Resolving Macrophages Counter Osteolysis by Anabolic Actions on Bone Cells

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Appendix

Supplemental Methods

Flow Cytometry

The surface marker expression of activated primary bone marrow macrophages was analyzed with an LSR Fortessa (BD Biosciences) flow cytometer. Cells were probed with antibodies against F4/80 (clone BM8), CD80 (clone 16-10A1), CD206 (clone C068C2) (Biolegend) fixed (4% PFA) and washed. For each of the antibodies used, appropriate fluorescently tagged isotype control antibodies were used to determine non-specific binding, and live cells selected using the Fixable Viability Dye eFluor 506 (eBioscience). Compensation was performed with single-stained OneComp eBeads (eBioscience) and doublets excluded on side scatter SSC-W vs SSC-H dot plots. Data were analyzed in FlowJo software (Tree Star, Ashland, OR).

Immunohistochemistry and TRAP quantification

Immunohistochemistry was completed at the Toronto Center for Phenogenomics (Mount Sinai Hospital, Toronto, ON, Canada). Coronal sections of healthy and diseased areas were processed for antigen retrieval (10 nM sodium citrate, baking) for TNF-α, F4/80, CD206, and cystatin C. Endogenous peroxidases were blocked with 3% hydrogen peroxide in methanol and non-specific binding with protein block serum free (Dako) or normal horse serum (Vector Labs) (osterix). Samples were probed with primary mouse anti-TNF-α antibody (1:100) (LifeSpan Biosciences), rat-anti-mouse F4/80 (1:100) (Vector Labs), polyclonal rabbit-anti-CD206 antibodies (1:100) (LifeSpan Biosciences) overnight at 4°C, followed by incubation with biotin conjugated rabbit anti-rat, horse anti-mouse or goat anti-rabbit secondary antibodies (Vector labs) for 1h at room temperature, and stained using an avidin/biotin tissue staining system (VECTASTAIN Elite; Vector Labs) and hematoxylin and eosin counterstaining. Ten micrographs of TRAP-stained coronal sections of maxillae per condition were taken in 40X magnification along the bone crest at ligated and contralateral non-ligated second molars. Quantification of osteoclastic activity was performed in ImageJ software after transformation to green channel and thresholding of

positive area relative to TRAP-negative control, as the fold change in TRAP-positive area at ligated vs non-ligated side.

Mass Spectrometry

Primary M1-like and M2-like macrophages were polarized as described above and cells were washed and plated for 24 hours in fresh media without FBS (DMEM, 1% penicillin/ streptomycin). The resulting conditioned media supernatants were analyzed by mass spectrometry. Samples were resupsended in 50 µL of 50 mM NH4HCO3 (pH=8.3), and DTT was added to reduce cysteines at a final concentration of 10 mM. Cysteines were reduced at 60°C for 1 hour. Sample was cooled to room temperature and iodoacetamide was added to a final volume of 20 mM for incubation in the dark for 30 minutes, followed by inactivation with DTT to a final concentration of 40 mM. MS grade TPCK-treated trypsin (Promega) was added to a final protease-to-protein ratio of 1:50-1:100 and samples were digested overnight at 37°C. Supernatant was removed from beads, lyophilized and re-suspended in 1% TFA. Peptides were purified by homemade C18 tips, and then lyophilized. Samples were analyzed on a linear ion trap-Orbitrap hybrid analyzer (LTQOrbitrap, ThermoFisher) outfitted with a nanospray source and EASY-nLC split-free nano-LC system (ThermoFisher). Lyophilized peptide mixtures were dissolved in 0.1% formic acid and loaded onto a 75 µm x 50cm PepMax RSLC EASY-Spray column filled with 2 µM C18 beads (ThermoFisher) at a pressure of 800 BAR. Peptides were eluted over 60 min at a rate of 250 nL/min using a 0 to 35% acetonitrile gradient in 0.1% formic acid. Peptides were introduced by nano-electrospray into an LTQ-Orbitrap hybrid mass spectrometer (Thermo-Fisher). The instrument method consisted of one MS full scan (400–1500 m/z) in the Orbitrap mass analyzer, an automatic gain control target of 1e6 with a maximum ion injection of 200 ms, one microscan, and a resolution of 240,000. Ten data-dependent MS/MS scans were performed in the linear ion trap using the ten most intense ions at 35% normalized collision energy. The MS and MS/MS scans were obtained in parallel fashion. In MS/MS mode automatic gain control targets were 3e5 with a maximum ion injection time of 50 ms. A minimum ion intensity of 5000 was required to trigger an MS/MS spectrum. The dynamic exclusion was applied using a maximum exclusion list of 500 with one repeat count with a repeat duration of 30 s and exclusion duration of 15 s.

RNA extraction and RT-qPCR

Total RNA was isolated using TRIzol Reagent, as described by the manufacturer. 20 µg of molecular biology grade glycogen was added into the aqueous phase to facilitate RNA recovery. Isolated total RNA was stored at -20°C. RNA concentration was estimated using a Nanodrop 1000 fiber optic spectrophotometer (Thermo Scientific) and analyzed on an Agilent 2100 Bioanalyzer using “nano” chip. Samples with an RNA integrity number (RIN) of 6-8 were used without further purification for

quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was reverse transcribed into cDNA using a genetically modified Moloney murine leukemia virus reverse transcriptase (Transcriptor, Roche Diagnostics), using the manufacturer's recommended reaction composition in a 20 µl volume, wherein cDNA synthesis from 1 µg of RNA was primed by 20 pmol of oligo-dT24VN primer (Thermo Fisher) by incubation for 15 min at 42° C followed by 1 hour at 50° C. A small aliquot of the cDNA reaction was diluted 1:25 with reagent-grade water for initial testing, while the remainder of the reaction was phenol-chloroform extracted, ethanol precipitated in the presence of 20 µg of glycogen and taken up in 100 µl of water for storage at -20 °C. Five µl aliquots of appropriate dilutions of the purified cDNA, typically from 1:5 to 1:25, were added to a 20-µl qRT-PCR reaction, along with 5 µl of a 1 µM primer pair and 10 µl of SYBR Select reagent master mix (Applied Biosystems).

Western Blotting

Primary BMM were polarized as previously described into M1-like and M2-like cells. The cells were lysed with RIPA buffer containing proteinase inhibitors and 20 µg/lane samples were boiled in Laemilli buffer for 5 minutes at 100°C. After electrophoresis, the proteins were transformed onto nitrocellulose membrane, blocked (5% milk/TBST) for 1 hour, and incubated in rabbit anti-mouse cystatin C (1:7000; R&D) and anti-mouse beta-actin (1:5000, Sigma) at 4°C overnight. After washing with TBST, the membrane was incubated in 5% milk/TBST containing IRDye 800CW goat anti-Rabbit IgG (H, L) and goat anti-mouse IgG CW 680 for 1 hour at room temperature and imaged using Li-Cor (Li-cor Biosciences). For cystsin C immunodepletion, anti-cystatin C antibodies from two companies were tested and the most efficient was used for the osteoblast and osteoclast experiments as described.

Phagocytosis and Migration Assays

Neutrophils were recruited in the peritoneal cavity of mice with Zymosan A (Sigma-Aldrich), aged overnight at room temperature in dark, stained with APC conjugated anti-Ly6G antibody (1:100) (eBioscience) and incubated 1:1 with M1 (IFN-γ + LPS) and M2 (IL-4) activated macrophages for 4 hours. Cells were then incubated with phalloidin-Alexa Fluor488 (eBioscience), stained with DAPI, and Ly6C positive particles inside macrophages quantified by confocal microscopy. To assess migration, attached primary M1 or M2 activated cells were incubated with the chemoattractant (20 ng/mL M-CSF) in Costar transwells (Corning Inc) for 2 hours. Migrated cells were washed, fixed, stained with DAPI and counted.

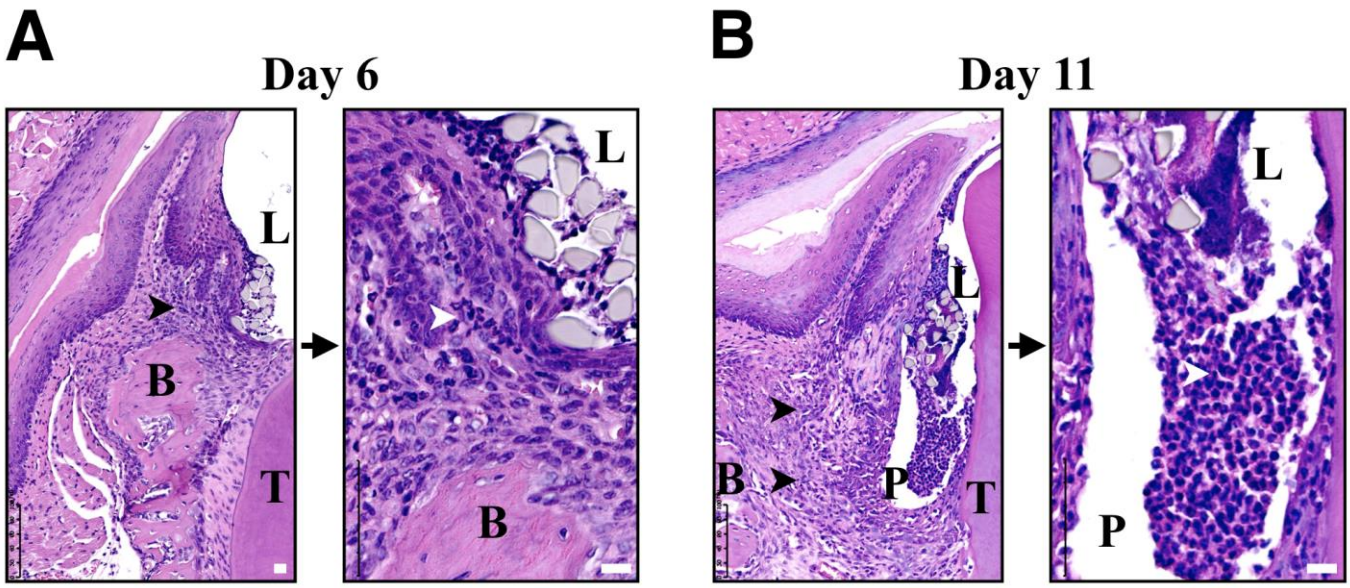
Osteoclast and Osteoblast Activity Assays

To assess the actions of polarized macrophages on osteoblast and osteoclast function, washed M1 and M2 free of activation factors were counted, plated for 24 hours in D-MEM, and the resulting conditioned media added to primary osteoblast and osteoclast cultures at different macrophage-to-bone cell ratios (1:1, 1:5 and 1:10). Bone marrow derived osteoclast precursors were collected as previously described (Jiang et al. 2015). The non-adherent cells were plated with different ratios (1:10, 1:5, 1:1) of activated cells (M1, M2) for 8 days or their supernatants every 2 days for 8 days, supplemented with 20 ng/ml M-CSF and 60 ng/ml sRANKL every 2 days. Osteoclast activity was assessed as previously described (Tintut et al 2002) by measurement of tartrate resistant acid phosphatase (TRAP) expression (naphthol AS-BI phosphate, fast red TR salt and sodium tartrate) and activity (citrate buffer, sodium tartrate, Triton X-100, p-nitrophenyl phosphate buffer) read at a 405 nm wavelength. Mineral deposition by osteoblasts was assessed in MC3T3-E1 cells and primary murine osteoblasts differentiated from calvarial precursors. MC3T3-E1 cells were differentiated for 3 weeks (α-MEM, 50 μg/ml Ascorbic acid, 10 mM β-glycerophosphate, 10% FBS and 1% penicillin/ streptomycin) and incubated at different ratios (1:10, 1:5, 1:1) with activated M1-like or M2-like supernatants every 3 days. The supernatant concentration proportional to desired cell:cell ratios was obtained after quantifying M1 and M2 macrophages washed of activation factors and plating them 24 hours followed by addition of M supernatant volumes proportional to the number of cells required for each ratio, to osteoblasts and osteoclast cultures. For osteoblast mineralized nodule quantification wells were stained with stained 1 ml of 2% Alizarin Red staining solution (40 mM) followed by 20 minutes incubation in room temperature. 15 random areas were photographed with 3 samples per condition. The images were converted to a green channel using ImageJ software and the threshold set to 118 (118-255). Mineralized nodule areas were quantified in ImageJ.

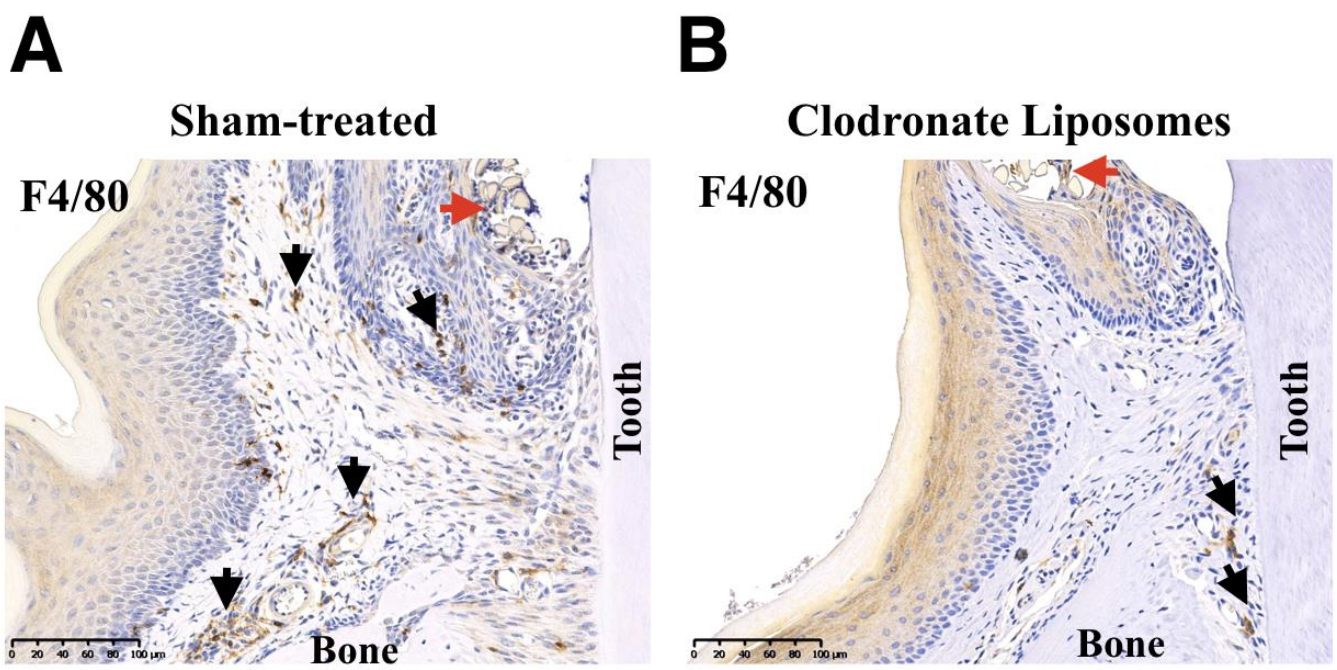
Reference:

Jiang H, Wang Y, Viniegra A, Sima C, McCulloch CA, Glogauer M. 2015. Adseverin plays a role in osteoclast differentiation and periodontal disease-mediated bone loss. The FASEB journal 29:2281–2291.

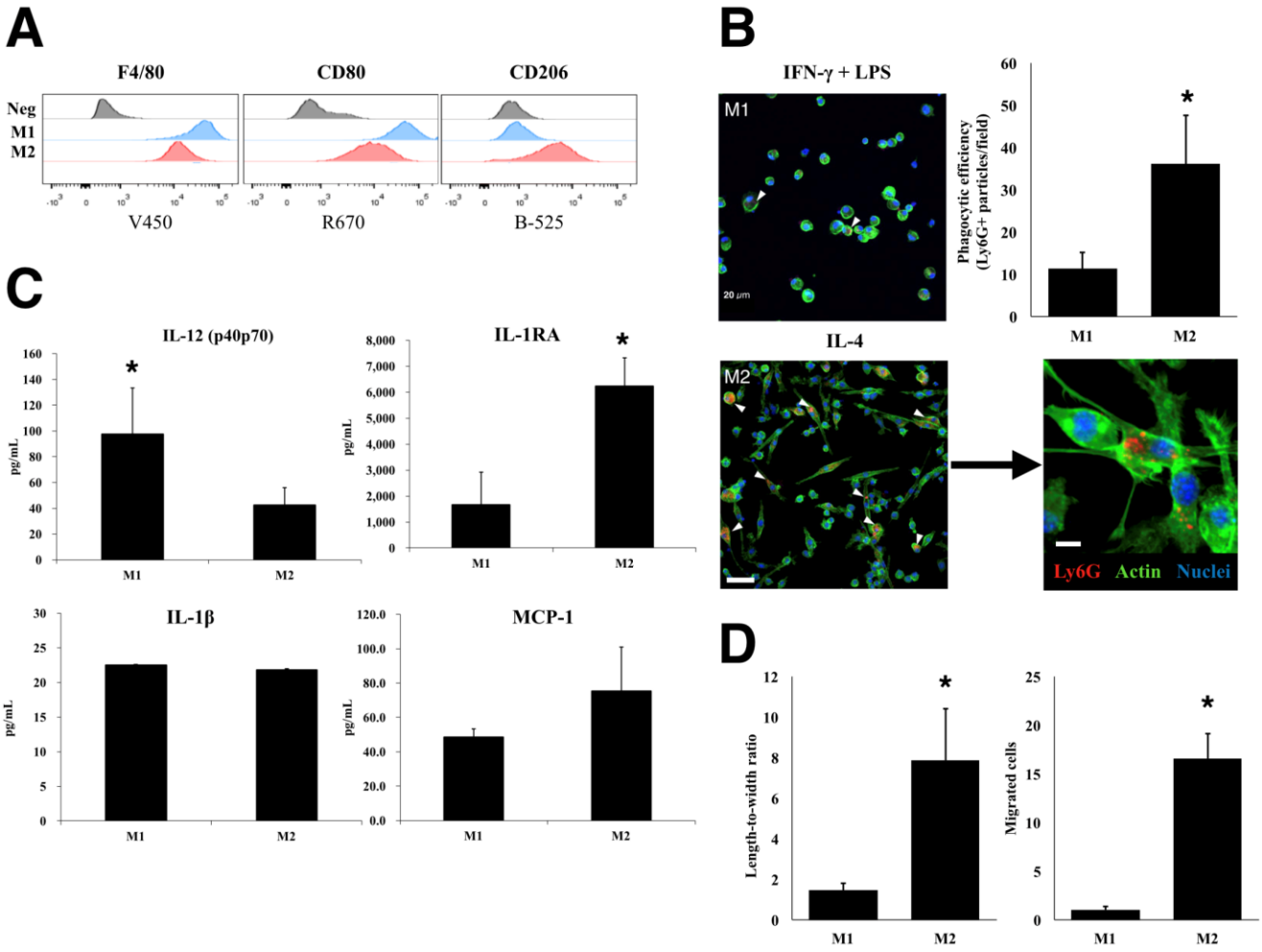
Y. Tintut, F. Parhami, A. Tsingotjidou, S. Tetradis, M. Territo, L.L. Demer. (2002) 8-Isoprostaglandin E2 enhances receptor-activated NFkappa B ligand (RANKL)-dependent osteoclastic potential of marrow hematopoietic precursors via the cAMP pathway. The Journal of biological chemistry 277:14221-14226.



Appendix Figure 1. Periodontal inflammatory cell infiltration is associated with pocket formation within 11 days of ligature placement. Coronal sections of ligated maxillary second molars were stained with hematoxylin and eosin for assessment of tissue changes. (**A**) Left: Within 6 days of periodontitis induction, proliferation of sulcular epithelial cells and infiltration of inflammatory cells are observed within 100 µm form the bone crest (*black arrowhead*). Magnification 20 x. Scale bar, 10 µm. Right: Most inflammatory cells infiltrating the gingiva and surrounding silk filaments and associated biofilm are polymorphonuclear neutrophils (*white arrowhead*). Magnification: 60 x. Scale bar, 10 µm. (**B**) Left: By day 11 a periodontal pocket is formed, and a large number of inflammatory cells is observed in the tissue (*black arrowheads*) and inside the pocket. Right: Most inflammatory cells inside the pocket are polymorphonuclear neutrophils (*white arrowhead*). Magnification: 60 x. Scale bar, 10 µm. B, bone; L, ligature consisting of 10-20 individual silk filaments; P, pocket; T, tooth.



Appendix Figure 2. Depletion of macrophages with clodronate liposomes. Clodronate liposomes (1 µg/g body weight) were delivered intraperitoneally every 2 days for the duration of periodontitis induction. Representative coronal sections of periodontal tissues of ligated molars for 21 days stained for macrophage marker F4/80. (**A**) In sham treated mice, F4/80 macrophages were found throughout the tissue including epithelium, connective tissue stroma and perivascular areas (black arrows), around the silk filaments of ligature (red arrows), and on the bone surface (green arrows). (**B**) Almost complete depletion of macrophages was achieved with clodronate liposome treatment. Few F4/80 positive cells were noted in perivascular areas (black arrows) and around silk filaments of the ligature (red arrow).



Appendix Figure 3. Pro-resolving functions of IL-4 activated macrophages. (**A**) Representative flow cytometric histograms of F4/80 pan-macrophage marker, CD80 and CD206 expression on M1 (IFN-γ and LPS) and M2 (IL-4) activated murine primary bone marrow macrophages (BMM). (**B**) BMM activated to either M1 or M2 phenotype were incubated with 24-h aged peritoneal recruited and labeled neutrophils (Ly6G, *red*). *Green*, actin; *Blue*, nuclei. Phagocytic efficiency was quantified as numbers of Ly6G positive particles inside activated macrophages (n=4, experiment run twice in duplicate, *p<0.05, unpaired *t*-test). **(C)** BMM were activated with either M1 or M2 as described. The supernatants were collected after 48 hours and analyzed using a RayBiotech custom multiplex ELISA detecting IL-1β, IL-1RA, IL-12 (p40, p70), CCL2 (MCP-1) (n=3 independent experiments run in duplicate, *p< 0.05 unpaired t-test). (**D**) eGFP-RAW264.7 macrophages activated M1 or M2 for 48 h were used to quantify the shape and size differences, expressed as ratio between cell length and width (n=4, *p<0.05, unpaired *t*-test). Migration of M1 and M2 activated cells was measured after incubation of BMM with M-CSF as chemoattractant (n=3 experiments run in duplicate, *p<0.05, unpaired *t*-test). Bars represent mean±SEM.

Appendix Table. Proteins listed in descending order of relative abundance in activated macrophage media (M2/M1 ratio; >5 fold threshold, delineated as 5-10, 10-50, 50-100, >100)

Protein (*encoding gene*)	Accession	Avg. Mass (kDa)	M0 Area (x1000)	M1 Area (x1000)	M2 Area (x1000)	M2/M1 ratio
Collagenase 3 (*Mmp13*)	P33435	54	0	18.1	32400	1790
Chitinase-like protein 4 (*Chil4*)	Q91Z98	45	0	27.8	18800	676
Cystatin C (*Cst3*)	Q3U5K7	15	0	177	102000	576
Chitinase-like protein 3 (*Chil3*)	O35744	44	1210	486	229000	471
Cathepsin L (*Ctsl*)	Q3UHZ4	38	0	392	81500	208
Amyloid precursor protein (*App*)	Q3TWF3	85	0	219	21800	100
Glypican 1 isoform CRA_c (*Gpc1*)	Q3U379	61	0	20.9	1970	94
Transcobalamin-2 (*Tcn2*)	O88968	48	0	86.7	7340	85
Complement C1q subunit C (*C1qc*)	Q02105	26	0	24.8	1670	67
Signal-regulatory protein alpha (*Sirpa*)	Q6P6I8	56	0	129	7740	60
Procollagen-lysine 2-oxoglutarate 5-dioxygenase 1 (*Plod1*)	Q9R0E2	84	0	42.7	2220	52
Macrophage metalloelastase (*Mmp12*)	P34960	55	0	2260	115000	51
Arginase-1 (*Arg 1*)	Q61176	35	0	598	19000	32
Uncharacterized protein (*Col1a2*)	Q3TU64	130	3040	197	5450	28
Osteonectin (*Sparc*)	Q5NCU4	34	0	351	9270	26
Cathepsin B (*Ctsb*)	P10605	37	0	14600	372000	25
Cytochrome c oxidase subunit 6B1 (*Cox6b1*)	P56391	10	0	146	3500	24
Leukocyte elastase inhibitor A (*Serpinb1a*)	Q9D154	43	0	164	3430	21

Group XV phospholipase A2 (*Pla2g15*)	Q8VEB4	47	0	400	8050	20
Uncharacterized protein (*Nrp1*)	Q3UFP7	94	0	77.6	1470	19
Serpin B6 (*Serpinb6*)	Q60854	43	0	503	9440	19
Farnesyl pyrophosphate synthase (*Fdps*)	A0A0G2JDJ5	14	0	114	2120	19
Biglycan (*Bgn*)	P28653	42	0	547	9530	17
Cathepsin Z (*Ctsz*)	Q9WUU7	34	0	4010	67700	17
Argininosuccinate lyase (*Asl*)	E0CY49	29	0	26	413	16
Cathepsin D (*Ctsd*)	Q3TWR6	45	0	13500	196000	15
Uncharacterized protein (*Ldlr*)	Q3TVR4	95	0	68.9	982	14
Extracellular matrix protein 1 (*ECM1*)	Q9QX30	63	0	62.6	809	13
Carboxypeptidase (*Ctsa*)	Q9D2D1	54	932	4110	48700	12
Reticulocalbin-1 (*Rcn1*)	Q05186	38	0	90.9	990	11
Platelet-activating factor acetylhydrolase (*Pla2g7*)	Q60963	49	0	419	4340	10
Tumor necrosis factor alpha (*Tnfa*)	Q0X0E6	15	0	180	1860	10
Lipase (*Lipa*)	Q6PDR1	45	0	2230	22500	10
Lysosomal alpha-mannosidase (*Man2b1*)	O09159	115	0	2930	27800	9
Biliverdin reductase (*Blvra*)	Q9CY64	34	0	251	2340	9
Gamma-interferon-inducible lysosomal thiol reductase (*Ifi30*)	Q9ESY9	28	1260	1250	11300	9
Follistatin-related protein 1 (*Fstl1*)	Q62356	35	0	132	1160	9
Beta-2-microglobulin (*B2m*)	P01887	14	0	26200	229000	9
Creatine kinase B-type (*Ckb*)	Q04447	43	0	4890	40800	8
Keratin type I cytoskeletal 17 (*Krt17*)	Q9QWL7	48	7010	1020	7430	7

Lipoprotein lipase (*Lpl*)	Q3UAX2	52	237	1400	10000	7
Calumenin (*Calu*)	Q6XLQ8	37	0	1070	7470	7
40S ribosomal protein S21 (*mCG_6739*)	Q8C1L7	9	0	239	1660	7
Galectin-3-binding protein (*Lgals3bp*)	Q07797	64	0	2150	14900	7
Coiled-coil domain-containing protein 58 (*Ccdc58*)	F8WJI3	16	0	109	755	7
Rab GDP dissociation inhibitor beta (*Gdi2*)	Q61598	51	0	1380	9250	7
Neurolysin (Metallopeptidase M3 family) (*Nln*)	Q3UUI1	78	0	216	1440	7
Tropomyosin alpha-1 chain (*Tpm1*)	E9Q450	33	0	56.3	367	7
Uncharacterized protein (*Tpp1*)	Q3U2Z6	61	0	1390	8920	6
Isoform 2 of Splicing factor 3B subunit 3 (*Sf3b3*)	Q921M3-2	125	0	170	1030	6
Prostaglandin reductase 1 (*Ptgr1*)	Q4FJY5	36	0	163	967	6
Proteasome subunit beta type-5 (*Psmb5*)	O55234	29	0	249	1470	6
Syndecan 4 (*Sdc4*)	Q3U5S6	21	0	257	1510	6
Apolipoprotein E (*Apoe*)	Q3TXU4	36	0	4660	26400	6
Prolow-density lipoprotein receptor-related protein 1 (*Lrp1*)	Q91ZX7	505	1540	1070	5830	5
Galectin-1 (*Lgals1*)	P16045	15	0	12800	69700	5
Isoform 2 of Adenylate kinase 2 mitochondrial (*Ak2*)	Q9WTP6-2	26	0	5600	30100	5
Metalloproteinase inhibitor 1 (*Timp1*)	P12032	23	0	123	634	5