST	CST	CXCL2
T=0	Ns	ns	Ns
T=30	**	**	**
T=60	Ns	*	**
T=90	Ns	*	*

C

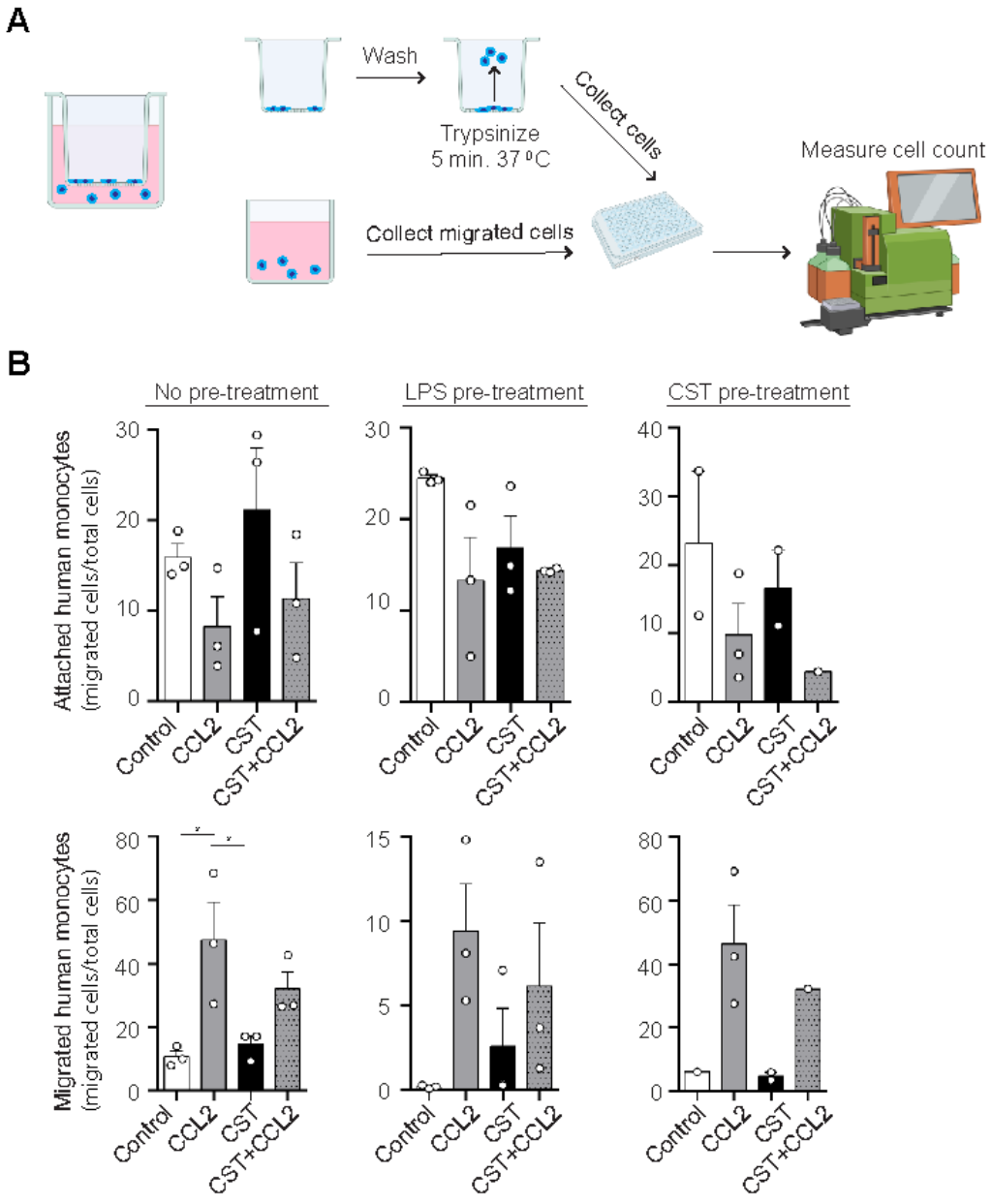


CTRL vs.	CXCL2+CST	CST	CXCL2
T=0	ns	ns	Ns
T=30	****	****	****
T=60	****	***	***
T=90	**	**	**

Sup. Fig. 5: The combination of CST and MIP-2 reduces chemotaxis. Venules of the cremaster muscle were overflown with bicarbonate-buffered saline buffer (buffer only control), the chemoattractant MIP-2 or CST, as shown in main Fig. 1D. Graph shows quantification of tissue migration **(A)**, rolling cells (cells/min) **(B)** and velocity **(C)** upon stimulation with CST, MIP-2 or stimulation with both (N=3, two-way ANOVA) *: P<0.05; **: P<0.01; ***P<0.001; ****P<0.0001; ns: not significant.



Sup. Fig. 6: Migration of phagocytes towards fMLP and IL-8. (A) Representative HE images of migrated phagocytes towards fMLP, CST and CST+fMLP. **(B)** Graphs displaying migrated mouse granulocytes (left) and monocytes (right) towards lower compartment of the transwell assay filled with only medium or medium containing fMLP, CST or both. **(C)** Percentages of attached (grey) and migrated (black) human monocytes towards lower compartment of the transwell assay filled with only medium or medium containing only CST, fMLP, IL-8 or combined with CST.



Sup. 7: Human monocyte migration and attachment after pre-treatment with LPS or CST. (A) Set-up of transwell assay for collection of migrated and attached cells. Cells were seeded in the upper well. After 3 hours the upper well was removed and cells were trypsinized after PBS washes. Cells from the bottom part were collected, washed and all cells were seeded in a 96 wells plate for measurement on the MacsQuant. Image created with Biorender **(B)** Graphs displaying human monocyte attachment (up) and migration (below) towards CCL2, CST and CCL2+CST after pre-treatment with buffer, LPS or CST for 30 minutes.