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Supplemental Information

Cystatin C Plays a Sex-Dependent Detrimental Role in Experimental Autoimmune Encephalomyelitis

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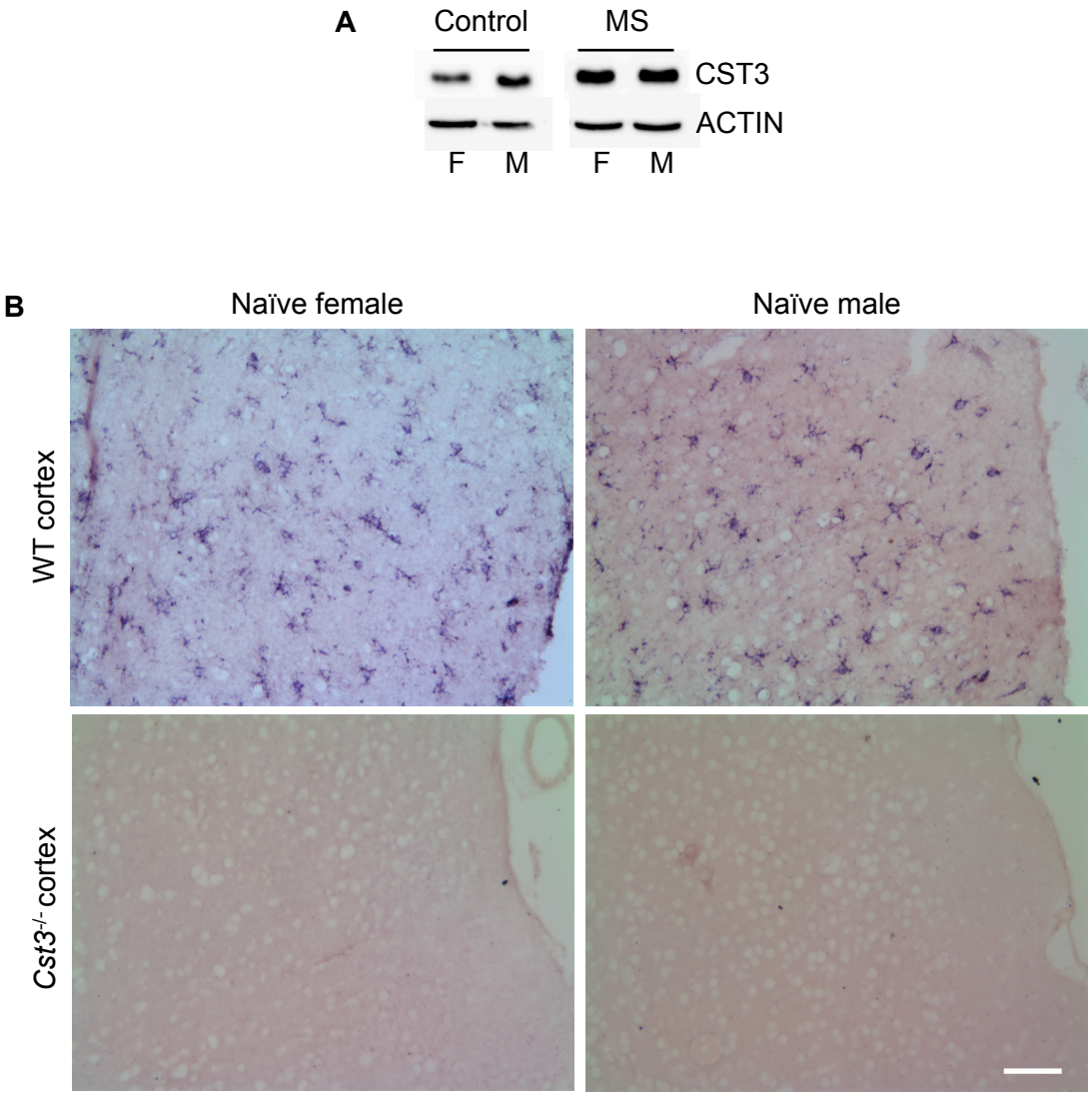


Figure S1. CST3 protein and *Cst3* mRNA in MS and mouse brains, Related to Figure 1

(A) CST3 protein level in the brains of a female (F) and a male (M) control subject and MS patient.

(B) Micrographs (Bar = 50μm) of *Cst3* mRNA expression in the cortex of naive female and male WT and *Cst3*$^{-/-}$ mice.

Figure S2

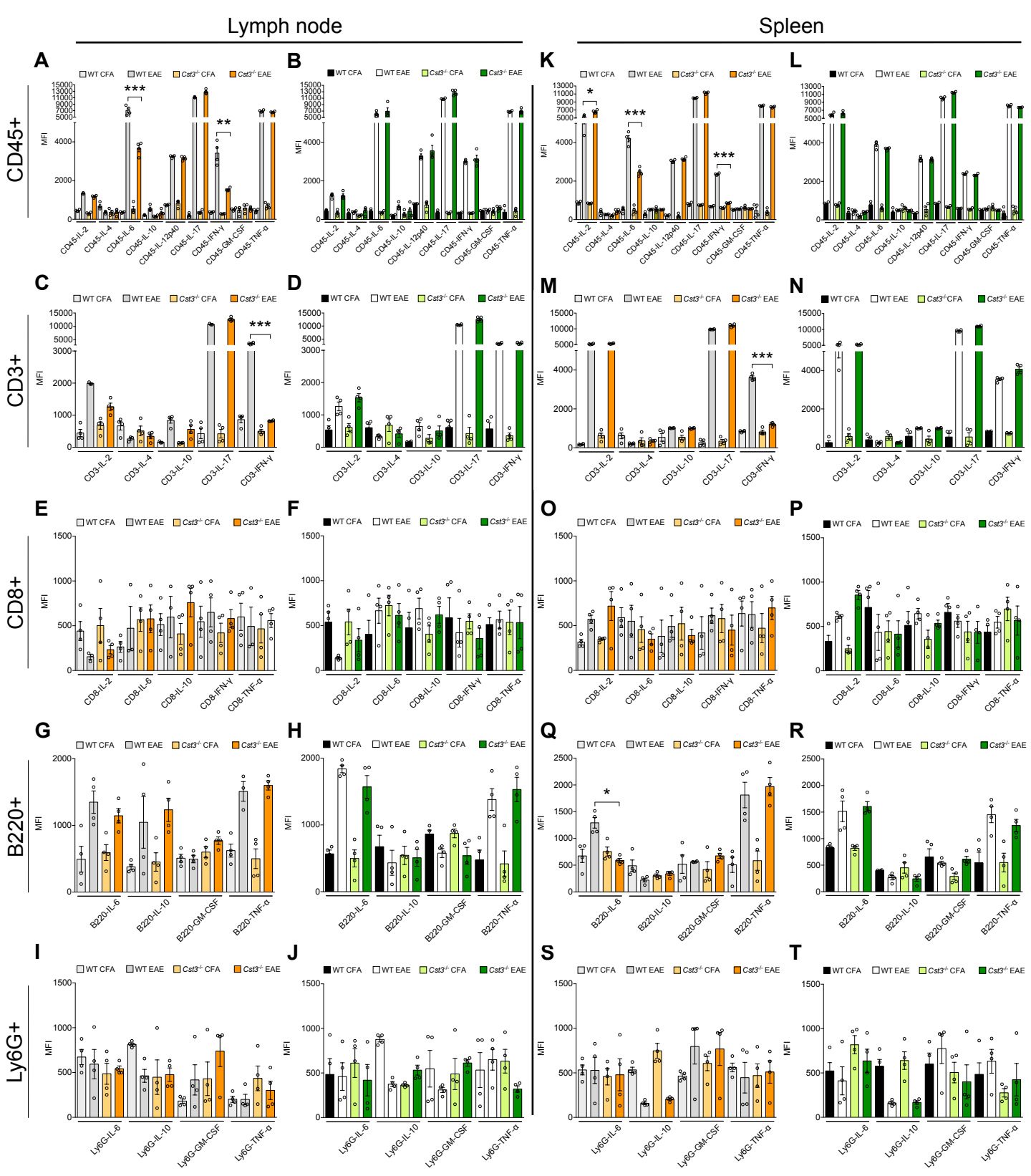

Lymph node
Spleen
CD45+
CD3+
CD8+
B220+
Ly6G+
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
WT CFA
WT EAE
Cst3-/- CFA
Cst3-/- EAE
MFI
CD45-IL-2
CD45-IL-4
CD45-IL-6
CD45-IL-10
CD45-IL-12p40
CD45-IL-17
CD45-IFN-γ
CD45-GM-CSF
CD45-TNF-α
CD3-IL-2
CD3-IL-4
CD3-IL-10
CD3-IL-17
CD3-IFN-γ
CD8-IL-2
CD8-IL-6
CD8-IL-10
CD8-IFN-γ
CD8-TNF-α
B220-IL-6
B220-IL-10
B220-GM-CSF
B220-TNF-α
Ly6G-IL-6
Ly6G-IL-10
Ly6G-GM-CSF
Ly6G-TNF-α

Figure S2. CST3 promotes IL-6 and IFN-γ cytokine expression in lymph node and spleen-derived immune cells from female mice with EAE, Related to Figure 3**.**
(A-T) Mean fluorescent intensity of various cytokines present within $CD45^+$ (A, B, K, L), $CD3^+$ (C, D, M, N), $CD8^+$ (E, F, O, P), $B220^+$ (G, H, Q, R), and $Ly6G^+$ (I, J, S, T) lymph node (A-J) and spleen-derived immune cells (K-T) from female (A, C, E, G, I, K, M, O, Q, S) and male (B, D, F, H, J, L, N, P, R, T) WT (grey/white/black bars) and *Cst3*$^{-/-}$ (orange/green bars) CFA and EAE mice. Data shown as mean ± SEM and represent 4 individual animals per group. One-way ANOVA with Šídák post hoc test, *P<0.05, **P<0.01, ***P<0.001.

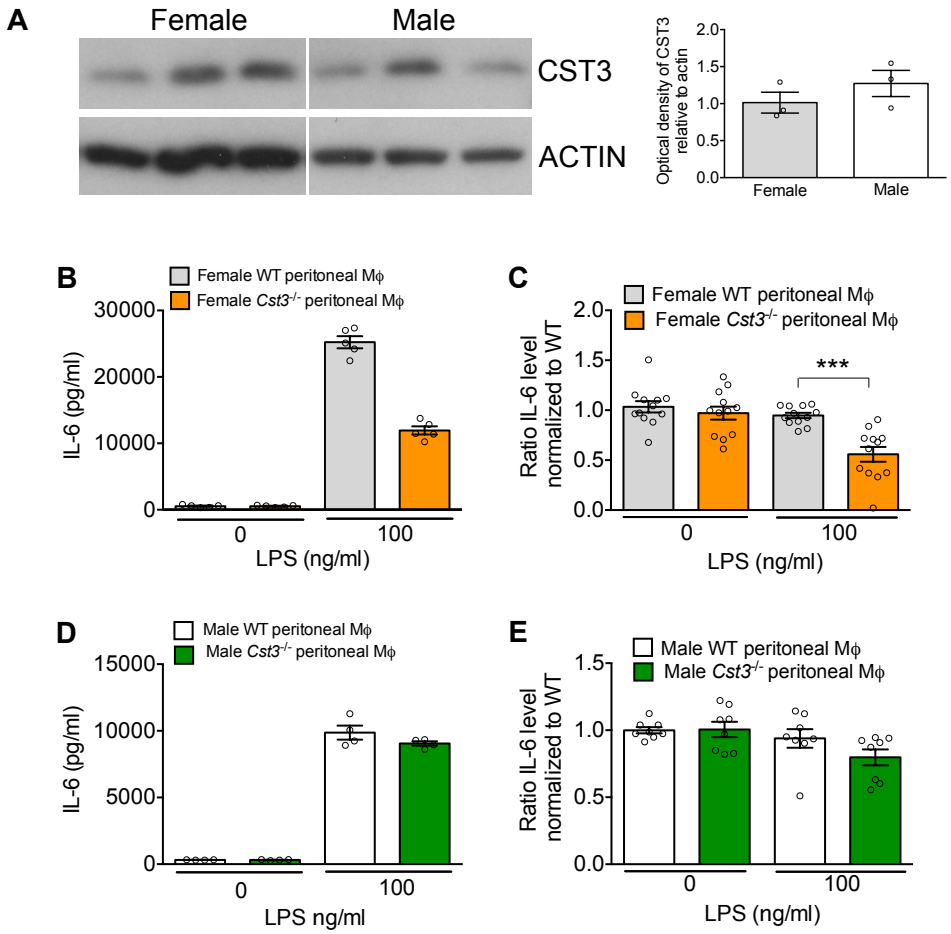


Figure S3. CST3 expression and IL-6 secretion by CD11b$^+$ cells, Related to Figure 5**.**
(A) Western blot and semi-quantification of CST3 protein level in female and male splenic EAE CD11b$^+$ cells. Each lane represents an individual mouse. Data represented as mean ± SEM, n=3 mice/group, 2-tailed unpaired Student's *t*-test.
(B-E) IL-6 production by female (B, C) and male (D, E) WT (grey/white) and *Cst3*$^{-/-}$ (orange/green) peritoneal macrophages stimulated with LPS. Data represent an individual experiment (B, D) or the combination of 2 separate experiments (C, E) with each experiment consisting of cells pooled from 3 or 5 mice for each group and triplicate wells plated for each condition. Each dot represents each well and indicates the ratio of that well normalized to its corresponding WT condition. Data represented as mean ± SEM, two-tailed unpaired Student's *t*-test, *P<0.05, **P<0.01, ***P<0.001.

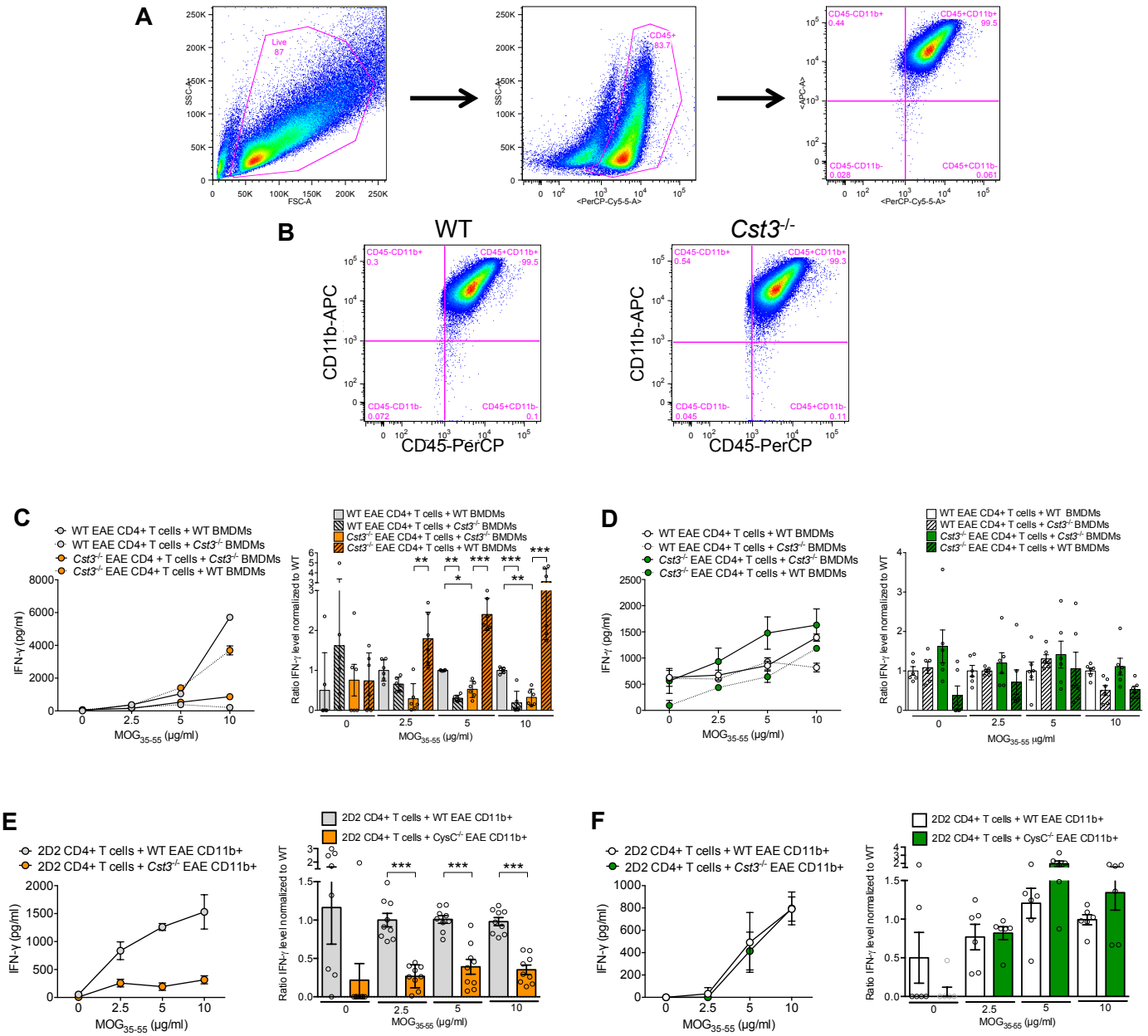


Figure S4. CST3 promotes activation of antigen presenting cells in female EAE mice, Related to Figure 5.

(A) Gating strategy and (B) purity of CD11b$^+$ BMDMs from WT and *Cst3*$^{-/-}$ female mice. Plots represent pooled cells from two mice from each genotype.

(C-F) IFN-γ secretion by female (grey/orange) and male (white/green) WT (grey/white) and *Cst3*$^{-/-}$ (orange/green) spleen-derived EAE CD4$^+$ T cells (C, D) or 2D2 CD4$^+$ T cells (E, F) co-cultured with syngeneic (solid bars) or alternate genotype (patterned bars) BMDMs (C, D) or EAE CD11b$^+$ cells (E, F). Line graphs depict an individual experiment that consisted of cells pooled from 3 or 5 mice for each group and triplicate wells plated for each condition. Bar graphs represent the combination of 3 separate experiments. Each dot represents each well and indicates the ratio of that well normalized to its corresponding WT condition. Data presented as mean ± SEM, one-way ANOVA with Šídák post hoc test, *P<0.05, **P<0.01, ***P<0.001.

Figure S5

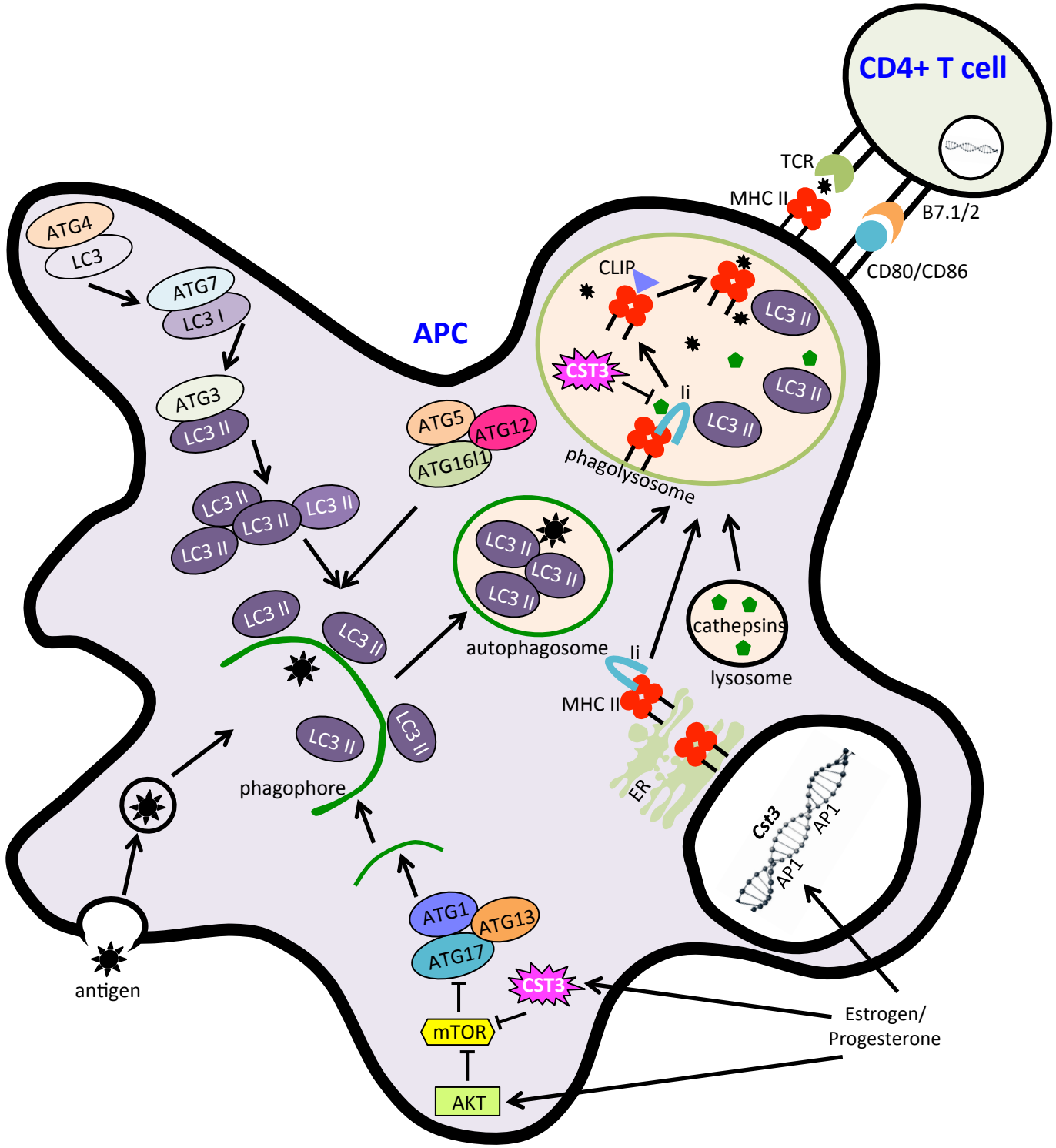

CD4+ T cell
TCR
MHC II
B7.1/2
CD80/CD86
APC
ATG4
LC3
ATG7
LC3 I
ATG3
LC3 II
ATG5
ATG12
ATG16l1
CLIP
CST3
Ii
phagolysosome
autophagosome
cathepsins
lysosome
phagophore
ER
antigen
ATG1
ATG13
ATG17
mTOR
AKT
Cst3
AP1
Estrogen/
Progesterone

Figure S5. Schematic diagram showing the molecular players in antigen presentation and autophagy-mediated antigen processing and loading, Related to Figure 6A, B**.** Macroautophagy is a mechanism that degrades damaged or aged organelles as well as intracellular or extracellular antigens. Inhibition of mTOR by AKT or CST3 mediates activation of the Atg13/Atg17/Atg1 complex that induces autophagosome formation. During phagophore elongation, an Atg4/Atg7/Atg3 complex promotes the conversion of LC3I to LC3II that relocates from the cytosol to the surfaces of autophagic membranes. Extracellular antigen is engulfed by endosomes or endocytic vesicles and transferred to autophagic membranes (autophagosomes) that fuse with lysosomes to form phagolysosomes. Here, antigens are processed by lysosomal enzymes (such as cathepsins B or S) and loaded unto MHC II molecules. CST3 controls the activity of cathepsins to prevent complete degradation of antigens. The autophagic cargo is then exocytosed to the plasma membrane and the antigen is presented via MHC II to TCRs on CD4+ T cells. Co-stimulation via binding of CD80 and CD86 to B7.1/2 co-stimulatory molecules is required to promote full activation of T cells. *(Patterson and Mintern, 2012, Protein and Cell; Wang and Muller, 2015, Front Immunol; Khan and Ahmed, 2015, Front Immunol).*

Figure S6

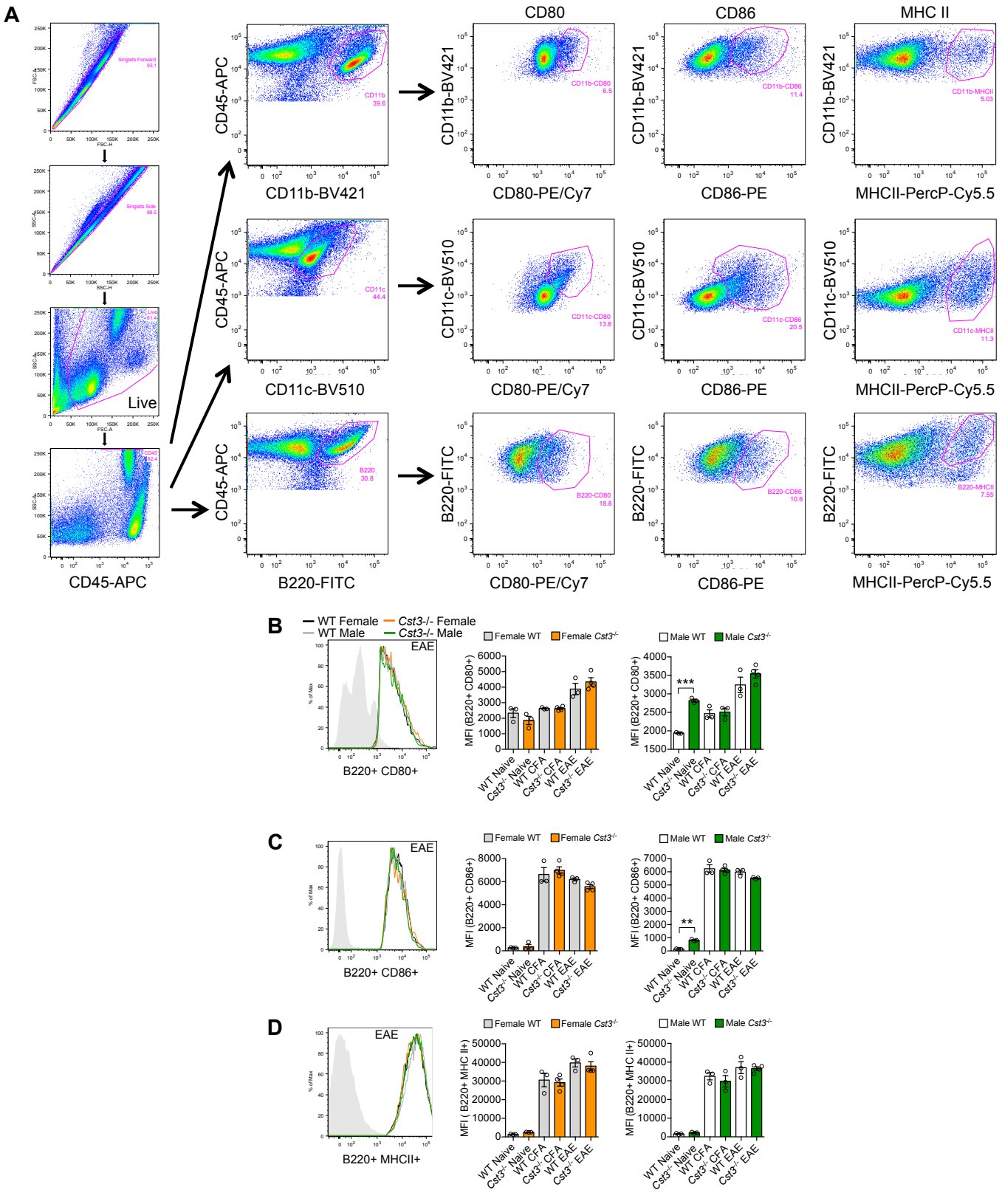
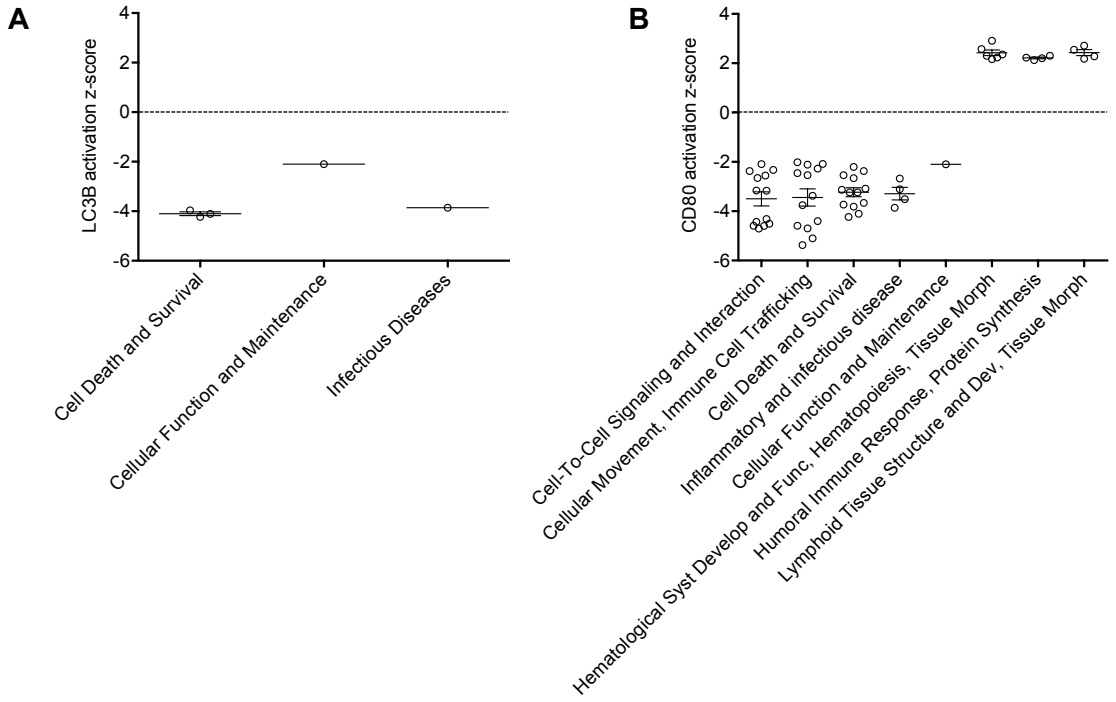

A
CD80
CD86
MHC II
Singlets Forward
Singlets Side
Live
CD45-APC
CD11b-BV421
CD11c-BV510
B220-FITC
CD80-PE/Cy7
CD86-PE
MHCII-PercP-Cy5.5
B
WT Female
WT Male
Cst3-/- Female
Cst3-/- Male
EAE
B220+ CD80+
Female WT
Female Cst3-/-
Male WT
Male Cst3-/-
MFI (B220+ CD80+)
WT Naive
Cst3-/- Naive
WT CFA
Cst3-/- CFA
WT EAE
Cst3-/- EAE
C
B220+ CD86+
MFI (B220+ CD86+)
D
B220+ MHCII+
MFI (B220+ MHC II+)

Figure S6. Gating strategy and expression of antigen presenting markers in B220^{+} cells, Related to Figure 6.

(A) Gating strategy for antigen presentation molecular markers.

(B-D) Mean fluorescent intensity of CD80 (B), CD86 (C) and MHC II (D) expression in female (grey/orange) and male (white/green) WT (grey/white bars) and *Cst3*$^{-/-}$ (orange/green bars) spleen-derived B220^{+} cells from naïve, CFA and EAE mice. Data represented as mean ± SEM and represent 3-4 individual animals per group. One-way ANOVA with Šídák post hoc test, **P<0.01, ***P<0.001.

Figure S7


A
LC3B activation z-score
4
2
0
-2
-4
-6
Cell Death and Survival
Cellular Function and Maintenance
Infectious Diseases
B
CD80 activation z-score
Cell-To-Cell Signaling and Interaction
Cellular Movement, Immune Cell Trafficking
Cell Death and Survival
Inflammatory and Infectious disease
Cellular Function and Maintenance
Hematological Syst Develop and Func, Hematopoiesis, Tissue Morph
Humoral Immune Response, Protein Synthesis
Lymphoid Tissue Structure and Dev, Tissue Morph

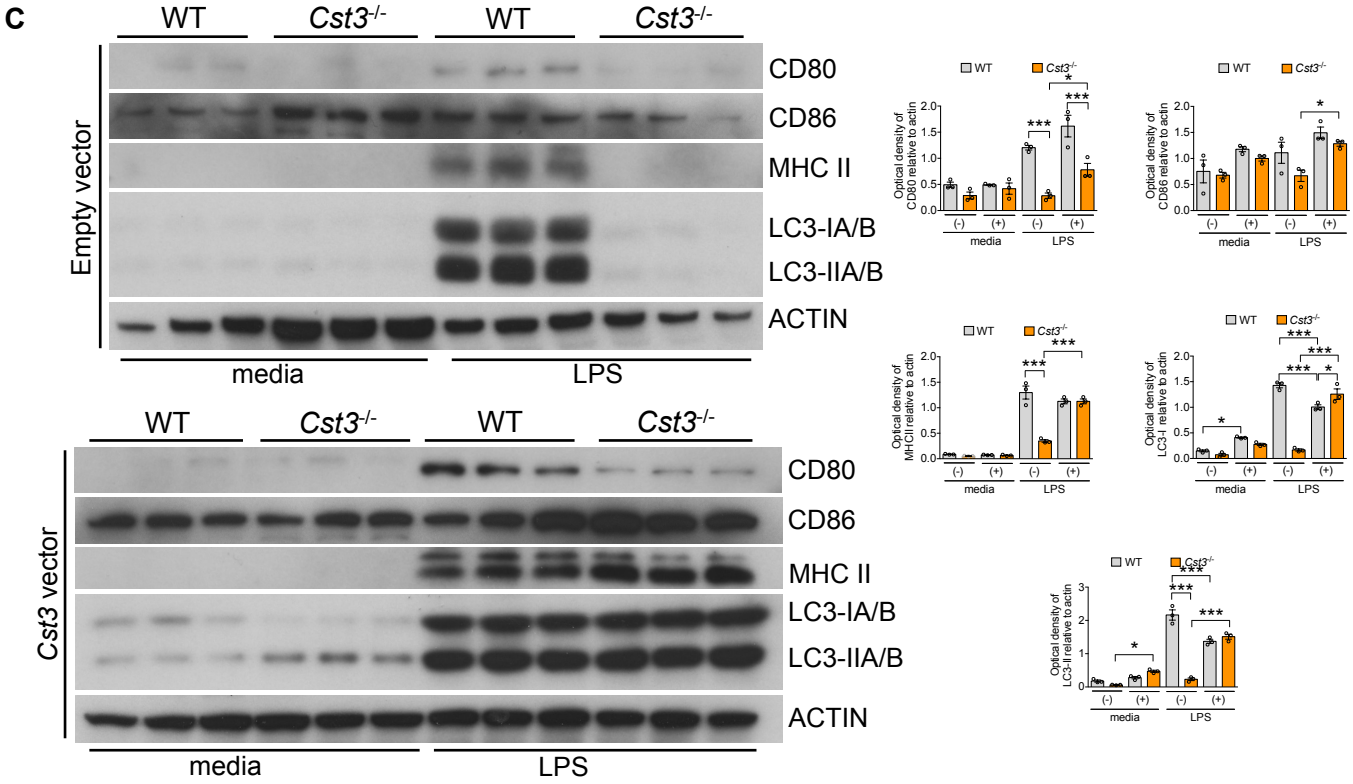

C
WT
Cst3-/-
WT
Cst3-/-
Empty vector
CD80
CD86
MHC II
LC3-IA/B
LC3-IIA/B
ACTIN
media
LPS
Cst3 vector
Optical density of CD80 relative to actin
Optical density of CD86 relative to actin
Optical density of MHCII relative to actin
Optical density of LC3-I relative to actin
Optical density of LC3-II relative to actin

Figure S7. CST3 regulates expression of antigen presentation markers in female CD11b^{+} cells, Related to Figure 6, Table S4**.**

(A-B) The number of times that LC3B (A) and CD80 (B) were present in functional pathways that were decreased (negative z-score) or increased (positive z-score) in female *Cst3*$^{-/-}$ EAE CD11b^{+} cells relative to their male counterparts.

(C) Western blots and semi-quantification of CD80, CD86, MHC II and LC3A/BI/II levels in female WT and *Cst3*$^{-/-}$ BMDMs transfected with empty or *Cst3* vector ± LPS. Data represent one of two experiments with graphs displaying the mean ± SEM of 3 individual mice. Two-way ANOVA with Tukey post-hoc test, *P<0.05, **P<0.01, ***P<0.001.

Figure S8

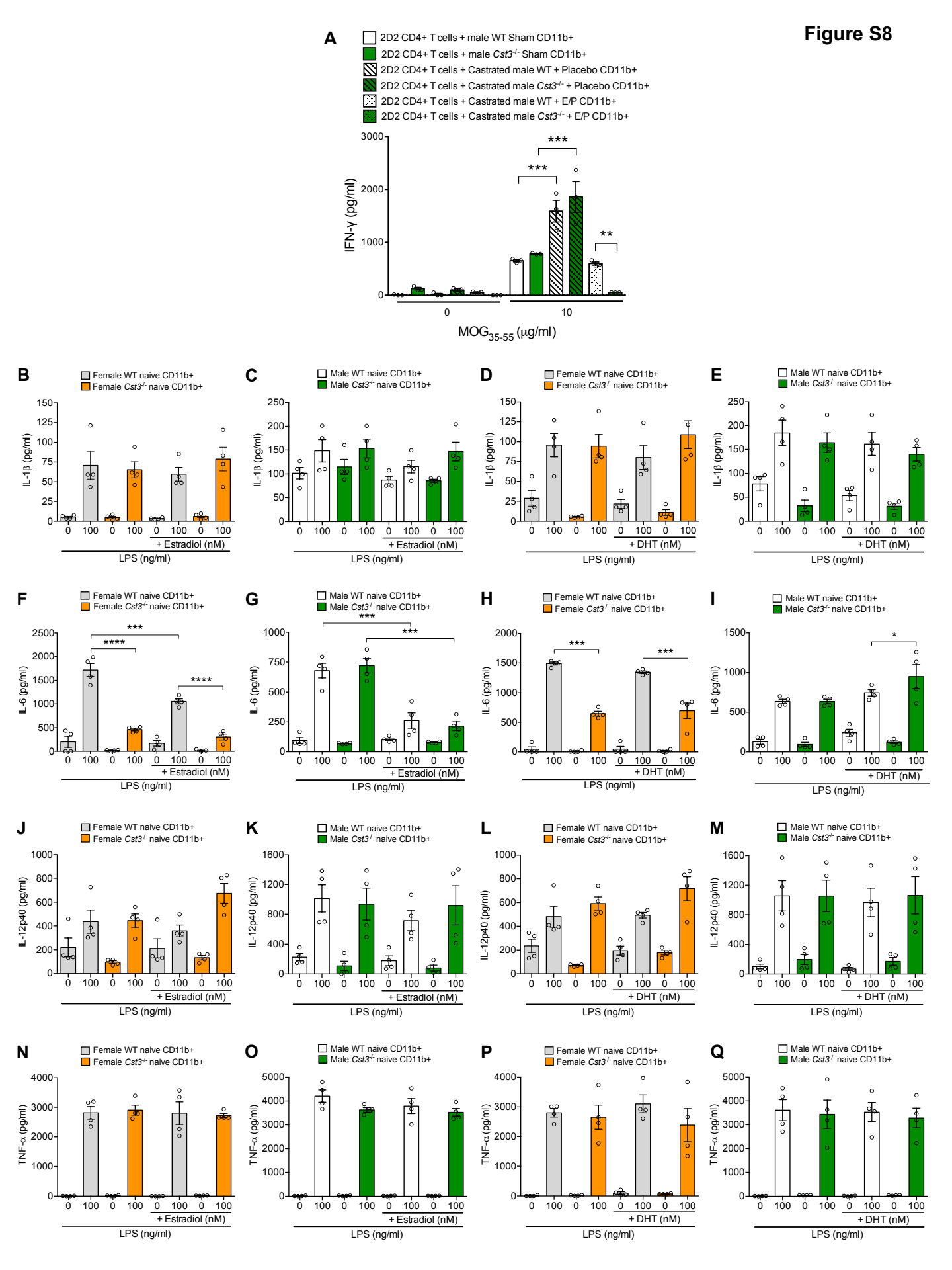

A
2D2 CD4+ T cells + male WT Sham CD11b+
2D2 CD4+ T cells + male Cst3-/- Sham CD11b+
2D2 CD4+ T cells + Castrated male WT + Placebo CD11b+
2D2 CD4+ T cells + Castrated male Cst3-/- + Placebo CD11b+
2D2 CD4+ T cells + Castrated male WT + E/P CD11b+
2D2 CD4+ T cells + Castrated male Cst3-/- + E/P CD11b+
IFN-γ (pg/ml)
MOG35-55 (μg/ml)
B
Female WT naive CD11b+
Female Cst3-/- naive CD11b+
IL-1β (pg/ml)
+ Estradiol (nM)
LPS (ng/ml)
C
Male WT naive CD11b+
Male Cst3-/- naive CD11b+
D
+ DHT (nM)
E
F
IL-6 (pg/ml)
G
H
I
J
IL-12p40 (pg/ml)
K
L
M
N
TNF-α (pg/ml)
O
P
Q

Figure S8. Estrogen contributes to CST3's sex-dependent effect on activation of CD11b$^+$ cells, Related to Figure 7.

(A) Secretion of IFN-γ by MOG_{35-55}-stimulated male 2D2 $CD4^+$ T cells co-cultured with splenic-derived $CD11b^+$ cells from sham (open bars), castrated + placebo (stripes) or castrated + estrogen/progesterone (dots)-treated male WT (grey) and *Cst3*$^{-/-}$ (green) EAE animals at d40 post-immunization. Data presented as mean ± SEM with three individual mice per condition and triplicate wells plated for each mouse; one-way ANOVA with Šídák post-hoc test.

(B-Q) IL-1β (B-E), IL-6 (F-I), IL-12p40 (J-M) and TNF-α (N-Q) production by splenic $CD11b^+$ cells from naive female (B, D, F, H, J, L, N, P) and male (C, E, G, I, K, M, O, Q) WT (grey/white) and *Cst3*$^{-/-}$ (orange/green) mice cultured with 17-β estradiol or DHT ± LPS. Data represented as mean ± SEM with four individual mice per condition; two-way ANOVA with Šídák post hoc test; *P<0.05, **P<0.01, ***P<0.001.

Table S1. Characteristics of MS brain samples acquired from the UK MS Tissue Bank, Related to Figure 1

MS Sample	Sex	Age	Type of MS	Brain area	Lesion Type
CO26	F	78	control	Occipital cortex	
CO25	M	35	control	Occipital periventricular white matter	
MS562	F	62	SPMS	Frontal cortex with underlying white matter	Late chronic active to inactive; shadow plaques
MS335	M	62	SPMS	Frontal cortex and white matter	Inactive; shadow plaques

Table S2: Incidence, mean day of onset and cumulative mean score of WT vs *Cst3*$^{-/-}$ EAE mice, Related to Figure 1

	Incidence	Mean day of onset	Cumulative mean score
Female WT	29/29	10.5 ± 0.75	2.87 ± 0.87
Female *Cst3*$^{-/-}$	26/29	16.4 ± 1.65	0.88 ± 0.34***
Male WT	25/25	9.4 ± 0.63	2.40 ± 0.38
Male *Cst3*$^{-/-}$	25/25	12.1 ± 0.6	1.94 ± 0.44^^

Values are means ± SEM; **/^^P<0.01, ***/^^^P<0.001, 2-tailed unpaired Student's *t*-test, n=25-29 mice per group; Comparisons made: *=female WT vs female *Cst3*$^{-/-}$, ^=male WT vs male *Cst3*$^{-/-}$

Table S3: Incidence, mean day of onset and cumulative mean score of WT vs *Cst3*Tg EAE mice, Related to Figure 1

	Incidence	Mean day of onset	Cumulative mean score
Female WT	12/15	16.14 ± 1.33	1.03 ± 0.29
Female *Cst3*Tg	15/15	10.62 ± 0.86	1.76 ± 0.34***
Male WT	14/15	10.12 ± 0.9	1.67 ± 0.37
Male *Cst3*Tg	15/15	9.75 ± 0.63	1.78 ± 0.25

Values are means ± SEM; ***/^^^P<0.001, 2-tailed unpaired Student's *t*-test, n=15 mice per group; Comparisons made: *=female WT vs female *Cst3*Tg, ^=male WT vs male *Cst3*Tg

Table S4: Presence of CD80 and LC3B in functional pathways that were altered in female *Cst3*$^{-/-}$ EAE CD11b^{+} cells relative to their male counterparts, Related to Figure 6, Figure S7A, B

CD80			
DECREASED	**FUNCTION**	**P VALUE**	**ACTIVATION Z SCORE**
Cell-To-Cell Signaling and Interaction	Activation of myeloid cells	1.57e-15	-4.701
	Activation of phagocytes	1.09e-17	-4.594
	Activation of blood cells	1.6e-17	-4.594
	Activation of leukocytes	8.35e-29	-4.507
	Activation of antigen presenting cells	4.14e-09	-4.432
	Activation of cells	1.14e-27	-4.314
	Immune response of leukocytes	4.83e-19	-3.17
	Stimulation of cells	7.39e-09	-3.163
	Interaction of tumor cell lines	2.48e-11	-2.656
	Binding of tumor cell lines	1.01e-11	-2.551
	Response of mononuclear leukocytes	1e-11	-2.328
	Activation of mononuclear leukocytes	1.18e-20	-2.328
	Interaction of blood cells	6e-19	-2.091
Cell Death and Survival	Cell viability	6.63e-17	-4.23
	Cell survival	2.46e-18	-4.106
	Cell death of lymphocytes	4.54e-15	-3.812
	Cell death of mononuclear leukocytes	4.56e16	-3.728
	Cell death of lymphoid cells	2.2e-15	-3.663
	Apoptosis of lymphocytes	6.28e-13	-3.236
	Cell death of lymphatic system cells	2.44e-16	-3.237
	Apoptosis of mononuclear leukocytes	6.5e-14	-3.13
	Apoptosis of lymphoid cells	2.7e-13	-3.085
	Apoptosis of lymphatic system cells	4.96e-14	-2.658
	Cell death of immune cells	3.12e-28	-2.538
	Apoptosis of leukocytes	4.93e-18	-2.372
	Apoptosis of blood cells	2.22e-19	-2.204
Cellular Movement, Immune Cell Trafficking	Migration of cells	2.99e-27	-5.37
	Cell movement	4.73e-30	-5.103
	Cell movement of phagocytes	3.15e-18	-4.694
	Leukocyte migration	5.62e-30	-4.694
	Cell movement of leukocytes	3.31e-24	-4.402
	Cell movement of mononuclear leukocytes	5.82e-10	-3.758
	Migration of mononuclear leukocytes	2.42e-08	-3.372
	Migration of phagocytes	1.76e-09	-2.538
	Cellular infiltration by leukocytes	8.29e-13	-2.453
	Infiltration by leukocytes	2.38e-13	-2.275
	Cell movement of lymphatic system cells	1.48e-08	-2.11
	Cell movement of lymphoid cells	1.88e-08	-2.082
	Cellular infiltration	7.55e-16	-2.017
INCREASED			
Hematological System Development and Function, Lymphoid Tissue Structure and Development, Tissue Morphology	Quantity of T lymphocytes	1.54e-20	2.912
	Quantity of blood cells	9.87e-47	2.575
	Quantity of hematopoietic progenitor cells	1.35e-24	2.352
	Quantity of lymphocytes	7.59e-36	2.299
	Quantity of mononuclear leukocytes	1.65e-6	2.231
	Quantity of leukocytes	4.83e-41	2.168
Humoral Immune Response, Protein Synthesis	Production of antibody	1.86e-20	2.3
	Quantity of IgG	1.35e-15	2.228
	Quantity of IgG2a	2.89e-09	2.201
	Quantity of immunoglobulin	7.63e-21	2.13

Table S4: cont.

Lymphoid Tissue Structure and Development, Tissue Morphology	Quantity of lymphatic system cells	6.13e-40	2.715
	Quantity of lymphoid organ	4.62e-16	2.543
	Quantity of lymphoid tissue	8.96e-22	2.282
	Quantity of lymphoid cells	3.41e-36	2.181
LC3B			
DECREASED	**FUNCTION**	**P VALUE**	**ACTIVATION Z SCORE**
Cell Death and Survival	Cell viability	3.91e-11	-3.965
	Cell survival	2.46e-18	-4.106
Cellular Function and Maintenance	Cellular homeostasis	1.79e-21	-2.101
Infectious Diseases	Viral Infection	2.67e-20	-3.858

Table S5: Incidence, mean day of onset and cumulative mean score of ovariectomized female EAE mice administered testosterone, Related to Figure 7A, B

	Incidence	Mean day of onset	Mean score
Female WT sham	10/10	8.90 ± 0.33	2.37 ± 0.25
Ovariectomized female WT + Placebo	10/10	10.2 ± 0.80	2.79 ± 0.43**
Ovariectomized female WT + Testosterone	10/10	10.8 ± 0.66	2.62 ± 0.47
Female *Cst3*$^{-/-}$ sham	4/8	15 ± 3.74	0.59 ± 0.35***
Ovariectomized female *Cst3*$^{-/-}$ + Placebo	7/8	12.83 ± 1.68	1.67 ± 0.62***
Ovariectomized female *Cst3*$^{-/-}$ + Testosterone	8/8	13 ± 1.27	1.94 ± 0.55**

Values are means ± SEM; ** P<0.01, ***P<0.001, 2-tailed unpaired Student's *t*-test, n=8-10 mice per group; each group is compared to WT sham

Table S6: Incidence, mean day of onset and cumulative mean score of castrated male EAE mice administered estrogen/progesterone, Related to Figure 7A, B

	Incidence	Mean day of onset	Mean score
Male WT sham	10/10	7.7 ± 0.16	2.48 ± 0.21
Castrated male WT + Placebo	10/10	7.8 ± 0.14	2.70 ± 1.09*
Castrated male WT + Estradiol/ Progesterone	9/10	23 ± 1.93	1.09 ± 0.48***
Male *Cst3*$^{-/-}$ sham	8/9	13.25 ± 1.86	1.86 ± 0.48***
Castrated male *Cst3*$^{-/-}$ + Placebo	9/9	15.44 ± 1.91	1.90 ± 0.48***
Castrated male *Cst3*$^{-/-}$ + Estradiol/ Progesterone	5/9	30 ± 0.35	0.35 ± 0.34***

Values are means ± SEM; *P<0.05, ***P<0.001, Student's *t*-test, n=8-10 mice per group; each group is compared to WT sham