Neurotensin increases mortality and mast cells reduce neurotensin levels in a mouse model of sepsis

Supplementary Methods (A. M. Piliponsky *et al*.)

Lentiviral vector production

pLentiLox 3.7 (pLL3.7), a vector engineered to co-express enhanced green fluorescent protein (GFP) as a reporter gene, that permits infected cells to be tracked by flow cytometry,[1] was digested with XhoI and HpaI and the annealed oligos 5'-t-GAAACAGTTTGATGTGAAA-ttcaagaga-TTTCACATCAAACTGTTTC-ttttttc-3' and 5'-tcgagaaaaaa-GAAACAGTTTGATGTGAAA-tctcttgaa-TTTCACATCAAACTGTTTC-a-3' for mMC-CPA and 5'-t-GGAATGCGAAGAGCGAGGT-ttcaagaga-ACCTCGCTCCTTAAAATTCC-ttttttc-3' 5'-tcgagaaaaaa-GGAATGCGAAGAGCGAGGT-tctcttgaa-CTCGCTCTTCGCATTCCA-a-3' for NLN were ligated into pLL3.7 to yield a directed shRNA-producing vector. The 19 nt target sequences are indicated in capitals in the oligonucleotide sequence. Active viral stocks were created and concentrated as previously described.[2] Briefly, 293T cells were transfected with the transfer vector plasmid pLL3.7-mMC-CPA or pLL3.7-NLN or pLL3.7 (empty vector), the VSV-G envelope-encoding plasmid pMD.G, and the packaging plasmid CMVΔR8.74 using the calcium phosphate method. The supernatants were harvested 48 or 72 h post-transfection, pooled, passed through a 0.45 μm filter, ultracentrifuged for 2 h 20 min at 19,200 rpm in an SW28 rotor, re-suspended in 100 μl of 0.1% BSA in PBS and stored at -80 °C.

Preparation of NLN shRNA- or mMC-CPA shRNA-containing mast cells (MCs)

2-5 week old bone marrow-derived cultured MCs (BMCMCs) were infected with virus carrying the NLN- or mMC-CPA-targeting shRNA or the empty vector. Since pLL3.7 carries a CMV-GFP cassette, BMCMCs were sorted for GFP expression at 72-96 h after infection using FACS Aria (Becton Dickinson) and then were cultured in IMDM + 10 ng/ml mouse recombinant IL-3 (Peprotech) + 10 ng/mL rat recombinant SCF (Amgen). 1.0×10^6 infected BMCMCs were injected i.p. into $Kit^{W/W\text{-}v}$ mice and experiments were performed 4 weeks later.

RT-PCR

For RT-PCR, RNA (50 ng) was isolated from cells with an RNeasy mini kit (Qiagen) and converted to first-strand cDNA with oligo(dT) primers (Ambion) and Sensiscript reverse transcriptase (Qiagen) before amplification with specific primers using RETROscriptTM (Ambion). The resulting PCR products were resolved on 1.5% agarose gels. The primer pairs used for amplification were the following: 5'-TGGGACCTCCATTACTACATGACC-3' (forward) and 5'-CCATAATACTGGCCGTCATACCCT-3' (reverse) for NLN; 5'-GCCACAGCCCTCAATGTAGCC-3' (forward) and 5'-GACGGTCAGTTTGTTGGCTAT-3' (reverse) for Ntsr1; 5'-ACAGAAGCACGAAATGGAGAGG-3' (forward) and 5'-CACCTGGAATGTAGACCTGGAG-3' (reverse) for Ntsr2; 5'-

CAACAATACGCACCAGCATGT-3' (forward) and 5'-CTTGGAAAGTGGTCAGGACGAG-3' (reverse) for Ntsr3.

Membrane preparations
Frozen cell pellets were resuspended in hypotonic lysis buffer (20mM HEPES, pH 7.2, 100 mM KCl, 1mM DTT, Complete Mini Protease Inhibitor tablets–EDTA [Roche]), lysed by sonication, and centrifuged for 5 min at 3000 rpm (960g) to remove intact cells and nuclei. Post nuclear supernatants were centrifuged at 50,000 rpm (70,000g) in a TLA55 rotor for 15 min to separate membrane from cytosol fractions.

Western blot analysis
Cells, membranes, and supernatant fractions were denatured by boiling 1 min with sample buffer (SB) (2.5% SDS 10% glycerol and 5% mercaptoethanol). Lysates were separated by SDS/PAGE, electroblotted onto Invitrolon poly (vinylidene difluoride) membranes (Invitrogen), then probed with antibodies against NLN, mMCP-4, mMCP-5 and GAPDH (Research Diagnostics).

CPA enzymatic activity assay
CPA enzymatic activity was assessed by measuring reduction of absorbance of a chromogenic substrate (N-[4-Methoxyphenylazoformyl]-Phe-OH) (Bachem Peninsula Labs) specific for CPA as previously described.[3]

Beta-hexosaminidase release
Beta-hexosaminidase release by MCs was measured on an enzyme-linked immunosorbent assay "reader" that detects the hydrolysis product of p-nitrophenyl-N-acetyl-b-D-glucosamine (Sigma) used as a substrate.[4]

Serotonin release
Serotonin release by MCs was assessed by the specific release, by 15 min after MC stimulation at 37 °C, of [^{3}H]-hydroxytryptamine creatinine sulfate (^{3}H-5HT [or ^{3}H-serotonin]; Perkin Elmer) from MCs pre-loaded with ^{3}H-5HT for 2 h at 37 °C.[5]

NT and ET-1 measurements
Concentrations of NT and ET-1 were measured by ELISA (Bachem Peninsula Labs for NT; Biomedica for ET-1). The NT ELISA kit does not cross-react with the following NT fragments: NT (1-12), NT (1-11) and NT (1-10). The detection limits for the ELISA kits were 20-35 fmol/ml for NT and 0.048 fmol/ml for ET-1.

Evaluation of MCs in the peritoneal cavity and mesenteric windows
To harvest peritoneal cells, 2 ml of HBSS buffer containing 10 u/ml heparin and 1% FCS were injected into the peritoneal cavity, and the abdomen was massaged gently for 30 s. Fluid containing peritoneal cells was aspirated and the cells were cytocentrifuged onto glass slides and stained with May-Grunwald-Giemsa to identify and quantify the % of various cell populations and to assess the degranulation status of MCs. Evaluation of MCs in the mesenteric windows was performed as previously described.[6,7] Briefly, ~ 4-5

mesenteric windows from approximately the same locations in each mouse were arranged onto slides and fixed for 1 h in Carnoy's solution (3:2:1 v/v/v of ethanol, chloroform, and acetic acid). Tissues were stained with Csaba stain.[6,7] Csaba stain contains both safranin (red, identifying mature MCs) and alcian blue (blue, identifying less mature MCs), which bind to MC cytoplasmic granules. Cytocentrifuge preparations were evaluated (for % of MCs and quantification of MC degranulation), and slides of meseneteric windows were examined (to assess presence, numbers and distribution of MCs in the mesentery), by an observer not aware of the identity of the individual mice.

Human umbilical cord blood-derived MCs

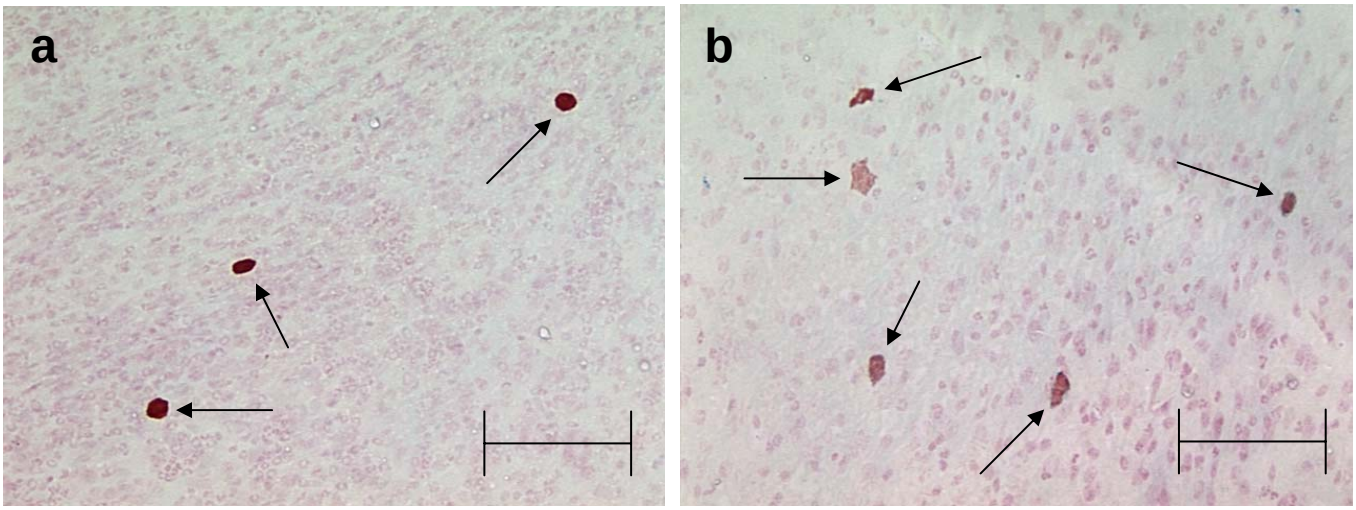
Human umbilical cord blood–derived MCs were obtained essentially as previously described.[8] Briefly, mononuclear cells were collected and $CD34^+$ cells were isolated using a magnetic separation column and a CD133 cell isolation kit (Miltenyi Biotec). Cells were maintained in Iscove's modified Dulbecco medium supplemented with 100 ng/mL recombinant human $rSCF^{164}$ (rhSCF; Amgen); 1 ng/mL rhIL-3; and 50 ng/mL rhIL-6 (rhIL-6; Amgen); and half of the culture medium was changed weekly. Cells were incubated with IL-3 during the first week of culture. The preparations used in all of the experiments consisted of cell populations that were at least 10 weeks old and that expressed high levels of c-Kit as assessed by flow cytometry. Umbilical cord blood was obtained with the approval of the Stanford University Institutional Review Board.

References

1. Rubinson, D.A. et al. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet* **33**, 401-6 (2003).
2. Lois, C., Hong, E.J., Pease, S., Brown, E.J. & Baltimore, D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* **295**, 868-72 (2002).
3. Tchougounova, E., Pejler, G. & Abrink, M. The chymase, mouse mast cell protease 4, constitutes the major chymotrypsin-like activity in peritoneum and ear tissue. A role for mouse mast cell protease 4 in thrombin regulation and fibronectin turnover. *J Exp Med* **198**, 423-31 (2003).
4. Ortega, E., Hazan, B., Zor, U. & Pecht, I. Mast cell stimulation by monoclonal antibodies specific for the Fc epsilon receptor yields distinct responses of arachidonic acid and leukotriene C4 secretion. *Eur J Immunol* **19**, 2251-6 (1989).
5. Coleman, J.W., Holliday, M.R., Kimber, I., Zsebo, K.M. & Galli, S.J. Regulation of mouse peritoneal mast cell secretory function by stem cell factor, IL-3 or IL-4. *J Immunol* **150**, 556-62 (1993).
6. Maurer, M. et al. Mast cells promote homeostasis by limiting endothelin-1-induced toxicity. *Nature* **432**, 512-6 (2004).
7. Metz, M. et al. Mast cells can enhance resistance to snake and honeybee venoms. *Science* **313**, 526-30 (2006).

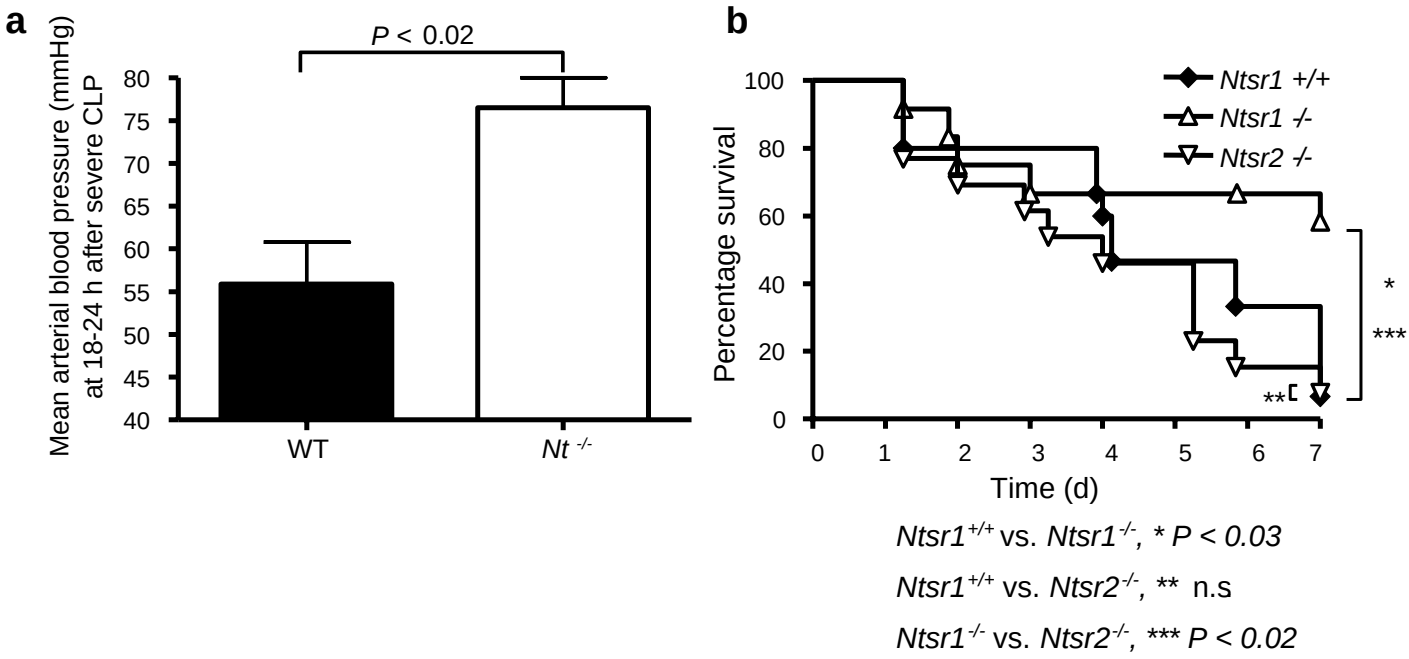
8. Matsuda, K. et al. Monomeric IgE enhances human mast cell chemokine production: IL-4 augments and dexamethasone suppresses the response. *J Allergy Clin Immunol* **116**, 1357-63 (2005).

Supplementary Figure 1, A. M. Piliponsky *et al*.



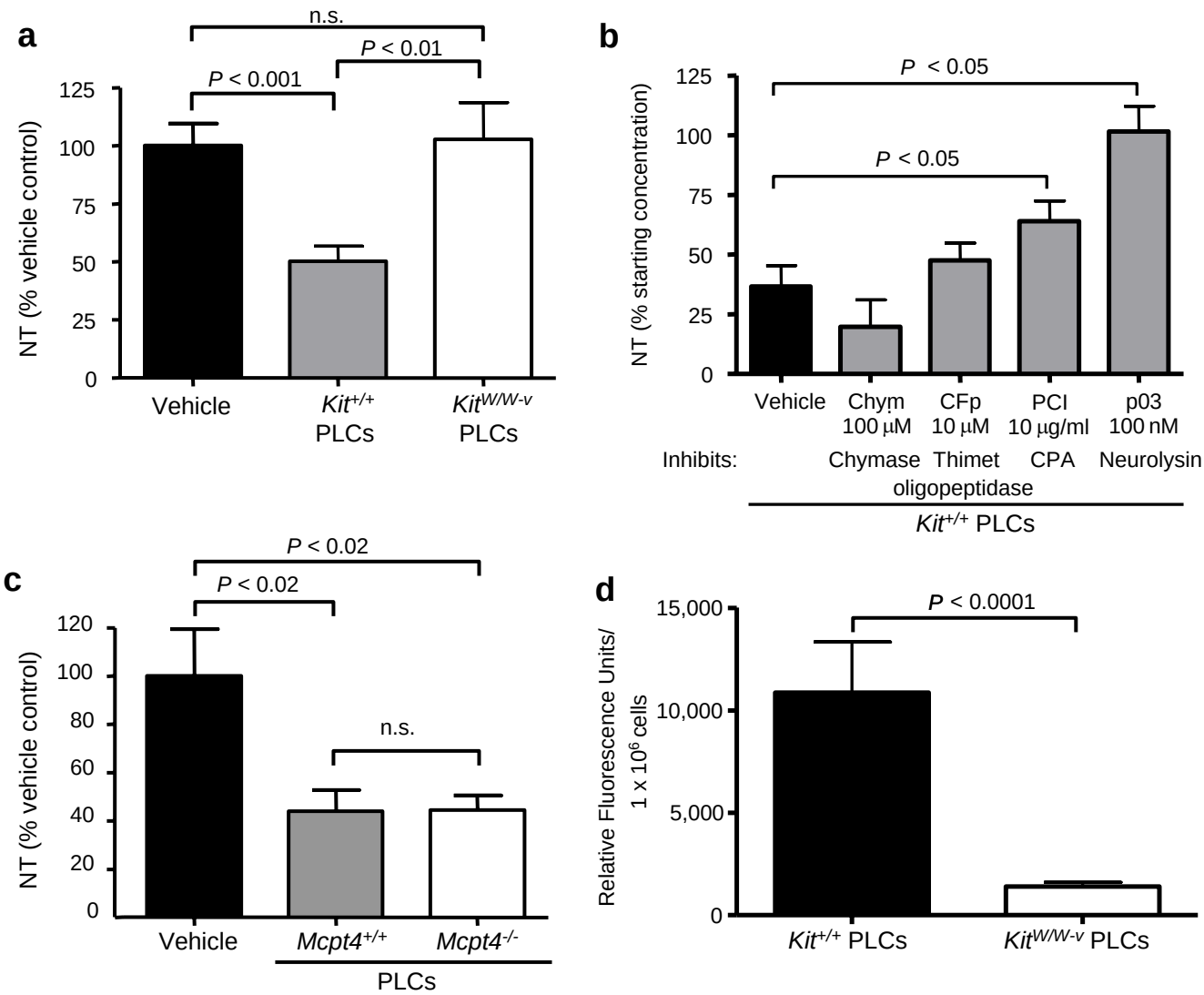
Supplementary Figure 1. Distribution of mast cells (MCs) in the mesentery of $Nt^{+/+}$ or $Nt^{-/-}$ mice. MCs in the mesentery (arrows, MCs; scale bars, 100 μm; Czaba stain) of (**a**) an $Nt^{+/+}$ mouse and (**b**) an $Nt^{-/-}$ mouse.

Supplementary Figure 2, A. M. Piliponsky *et al*.



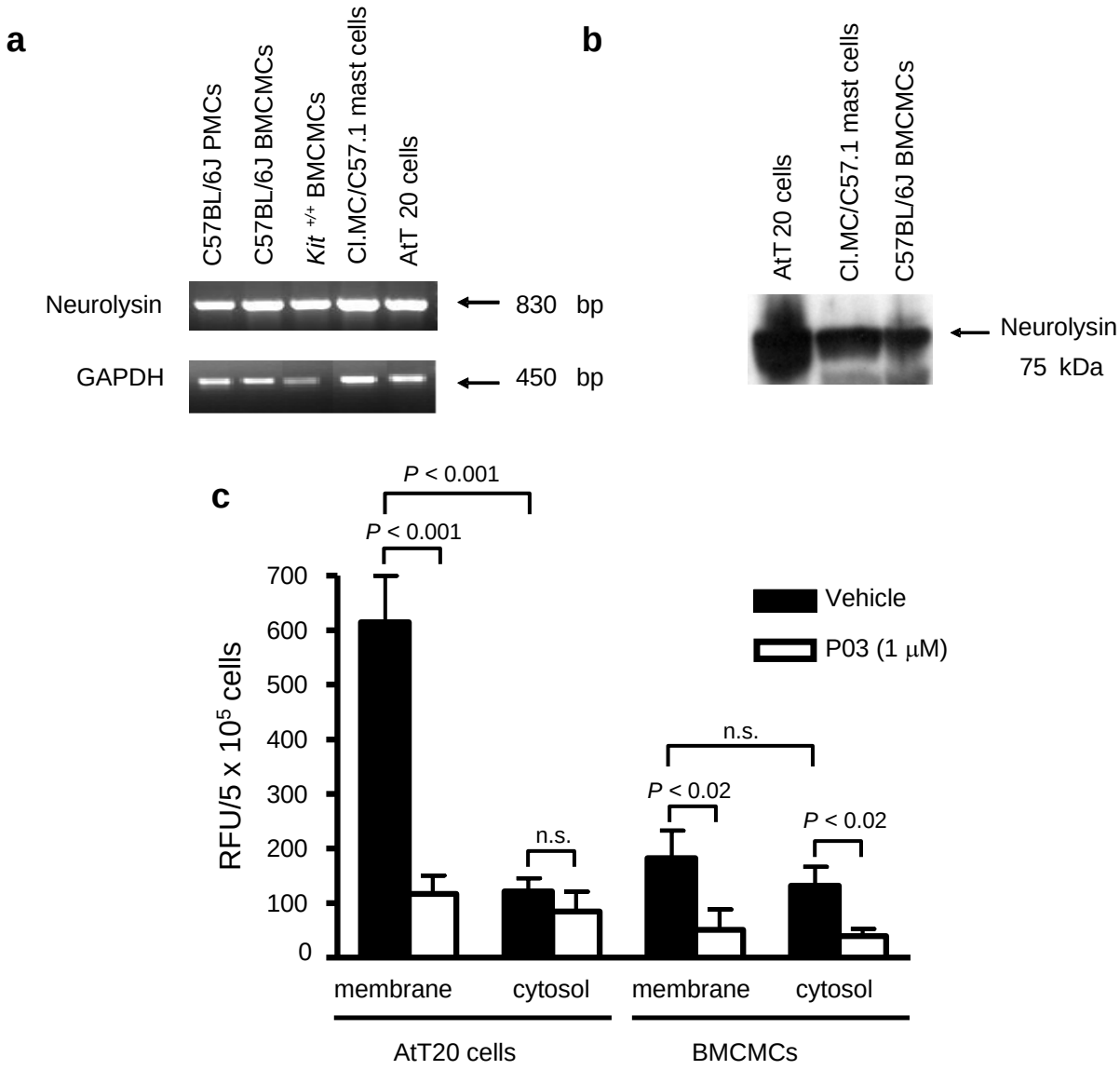
Supplementary Figure 2. Neurotensin (NT) contributes to hypotension, and Ntsr1 contributes to mortality, during severe CLP. (**a**) Mean arterial blood pressure in $Nt^{+/+}$ and $Nt^{-/-}$ mice (n = 5/group) at 18-24 h after severe CLP (ligation of distal half of caecum; one puncture with a 20G needle). (**b**) Survival after severe CLP (ligation of distal half of caecum; one puncture with a 20G needle) in 8-12-week old female or male wild type ($Ntsr^{+/+}$) (n = 15), Ntsr1-deficient ($Ntsr1^{-/-}$) (n = 12) and Ntsr2-deficient ($Ntsr2^{-/-}$) (n = 13) mice. Data were pooled from the three experiments performed, each of which gave similar results. n.s. = not significant (P > 0.05).

Supplementary Figure 3, A. M. Piliponsky *et al*.



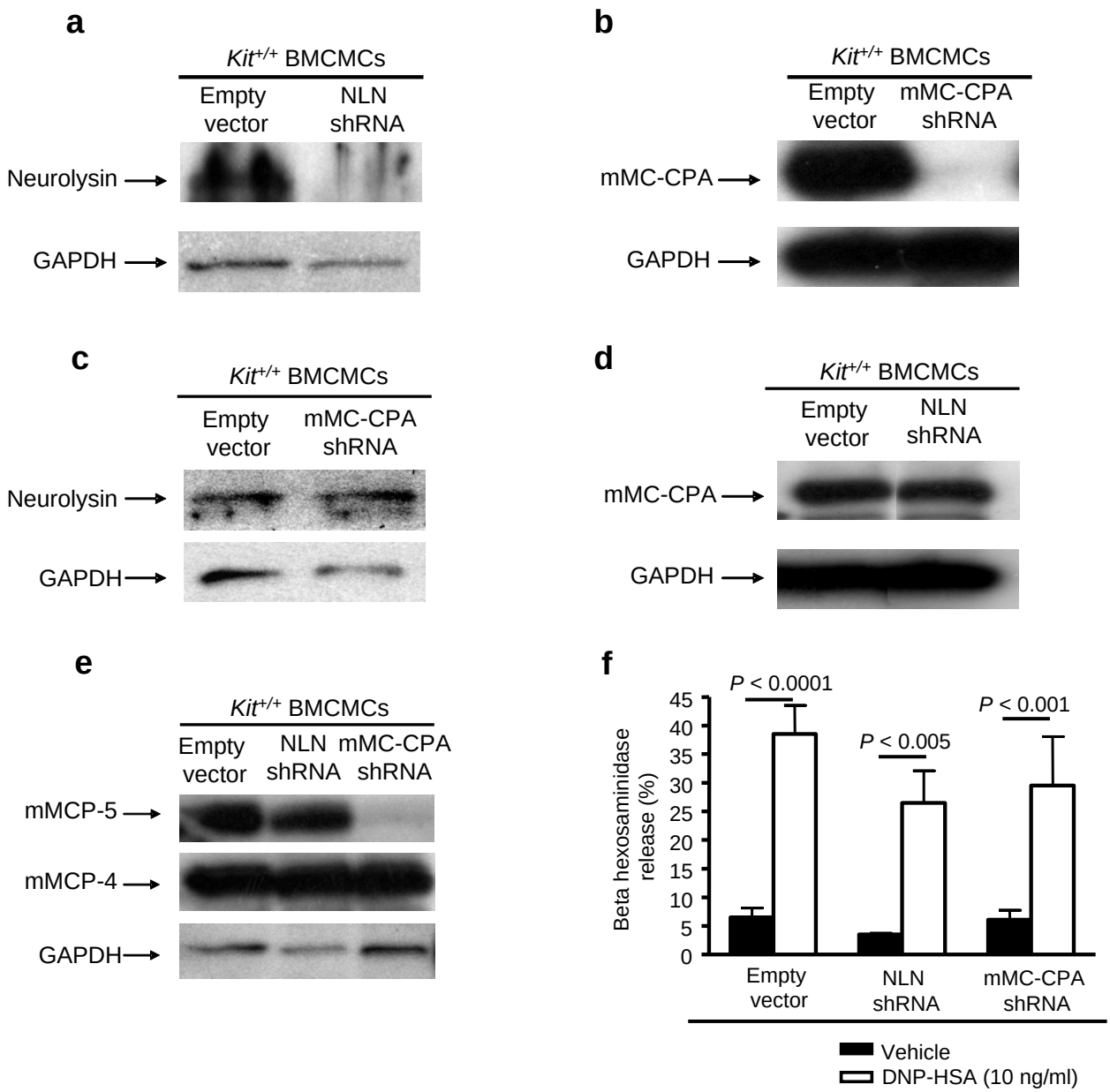
Supplementary Figure 3. Evidence that MCs importantly contribute to the ability of peritoneal lavage cells (PLCs) to degrade NT. NT (10 μM) was incubated for 30 min at 37 °C with 1 x 10^6 PLCs from *Kit*$^{+/+}$ mice (containing ~ 5 x 10^4 peritoneal MCs) (**a, b**), with 1 x 10^6 PLCs from *Kit*$^{W/W-v}$ mice (containing virtually no MCs) (**a**), from wild type (*Mcpt4*$^{+/+}$) or mMCP-4-deficient (*Mcpt4*$^{-/-}$) mice (**c**) or with vehicle alone (**a, b, c**). In **b**, cells were pre-treated for 15 min at 37 °C with one of the following protease inhibitors: chymostatin (Chym, a chymase inhibitor, at 100 μM), CFp-Ala-Ala-Phe-pAB (CFp, a thymet oligopeptidase inhibitor, at 10 μM), potato carboxypeptidase inhibitor (PCI, a carboxypeptidase inhibitor, at 10 μg/ml) and phosphodiepryl 03 (p03, a neurolysin inhibitor, at 100 nM). In **a**, **b** and **c**, results are expressed as the percentage of NT remaining in the samples incubated with cells compared to that at the start of the incubation (**b**) or compared to that in samples of NT incubated in vehicle alone (**a, c**) (n = 4-9/group; data were pooled from the two-three independent experiments performed, each of which gave similar results). n.s. = not significant ($P > 0.05$). (**d**) Quenched Fluorescence Substrate (QFS) assay for the assessment of NLN activity in PLCs of *Kit*$^{+/+}$ mice or *Kit*$^{W/W-v}$ mice. Results are expressed in relative fluorescence substrate units (RFU) generated by incubation of the quenched fluorescence substrate (QFS) for 1 h at 37 °C with 2 x 10^6 cells activated by A23187 (5 μM) (n = 8-12 replicates/group, pooled from two experiments, each of which gave similar results).

Supplementary Figure 4, A. M. Piliponsky *et al*.



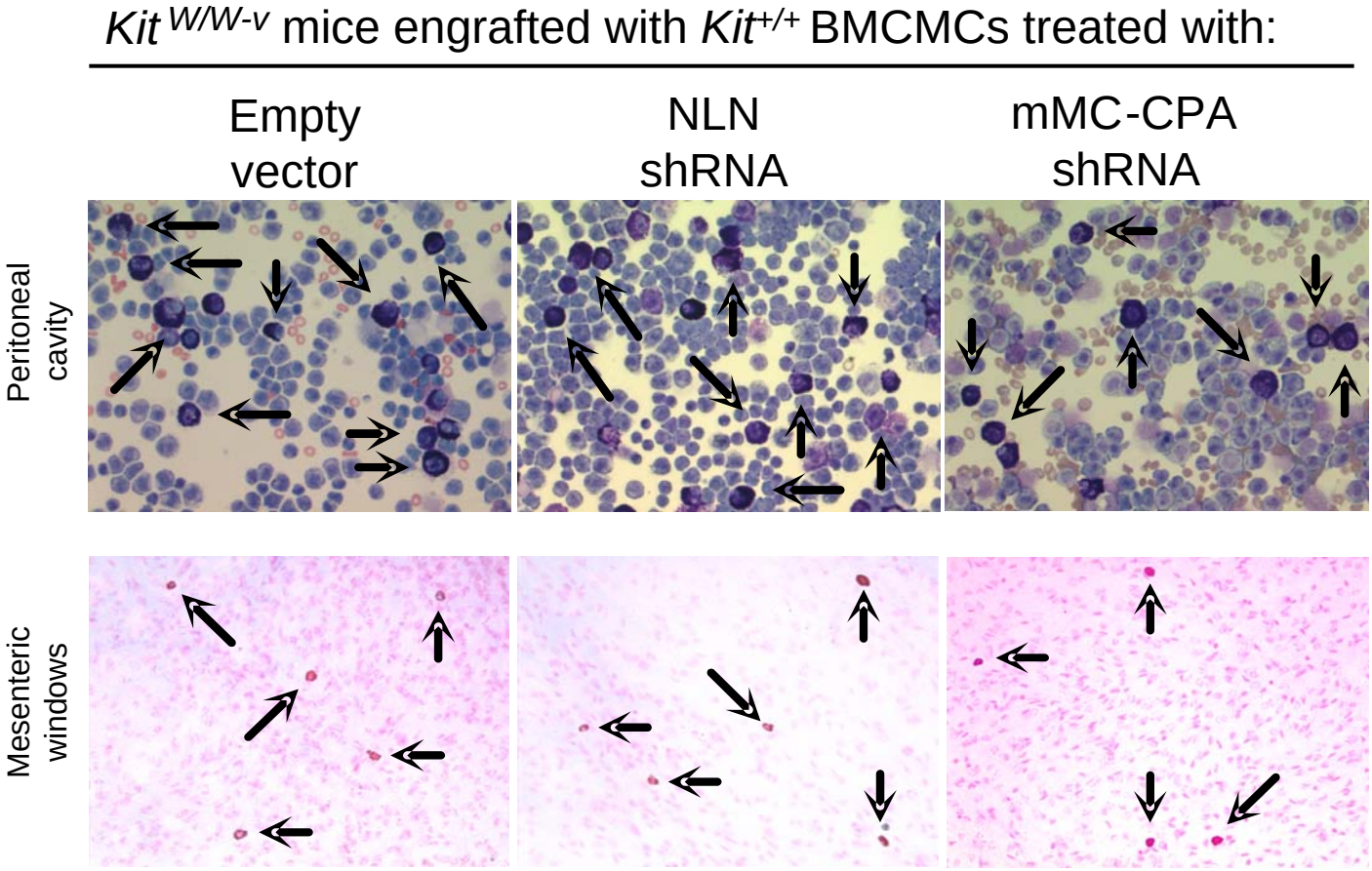
Supplementary Figure 4. MCs express neurolysin. (**a**) Expression of mRNA for neurolysin (NLN) in the AtT20 neuroendocrine cell line (used as a positive control), the transformed MC line Cl.MC/C57.1, C57BL/6J BMCMCs, *Kit*$^{+/+}$ BMCMCs and peritoneal MCs (PMCs) purified from *Kit*$^{+/+}$ mice (PMCs). (**b**) Western blot analysis for NLN in lysates of AtT20 cells, Cl.MC/C57.1 MCs and C57BL/6J BMCMCs. (**c**) Quenched Fluorescence Substrate (QFS) assay for the assessment of NLN activity in membrane and cytosol preparations obtained from AtT20 neuroendocrine cells and C57BL/6J BMCMCs that were pre-treated with either vehicle or with p03 (1 μM). Results are expressed in relative fluorescence substrate units (RFU) generated by incubation of the quenched fluorescence substrate (QFS) for 1 h at 37 °C with membrane or cytosol preparations obtained from 5 x 10^5 cells (n = 4/group); data were pooled from the two independent experiments performed, each of which gave similar results). n.s. = not significant (P > 0.05).

Supplementary Figure 5, A. M. Piliponsky *et al*.



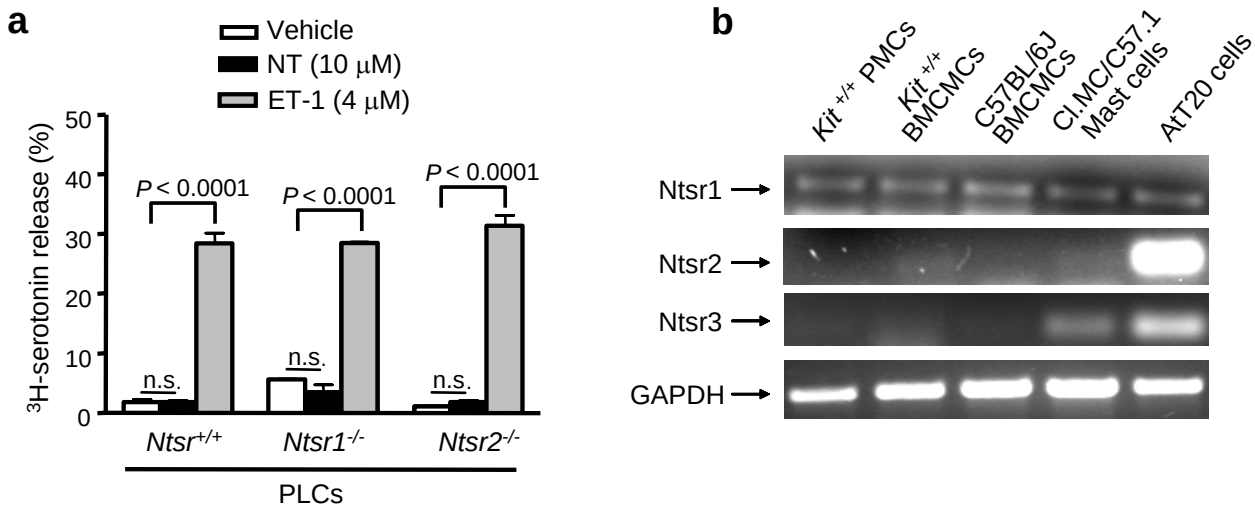
Supplementary Figure 5. Effects of treatment of BMCMCs with NLN shRNA or mMC-CPA shRNA. Protein expression for: (**a, c**) NLN or (**b, d**) mMC-CPA in *Kit*$^{+/+}$ BMCMCs treated with either empty vector or shRNAs. (**e**) mMCP-4 and mMCP-5 protein expression in *Kit*$^{+/+}$ BMCMCs treated with empty vector, or with NLN shRNA or mMC-CPA shRNA. Results in **a**-**e** are representative of those obtained in each of the three to five experiments performed. (**f**) Beta-hexosaminidase release by *Kit*$^{+/+}$ BMCMCs treated with empty vector, NLN shRNA or mMC-CPA shRNA. BMCMCs (1.25×10^5) were sensitized with IgE mAb to DNP (2 μg/ml) overnight at 37 °C and then were challenged with DNP-HSA (10 ng/ml) for 30 min at 37 °C (n = 4-8/group; data were pooled from the three independent experiments performed, each of which gave similar results).

Supplementary Figure 6, A. M. Piliponsky *et al*.



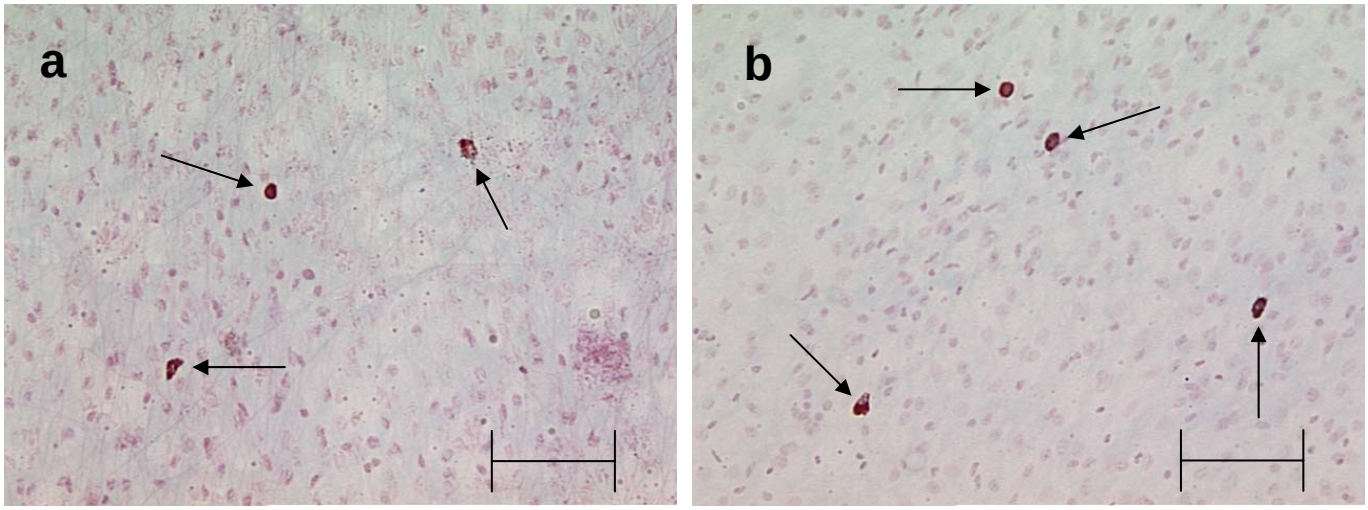
Supplementary Figure 6. Distribution of MCs in the mesentery of *Kit*$^{W/W-v}$ mice engrafted with various BMCMC preparations. MCs (indicated by arrows) in the peritoneal cavity or mesentery ("Mesenteric windows") of *Kit*$^{W/W-v}$ mice which had been engrafted i.p. with *Kit*$^{+/+}$ BMCMCs treated with either empty vector (empty vector BMCMCs→*Kit*$^{W/W-v}$), NLN-shRNA (NLN-shRNA BMCMCs→*Kit*$^{W/W-v}$) or mMC-CPA-shRNA (mMC-CPA-shRNA BMCMCs→*Kit*$^{W/W-v}$) (n = 4/group). In photomicrographs of cells from the peritoneal cavity (May Grunwald-modified Giemsa stain) or of the mesenteric windows (Csaba stain), scale bars = 100 μm.

Supplementary Figure 7, A. M. Piliponsky *et al*.



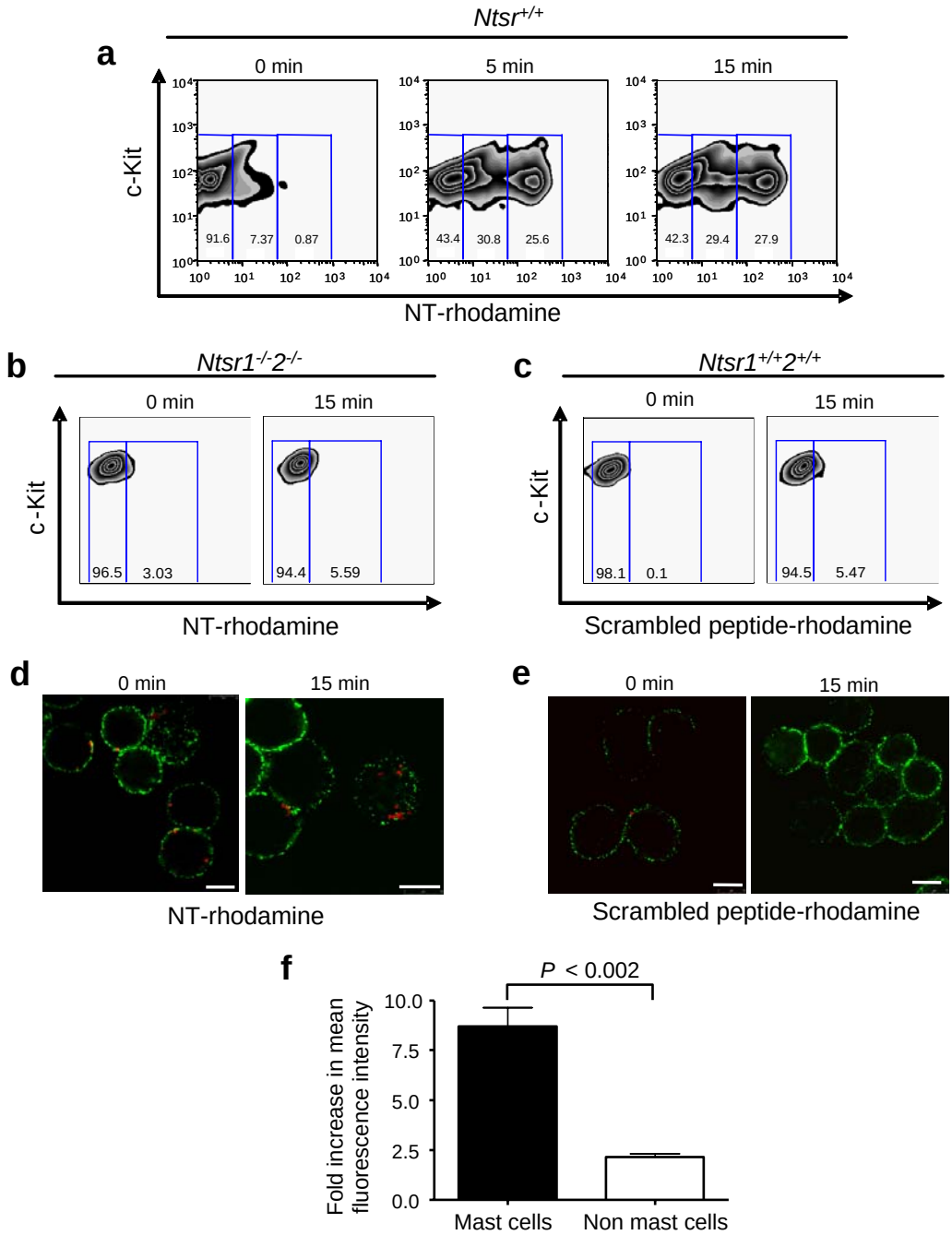
Supplementary Figure 7. Endothelin-1 (ET-1)-induced ^{3}H-serotonin release and NT receptor (Ntsr) expression in MCs. (**a**) 1 x 10^6 peritoneal lavage cells (PLCs) containing ~ 1 x 10^4 peritoneal MCs (PMCs) obtained from either wild type C57BL/6 (*Ntsr*$^{+/+}$) mice, Ntsr1-deficient (*Ntsr1*$^{-/-}$) mice or Ntsr2-deficient (*Ntsr2*$^{-/-}$) mice were incubated for 2 h with ^{3}H-5-hydroxytryptamine (^{3}H-serotonin) at 37 °C and stimulated for 15 min at 37 °C with either ET-1 (4 μM) or NT (10 μM) (n = 4/group; data were pooled from the two independent experiments performed, each of which gave similar results). (**b**) mRNA expression for NT-receptors in the AtT20 neuroendocrine cell line, the transformed MC line Cl.MC/C57.1, C57BL/6J BMCMCs, *Kit*$^{+/+}$ BMCMCs and PMCs purified from *Kit*$^{+/+}$ mice. n.s. = not significant (P > 0.05).

Supplementary Figure 8, A. M. Piliponsky *et al*.



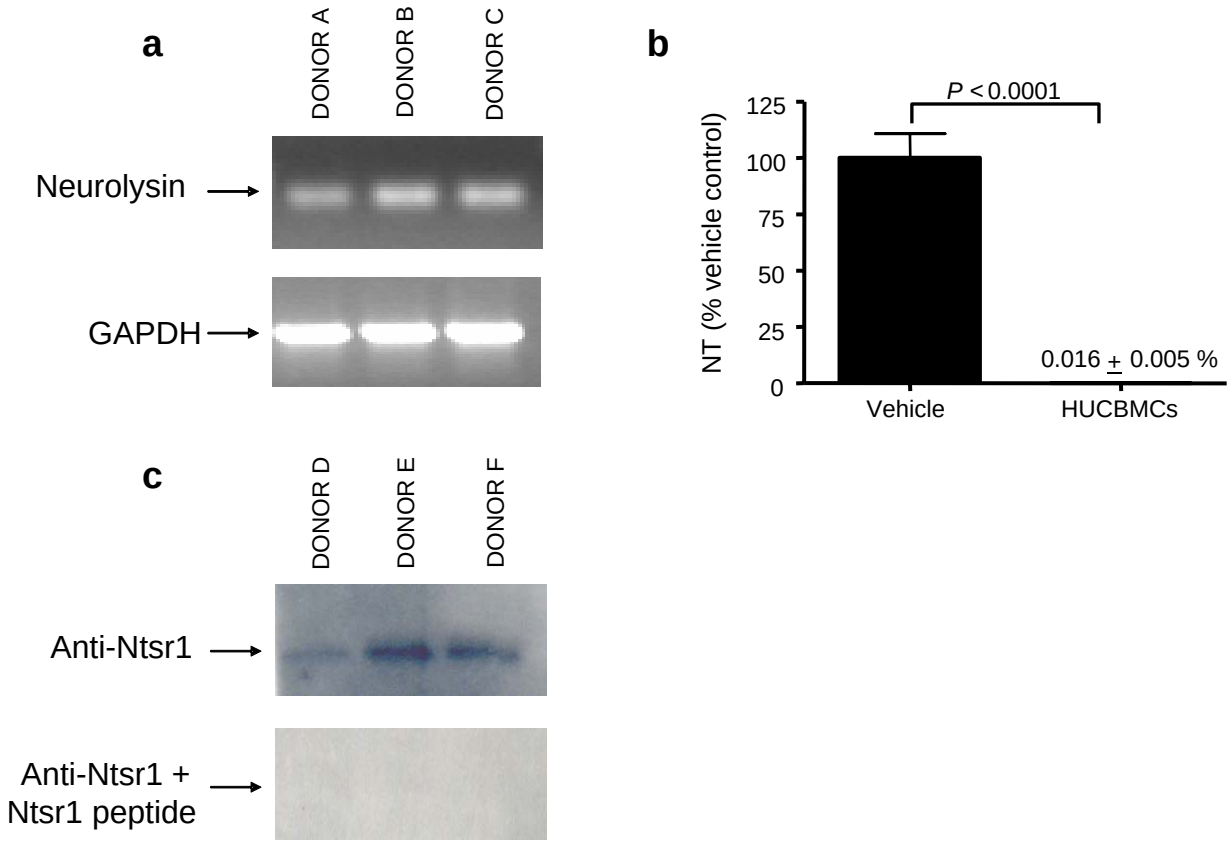
Supplementary Figure 8. Distribution of MCs in the mesentery of wild type (*Ntsr*$^{+/+}$) BMCMC-engrafted or *Ntsr1*$^{-/-}$ BMCMC-engrafted *Kit*$^{W/W\text{-}v}$ mice. MCs in the mesentery (arrows, MCs; scale bar, 100 μm; Czaba stain) of (**a**) an *Ntsr*$^{+/+}$ BMCMCs→*Kit*$^{W/W\text{-}v}$ mouse or (**b**) an *Ntsr1*$^{-/-}$ BMCMCs→*Kit*$^{W/W\text{-}v}$ mouse.

Supplementary Figure 9, A. M. Piliponsky *et al*.



Supplementary Figure 9. Evidence that mast cells internalize NT through Ntsrs. (**a-c**) Flow cytometry analysis and (**d, e**) confocal microscopy of C57BL/6 wild type (*Ntsr*$^{+/+}$) (**a, c**) or *Ntsr1*$^{-/-}$ *2*$^{-/-}$ (**b**) peritoneal MCs (PMCs) pre-loaded with either NT-rhodamine (**a, b, d**) or scrambled peptide-rhodamine (**c, e**) (10 μM) (red) that were placed at 37 °C for the indicated times. Control cells pre-loaded with either NT-rhodamine or scrambled peptide-rhodamine were maintained at 4 °C ("0 min"). PMCs were identified among other peritoneal lavage cells (PLCs) in confocal microscopy by green staining of IgE bound to the PMC surface and in flow cytometry by staining of c-Kit receptors. (**f**) Fold increase in mean fluorescence intensity by flow cytometry in PMCs versus other (non mast cell) PLCs obtained from *Ntsr*$^{+/+}$ mice incubated for 15 min at 37 °C with NT-rhodamine when compared to control cells that were maintained at 4 °C for 15 min. Data are representative of (**a-e**) or pooled from (**f**) the three experiments performed, each of which gave similar results.

Supplementary Figure 10, A. M. Piliponsky *et al*.



Supplementary Figure 10. **Human mast cells degrade NT and express NLN mRNA and Ntsr1 protein**. (**a**) Expression of mRNA for neurolysin by RT-PCR in human umbilical cord blood-derived MCs (HUCBMCs, purity >99%) obtained from three different donors. (**b**) Degradation of NT (10 μM) by A23187 (5 μM)-activated HUCBMCs (2 x 10^5). Cells were incubated with NT for 30 min at 37 °C. Results are expressed as the percentage of NT remaining in the samples compared to that in samples of NT incubated in vehicle alone at 37 °C (n = 3; data were pooled from the three experiments performed, each of which gave similar results). (**c**) Western blot analysis for Ntsr1 in lysates of 5 x 10^5 HUCBMCs obtained from three different donors. Blots were incubated with Ntsr1 antiserum (anti-Ntsr1) or with Ntsr1 antiserum that had been pre-absorbed overnight with the cognate peptide antigen (rat c-terminus of Ntsr1) (Anti-Ntsr1 + Ntsr1 peptide).

Supplementary Table 1, A. M. Piliponsky *et al*.

	Healthy controls (n = 14)	Patients with septic shock (n = 17)	Patients with cardiogenic shock (n = 6)
Age*	**50.7 ± 1.7**	**55.1 ± 4.3**	**63.5 ± 8.6**
Gender (%M/%F)	**64/36**	**71/29**	**67/33**
Etiological agent of sepsis: G+ bacteria/ G- bacteria/ unknown (n)	**n/a**	**6/7/5**	**n/a**
APACHE II score* #	**n/a**	**24.6 ± 2.0**	**19.2 ± 3.1**

n/a: not applicable.
***** Data are mean ± SEM.
The APACHE II score is based on physiological measurements, age and prior health status. In addition to representing an attempt to derive a general measure of the severity of disease in individual patients, the score can be used, together with the diagnosis on admission, to estimate the disease-specific probability of mortality (Knaus, W.A., Draper, E.A., Wagner, D.P. & Zimmerman, J.E. APACHE II: A severity of disease classification system. *Crit Care Med* 13, 818-29 (1985); Vincent, J.-L., Ferreira, F. & Moreno, R. Scoring systems for assessing organ dysfunction and survival. *Sepsis and Septic Shock* 16, 353-366 (2000)).

Supplementary Table 1. Characteristics of healthy control subjects and patients with severe sepsis or cardiogenic shock (as assessed at time of admission to the intensive care unit). Blood samples were collected within 24 h of admission to the ICU. Briefly, blood samples were collected in EDTA-treated tubes and were centrifuged at 1600g for 20 min at 4 ° C. Plasma was separated and stored at – 80 °C. Blood was obtained with the approval of the Stanford University Institutional Review Board. Blood samples from 14 healthy volunteers ("Healthy controls") were provided by the Stanford Blood Center; these subjects were instructed to be on a low fat diet before donating blood.