The neuropeptide cortistatin attenuates experimental autoimmune myocarditis via inhibition of cardiomyogenic T cell-driven inflammatory responses

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SUPPLEMENTARY INFORMATION

Table S1. Sequences of primers and their temperature and time of annealing used for quantitative real-time PCR analysis.

Name	**Sequence 5´---3´**	**Annealing Temperature Time**
TNFα-FW	GCGACGTGGAACTGGCAGAAGAG	64ºC
TNFα-RV	TGAGAGGGAGGCCATTTGGGAAC	30 sec
IFNγ-FW	ACACTGCATCTTGGCTTTGC	58ºC
IFNγ-RV	TTGCTGATGGCCTGATTGTC	30 sec
CST-FW	GCCTTCTGACTTTCCTTGCC	60ºC
CST-RV	GAAAGCTCCCCGCTGATTGA	30 sec
IL1β-FW	CTCCATGAGCTTTGTACAAGG	60ºC
IL1β-RV	TGCTGATGTACCAGTTGGGG	45 sec
CCL5-FW	CATATGGCTCGGACACCACT	58ºC
CCL5-RV	GCGGTTCCTTCGAGTGACAA	30 sec
IL6-FW	CAACGATGATGCACTTGCAGA	62ºC
IL6-RV	TGTGACTCCAGCTTATCTCTTGG	60 sec
GAPDH-FW	AACTTTGGCATTGTGGAAGG	60ºC
GAPDH-RV	ACACATTGGGGGTAGGAACA	30 sec

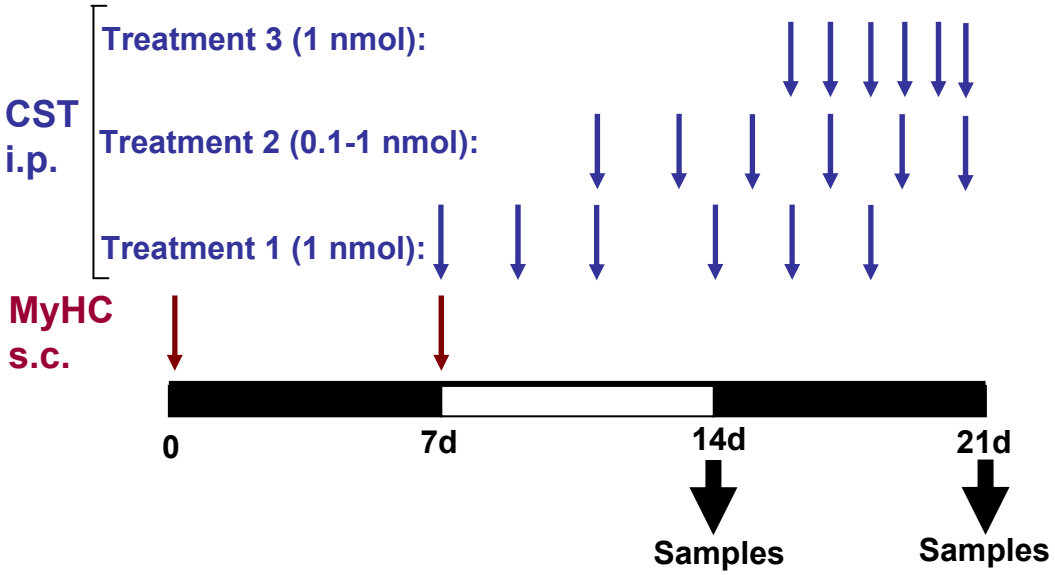

Figure S1.- Scheme illustrating the experimental procedure used to induce and treat mice with experimental autoimmune myocarditis (EAM). Mice were injected s.c. with $MyHC_{614-629}$ on days 0 and 7 (red arrows) and were then randomly distributed in different experimental groups that were treated i.p. with PBS (controls) or with cortistatin (CST, blue arrows) following three different profiles: treatment 1 consisted in six injections of 1 nmol of cortistatin starting at day 7 (early at the effector phase); treatment 2 consisted in six injections of 1, 0.5 or 0.1 nmol of cortisatin starting at day 11 (during the effector phase); and treatment 3 consisted in six injections of 1 nmol of cortistatin starting at day 15 (late during the effector phase). Samples were collected at day 14 (lymph nodes and spleen) or at day 21 (hearts, sera, lymph nodes and spleen) from each experimental group for analysis.

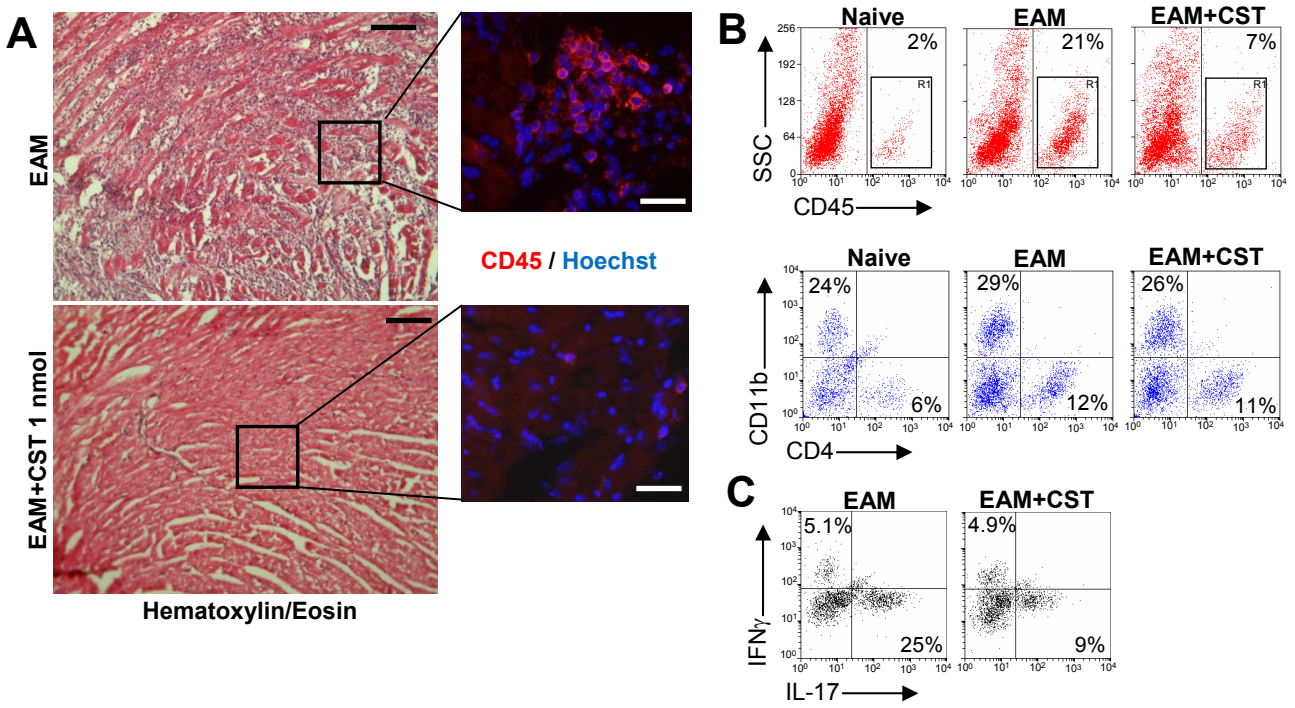

Figure S2.- Cortistatin reduces inflammatory infiltration in EAM. Mice with $MyHC_{614-629}$-induced EAM were treated i.p. with PBS (EAM) or cortistatin (EAM+CST) three times per week during two weeks. At day 21, hearts were obtained from each experimental group for analysis. Naïve mice were used as reference. **A,** Identity of inflammatory infiltrates in myocardium was revealed by immunefluorescence for $CD45^+$ leukocytes in heart sections. Nuclei were Hoechst-counterstained. Scale bars: 100 µm. **B,** Inflammatory cells infiltrating the heart were isolated and analyzed by flow cytometry. Representative dot plots showing flow cytometric analysis of $CD45^+$ leukocytes in live cells are shown (upper plots) and of $CD11b^+$ monocytes and $CD4^+$ lymphocytes in gated $CD45^+$ cells (lower plots). **C,** Infiltrating inflammatory cells isolated from hearts were activated with phorbol 12-myristate 13-acetate in the presence of monensin and analyzed by flow cytometry for the expression of intracellular IFNγ and IL-17 in gated $CD4^+$ lymphocytes. Numbers in dot plots correspond to the percentage of positive cells in each quadrant and the mean of six experiments is shown in Figure 2.

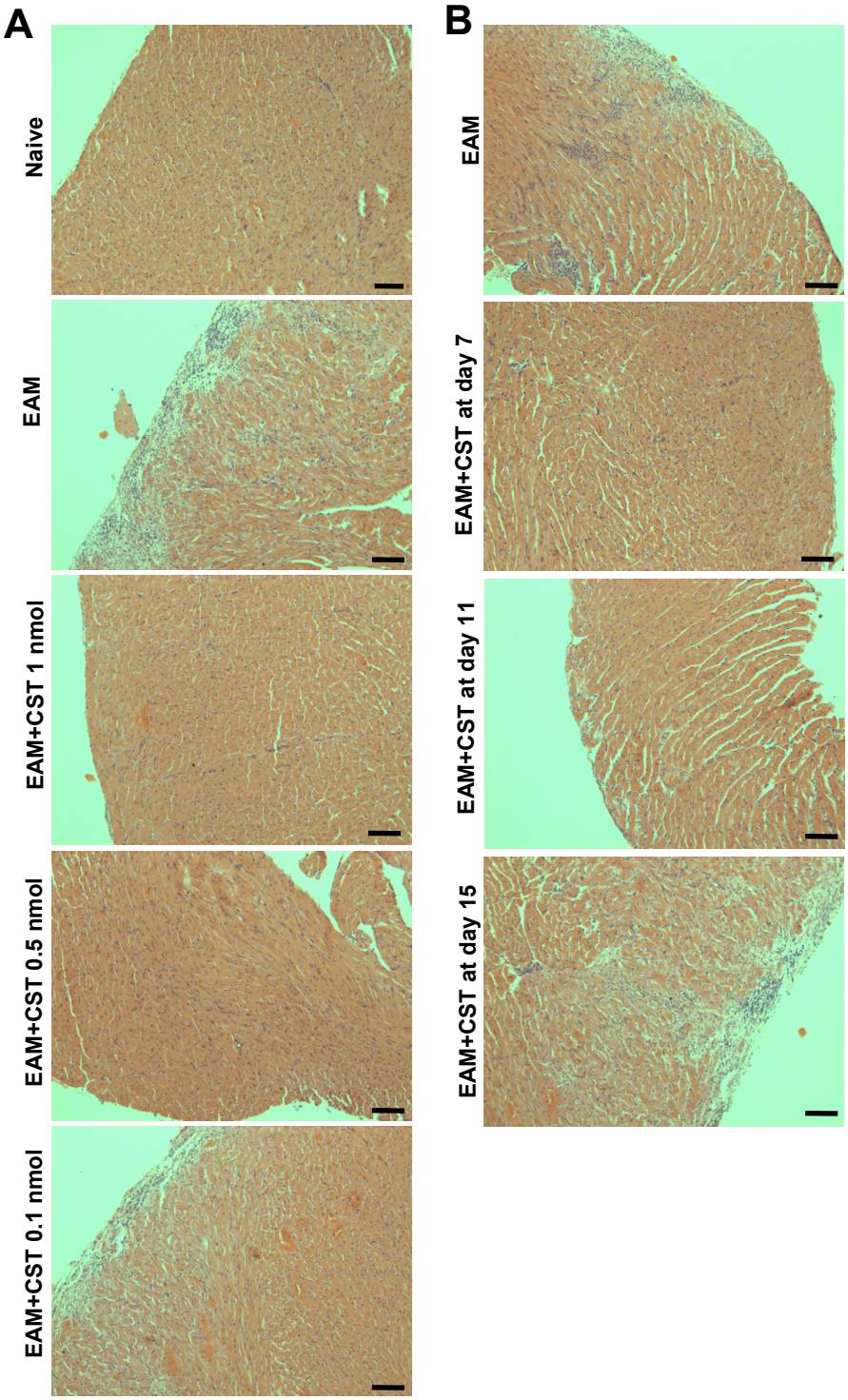

Figure S3.- Cortistatin alleviates clinical signs in EAM. Mice with $MyHC_{614-629}$-induced EAM were treated i.p. with PBS (EAM) or cortistatin (EAM+CST) at different doses (**A**) or at 1 nmol per mouse (**B**) starting at day 11 (**A**) or at the indicated time points (**B**) as depicted in Figure S1. At day 21, hearts were obtained, sectioned and stained with hematoxylin-eosin to determine the extension of myocardial area with inflammatory infiltration and cardiomyocyte necrosis (see Figure 3 for quantitative results). Images are representative of 7 mice per group. Scale bars: 100 μm.

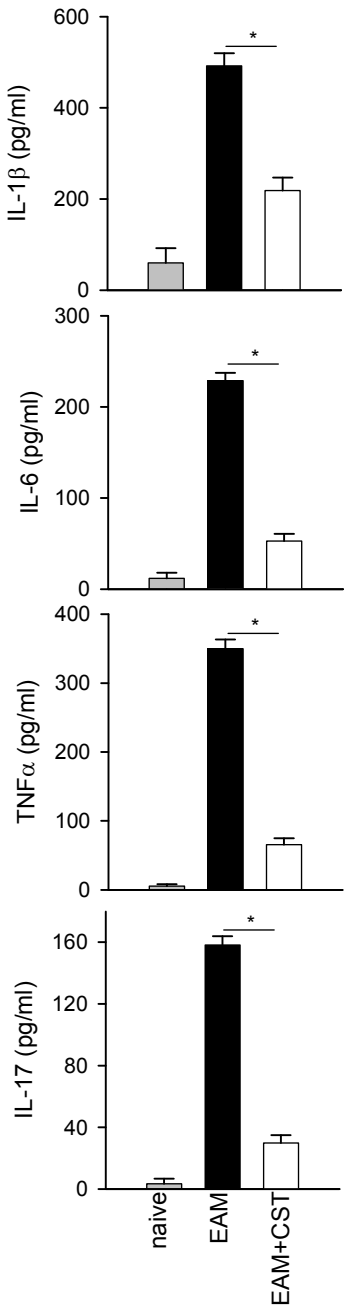

Figure S4.- Cortistatin decreases inflammatory response in EAM. Mice with $MyHC_{614-629}$-induced EAM were treated i.p. with PBS (EAM) or cortistatin (EAM+CST) three times per week during two weeks. Sera were isolated at day 21, and the content of cytokines was assayed by ELISA. n=11 mice per group, performed in two independent experiments. *$p<0.5$.

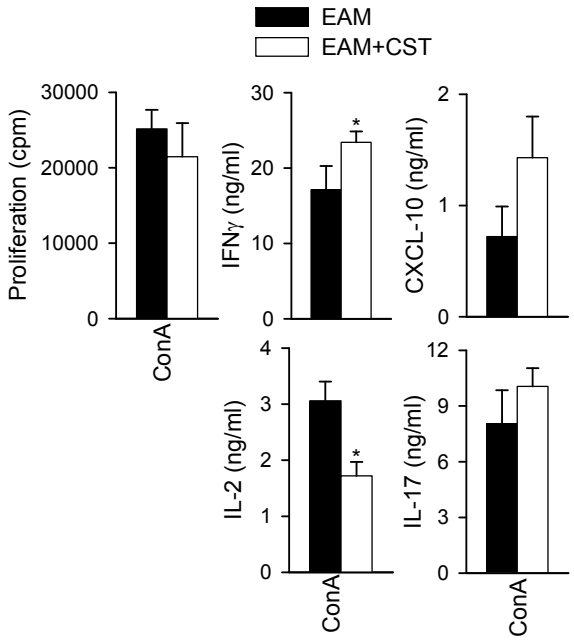

Figure S5. Proliferation and cytokine production by draining lymph node cells isolated at day 21 from untreated (EAM) or cortistatin-treated (EAM+CST) mice with EAM and stimulated *ex vivo* with Concanavalin A (Con A). We obtained similar results with spleen cells stimulated with ConA and with draining lymph node cells stimulated with an anti-CD3 antibody. n=10 mice/group, performed in two independent experiments. *$p<0.05$ *vs* untreated EAM mice.

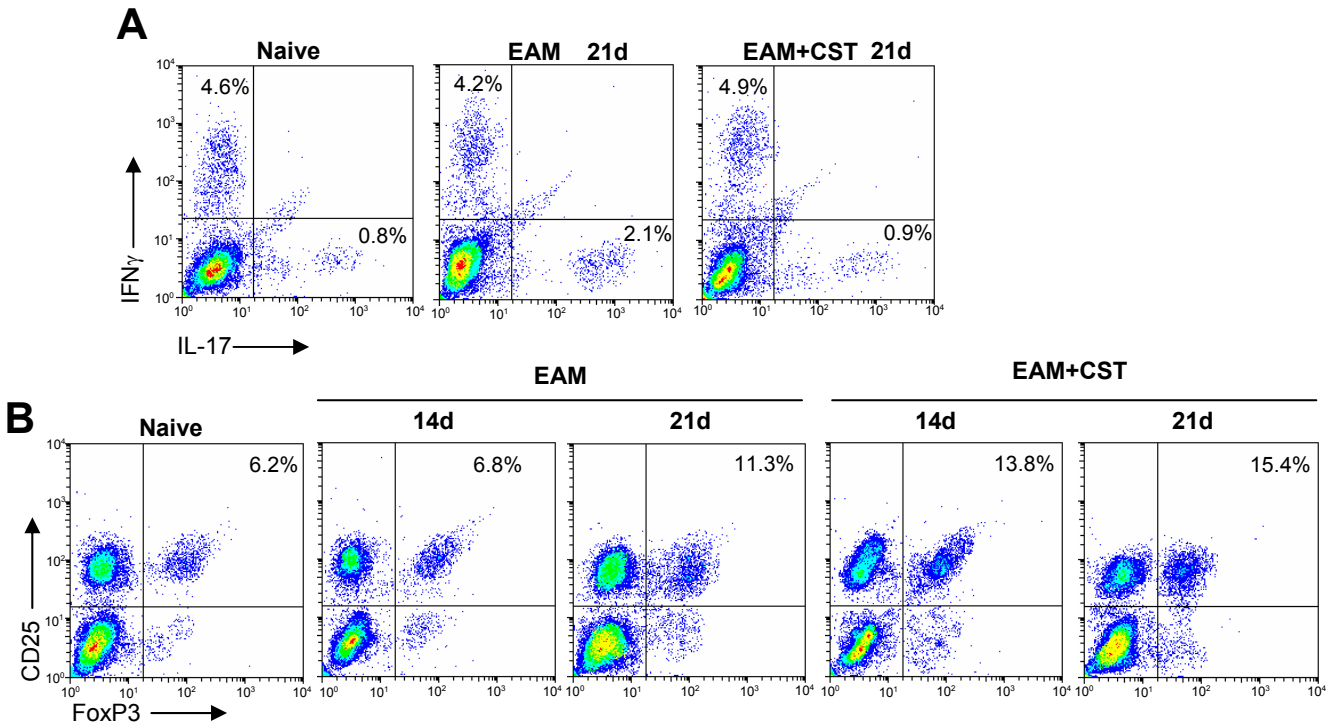

Figure S6.- Effect of cortistatin in the generation of Th1, Th17 and Treg cells in EAM. Mice with $MyHC_{614-629}$-induced EAM were treated i.p. with PBS (EAM) or cortistatin (EAM+CST) three times per week during two weeks. At days 14 or 21 after EAM induction, draining lymph node cells were isolated and analyzed by flow cytometry for intracellular cytokine expression (**A**) or for CD25 and FoxP3 (**B**) expression in gated $CD4^+$ cells. Naïve mice were used as reference. Numbers in plots correspond to the percentage of positive cells in each quadrant and the means of 8-12 mice per group are shown Figure S6A and S6B.

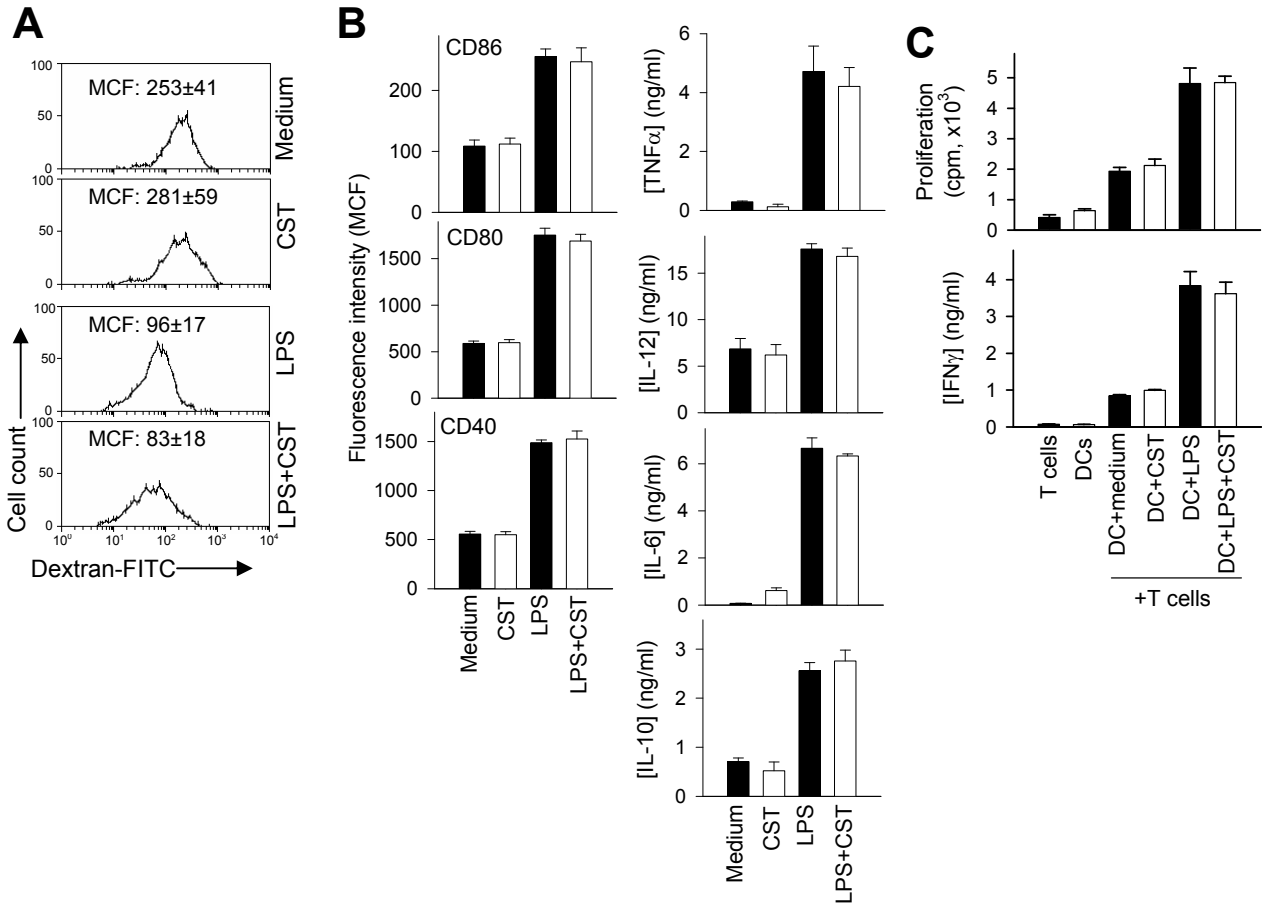

Figure S7.- Cortistatin does not affect dendritic cell (DC) function. **A** and **B,** Phagocytosis of FITC-labeled dextran (**A**) and expression of costimulatory molecules and production of cytokines (**B**) by DCs cultured with medium or matured/activated with LPS in the absence or presence of 100 nM cortistatin. MCF: mean channel fluorescence. n=6 experiments performed in duplicates. **C,** DCs isolated form C57Bl/6 mice were cultured with medium or matured with LPS in the absence or presence of cortistatin for 24 hours and then co-incubated with allogeneic T cells isolated from spleens of Balb/c mice. Cell proliferation and the production of IFNγ were determined 72 hours and 48 hours later, respectively. Cultures of T cells and DCs alone were used as basal controls. n=5 experiments performed in duplicates.

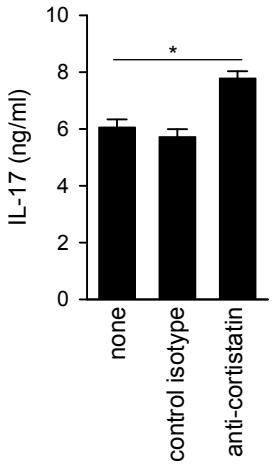

Figure S8. Role of endogenous cortistatin in the control of cardiomyogenic T cell responses *in vitro*. Production of IL-17 by DLN cells isolated from mice with EAM at day 21 and restimulated *ex vivo* with $MyHC_{614-629}$ in the absence (none) or presence of a neutralizing anti-cortistatin antibody or a control IgG antibody (control isotype). n=5 mice per group, performed in duplicate. *$p<0.05$.