

# The anti-inflammatory peptide Catestatin blocks chemotaxis

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15 **Chemotaxis<sup>6</sup>, Migration<sup>7</sup>**

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18 **Abstract**

19 Increased levels of the anti-inflammatory peptide catestatin (CST), a cleavage product of the  
20 pro-hormone chromogranin A, correlates with less severe outcomes in hypertension, colitis and  
21 diabetes. However, it is unknown how CST reduces the infiltration of monocytes and  
22 macrophages in inflamed tissues. Here, we report that CST blocks leukocyte migration towards  
23 inflammatory chemokines. By *in vitro* and *in vivo* migration assays, we show that although CST  
24 itself is weakly chemotactic, it blocks migration of monocytes and granulocytes to  
25 inflammatory attracting factor CC-chemokine ligand 2 (CCL2) and macrophage inflammatory  
26 protein 2 (MIP-2). Moreover, it directs CX<sub>3</sub>CR1<sup>+</sup> macrophages away from pancreatic islets.  
27 These findings support the emerging notion that CST is a key anti-inflammatory modulator.  
28

29 **1. Introduction**

30  
31 As an immunological response to inflammation, monocytes, granulocytes and leukocytes  
32 are attracted to inflamed tissues by chemokines such as CC-chemokine ligand 2 (CCL2,  
33 a.k.a. MCP-1) and macrophage inflammatory protein 2 (MIP-2, a.k.a. CXCL2) (1).  
34 However, to avoid an excessive response, leukocyte infiltration should be halted for  
35 resolution of inflammation, but the mechanisms that govern this are unknown (2). Here, we  
36 addressed the potential chemotactic effect of chromogranin A (CgA)-derived peptide  
37 Catestatin (CST: hCgA<sub>352-372</sub>) (3). While CST circulates at low nM range, the local  
38 concentrations were detected in the  $\mu$ M range in mouse tissues (3–6).

39 Being an anti-inflammatory peptide, CST reduces inflammation in cardiac and chronic  
40 inflammatory diseases (3,7–9). Despite the chemotactic effects of CST (7,10,11),  
41 administration of exogenous CST reduces monocyte and macrophage infiltration in the  
42 liver, heart and gut in mouse models of type II diabetes, hypertension, atherosclerosis and  
43 colitis (4,7,8,12,13). In a colitis model, CST also reduced granulocyte infiltration in the  
44 colon (8). In line with this, the adrenal gland, heart, and gut of CST knockout mice display  
45 increased macrophage infiltration (4,7,12). In this study, we show that while CST itself is  
46 weakly chemotactic, it blocks the extravasation and migration of phagocytes both *in vitro*  
47 and *in vivo*. Thus, the anti-inflammatory effects of CST are partly the result of redirecting  
48 monocytes and granulocytes away from the inflammation sites.  
49

## 50 **2. Methods; experimental procedures**

### 51 *2.1 Animals and human bloods samples*

52 Male and female C57BL/6J (Taconic, Denmark) and *Cx3cr1<sup>GFP</sup>* (14) mice weighing 20-26  
53 g were used. All animal experiments were approved by the Regional Animal Ethics  
54 committee in Uppsala, Sweden. The research with human blood samples at the Department  
55 of Tumor Immunology complies with all institutional and national ethics regulations and  
56 has been approved by the ethics committee of Sanquin blood bank. All blood donors were  
57 informed of the research and have granted their consent.  
58

### 59 *2.2 Gradientech assay*

60 A CellDirector 2D device (Gradientech) was coated with bovine serum overnight. Human  
61 peripheral blood monocytes were isolated from buffy coats of healthy donors as described  
62 (15), followed by human microbead CD14<sup>+</sup> isolation of monocytes according to  
63 manufactures' instructions (130-050-201, Milteny Biotec). Monocytes were activated with  
64 LPS for 1 h, washed with PBS, and seeded in the device in 200  $\mu$ l RPMI-1640 medium.  
65 After one hour at 37°C, the two supplied syringes with 1 ml of RPMI-1640 medium, with  
66 one containing 5  $\mu$ M CST were attached to the CellDirector and a flow rate of 5  $\mu$ l/min was  
67 applied. Monocyte movement was visualized with an Axiovert 200 M microscope with a  
68 5x objective (Zeiss, Jena, Germany). Movies were recorded at 2 frames/min for 3 hours.  
69 Cell movement was analysed using the Tracking Tool PRO software (Gradientech). 0.5 nM  
70 CCL2 (300-04, PeproTech) was used as a positive control.  
71

### 72 *2.3 Cremaster muscle imaging*

73 Monocyte and granulocyte (Ly6G-mAb) migration was imaged in the cremaster muscle of  
74 mice superfused with pre-warmed (37°C) bicarbonate-buffered saline solution (pH 7.4) (16)  
75 containing CST (5  $\mu$ M) and/or MIP-2/CXCL2 (0.5 nM) (250-15, PeproTech) was used as  
76 a positive control. A bright-field intravital microscope (Leica DM5000B) with a 25 $\times$ /0.6W  
77 (Leica) objective and connected to an Orca R2 camera (Hamamatsu; Volocity acquisition  
78 software) was used to record movies of five minutes at 0, 30, 60, 90 min after cytokine  
79 addition. Venules with diameter range of 20-30  $\mu$ m were imaged. Movies were analysed  
80 using ImageJ and corrected using the Hyperstackreg ImageJ macro. For rolling flux, all  
81 cells rolling in the vessel were counted. For rolling speed, velocity over a 100  $\mu$ m section  
82 of the vessel was analysed. In the same 100  $\mu$ m section, cells were considered adherent if  
83 they remained stationary for at least 3 min.  
84

### 85 *2.4 Aortic ring assay with pancreatic islet culture*

86 Aortic ring isolation was carried out as previously described (17). Briefly, 13-16-week-old  
87 *Cx3cr1<sup>GFP</sup>* mice were euthanized, followed by dissection of the thoracic aorta. Under a  
88 stereo-microscope, extraneous fat, tissue, and branching vessels were carefully removed,

89 and perfused with serum-free OptiMEM medium (Thermo Fisher) with penicillin-  
90 streptomycin solution. The aorta was sectioned into 1 mm thick rings. After overnight  
91 starvation in serum-free Opti-MEM medium, rings were embedded in 1 mg/ml rat tail  
92 collagen I (#ALX-522-435-0100, Enzo Life sciences) adjacent to pancreatic islets (2-5 islets  
93 per ring), which were isolated from C57BL/6 mice as described before (18), in 8 well Nunc  
94 Lab-Tek II microscope chambers (Thermo Fisher). After 1 h, embedded rings were cultured  
95 with 300  $\mu$ l of OptiMEM with 2.5% FBS, 11.1 mM glucose, penicillin-streptomycin, M-  
96 CSF (40 ng/ml) to stimulate CX<sub>3</sub>CR1<sup>GFP+</sup> macrophage survival and 5  $\mu$ M CST for six days.  
97 On day six, rings were imaged using a Zeiss LSM700 (Carl Zeiss) confocal microscope.  
98 The numbers of CX<sub>3</sub>CR1<sup>GFP+</sup> cells were quantified using the image analysis software Imaris  
99 (Bitplane). The location of the CX<sub>3</sub>CR1<sup>GFP+</sup> cells was determined using the Surface Center  
100 of Mass Position to Spots object plugin after manually defining the aorta. For analyzing  
101 angiogenesis, staining with anti-CD31 antibody conjugated to Alexa Fluor 647 (#102515,  
102 Biologend) was carried out prior to imaging. Aortic rings that did not show any sprouting  
103 were excluded from further analysis. Vessels were analyzed using Fiji image analysis  
104 software (19). Sprouts that originated directly from the ring endothelium were considered  
105 main sprouts, and branches as divarications from main sprouts.  
106

### 107 2.5 Statistical data analysis

108 Data are expressed as mean  $\pm$  SEM. One-way ANOVA with Bonferroni post-hoc tests or  
109 non-parametric Mann-Whitney test were applied for multiple comparisons. Outliers were  
110 identified using ROUT test (Q=1%). A value of  $p < 0.05$  was considered statistically  
111 significant.  
112

### 113 3. Results & Discussion

114 Although human blood monocytes migrated towards a high (but physiological)  
115 concentration of CST (5  $\mu$ M), this was less efficient compared to the canonical  
116 inflammatory chemokine CCL2 (0.5 nM) (Fig. 1A-C), reinforcing a weak chemoattractive  
117 effect of CST (7,10,11). To confirm this *in vivo*, we performed imaging of the cremaster  
118 muscle (Fig. 1D) (16). Upon perfusion of the muscle with CST (5  $\mu$ M), phagocytes  
119 (monocytes and granulocytes) decreased their speed and attached to the vessel wall with  
120 similar efficiency as of the inflammatory chemotactic agent MIP-2 (0.5 nM) (Fig. 1D-F,  
121 Fig. S1). Thus, both our *in vivo* and *in vitro* migration assays show that CST is weakly  
122 chemotactic, raising the question how CST can reduce monocyte and granulocyte  
123 infiltration in inflamed tissues such as the liver (diet induced obese mice), intestine (colitis  
124 model), heart (hypertension model) and atheromatous plaques (atherosclerosis model)  
125 (4,7,8,12,13).  
126

127 To address how CST affects macrophage chemotaxis to inflamed tissues, we used the aortic  
128 ring vessel model (17) (Fig. 2A), which is based on the co-embedding of part of the aorta  
129 of *Cx3cr1<sup>+gfp</sup>* transgenic mice adjacent to isolated pancreatic islets (20). These islets secrete  
130 chemokines, such as vascular endothelial growth factor (VEGF)-A, resulting in the  
131 directional macrophage migration from the aortic ring as well as vessel growth towards the  
132 pancreatic islets. Migration of CX<sub>3</sub>CR1<sup>+</sup> macrophages from the aortic ring was visualized  
133 by fluorescence microscopy (19) (Fig. 2B, S2). As expected, the CX<sub>3</sub>CR1-macrophages  
134 moved towards the pancreatic islets in absence of CST (Fig 2B). However, perfusing the  
135 aortic ring with CST (5  $\mu$ M) resulted in a lower number of CX<sub>3</sub>CR1<sup>+GFP</sup> macrophages  
136 migrating towards the pancreatic islets (Fig 2B), indicating that CST blocked directional  
137 migration. Interestingly, we also observed that CST is pro-angiogenic, as it increased both

138 the amount and length of the sprouts and branches emanating from the aortic rings (Fig. 2C-  
139 D, S3).

140

141 The loss of directional cell migration to the pancreatic islets might be caused by blockage  
142 of chemokine-induced cell migration by CST. To investigate this possibility, we performed  
143 intravital imaging of the cremaster muscle, but this time for CST in combination with MIP-  
144 2. This resulted in the inverse effect compared to CST or MIP-2 alone: release of attached  
145 cells from the vessel wall and reduced migration of cells into the tissue (Fig. 2E, S4),  
146 indicating that despite being weakly chemotactic, CST blocks MIP-2 elicited phagocyte  
147 recruitment. To further confirm this, we performed an *in vitro* migration assay, where  
148 human monocytes were stimulated with a gradient of CCL2 in presence of CST (Fig. 2F).  
149 Similar to our findings with the intravital imaging, CST blocked monocyte migration  
150 towards the CCL2.

151

152 Although CST counteracts the chemoattraction by inflammatory cytokines (Fig. 2G), the  
153 question remains open which receptor(s) CST utilize to exert these effects on cell migration.  
154 We speculate that this might be a G-protein coupled receptor (GPCR), since GPCRs are  
155 actively involved in leukocyte migration (21) coupled with expression of GPCRs in all cell  
156 types responsive to catenastatin (e.g. monocytes (10), neutrophils (22,23), macrophages  
157 (4,7,8,12,13), endothelial (13,24) and mast cells (11)), we speculate that CST might act  
158 through this receptor type. We have not only shown how CST reduces the infiltration of  
159 monocytes and macrophages in inflamed tissues (4,7,8,12,13), but offer a possible  
160 mechanistic explanation for the correlation of CST levels with improved disease outcome  
161 in patients suffering from chronic diseases (4–6), reinforcing CST as a therapeutic target  
162 for treatment of diseases associated with chronic inflammation.

163

#### 164 4. Author contributions

165 E.M.M., G.C., S.K.M. and G.v.d.B. designed the study. E.M.M, K.P., G.C. designed and  
166 performed the experiments. E.M.M. and G.v.d.B wrote the manuscript and all authors  
167 participated in discussing and editing of the manuscript.

168

#### 169 5. Disclosure of conflict of interest

170 The authors declare that the research was conducted in the absence of any commercial or  
171 financial relationships that could be construed as a potential conflict of interest.

172

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#### 185 7. References

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272  
273 **8. Figure legends**  
274

275 **Fig. 1: CST is weakly chemotactic.** (A) Scheme showing set-up of Gradientech migration  
276 assay. Two syringes filled with buffer +/- chemoattractant were connected to the device  
277 (green) to create a flow (*x*-direction) and perpendicular (*y*) cytokine gradient. The inset  
278 shows migration of monocytes along the flow and towards the chemoattractant. (B)  
279 Representative tracks of human monocytes showing the *x*- and *y*-movement of individual  
280 cells upon exposure to the indicated buffer, 5  $\mu$ M CST or 0.5 nM CCL2. (C) Quantification  
281 of panel B (N=3). (D) Scheme showing set-up of cremaster muscle imaging in mice to  
282 visualize phagocyte (monocytes and granulocytes) extravasation *in vivo*. (E) Phagocyte  
283 rolling velocity (top) and attachment (bottom) upon overflowing the muscle with buffer  
284 (control, gray), 0.5 nM MIP-2 (blue) or 5  $\mu$ M CST (black) (N=3, two-way ANOVA). (F)  
285 Representative images of granulocyte attachment as visualized by Ly6G-mAb (green) to  
286 the vessel wall upon only buffer, MIP-2 or CST stimulation. \*: P<0.05; \*\*: P<0.01;  
287 \*\*\*P<0.001; ns: not significant.  
288

289 **Fig. 2: CST blocks migration induced by inflammatory chemokines and promotes**  
290 **angiogenesis.** (A) Scheme showing set-up of aortic ring assay. Aortic ring was isolated  
291 from CX<sub>3</sub>CR1-GFP mice and embedded adjacent to pancreatic islets in collagen I. Image  
292 shows islets (blue), CD31 (red) and CX<sub>3</sub>CR1 (green). (B) Representative images of  
293 CX<sub>3</sub>CR1-macrophage migration upon control or CST stimulation of the aortic ring. The  
294 graph shows the percentage of cells above (yellow) the center of mass (N=8). (C)  
295 Representative images of vessels by CD31 (red) upon control or CST stimulation of the  
296 aortic ring. (D) Quantification of angiogenesis. Total number of sprouts and branches (left)  
297 and their length (right) (N=5-6). (E) Cremaster muscle imaging. Phagocyte attachment to  
298 vessel wall upon overflowing the muscle with buffer (control, gray) and buffer with the  
299 chemoattractant MIP-2 (blue), CST (black) or both (red) (N=3, two-way ANOVA). (F)  
300 Gradientech migration assay. Representative *x*- and *y*-movement of human monocytes  
301 exposed to opposite gradients of CST and CCL2 (N=3). (G) Model showing leukocyte  
302 extravasation in presence of low and high concentrations of CST. Mann-Whitney test \*:  
303 P<0.05; \*\*: P<0.01; \*\*\*P<0.001; \*\*\*\*P<0.0001; ns: not significant.  
304

305 **Sup. 1: Attachment of granulocytes and monocytes to vessel wall.** (A) Venules of the  
306 cremaster muscle were overflowed with bicarbonate-buffered saline buffer (buffer only  
307 control), the chemoattractant MIP-2 or CST as shown in main Fig. 1D-F. Graph shows  
308 quantification of rolling cells (cells/min). (B) Quantification of cell in tissue. (C)  
309 Representative brightfield snapshots of *in vivo* cremaster muscle imaging as in main figure  
310 1D-F. (C) Quantification of adherent granulocytes (visualized by Ly6G-mAb, main Fig.  
311 1F) and monocytes (brightfield, panel C) after 0, 30, 60 and 90 minutes (N=1-2).  
312

313 **Sup. 2: Quantification of CX<sub>3</sub>CR1+ cell movement in the aortic ring model.** (A)  
314 Brightfield image of the aortic ring with islets. (B) Description of CX<sub>3</sub>CR1-cell movement  
315 quantification by determination of total amount outside the aortic ring (endothelium), center  
316 of mass (red spot) and the islet side (black arrow).

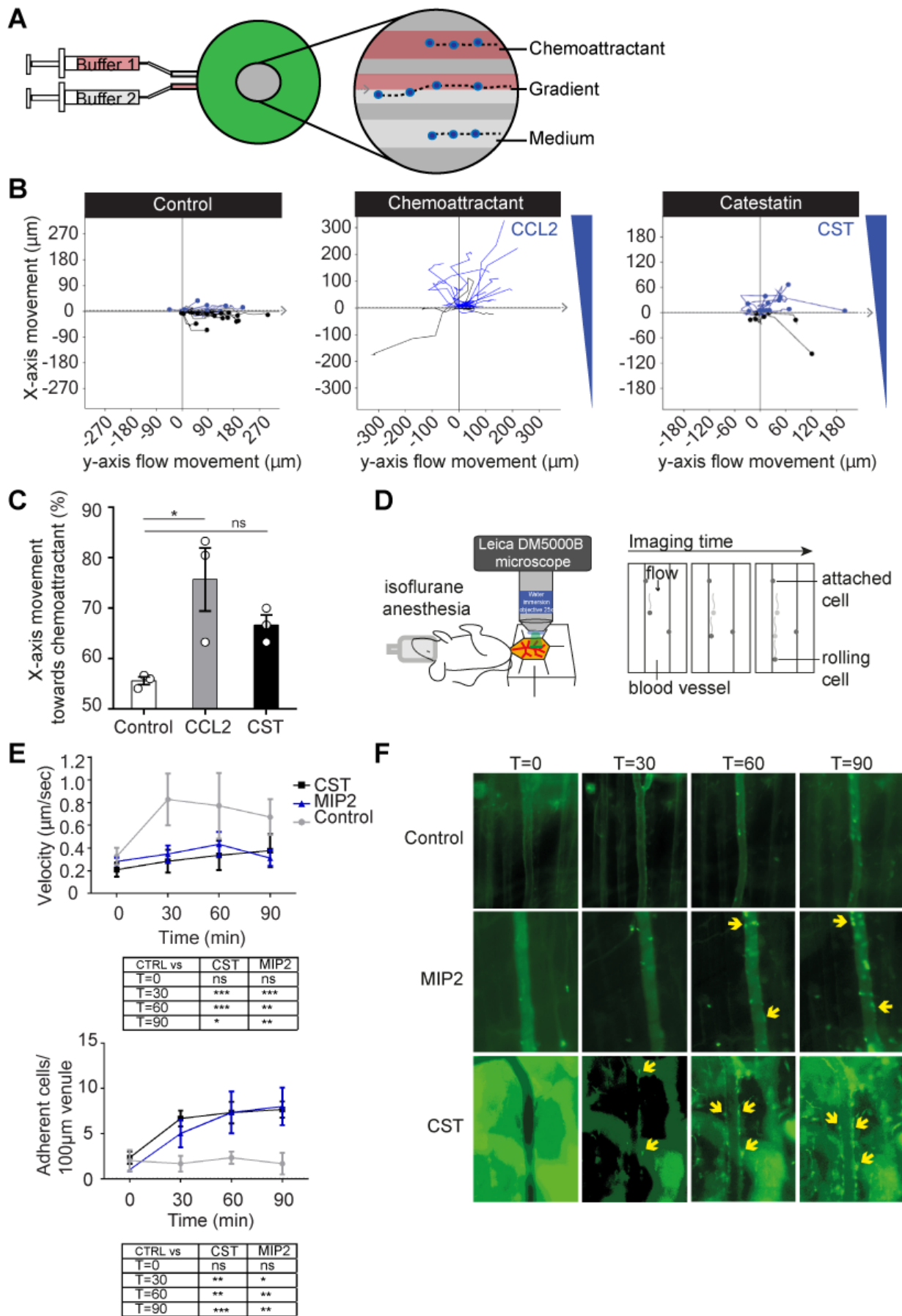
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**Sup. 3: Branches and sprouts in the aortic ring assay.** (A) Representative images of angiogenesis quantification of main figure 2C-D. The images show the ring endothelium, main sprout (red), branch (gray) (B) Quantification of total number of sprouts and branches separately and their length (N=5-6). Mann-Whitney test \*:  $P < 0.05$ ; \*\*\* $P < 0.001$ ; ns: not significant.

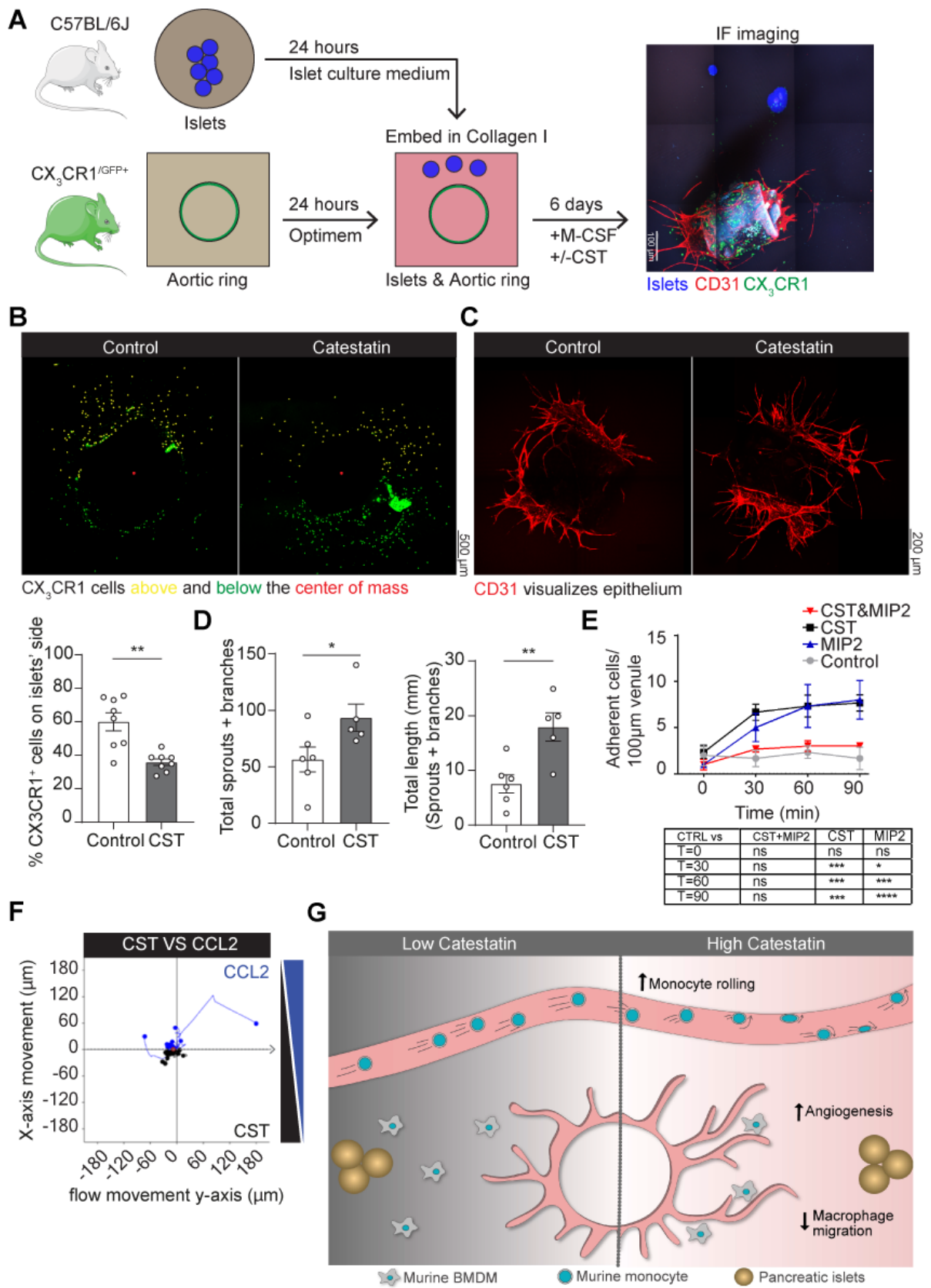
**Sup. 4: The combination of CST and MIP-2 reduced chemotaxis.** Venules of the cremaster muscle were overflowed with bicarbonate-buffered saline buffer (buffer only control), the chemoattractant MIP-2 or CST, as shown in main Fig. 1D. Graph shows quantification of tissue migration (A), rolling cells (cells/min) (B) and velocity (C) upon 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.

## 9. Figures





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**Fig. 2**