SUPPLEMENTAL METHODS

Radiolabeling of asparaginase

Asparaginase (4 mg, Prospec, Ness-Ziona, Israel) was conjugated under strict metal-free conditions with isothiocyanatobenzyl–diethylenetriaminepentaacetic acid (ITC-DTPA, Macrocyclics, Dallas, TX) in 0.1 M $NaHCO_3$, pH 9.5, using a 10-fold molar excess of ITC-DTPA. After 1 hour incubation at RT the conjugation mixture was dialyzed in a dialysis cassette with a molecular cut-off value of 20 kDa (Slide-A-Lyzer 20K, Thermo Scientific, Waltham, MA) against 0.25 M Ammonium Acetate, pH 5.5 to remove the unconjugated ITC-DTPA. The substitution ratio, as determined by labeling the conjugation mixture with $^{111}InCl_3$ (600-800 MBq, Mallinckrodt, Petten, The Netherlands), was 0.8 DTPA/asparaginase molecule. $^{111}InCl_3$ was added to DTPA-asparaginase (50-65 µg) for 1 h at RT in 0.1 M 2-(N-morpholino)ethanesulfonic acid buffer, pH 5.4. Free In-111 was complexed by adding 50 mM ethylenediaminetetraacetic acid (EDTA; final concentration of 5 mM). Quality control was performed using instant thin-layer chromatography. To obtain a radiochemical purity ≥95%, ^{111}In-labeled protein was purified by gel filtration on a disposable PD10 column (GE Healthcare Life Sciences, Eindhoven, the Netherlands), eluted with PBS containing 0.5% bovine serum albumin (BSA). The specific activity of the radiolabeled asparaginase preparations used in the experiments was (5-12 MBq/µg). The labeling procedure did not affect asparaginase activity, as determined by a Nessler assay (Supplemental Fig. 1A).

Autoradiography

Formalin-fixed, paraffin-embedded tissue sections (4 µm) from mice injected intravenously with ^{111}In-asparaginase were dewaxed, rehydrated and exposed to a phosphor imaging screen for up to 7 days. The screen was scanned in a phosphor imager system (Bioimaging Analyzer System 1800-II; Fujifilm, Tilburg, the Netherlands) at a pixel size of 50 × 50 µm.

Asparaginase binding assay

Human peripheral blood mononuclear cells (PBMCs) were isolated from blood obtained from healthy volunteers upon informed written consent, using density gradient centrifugation (Lymphoprep; Axis-Shield, Dundee, Scotland). Asparaginase (Takeda, Hoofddorp, the Netherlands) was conjugated with Fluorescein isothiocyanate according to manufacturer's instructions (Pierce / Life Technologies, Carlsbad, CA). PBMCs or THP-1 cells were incubated with fluorescein isothiocyanate-labeled asparaginase in culture medium for 1 hour at the indicated temperatures. After incubation, PBMCs were stained for expression of lineage markers with anti-CD14, -CD45, -CD3 and -CD19 antibodies purchased form BD Biosciences and analyzed using a LSRII flow cytometer (BD Biosciences, Breda, the Netherlands). The data were collected and analyzed by FlowJo software version 8.8.7 FlowJo, Ashland OR).

Immunohistochemistry

For histological analysis, tissues and organs, including spleen, lymph nodes, thymus, liver and femur/tibia were fixed in 4% formalin during 24 hours. Bone containing tissues were decalcified using Osteosoft (Merck Laboratories). Specimens were embedded in paraffin, 4 µM serial sections were cut and stained with hematoxylin and eosin (HE) following standard procedures. After dewaxing, rehydration and antigen retrieval using sodium citrate, slides were blocked with 2% normal goat serum (Vector Laboratories, Burlingame, CA) and 2% BSA in 0.05% PBS, Tween20 prior to overnight staining with F4/80 (MCA497GA, AbD Serotec, Oxford, United Kingdom) and Asparaginase (ab21013, Abcam, Cambridge, United Kingdom) antibodies. Appropriate biotinylated antibodies (Vector Laboratories) were used as secondary antibody and staining was revealed by incubation with metal-enhanced diaminobenzidine in stable peroxide substrate buffer (Thermo Fisher Scientific Inc.). After

counterstaining with hematoxylin (1:3) sildes were dehydrated and coverslipped using entellan (Merck Laboratories). Images were captured using a VisionTek digital microscope (Sakura Finetek, Torrance, CA).

Cell culture

The human acute monocytic leukemia cell line THP-1 was obtained from the American Type Culture Collection (ATCC, #TIB-202) cultured in RPMI 1640 containing L-glutamine (Invitrogen /Life technologies), supplemented with 10% heat-inactivated fetal bovine serum (Greiner Bio-One), and 100 units/ml penicillin/streptomycin (Invitrogen). The mouse leukemic monocyte macrophage cell line RAW264.7 (ATCC #TIB-71) and HEK293 FT (Invitrogen) cells were cultured in DMEM containing L-glutamine (Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin/streptomycin, and 1% non-essential amino acids (Invitrogen). All cells were incubated at 37°C in a 5% CO_2 humidified atmosphere. All cell Lines were used within 3 months of culture. THP-1 differentiation was induced by culturing 5 – 8 x 10^6 cells in the presence of 100 nM phorbol myristate acetate (PMA; Sigma-Aldrich) for three days. After three days the medium was refreshed by medium without PMA and terminal differentiation was continued for an additional three days.

Real-time Quantitative Polymerase Chain Reaction

Total RNA was extracted using the RNeasy mini-kit (Qiagen, Venlo, the Netherlands) according to manufacturer's instructions. Subsequently, cDNA was synthesized of 500 ng RNA template using the iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA). mRNA expression levels were determined by use of Power SYBR® Green PCR master mix using gene-specific primers (sequences are listed in Supplemental Table 2) and the CFX96 TouchTM Real-Time PCR detection system (Bio-Rad). TBP mRNA expression was used as a reference to obtain the relative fold expression of target genes using the comparative cycle threshold $2^{(-\Delta\Delta Ct)}$ method. Prism software

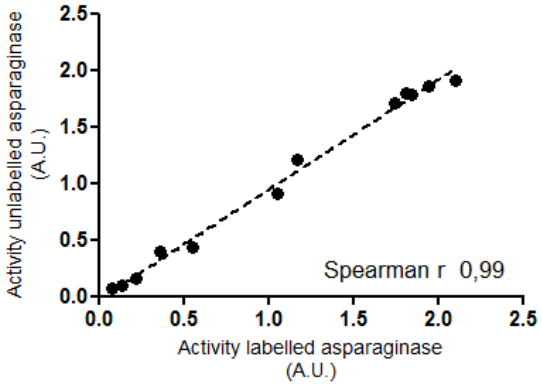
(Graphpad, La Jolla, CA) was used to plot the data and for statistical analysis. $p < 0.05$ was considered a significant difference.

Immunoblotting

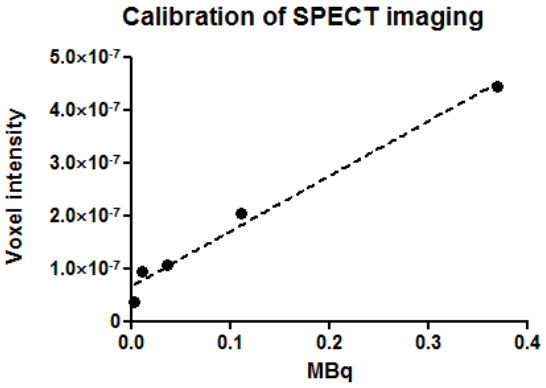
Whole cell extracts were prepared in NP-40 lysis buffer (200 mM NaCl, 0.5% NP-40, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA) and cleared by centrifugation. Protein lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were probed with primary antibodies against cathepsin B (sc-13985, Santa Cruz Biotechonology, Dallas, TX) or β tubulin (#2128, Cell Signalling Technology, Leiden, the Netherlands), followed by horseradish peroxidase (HRP) conjugated secondary antibody (DAKO, Heverlee, Belgium). Proteins were visualized with ECL reagent (GE Healthcare) and expression was detected with Fluorchem (Protein Simple, San Jose, CA).

Supplemental Figure 1

A



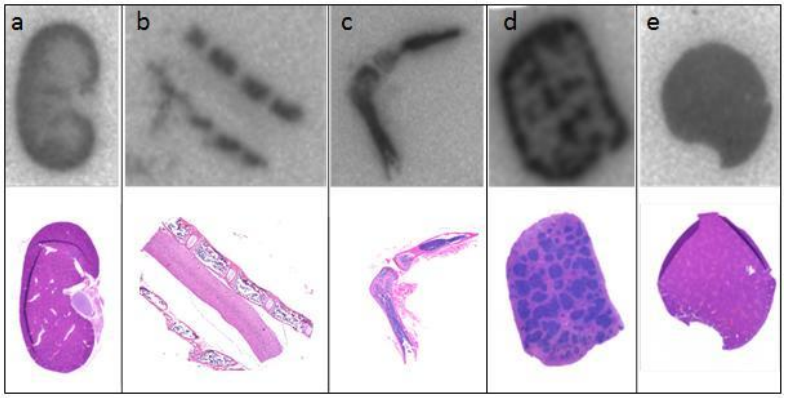
B



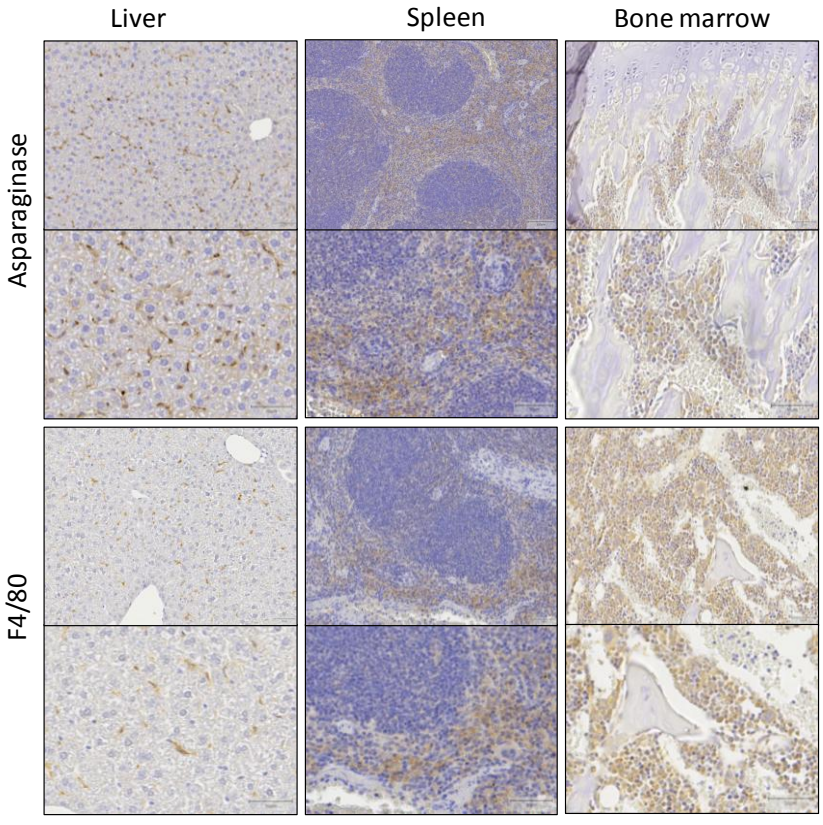
Supplemental Figure 1. Validation of asparaginase activity and calibration of qSPECT quantification
A Asparaginase activity of diluted samples of ^{111}In-labeled asparaginase plotted against unlabelled asparaginase.
B Calibration curve of a dilution series of ^{111}Indium-labeled asparaginase, determining the voxel intensity per MBq of activity.

Supplemental Figure 2

A

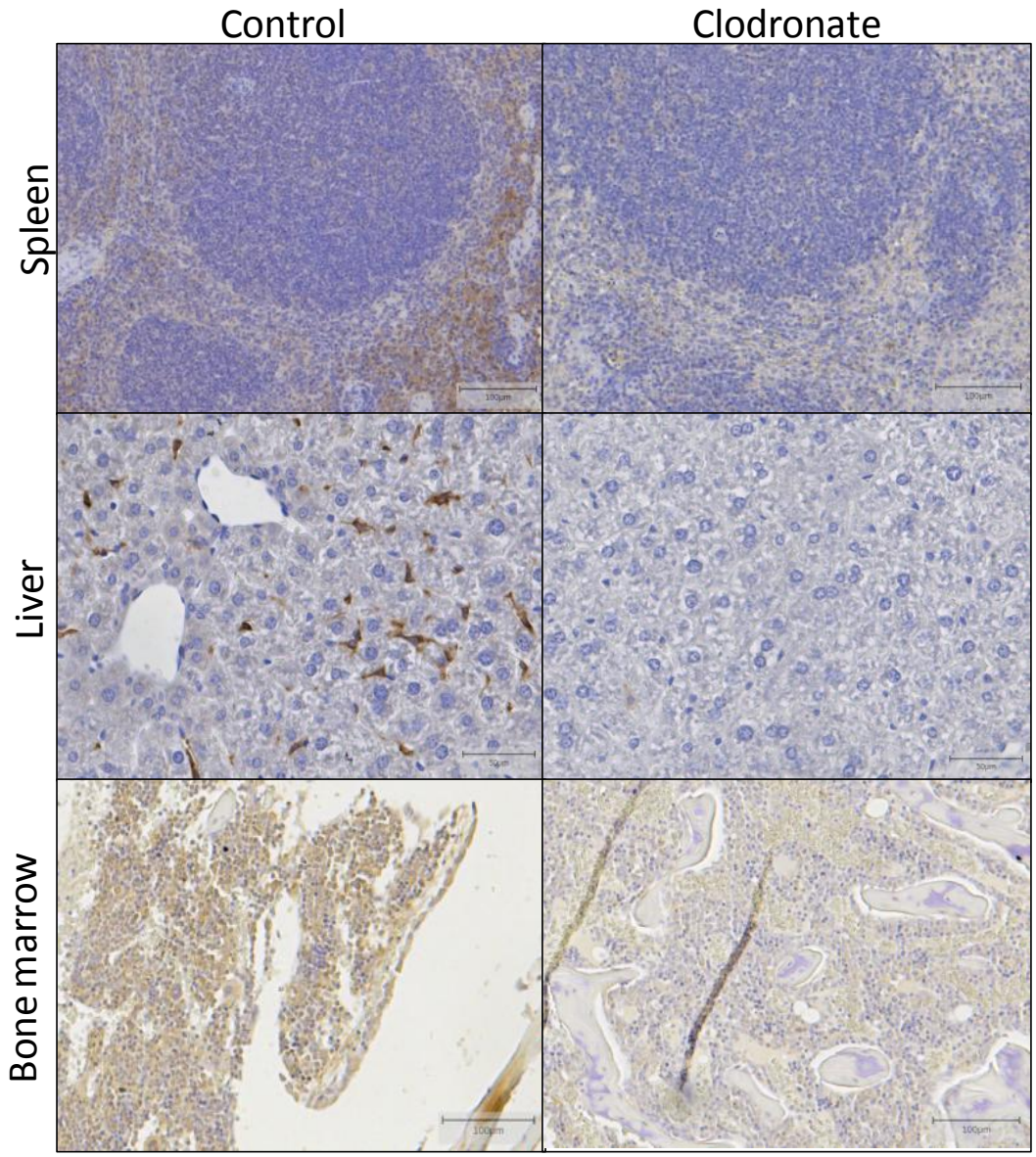


B



Supplemental Figure 2. Asparaginase is taken up in macrophage-rich parts of spleen, liver and bone marrow
A Autoradiography (upper image) and hematoxylin and eosin stain staining (lower image) of ^{111}Indium-labeled asparaginase. Images show kidney (a), spine (b), hind leg bones (c), spleen (d) and liver (e).
B Immunohistochemical staining of asparaginase and macrophage marker F4/80 in organs dissected from mice 9-15 hours post injection of unlabelled asparaginase. Images show two representative magnifications of liver, spleen and femoral bone marrow.

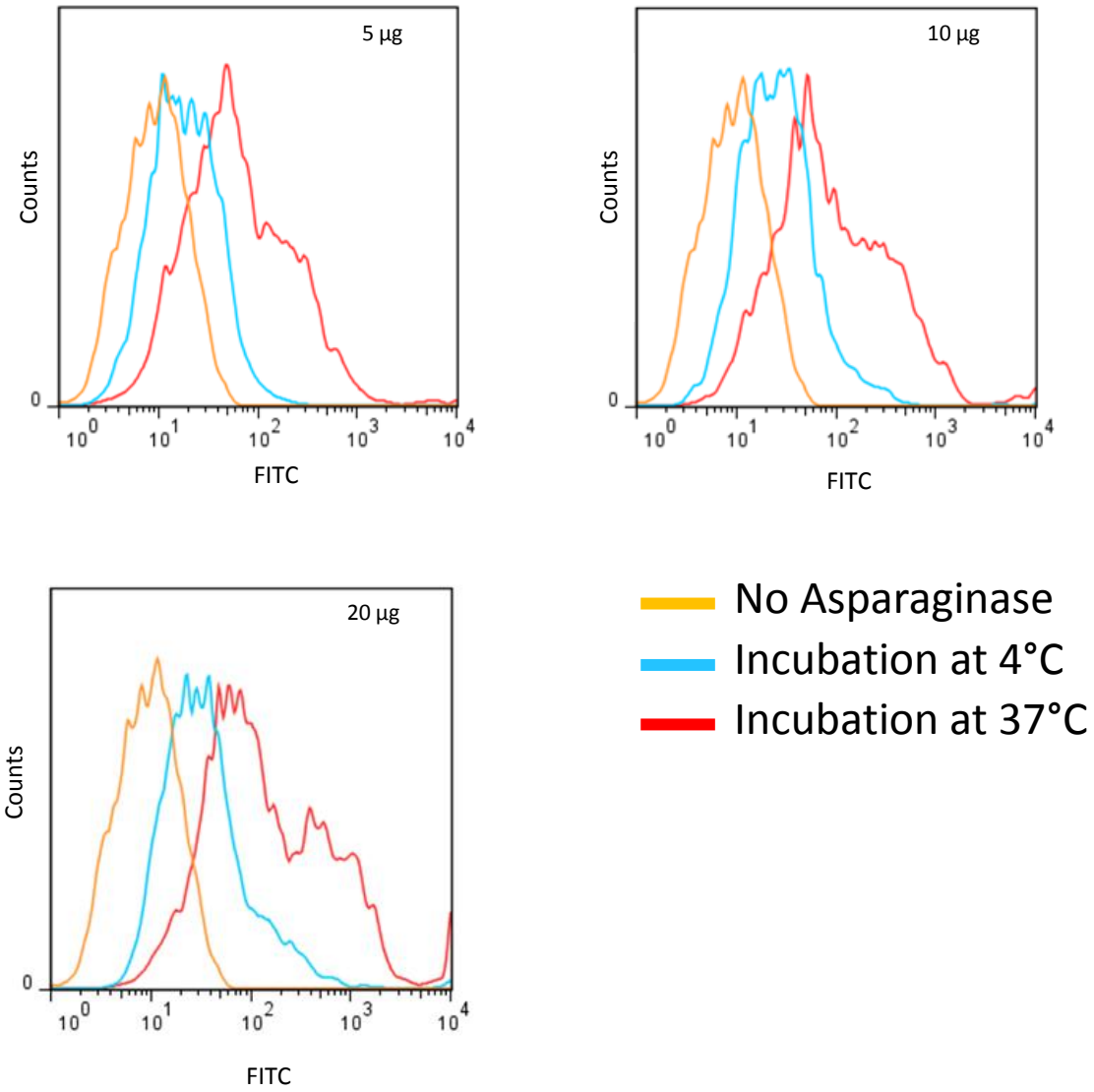
Supplemental Figure 3



Supplemental Figure 3. Immunohistochemical analysis of clodronate mediated macrophage depletion

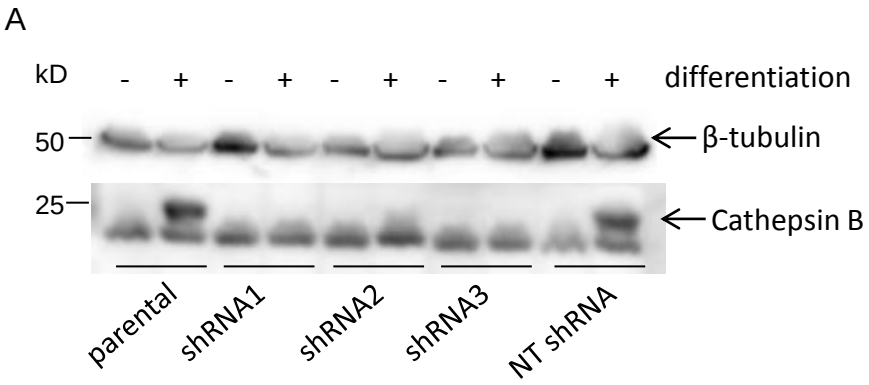
Immunohistochemical staining of macrophage marker F4/80 in organs dissected from mice 9-15 hours post injection of control and clodronate liposomes. Representative images of, spleen ,liver and femoral bone marrow are shown.

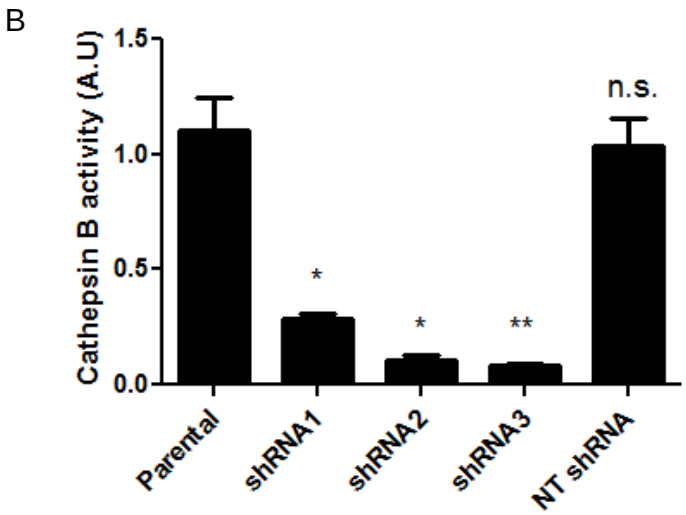
Supplemental Figure 4



Supplemental Figure 4. Differentiated THP1 cells bind asparaginase
Histograms showing binding of asparaginase to differentiated THP-1 cells. PMA induced differentiated cells were incubated with different concentrations of FITC-labeled asparaginase at different temperatures. Binding was analyzed by flowcytometry.

Supplemental Figure 5





Supplemental Figure 5. Cathepsin B protein expression and activity in THP-1 cells
A Western blot analysis of Cathepsin B protein expression in undifferentiated and PMA induced differentiated THP-1 cells transduced with shRNAs targeting Cathepsin B or control, non-targeting (NT) shRNA. β-tubulin was used as a control for equal loading.
B Cathepsin B activity in lysates of differentiated THP-1 cells transduced with control or Cathepsin B targeting shRNAs, was determined by measuring cleavage of the fluorescent substrate Ac-RR-AFC. The plot shows an average of 2 experiments with standard deviation. One of the control samples was set to 1 and all samples were correlated to this sample. Unpaired two-tailed t-test was used to determine significance compared to parental cells * = $p < 0.05$, ** = $p < 0.01$.

Supplemental Table 1

Sequences of the shRNA constructs. Seed sequences are underlined.

shRNA	Hairpin sequence
Non-targeting shRNA	CACCCAACAAGATGAAGAGCACCAACGAATTGGTGCTCTTCATCTTGTTG
Cathepsin B shRNA #1	CCGGCCAGAGAGTTATGTTTACCGACTCGAGTCGGTAAACATAACTCTCTGGTTTTT
Cathepsin B shRNA #2	CCGGGACAAGCACTACGGATACAATCTCGAGATTGTATCCGTAGTGCTTGTCTTTTT
Cathepsin B shRNA #3	CCGGCCAACACGTCACCGGAGAGATCTCGAGATCTCTCCGGTGACGTGTTGGTTTTT

Supplemental table 2

Sequences of RT-qPCR primers

Gene		Sequence
***CTSB* (human)**	Forward	CTTGAAGAGGCTATGTGGTACC
	Reverse	CCCTGGTCTCTGATCTCTTTG
***TBP* (human)**	Forward	GCACAGGAGCCAAGAGTGAA
	Reverse	ACATCACAGCTCCCCACCAT
***Ctsb* (mouse)**	Forward	ACAAGCCTTCCTTCCACCCG
	Reverse	TGTCCTCACCGAACGCAACC
***Hprt* (mouse)**	Forward	GGGGGCTATAAGTTCTTTGCTGACC
	Reverse	TCCAACACTTCGAGAGGTCCTTTTCAC