The immunosuppression of macrophages underlies the cardioprotective effects of catestatin (CST)

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ABSTRACT

Hypertension (HTN) is a pandemic associated with inflammation and excessive production of catecholamines. Previous work has shown that hypertensive patients have reduced plasma levels of Catestatin (CST), a bioactive cleavage product of the prohormone Chromogranin A (CgA). Similarly, in mouse models, HTN symptoms can be reduced by administration of CST, but the role of CST in the regulation of cardiovascular function is unknown. In the present study, we generated mice with knockout (KO) of the region of the CgA gene coding for CST (CST-KO) and found that CST-KO mice are not only hypertensive as predicted, but also display left ventricular hypertrophy, have marked macrophage infiltration of the heart and adrenal gland, and have elevated levels of pro-inflammatory cytokines and catecholamines. Additionally, intraperitoneal injection with CST reverses these phenotypes, and ischemic pre-conditioning-induced cardioprotection was also abolished in CST-KO mice. To further explore the relationship between HTN and CST/macrophages, experiments with chlodronate depletion of macrophages and bone-marrow transfer showed that macrophages produce CST and that the anti-hypertensive effects of CST are mediated in part via CST's immunosuppression of macrophages as a form of feedback inhibition. The data thus implicate CST as a key autocrine attenuator of the cardiac inflammation in HTN by reducing macrophage inflammation.

53 **ABBREVIATIONS**
54

55 BMDM: bone marrow-derived macrophage;
56 BP: blood pressure;
57 BMT: bone marrow transfer;
58 CCL: C-C motif chemokine ligand
59 CXC: C-X-C motif chemokine ligand
60 CDN: chlodronate;
61 CgA: chromogranin A;
62 CST: catestatin;
63 DA: dopamine;
64 EPI: epinephrine;
65 HTN: hypertension;
66 IFN: interferon;
67 IL: interleukin;
68 IPC: ischemic preconditioning;
69 IR: ischemia/reperfusion;
70 KO: knockout;
71 LV: left ventricular;
72 LVDP: LV developed pressure;
73 LVEDP: LV end diastolic pressure;
74 LVPWd: LV posterior wall thickness;
75 MAP: mean arterial pressure;
76 IVSd: interventricular septum wall thickness;
77 NE: norepinephrine;
78 SBP: systolic blood pressure;
79 TEM: transmission electron microscopy;
80 TNF: tumor necrosis factor;
81 WT: wild-type;
82

INTRODUCTION

Hypertension (HTN) is an important risk factor for cardiovascular disease and mortality [1]. The burden of HTN and the estimated HTN-associated deaths have increased substantially over the past 25 years. The immune system is well recognized for the genesis and progression of HTN [2, 3] . Elevated levels of pro- and reduced levels of anti-inflammatory cytokines have been reported in hypertensive or pre-hypertensive patients compared to healthy individuals [4-6] . These inflammatory cytokines can lead to vascular and renal dysfunction and progression of HTN [7]. Moreover, inflammatory cytokines can increase blood pressure (BP) by increasing the production of catecholamines in the adrenal gland. Specifically, studies in animal models and cultured neuroendocrine cells show that inflammatory cytokines such as interleukin (IL)-1β, interferon (IFN)-α, IL-6 and tumor necrosis factor (TNF)-α can elevate production of dopamine (DA), norepinephrine (NE), and epinephrine (EPI) [3, 8, 9]. The dysregulation of the production of catecholamines has been well recognized in HTN [10, 11].

Here, we reveal an unexpected finding of how catecholamine production is attenuated by another secretion product of neuroendocrine cells: the peptide catestatin (CST). Previous work has shown that CST is a bioactive proteolytical fragment from the pro-hormone Chromogranin A (CgA; $hCgA_{352-372}$) [12], which is co-stored and co-released with catecholamines in neuroendocrine cells [13]. Likely as a consequence of higher catecholamine production [3], CgA levels are elevated in humans with essential HTN [14] and in rodent genetic models of HTN [14]. However, unlike CgA, plasma CST levels are diminished not only in essential HTN [14, 15], but also in the normotensive offspring of patients with HTN [15], suggesting dysregulation in the processing of CgA to CST in HTN [14]. Moreover, HTN-associated single nucleotide polymorphisms within the CST segment of CgA have been identified [16-18].

Animal experiments also indicate a role for CST in HTN: both CgA heterozygote and complete knockout (KO; CgA-KO) mice are hypertensive, and treatment with CST decreases the BP and the levels of plasma catecholamines to that seen in control littermates [19]. It is increasingly clear in mouse models of diabetes [20], colitis [21] and atherosclerosis [22] that CST exerts anti-inflammatory effects by inhibiting the activation of

macrophages and shifting their differentiation to more anti-inflammatory phenotypes [23]. Therefore, we hypothesized that CST exerts its cardioprotective role by skewing macrophages to more anti-inflammatory phenotypes, thereby resulting in lower catecholamine production.

To directly discern the role of CST in the regulation of the cardiovascular system, we generated a precise tool: CST-KO mice, which lack only the CST-coding region of the *Chga* gene. As predicted, CST-KO mice display a hypertensive, hyperadrenergic, and inflammatory phenotype which is rescued by exogenous addition of CST. Thus, by exploring our CST-KO mice in conjunction with macrophage depletion via two methods, chlodronate (CDN) liposomes and from bone-marrow transfer (BMT) between CST-KO and wild-type (WT), this study sought to elucidate the neuroendocrine relationship between CST, catecholamine production, and ultimately the anti-inflammatory/anti-HTN functions of macrophages. With an increased understanding of the CST to HTN pathway, this could be an important advance to eventually utilizing CST as a novel target for the treatment and prevention of HTN.

130 **METHODS**

131

132 An expanded Materials and Methods section is available in the Data Supplement. Further
133 data and protocols are also available upon reasonable request from the corresponding
134 author.

135

136 **Mice**. We used male WT and CST-KO (20-24 weeks old) in C57BL/6 background unless
137 indicated otherwise. Since CgA is especially overexpressed in male patients with
138 hypertension [24], we used only male mice in this study. Further studies will look into female
139 mice. Mice were kept in a 12 hr dark/light cycle and fed a normal chow diet (NCD: 13.5%
140 calorie from fat; LabDiet 5001, TX). Animals were age and sex-matched, and randomly
141 assigned for each experiment. Control and experimental groups were blinded. Power
142 calculations were conducted to determine the number of mice required for each
143 experiment. For rescue experiments with exogenous CST, mice were injected
144 intraperitoneally with CST (2 μg/g body weight) at 9:00 AM for 2-4 weeks before collecting
145 feces or harvesting tissues. All studies with mice were approved by the UCSD and
146 Veteran Affairs San Diego Institutional Animal Care and Use Committees and conform to
147 relevant National Institutes of Health guidelines.

148

149 **Statistics.** Statistics were performed with PRISM 8 (version 8.4.3) software (San Diego,
150 CA). Data were analyzed using either unpaired two-tailed Student's *t*-test for comparison
151 of two groups or one-way or two-way analysis of variance (ANOVA) for comparison of
152 more than two groups followed by Tukey's *post hoc* test if appropriate. All data are
153 presented as mean ± SEM and significance was assumed when $p<0.05$.

154

155 **RESULTS**

156

157 **Generation and validation of CST-KO mice.** The CST coding region ($mCgA_{364-384}$; 63
158 bp) was removed from Exon VII of the *Chga* gene (Figure 1A&B). Using a mouse
159 monoclonal antibody (5A8), we detected full-length CgA (~70 kDa) in WT mice and a
160 proteoglycan form of CgA in CST-KO mice in adrenal gland lysates (Figure 1C), indicating
161 the presence of CgA in CST-KO mice. Blots using a polyclonal antibody directed against
162 the C-terminal domain of CST (CT-CST) showed a proteolytically processed CgA (~46
163 kDa) corresponding to $mCgA_{1-385}$ in WT littermates, but not in CST-KO mice (Figure 1C).
164 Because this antibody detects synthetic CST (positive control for antibody specificity), we
165 conclude that CST-KO mice indeed lack CST. Adrenal CgA content was comparable in
166 WT and CST-KO mice (Figure 1D). CST was not detectable in CST-KO mice (Figure 1D).

167

168 **CST-KO mice are hypertensive.** Consistent with the anti-HTN functions of CST [19, 25, 26],
169 we found that the CST-KO mice are hypertensive and display diurnal increases in both
170 systolic and mean arterial BP (Figure 2A & S1). The high BP in CST-KO mice is rescued
171 by intraperitoneal injection of exogenous CST (2 μg/g body weight for 15 days), whereas
172 CST had no impact on normotensive BP in WT mice (Figure 2B). In WT mice, the plasma
173 CST level was 0.86 nM, which increased to 1.72 nM 24 hrs after administration of CST
174 (Figure 2C). In CST-KO mice, plasma CST was 1.17 nM after 24 hr of CST
175 supplementation, indicating that CST supplementation provided a near physiological
176 concentration of CST.

177

178 **Ischemic pre-conditioning-induced cardioprotection is impaired in CST-KO mice.**
179 Since CST promotes cardioprotection in rats [27], we tested whether pre-conditioning-
180 induced cardioprotection is affected in CST-KO mice. We subjected WT and CST-KO
181 hearts to ischemia/reperfusion (IR) followed by ischemic preconditioning (IPC). IPC
182 significantly increased the post-ischemic left ventricular developed pressure (LVDP) and
183 lowered the left ventricular end diastolic pressure (LVEDP) in WT hearts compared to
184 CST-KO hearts and their respective IR controls (Figure S2A). Furthermore, neither LVDP
185 nor LVEDP was significantly modified in IPC-treated CST-KO hearts compared to the

186 respective IR controls. In WT mice, but not in CST-KO mice, IPC also improved recoveries
187 of both the maximum and minimum rates of pressure development in the LV (dP/dt_{max}
188 and dP/dt_{min}) compared to the respective IR controls (Figure S2B). These data show that
189 whereas IPC conferred protection against IR damage in WT mice, with observed
190 improvements in all functional measures in the reperfusion period, CST-KO mice could
191 not be preconditioned.
192
193 **CST-KO mice have increased inflammation in heart and circulation.** CST is an anti-
194 inflammatory peptide [23 28], raising the possibility that CST might regulate cardiovascular
195 function via the immune system. Indeed, in plasma of CST-KO mice, we found increased
196 levels of proinflammatory cytokines TNF-α, IFN-γ, C-C motif chemokine ligand (CCL)-2
197 and -3, and C-X-C motif chemokine ligand (CXCL)-1 (Figure 3A). By contrast, the anti-
198 inflammatory cytokine IL-10 was decreased in CST-KO mice. Intraperitoneal injection with
199 exogenous CST in CST-KO mice reversed this phenotype: it decreased the levels of most
200 proinflammatory cytokines and increased anti-inflammatory cytokines in plasma of both
201 WT and CST-KO mice (Figure 3A). RT-PCR also revealed inflammation in the heart of
202 CST-KO mice: the expression of anti-inflammatory genes *IL10*, *IL4*, *Mrc1*, *Arg1*, *Clec7a*
203 and *Clec10a* was reduced, whereas the pro-inflammatory genes *Tnfa*, *Ifng*, *Emr1*, *Itgam*,
204 *Itgax*, *Nos2a*, *IL12b CcL2*, and *CxcL1* were upregulated (Figure 3B&C). LV protein levels
205 of the proinflammatory cytokines TNF-α, IFN-γ, CCL-2, CCL-3, CXCL-1, and IL-6 were
206 also elevated in CST-KO mice (Figure 3D). These phenotypes were also reversable by
207 intraperitoneal injection of CST. We also observed increased phosphorylation
208 (Ser177/181) of IKK-β, a component of the cytokine-activated intracellular signaling
209 pathway involved in triggering immune responses via NF-κB (Figure 4A). These findings
210 show that the immune system of CST-KO mice is skewed towards inflammation.
211
212 **CST reduces pro-inflammatory macrophages *in vitro*.** In the next set of experiments,
213 we addressed whether CST would directly shift macrophages to more anti-inflammatory
214 responses *in vitro*. Macrophages were derived from bone-marrow of WT mice (BMDM)
215 and differentiated to an either pro-inflammatory M1-like phenotype or to an anti-
216 inflammatory M2-like phenotype [29]. Culturing these macrophages for 24 hr with 100 nM

217 CST resulted in a small, but significant, reduction of the production of pro-inflammatory
218 cytokines TNF-α, CCL-2, CCL-3, CXCL-1 and IL-1β (Figure 4B&C). In contrast, the levels
219 of anti-inflammatory IL-10 were increased, especially for the M2-like macrophages.
220 Since macrophages are a secretory cell type, we also addressed whether
221 macrophages produce CgA and CST. Indeed, Western blotting analysis revealed the
222 presence of both CgA and CST in peritoneal macrophages (Figure 4D), which were
223 isolated after thioglycolate (3% solution in water) and cultured in DMEM with 10% FBS
224 for 48 hr with daily medium changes [29]. To assess the physiological relevance of CST-
225 production by macrophages, we performed BMT experiments in which we irradiated CST-
226 KO mice and then cross-transplanted the marrow from WT mice. We analyzed plasma
227 CST of these mice and found that WT bone-marrow recipient CST-KO mice, but not CST-
228 KO bone-marrow recipients, had near physiologic levels of plasma CST (0.52 nM) (Figure
229 4E). Thus, macrophages (and possibly other bone-marrow derived cell types) are major
230 producers of CST in circulation.
231
232 **Macrophages are key effector cells responsible for the anti-inflammatory actions**
233 **of CST.** TEM studies revealed abundant infiltration of macrophages and fibrosis in the
234 intercellular spaces between chromaffin cells in the adrenal medulla of CST-KO mice
235 (Figure S3 and S4**)**. Also, marked cardiac fibrosis and an increased presence of
236 macrophages were observed in the heart of saline-treated CST-KO mice, as shown by
237 TEM and flow cytometry (Figure 5A, S3, S5 and S6A). In both the adrenal gland and heart
238 of CST-KO mice, CST supplementation reduced the abundance of macrophage infiltrates
239 (Figure S3). This was supported by flow cytometry analysis showing a ~38% decrease of
240 $CD11b^{+}F4/80^{+}$ macrophages in CST-supplemented CST-KO heart (Figure 5A and S6B).
241 We assessed the functional role of the infiltrated macrophages in the heart and
242 adrenal gland of CST-KO mice using two independent approaches. First, we depleted
243 macrophages by CDN liposomes (Figure S6B), which not only depleted macrophages in
244 heart and adrenal gland (Figure S3), but also reversed the hypertensive phenotype of
245 CST-KO mice (Figure 5B). Second, we carried out BMT assays in which we irradiated
246 both WT and CST-KO mice and then cross-transplanted their marrows: bone-marrow
247 from CST-KO mice was transplanted into WT mice and *vice versa*. Both the inflammatory

248 and hypertensive phenotypes were transferred by BMT: while CST-KO bone-marrow
249 recipient WT mice showed increased BP; elevated levels in plasma and heart of TNF-α,
250 IFN-γ, CCL-2, CCL-3, and CXCL-1; and reduced levels of IL-10, WT bone-marrow
251 recipient CST-KO mice showed the opposite phenotypes (Figure 5B-D). Since WT bone-
252 marrow recipient CST-KO mice had near physiologic levels of plasma CST (Figure 4E),
253 we conclude that macrophages and other immune cells are not only key effectors of the
254 anti-hypertensive actions of CST but are also main producers of CST themselves.
255
256 **Heightened sympathetic stimulation and hypersecretion of catecholamines in**
257 **adrenal gland of CST-KO mice.** Prior studies in humans, mice, and rats have shown
258 that pro-inflammatory cytokines increase catecholamine production and secretion [3, 8, 9].
259 In line with this, we found that compared to WT littermates, CST-KO mice has elevated
260 levels of both adrenal and plasma catecholamine levels (Figure 6A). This phenotype was
261 also transferable by BMT: CST-KO bone-marrow recipient WT mice showed increased
262 levels of NE and EPI in the adrenal medulla and plasma, whereas WT bone-marrow
263 recipient CST-KO mice showed reduced levels of NE and EPI (Figure 6A).
264 Since heightened sympathetic nerve traffic has been documented in young,
265 middle-aged, and elderly hypertensives; in pregnancy-induced hypertension; and in
266 systo-diastolic hypertension or an isolated elevation of BP [30], we measured NE in the LV
267 and kidney of WT and CST-KO mice. In contrast to the adrenal medulla and plasma, we
268 observed reduced levels of NE in the heart and kidney of CST-KO mice (Figure 6A).
269 Decreased NE in CST-KO mice indicates increased cardiac and renal spillover of NE,
270 which is common in hypertensive and heart failure patients [31-34]. The CST-KO adrenal
271 medulla exhibited abundant docked chromaffin granules and decreased acetylcholine-
272 containing vesicles at the sympatho-adreno-medullary synapse (Figure 6B-C and S7),
273 implicating heightened sympathetic nerve activity leading to hypersecretion of
274 catecholamines. Supplementation of CST-KO mice with CST reversed this phenotype
275 (Figure 6B-C and S7) and led to a concomitant decrease in both plasma and adrenal
276 catecholamines (Figure 6A). The elevated BP in CST-KO mice was reversed by the
277 nicotinic acetylcholine receptor antagonist chlorisondamine (Figure S8).

To test whether lack of CST affected heart structure and function, we undertook gravimetry (Figure S9A) and echocardiographic ultrasound imaging (Figure S9B). CST-KO mice showed increased heart weights and sizes compared to WT mice (Figure S9A). Although CST-KO mice maintained a similar level of left ventricular (LV) function (fractional shortening) to age-matched WT mice, there were significant abnormalities in LV remodeling as evidenced by the significant increase in LV posterior wall thickness (LVPWd), which has been associated with high BP [35, 36] , and a trend towards an increase in interventricular septum wall thickness (IVSd; p=0.07) (Figure S9B). Heart rate, left ventricular internal diameter during systole, and left ventricular internal diameter during diastole were comparable between WT and CST-KO mice (Figure S9B).

DISCUSSION

Inflammation and hypertension. Inflammation is well understood to contribute to the development of hypertension by inducing vascular damage, renal damage, and/or abnormal central neural regulation [37-39] . For instance, a recent study in the Japanese population found that prolonged low-grade inflammation as evaluated by increased C-reactive protein (CRP) increases arterial stiffness and the consequent development of HTN [40] . CRP is also considered as an independent risk factor for the development of HTN [41] . Besides the importance of peripheral vascular inflammation in hypertension, it has been shown that inducing inflammation in the brainstem triggers hypertension in a normotensive rat [42] .

From this study's data, it is becoming increasingly clear that this inflammation and the development of HTN are counteracted by the anti-inflammatory peptide hormone CST through its feedback inhibition/regulation of macrophages. Previous studies showing low levels of CST in hypertensive subjects [15] and normalization of BP in CgA-KO mice by CST [19] as well as decreasing BP in spontaneously hypertensive rats [25] indicate that CST is sufficient to reverse HTN. The findings from this study that CST-KO mice are hypertensive with a skewed immune system towards inflammation, and that these phenotypes can be rescued by exogenous administration of recombinant CST, add to this and demonstrate that CST is not only sufficient but also necessary for regulating HTN. Since the inflammation and BP can be reduced by administration of exogenous CST, CST might be a therapeutic target for the treatment of HTN.

Suppression of the immune system attenuates the development of HTN when induced by Ang II or DOCA-salt, while dysregulation of it causes sensitization to these hypertensive challenges [43] . Surprisingly, CST-KO mice already show elevated BP in absence of an additional challenge, raising the question of other mechanisms in addition to the immune activation are involved in the HTN phenotype in these mice. However, since bone-marrow transplant from CST-KO to WT mice already suffices to elicit HTN, and these mice only harbor CST lacking immune cells while the autonomic system is normal, it might be that an abnormal activation of the immune system triggers HTN.

322 **Neurohumoral regulation of BP.** We found a neuro-adrenergic overdrive-induced HTN
323 in CST-KO mice. Existing literature reveals heightened sympathetic nerve activity in white
324 coat syndrome and borderline hypertensive subjects [44] as well as in established
325 hypertensive subjects of all ages, and the magnitude of this elevation is related to the
326 magnitude of HTN [45] . In addition, hypertensive patients with metabolic risk factors, such
327 as obesity, metabolic syndrome, or diabetes mellitus, also exhibit sympathetic overdrive
328 [46-48] . Like humans, spontaneously hypertensive rats show reduced cardiac
329 parasympathetic nerve activity, elevated sympathetic nerve activity and increased NE
330 release [49] .
331
332 **Immunoendocrine regulation of BP.** The augmented sympathetic nerve activity in HTN
333 is known to activate both myeloid cells and T cells [2], and circulating concentrations of pro-
334 inflammatory cytokines are increased in primary HTN [50] . T-lymphocytes are critical for
335 Angiotensin II and deoxycorticosterone acetate-salt-induced hypertension [51, 52] .
336 Intracerebroventricular administration of IL-6 increases splenic sympathetic nerve activity
337 [53] , while central administration of IL-1β increases adrenal, splenic and renal sympathetic
338 nerve activity [54] . Injection of TNF-α into central sympathetic nuclei, such as the
339 paraventricular nucleus increases sympathetic nerve activity, BP and heart rate in rats [55]
340 .
341 To our knowledge, the present study is the first to demonstrate increased
342 infiltration of macrophages in the adrenal medulla concomitant with increased secretion
343 of catecholamines and the consequent development of HTN in CST-KO mice, which were
344 normalized after CST supplementation. These findings imply that CST regulates the BP
345 through a novel immunoendocrine regulation of catecholamine secretion via
346 macrophages.
347 What causes the elevated BP in CST-KO mice? In these mice, the heart rate was
348 not increased, and fractional shortening was unaltered compared to WT, indicating that
349 the increased BP is not driven by elevated cardiac output. Consequently, it might be that
350 the LV hypertrophy in the CST-KO mice, and possibly also the increased posterior wall
351 thickness associated with high BP [35, 36] , is a secondary effect of the BP elevation. It
352 seems therefore likely that the elevated BP is caused by increased vascular resistance,

353 due to vasoconstriction and/or increased arterial stiffness [40] . It is possible that increased
354 cardiac and renal spillover of NE also contribute to the development of BP in CST-KO
355 mice [31-34] .
356
357 **Perspectives.** This study provides a key mechanism as to how CST regulates
358 inflammation. Cardiac macrophages are critical for myocardial homeostasis [56, 57] . While
359 a subset of macrophages orchestrate monocyte recruitment and contribute to heart failure
360 pathogenesis [58] , others are increased during diastolic dysfunction [59] , myocardial
361 infarction, and acute hemodynamic stress [60] . We found an abundance of infiltrated
362 macrophages in the heart and adrenal gland of CST-KO mice. Using *in vitro* experiments
363 with cultured BMDMs and two parallel approaches (CDN macrophage depletion and
364 BMT) to decrease macrophage activity *in vivo*, we found that macrophages are key
365 effector cells for the anti-hypertensive actions of CST. We also found that bone-marrow
366 originated cells, possibly macrophages, are the main source of circulating CST.
367 We therefore propose that the macrophages (and chromaffin cells) produce CST,
368 which reduces inflammation in an autocrine/feedback inhibition fashion. These anti-
369 inflammatory actions underlie the anti-hypertensive effects of CST, since without CST,
370 macrophages are more reactive, infiltrate the heart, and alter the ultrastructure,
371 physiological makeup, and molecular makeup of the myocardium. Additionally, the data
372 implicate CST as a key mediator of the observed crosstalk between systemic and cardiac
373 inflammation in HTN, which hence plays a central role in cardiovascular homeostasis by
374 regulating the immunoendocrine axis.
375
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378
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384

385 **FIGURE LEGENDS**
386

387 **Fig. 1. Generation of CST-KO mice. (A)** Schematic diagram showing the cloning
388 strategy for generating CST-KO mice. DTA, diphtheria toxin; FRT, *Flp* recognition target.
389 **(B)** Screening of CST-KO mice by PCR. Primer set 1 flanks the CST domain; expected
390 PCR products: 162 bp, CST-KO; 225 bp, WT mice. Reverse primer 2 binds within CST-
391 coding region; no band, CST-KO, 180 bp WT mice. **(C)** Western blots showing the
392 presence of CgA and CST in WT and CST-KO mice using monoclonal antibody (mAb)
393 5A8 that does not recognize CST, and rabbit polyclonal antibody directed against the C-
394 terminus of CST (pAb CT-CST), which does not recognize CgA beyond CST domain. A
395 truncated CgA (CgA_{1-384}) was present in WT mice but not in CST-KO mice, confirming
396 the deletion of CST domain. **(D)** Adrenal CgA content (n=12) and plasma CST levels
397 (n=6). Unpaired two-tailed *t*-test: ns, not significant; ***p<0.001.
398

399 **Fig. 2. Hypertension in CST-KO mice. (A)** Diurnal systolic (SBP), diastolic (DBP) and
400 mean arterial (MAP) blood pressure by telemetry in wild-type (WT) and CST-KO mice
401 (n=8). **(B)** Daytime SBP by tail-cuff (n=9) and **(C)** plasma CST levels (n=6) of WT and
402 CST-KO mice treated with CST or saline (Sal). Two-way ANOVA: ns, not significant;
403 **p<0.01; ***p<0.001.
404

405 **Fig. 3. CST-KO mice display systemic and cardiac inflammation, which can be**
406 **reversed with exogenous CST. (A)** Plasma cytokines in WT and CST-KO mice (n=8).
407 Sal: saline. **(B&C)** RT-qPCR data showing steady-state mRNA levels of anti- **(B)** and pro-
408 inflammatory **(C)** genes in left ventricle (n=8). **(B&D)** Protein levels of IL-10 **(B)** and pro-
409 inflammatory cytokines **(D).** One-way ANOVA: ns, not significant; *p<0.05; **p<0.01;
410 ***p<0.001.
411

412 **Fig. 4. CST exerts anti-inflammatory effects in isolated macrophages and**
413 **macrophages are major producers of CST. (A)** Western blot analysis of
414 phosphorylated (Ser177/181) and total IKK2 in heart of 4 WT mice, 5 CST-KO mouse
415 and 5 CST-KO mice with intraperitoneal injection of CST. **(B&C)** Protein levels of

416 cytokines (n=8) in supernatant of bone-marrow derived macrophages (M0-BMDMs)
417 differentiated to proinflammatory M1-type **(B)** and anti-inflammatory M2-type phenotypes
418 **(C)**. Cells were treated with 100 nM CST. **(D)** Western blots showing the presence of CgA
419 and CST in peritoneal macrophages (n=4). **(E)** Plasma CST levels in CST-KO mice which
420 received BMT from CST-KO or WT mice (n=6). Panel A-C: one-way ANOVA; panel E: *t*-
421 test; ns, not significant; *p<0.05; **p<0.01; ***p<0.001.
422
423 **Fig. 5. Macrophages mediate cardioprotective effect of CST. (A)** Flow cytometry data
424 showing $CD45^+F4/80^+CD11b^+$ macrophages in CST-KO heart (n=3). Sal: saline control.
425 **(B)** Systolic blood pressure (SBP) after depletion of macrophages by CDN (n=8) and
426 bone-marrow transfer (BMT) into irradiated mice (n=8). Levels of cytokines in plasma **(C)**
427 and heart **(D)** of mice with BMT (n=8). One-way ANOVA: ns, not significant; *p<0.05;
428 **p<0.01; ***p<0.001.
429
430 **Fig. 6. Increased catecholamine secretion in CST-KO mice. (A)** Plasma, adrenal,
431 heart and kidney norepinephrine (NE) and epinephrine (EPI) levels in WT and CST-KO
432 mice. Mice were treated with saline (Sal) or CST or underwent bone-marrow transfer
433 (BMT) into irradiated mice (n=8-12). **(B)** TEM micrographs of chromaffin granules (CG) in
434 the adrenal medulla of CST-KO mice. PM, plasma membrane. **(C)** TEM micrographs of
435 splanchnic-adrenomedullary synapse with acetylcholine vesicles (AChV) and dense core
436 peptidergic vesicles (PdV). CC, chromaffin cell; CG, chromaffin granule. Two-way
437 ANOVA: ns, not significant; *p<0.05; **p<0.01; ***p<0.001.
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634

635 **NOVELTY AND SIGNIFICANCE**

636

637 **What is new?**

638 - Mice that lack the peptide hormone catestatin (CST) are hypertensive.
639 - CST skews macrophages to an anti-inflammatory phenotype.
640 - CST reduces inflammation in heart and adrenal gland.

641 **What is relevant?**

642 - Hypertension is associated with inflammation.
643 - Hypertensive patients have reduced plasma levels of CST.
644 - In mouse models, hypertension can be reduced by exogenous CST.

645 **Summary**

646 - The anti-hypertensive effects of CST are mediated via CST's immunosuppression
647 of macrophages.
648 - CST is a key autocrine attenuator of the cardiac inflammation in hypertension.

649

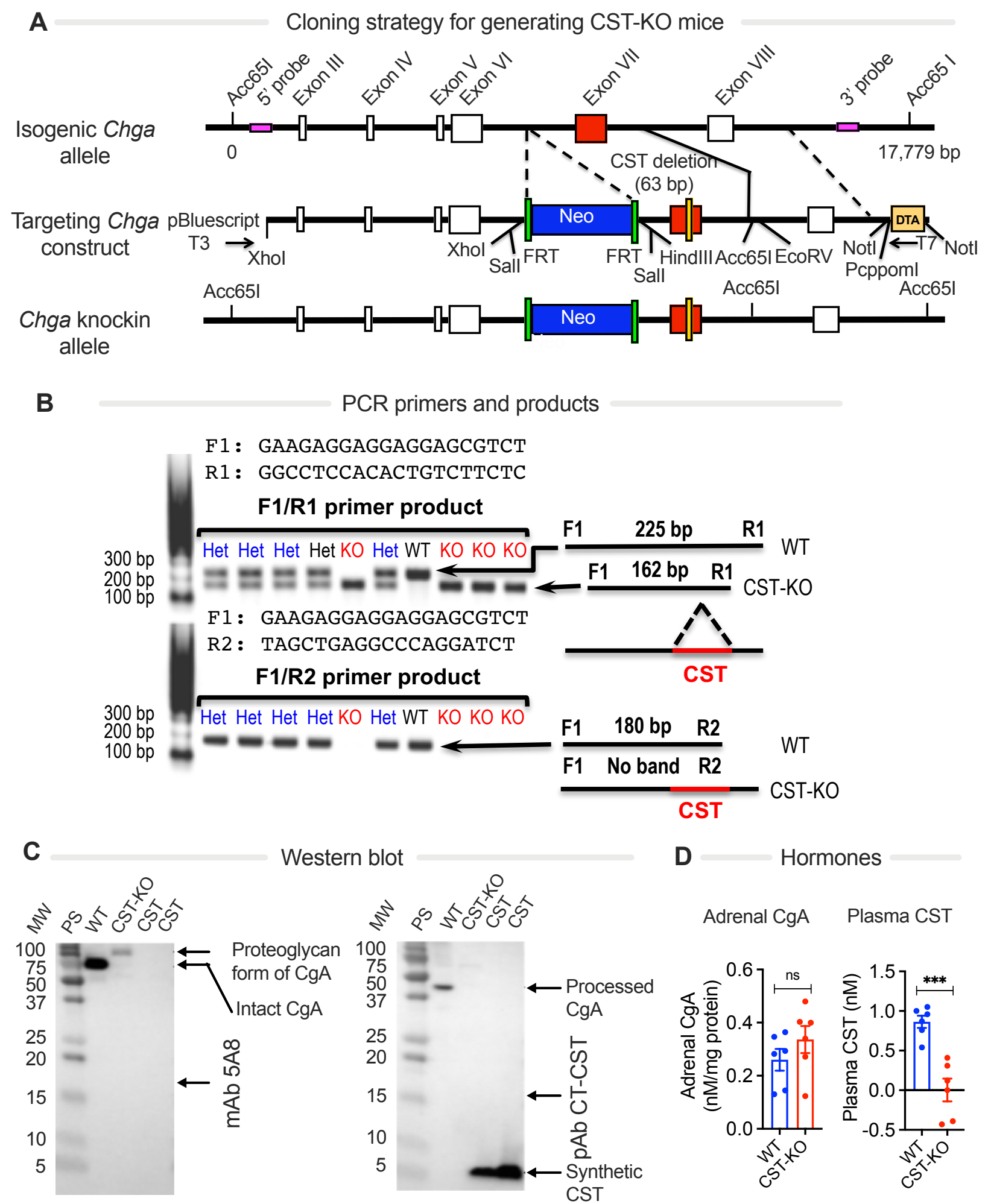
A
Cloning strategy for generating CST-KO mice
Isogenic Chga allele
Targeting Chga construct
Chga knockin allele
Acc65I
5' probe
Exon III
Exon IV
Exon V
Exon VI
Exon VII
Exon VIII
3' probe
Acc65 I
0
17,779 bp
CST deletion (63 bp)
pBluescript
T3
XhoI
Neo
SalI
FRT
HindIII
Acc65I
EcoRV
NotI
T7
PcppomI
DTA
B
PCR primers and products
F1: GAAGAGGAGGAGGAGCGTCT
R1: GGCCTCCACACTGTCTTCTC
F1/R1 primer product
Het Het Het Het KO Het WT KO KO KO
300 bp
200 bp
100 bp
F1 225 bp R1
WT
F1 162 bp R1
CST-KO
CST
F1: GAAGAGGAGGAGGAGCGTCT
R2: TAGCTGAGGCCCAGGATCT
F1/R2 primer product
Het Het Het Het KO Het WT KO KO KO
F1 180 bp R2
WT
F1 No band R2
CST-KO
C
Western blot
MW
PS
WT
CST-KO
CST
CST
Proteoglycan form of CgA
Intact CgA
mAb 5A8
Processed CgA
pAb CT-CST
Synthetic CST
D
Hormones
Adrenal CgA
Plasma CST
Adrenal CgA (nM/mg protein)
Plasma CST (nM)
ns
***
WT
CST-KO

Figure 1

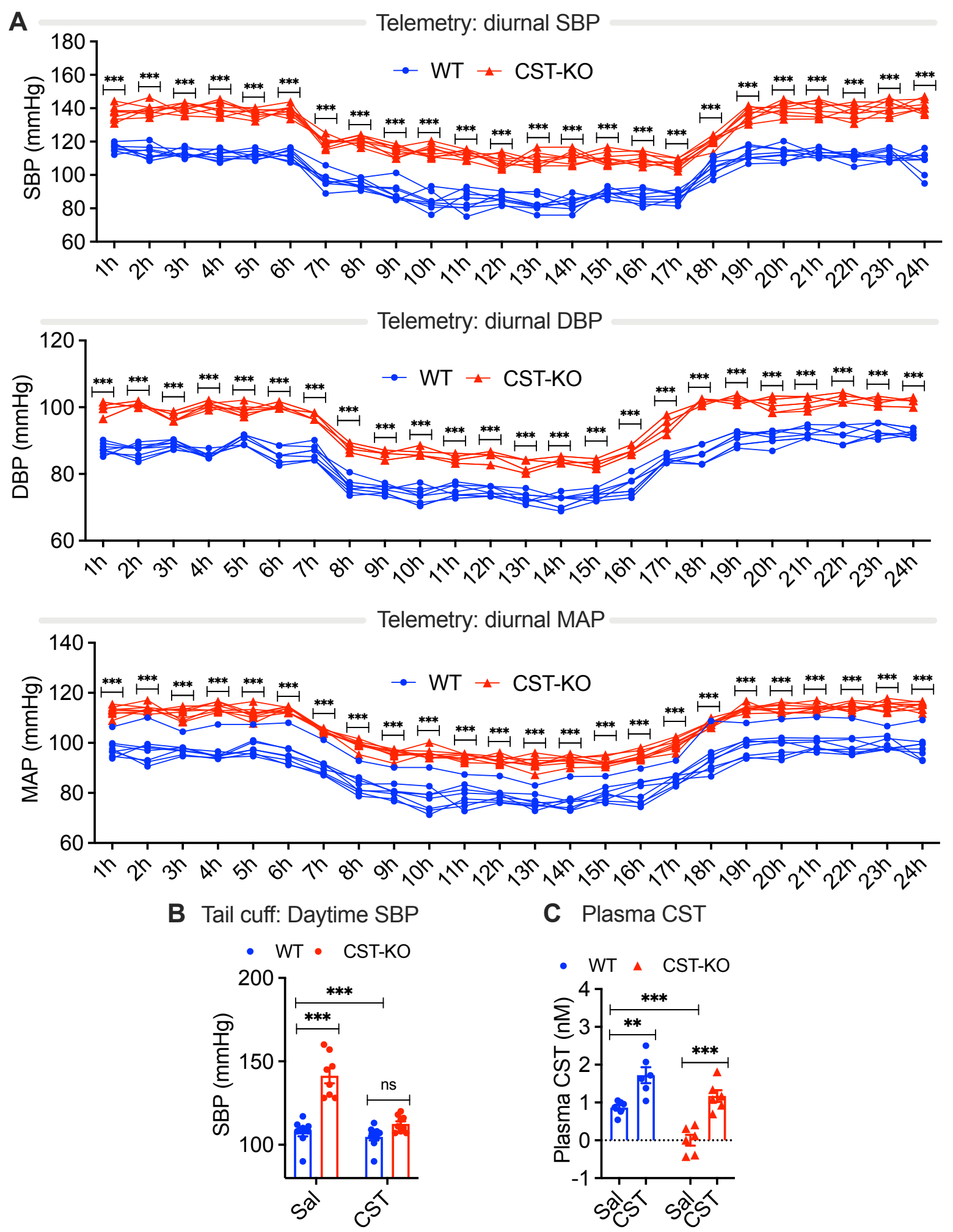
A
Telemetry: diurnal SBP
SBP (mmHg)
WT
CST-KO
Telemetry: diurnal DBP
DBP (mmHg)
Telemetry: diurnal MAP
MAP (mmHg)
1h 2h 3h 4h 5h 6h 7h 8h 9h 10h 11h 12h 13h 14h 15h 16h 17h 18h 19h 20h 21h 22h 23h 24h
B Tail cuff: Daytime SBP
SBP (mmHg)
ns
Sal
CST
C Plasma CST
Plasma CST (nM)

Figure 2

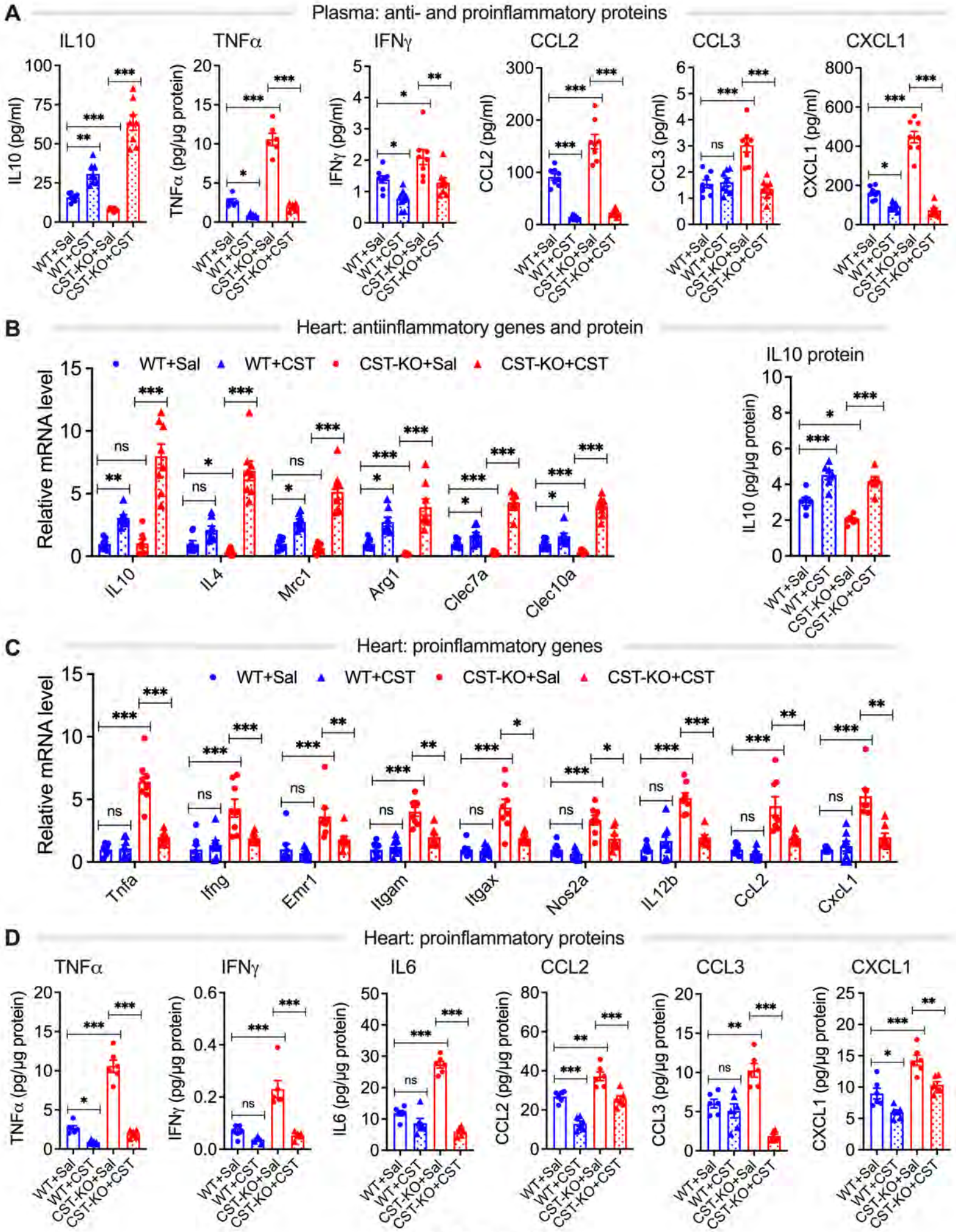
A
Plasma: anti- and proinflammatory proteins
IL10
TNFα
IFNγ
CCL2
CCL3
CXCL1
IL10 (pg/ml)
TNFα (pg/μg protein)
IFNγ (pg/ml)
CCL2 (pg/ml)
CCL3 (pg/ml)
CXCL1 (pg/ml)
WT+Sal
WT+CST
CST-KO+Sal
CST-KO+CST
B
Heart: antiinflammatory genes and protein
Relative mRNA level
IL10
IL4
Mrc1
Arg1
Clec7a
Clec10a
IL10 protein
IL10 (pg/μg protein)
C
Heart: proinflammatory genes
Tnfa
Ifng
Emr1
Itgam
Itgax
Nos2a
IL12b
CcL2
CxcL1
D
Heart: proinflammatory proteins
IL6
IL6 (pg/μg protein)

Figure 3

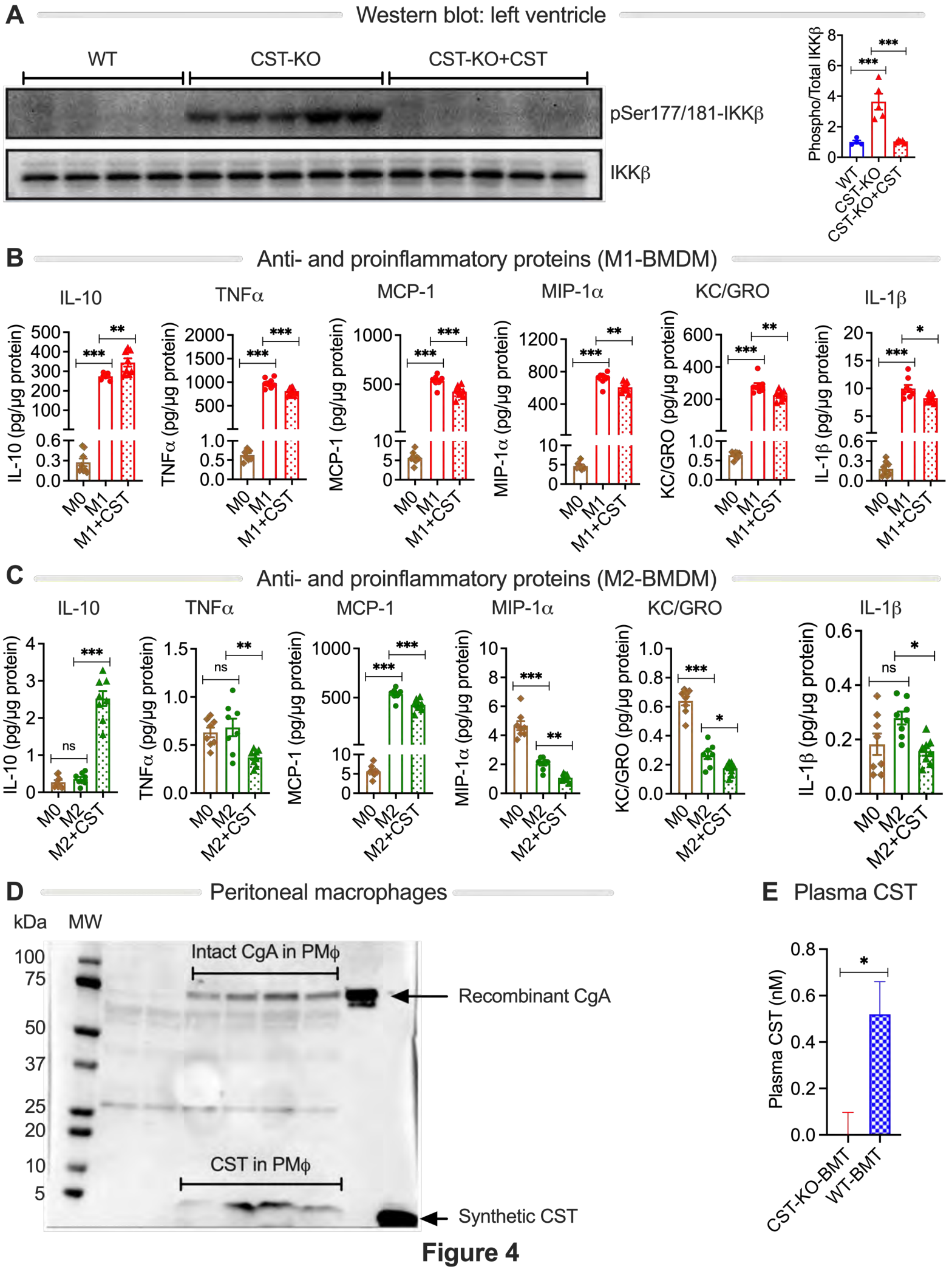
A
Western blot: left ventricle
WT
CST-KO
CST-KO+CST
pSer177/181-IKKβ
IKKβ
Phospho/Total IKKβ
B
Anti- and proinflammatory proteins (M1-BMDM)
IL-10
TNFα
MCP-1
MIP-1α
KC/GRO
IL-1β
IL-10 (pg/μg protein)
TNFα (pg/μg protein)
MCP-1 (pg/μg protein)
MIP-1α (pg/μg protein)
KC/GRO (pg/μg protein)
IL-1β (pg/μg protein)
M0
M1
M1+CST
C
Anti- and proinflammatory proteins (M2-BMDM)
M2
M2+CST
ns
D
Peritoneal macrophages
kDa
MW
Intact CgA in PMϕ
Recombinant CgA
CST in PMϕ
Synthetic CST
E
Plasma CST
Plasma CST (nM)
CST-KO-BMT
WT-BMT

Figure 4

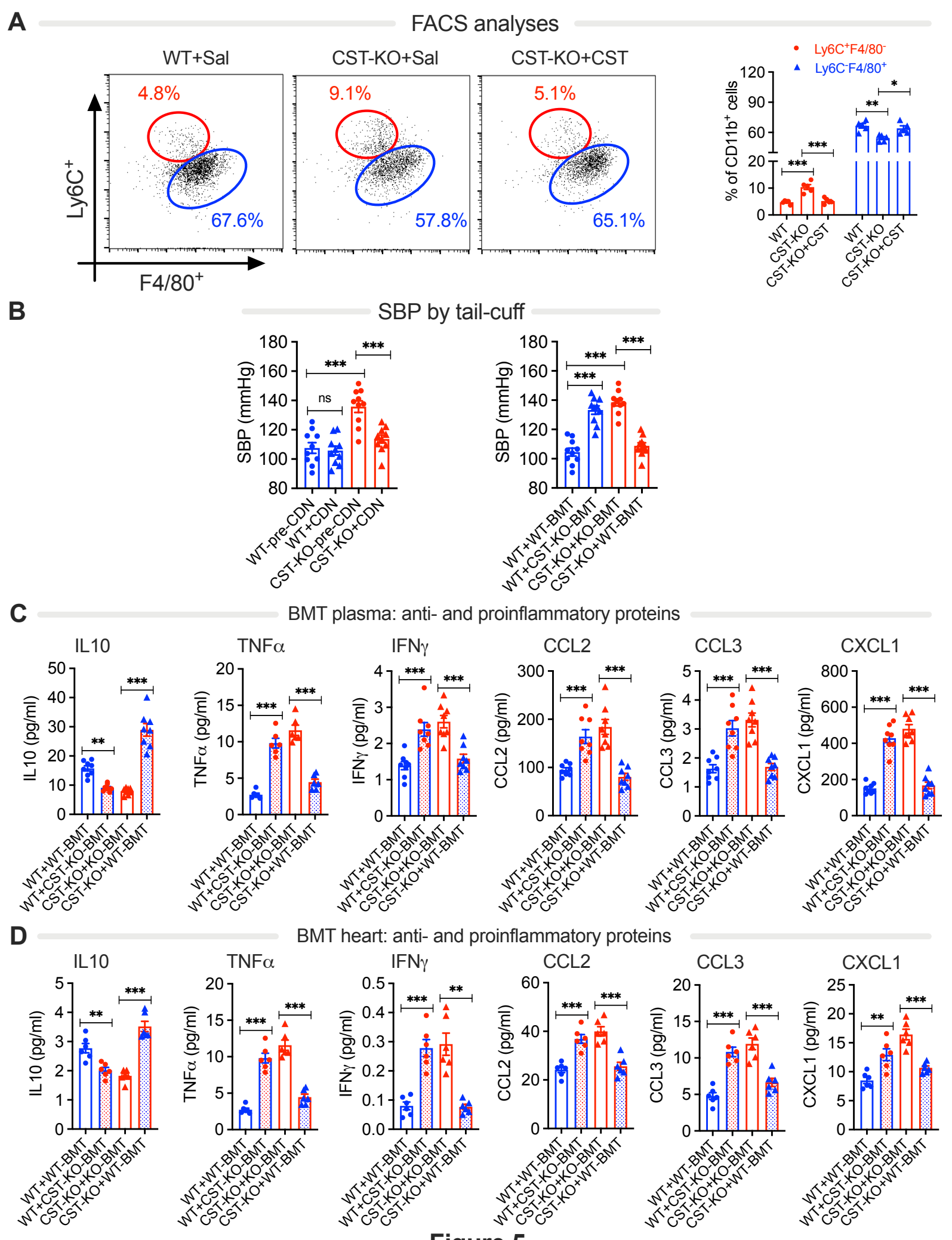
A
FACS analyses
WT+Sal
CST-KO+Sal
CST-KO+CST
4.8%
67.6%
9.1%
57.8%
5.1%
65.1%
Ly6C+
F4/80+
Ly6C+F4/80-
Ly6C-F4/80+
% of CD11b+ cells
WT
CST-KO
CST-KO+CST
B
SBP by tail-cuff
SBP (mmHg)
ns
WT-pre-CDN
WT+CDN
CST-KO-pre-CDN
CST-KO+CDN
WT+WT-BMT
WT+CST-KO-BMT
CST-KO+KO-BMT
CST-KO+WT-BMT
C
BMT plasma: anti- and proinflammatory proteins
IL10
TNFα
IFNγ
CCL2
CCL3
CXCL1
IL10 (pg/ml)
TNFα (pg/ml)
IFNγ (pg/ml)
CCL2 (pg/ml)
CCL3 (pg/ml)
CXCL1 (pg/ml)
D
BMT heart: anti- and proinflammatory proteins

Figure 5

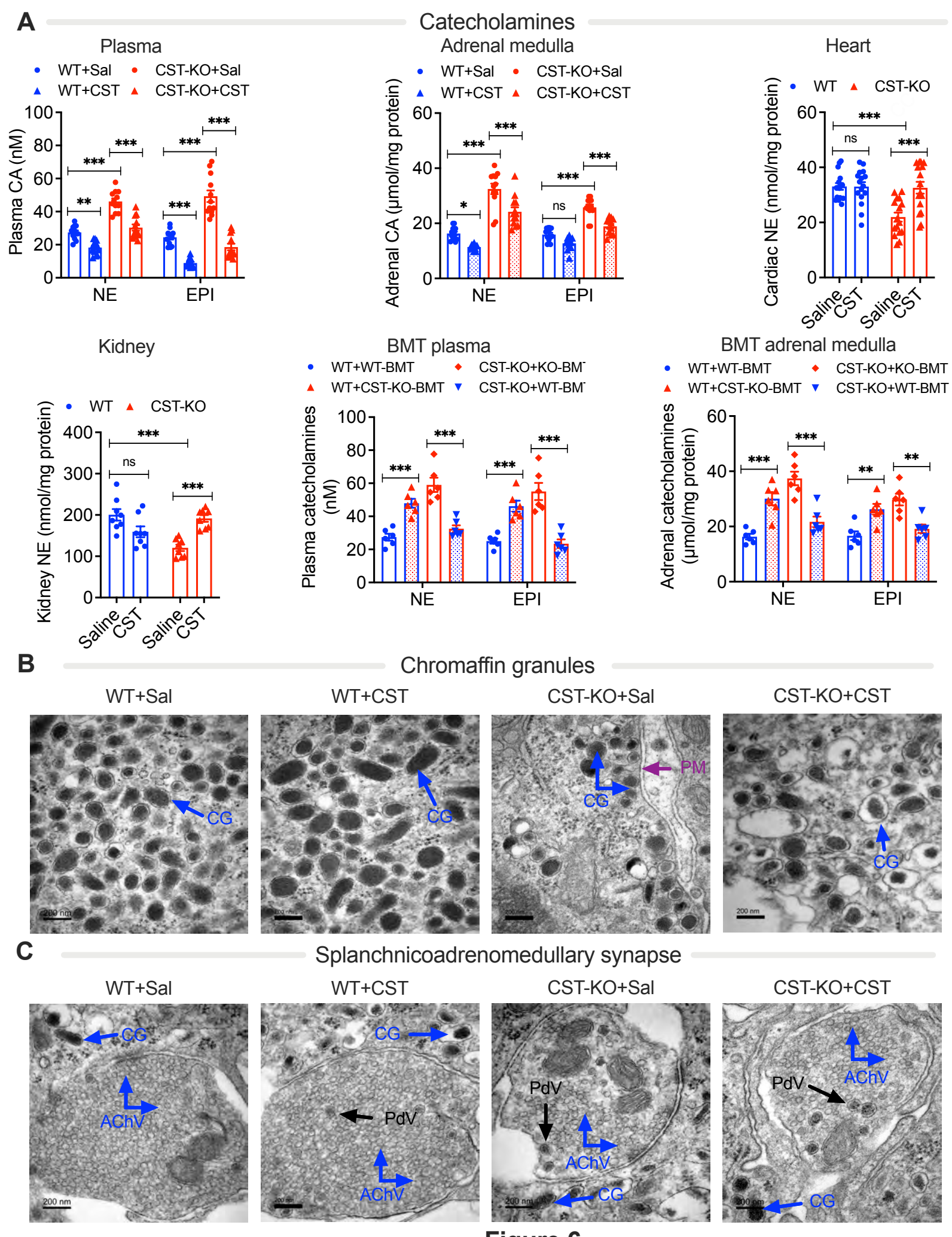
A
Catecholamines
Plasma
WT+Sal
CST-KO+Sal
WT+CST
CST-KO+CST
Plasma CA (nM)
NE
EPI
Adrenal medulla
Adrenal CA (μmol/mg protein)
Heart
WT
CST-KO
Cardiac NE (nmol/mg protein)
Saline
CST
Kidney
Kidney NE (nmol/mg protein)
BMT plasma
WT+WT-BMT
CST-KO+KO-BMT
WT+CST-KO-BMT
CST-KO+WT-BMT
Plasma catecholamines (nM)
BMT adrenal medulla
Adrenal catecholamines (μmol/mg protein)
B
Chromaffin granules
WT+Sal
WT+CST
CST-KO+Sal
CST-KO+CST
CG
PM
200 nm
C
Splanchnicoadrenomedullary synapse
AChV
PdV

Figure 6