Muscle Paralysis Induces Bone Marrow Inflammation and Predisposition to Formation of Giant Osteoclasts

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Running Title: Muscle Paralysis Induces Inflammation and Giant Osteoclasts

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Abstract

Transient muscle paralysis engendered by a single injection of botulinum toxin A (BTxA) rapidly induces profound focal bone resorption within the medullary cavity of adjacent bones. While initially conceived as a model of mechanical disuse, osteoclastic resorption in this model is disproportionately severe compared to the modest gait defect that is created. Preliminary studies of bone marrow following muscle paralysis suggested acute upregulation of inflammatory cytokines, including TNFα and IL-1. We therefore hypothesized that BTxA-induced muscle paralysis would rapidly alter the inflammatory microenvironment and the osteoclastic potential of bone marrow. We tested this hypothesis by defining the time course of inflammatory cell infiltration, osteo-inflammatory cytokine expression, and alteration in osteoclastogenic potential in the tibia bone marrow following transient muscle paralysis of the calf muscles. Our findings identified inflammatory cell infiltration within 24 hours of muscle paralysis. By 72 hours, osteoclast fusion and pro-osteoclastic inflammatory gene expression were upregulated in tibia bone marrow. These alterations coincided with bone marrow becoming permissive to the formation of osteoclasts of greater size and greater nuclei numbers. Taken together, our data are consistent with the thesis that transient calf muscle paralysis induces acute inflammation within the marrow of the adjacent tibia and that these alterations are temporally consistent with a role in mediating muscle paralysis-induced bone resorption.
Introduction

Transient muscle paralysis induced by a single injection of botulinum toxin A (BTxA) rapidly induces focal and profound bone resorption within the medullary cavity of adjacent bones. While initially conceived as a model of bone loss due to reduced gait-induced loading, osteoclastic resorption in this model is disproportionately severe compared to the modest gait defect that is created (19, 32, 49). Instead, the severe bone loss following BTxA-induced muscle paralysis is comparable in rapidity and magnitude to that induced by sciatic neurectomy and spinal cord injury (31, 34). Subsequent studies demonstrated that acute trabecular bone loss following muscle paralysis arises via profound RANKL mediated osteoclastogenesis, but the initial signaling cascade responsible for the rapid activation of this bone catabolic pathway is not known (2).

Preliminary studies to identify candidate signaling pathways responsible for activating RANKL-mediated osteoclastogenesis indicated that the inflammatory cytokines TNFα and IL-1 were upregulated prior to the onset of bone resorption and to altered bone marrow gene expression previously observed at 7 and 14 d post-paralysis (33, 53). As these cytokines have been shown to regulate osteoclast activity (1, 12, 27, 56), we speculated that inflammatory alterations within the marrow in response to muscle paralysis create a focal microenvironment that is permissive to osteoclastogenesis.

The linkage between inflammation, immune function, and bone catabolism is strongly supported by the literature. Predisposition to bone loss and osteoporotic fracture is observed in a number of chronic inflammatory diseases with immune components such as rheumatoid arthritis, inflammatory bowel disease, chronic obstructive pulmonary disease, and multiple sclerosis (8, 15, 17, 41). Inflammatory pathways have also been implicated in post-menopausal bone loss (40). When focal inflammatory disorders occur adjacent to bone surfaces (e.g., periodontitis or adjuvant arthritis), bone resorption is especially destructive (3, 29). One mechanism for this resorption is that RANKL can be highly expressed on activated CD4+ T cells and other inflammatory regulating cells (24, 25, 48), leading to enhanced osteoclast formation/recruitment and bone destruction. As well, inflammatory cytokines such as TNFα, IL-1 and IL-6 have been found to promote osteoclastogenesis (3, 20) and are implicated in post-menopausal bone loss (40). Lastly, both genetic and
pharmacologic inhibition of the immune response through T cell depletion and/or anti-inflammatory treatment reduces bone loss in models of ovariectomy and adjuvant arthritis (9, 40, 42).

In addition to clear evidence of immune system involvement in chronic inflammatory bone loss, the rapidity and magnitude of bone resorption induced by muscle paralysis implies that immune-inflammatory pathways may play a critical role in paralysis-induced bone loss. In this context, an advantage of exploring this phenomenon in the BTxA bone loss model is the brief, contained period of de novo osteoclast activity (6 to 13 days; (5)) induced by muscle paralysis. We therefore hypothesized that BTxA-induced muscle paralysis would rapidly alter the inflammatory microenvironment and the osteoclastic potential of bone marrow. We tested this hypothesis by defining the time course of inflammatory cell infiltration, osteoinflammatory cytokine expression, and alteration in osteoclastogenic potential in the tibia bone marrow following transient muscle paralysis of the adjacent calf muscle group.

Methods

This study was comprised of three complementary experiments. All mice were 16-wk-old female C57Bl/6 obtained from Jackson Laboratories. Each experiment utilized a single injection of botulinum toxin A (BTxA; 2U/100 g body weight; Allergan, Irvine, CA, USA) into the right calf muscle group to induce transient muscle paralysis on day 0 (39). Mice receiving BTxA treatment were allowed free cage activity for the remainder of each experiment. Calf paralysis was confirmed 24 hours post-injection by visual examination of reduced toe extension and ankle plantarflexion in the affected limb. We chose to use age matched naïve mice as controls in our studies for two primary reasons. First, while we have observed minimal alterations in RANKL protein levels, osteoclast numbers and bone volume alterations in control limbs following BTxA induced muscle paralysis (2, 50), we had no data confirming the absence of inflammatory gene expression alterations in contralateral limbs. Second, we had previously used naïve control mice to define the onset of osteoclastic resorption following transient muscle paralysis (4, 5). All experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee, University of Washington.
**Flow Cytometric Analysis of T cells**

Mice were euthanized 24 or 48 hours following BTxA injection (n=4 per group). Marrow within the proximal metaphysis of the right tibia (approximately 5 mm section distal to the growth plate) was flushed with RPMI and pelleted by centrifugation. Red blood cells in the cell pellet were lysed by incubation in ACK lysing buffer (BioWhittaker) for 2 minutes. Cells were resuspended in RPMI and aliquoted into FACS tubes (5 x 10^6 cells per sample). FACS samples were centrifuged and resuspended in surface antibody dilution (1:200 ratio of surface antibody to FACS buffer consisting of HBSS with 2% FBS) for 20 minutes at 4°C. Finally, samples were washed twice and resuspended in FACS buffer for flow cytometry analysis. Marrow from naïve mice (n=5) was identically collected and processed as the baseline control. Marrow cell populations were stained for T cell surface markers (TCRβ, CD4 and CD8 antibodies: Biolegend). Individual sample data were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar). Prior to analysis, one mouse from the BTxA group was removed due to a failed physical examination for muscle paralysis.

**Quantitative RT-PCR of Osteo-inflammation and Osteoclast Fusion Genes**

Mice in this study were sacrificed on day 1, 3 or 7 post BTxA injection (n=6/group). Whole marrow was flushed from the right tibia with RPMI. For quantitative RT-PCR, total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to manufacturer’s protocol and cDNA was synthesized using Super Script III reverse transcriptase (Invitrogen/Life Technologies). Control marrow from naïve mice was prepared identically (n=6). We identified a candidate panel of inflammatory and osteoclastogenic genes from the literature and based on our preliminary data (TNF, CD9, CD44, CD47, CD81, IL1a, IL1b, IL4, IL11, DC-STAMP, OC-STAMP). We analyzed gene expression with quantitative real-time PCR using SYBR green and the Applied Biosystems 7900 HT sequence detection system. Gene expression levels were quantified using the 2^(-2ΔΔCT) method relative to the inflammatory specific housekeeping gene HPRT (45). Primers used for this analysis are shown in Table 1.

**Primary Osteoclast Culture**
Tissue culture medium and supplements were purchased from Invitrogen (Life Technologies). Media were supplemented with heat-inactivated FBS (HyClone, Thermo Scientific). Marrow was collected from mouse tibiae at 1, 3 and 7 days post BTxA injection (n=2/gp). Marrow was flushed in culture medium (α-MEM with 10% FBS) and red blood cells were lysed as previously described above. Cells were seeded in duplicate at 1.5 x 10^4/cm² in chamber well slides (Thermo Scientific Inc.), supplemented with 50 ng/ml M-CSF (Peprotech) and 10 ng/ml RANKL (Biolegend) and incubated at 37°C and 5% CO₂. Cell media were refreshed on day 3 of culture and cells were fixed and stained for tartrate resistant acid phosphatase (TRAP) on day 8 of culture using the Leukocyte (TRAP) kit and protocol (Sigma-Aldrich). The experiment was replicated (total combined group size of n=4/grp), with data normalized within each experiment to identically treated marrow from treatment-naïve mice (n=2/exp). For each group, normal osteoclasts (i.e., TRAP-positive cells w/ 3+ nuclei) and giant osteoclasts (i.e., a subset of normal osteoclasts w/ 20+ nuclei) were quantified on a per well basis using bright field microscopy (Olympus BH2 microscope, 10X and 40X magnification). The cutoff for a giant osteoclast (20+ nuclei) was based on previous studies (22, 51) in order to distinguish an increase in cell size between experimental groups. Once this difference was identified, a secondary analysis was performed to quantify the cell size distribution within these identified samples to confirm that our original analysis was robust to our choice of what was considered a giant osteoclast. For this, we assessed osteoclast area in naïve and 3 days post-BTxA cultures via automated calculation of osteoclast TRAP+ cell area in two 10 mm² subsamples of the culture area (composite of eighty 20x light microscopy images, Ziess) using custom ImageJ software (FIJI). Specifically, the software identified individual cell perimeters through image thresholding, binarized the images and filled in the cell area for individual cell area calculations. The average size of a 3-nuclei osteoclast was quantified by imaging a series of 3-nuclei TRAP+ cells (n=20 cells across all groups).

**Statistical Analysis**

Due to non-homogeneity of variance, flow cytometry data were analyzed using Kruskal-Wallis rank sum tests with Dunn’s post-hoc analysis for pair-wise comparisons. Differential gene regulation in the quantitative RT-PCR study was determined using planned comparison one-way MANOVAs (i.e., gene expression on all
days compared to naïve mice) with Dunnett’s two-way post-hoc for multiple comparisons to controls (i.e., naïve). Multiple one-way ANOVAs with Tukey post-hoc analysis were used to determine significant differences in the primary osteoclast culture. To compare osteoclast size in cultures, a Kolmogorov-Smirnov (KS) test was first used to compare the distribution in cell areas. Next, normalized population distributions (i.e., percent of cell population at a given area) were determined and fit with separate power law curves using maximum likelihood estimation (MLE) and likelihood ratio testing (LRT) as described previously (46). Finally, we assessed whether the data sets could be fit by a single power law model via MLE and LRT. Statistical significance was determined to be p<0.05.

Results

CD4+ T Cells Are Acutely Upregulated Following Muscle Paralysis

Flow cytometry revealed rapid transient alterations in tibia marrow cell populations in the proximal tibia metaphysis following muscle paralysis (Fig. 1). The percentage of CD4+ T cells (mean ± s.e.) within the bone marrow was elevated 25% within 24 hours of BTxA injection (1.47 ± 0.07%) vs naïve (1.14 ± 0.10%, p<0.04; Fig. 1B), but returned to baseline levels with 48 hours after BTxA injection (1.14 ± 0.07%, p=0.75 vs naive; Fig. 1B). CD8+ populations were not significantly altered at any timepoint (2.36 ± 0.21% vs 2.32 ± 0.20% vs 2.52 ± 0.35 in day 1, day 2, and naïve, respectively, p>0.9; Fig. 1C). Thus, the ratio of CD4+/CD8+ T cells within the marrow was increased 34% on day 1 (0.63 ± 0.04) vs naïve (0.47 ± 0.04, , p<0.03; Fig. 1D), but not on day 2 (0.49 ± 0.01, p=0.82 vs naive; Fig. 1C). The overall T cell population size in naïve marrow (40.17 ± 1.78%) was not significantly altered at 24 hours (42.40 ± 2.91%, p=0.36 vs naive; Fig. 1E) or 48 hours (33.20 ± 2.24%, p=0.10 vs naive; Fig. 1E).

Osteoinflammatory Genes Are Rapidly Altered Following Muscle Paralysis

Temporal gene expression patterns within the tibia marrow following muscle paralysis were consistent with transient inflammatory signaling. While none of the panel of nine genes was significantly altered at 1 day following muscle paralysis (vs naïve controls), three genes showed statistically significant regulation by day 3.
Two genes were upregulated (TNF: 299%, IL-4: 243%; each p<0.02, Table 2), while one was downregulated (CD47: -88%; p<0.03, Table 2). Only IL-4 was significantly upregulated on day 7 (250%, p<0.02, Table 2). Additionally, three other genes (CD9, IL-1α and IL-1β; Table 2) were detectable in naïve and day 1 samples, but were undetectable on days 3 and 7. IL-6 gene expression was also assayed but the levels were undetectable in most samples and thus not reported.

The Osteoclastic Potential of Bone Marrow Is Altered by Muscle Paralysis

The culture conditions were permissive to the formation of osteoclasts with a small subset of giant osteoclasts (> 20 nuclei; Fig 2). Marrow from naïve mice generated 96.8 ± 26.7 (mean ± s.e.) normal osteoclasts/well, including 2.5 ± 1.0 giant osteoclasts/well. Transient muscle paralysis did not significantly alter the number of normal osteoclasts formed in marrow culture at any time point (Fig. 3A). However, the capacity to form giant osteoclasts was acutely upregulated 3 days following muscle paralysis, and was not significantly different from naïve mice by day 7. Marrow harvested 3 days after BTxA injection produced 268% more giant osteoclasts than naïve control marrow (p=0.008) and 352% more giant osteoclasts than day 1 marrow (p=0.003; Fig. 3B). Secondary analyses indicated that this observation was robust to the number of nuclei chosen to represent a giant osteoclast. A KS Test indicated that the distribution of osteoclast size arising from day 3 BTxA marrow was significantly different than that of naïve bone marrow (p<0.001). Additionally, we observed that two separate power law models (a* \( x^b \)) were required to describe the in vivo data (p=0.87, formulas in Fig 4). Given that the overall osteoclast numbers were not different in naïve and day 3 BTxA cultures (see above), this analysis indicates that the percentage of cells (and thus total cell number) in the day 3 BTxA culture were higher than in the naïve cultures regardless of the threshold chosen to classify a giant osteoclast.

Osteoclast Fusion Genes Are Upregulated 3 Days Following Muscle Paralysis

The elevated osteoclast size and number of nuclei generated by marrow following muscle paralysis might be explained by enhanced cell fusion. We therefore reassessed cDNA from the gene expression experiment and quantified alterations in the osteoclast fusion genes (DC-STAMP and OC-STAMP). Both DC-
STAMP (243% increase) and OC-STAMP (218% increase) were significantly upregulated in bone marrow removed 3 days post-paralysis as compared to naïve bone marrow (both p< 0.02, Fig 5). Consistent with the in vitro experiments, osteoclast fusion gene expression was not significantly altered on day 1 or day 7 following muscle paralysis compared to naïve samples.

Discussion

We used flow cytometry, quantitative RT-PCR, and primary osteoclast culture to explore the presence of inflammatory signaling within bone marrow following calf paralysis. We observed inflammatory cell infiltration within 24 hours and pro-inflammatory gene expression within 72 hours of muscle paralysis. Surprisingly, alterations in the osteoclastic potential of bone marrow extracted following muscle paralysis were not associated with increased numbers of osteoclasts, but instead manifested as an increase in osteoclast size and number of nuclei per osteoclast. The observation of enhanced numbers of giant osteoclasts was supported by simultaneous upregulation of the osteoclast fusion genes DC- and OC-STAMP. In combination, these data are consistent with muscle paralysis acutely precipitating a localized inflammatory response within bone marrow that is permissive to generating osteoclasts of greater size and nuclei number.

These observations must be considered in the context of the limitations of our approach. The impetus for this study was to assess our thesis that inflammatory signaling within bone marrow precedes or is coincident with the onset of bone resorption induced by muscle paralysis. To that end, we quantified inflammatory cell influx (FACS) and altered osteoinflammatory signaling (RT-PCR) within bone marrow. While recognizing that these outcomes are indirect and do not define a causal relationship between bone marrow inflammation and subsequent bone resorption, we believe this limitation is mitigated, in part, by the cadence of events underlying bone resorption following muscle paralysis. Specifically, we observed alterations in inflammatory cell populations (day 1) upstream of alterations in osteoinflammatory gene expression (day 3). The timing in which marrow became permissive to formation of giant osteoclasts (day 3), coupled with the time needed to form functioning osteoclasts from precursor populations (≈ 3-5 days (13, 47)) is congruent with the onset of
diaphyseal endocortical resorption following paralysis (which almost entirely occurs between days 6 and 13 post-paralysis) (5).

To further support our gene expression data, we performed a follow-on ELISA-based study to quantify protein level alterations in TNFα and IL4 (as both genes showed significant upregulation following muscle paralysis). Utilizing the same time points as the gene expression experiment, we did not observe elevated expression in either protein following muscle paralysis. There are many potential explanations for the lack of direct correlation between mRNA and protein levels, and the inflammatory literature illustrates this challenge even in a simple cell culture environment (e.g., (44)). One clear challenge in our system is that the temporal dynamics of protein expression and stability (e.g., half-life on the scale of minutes in an experiment of many days) is likely to be exceedingly non-linear. Though we believe the cumulated data across our studies suggest otherwise, it is possible that the inflammatory genes upregulation does not manifest in alteration in protein level expression. Regardless, these data support the need to further clarify the role of inflammatory mediation and suggest that either transgenic (6, 40) or pharmaceutical (9, 42) strategies would most effectively explore a mechanistic relation between inflammation and muscle paralysis-induced bone loss.

Using a primary culture system to explore whether bone marrow had altered osteoclastic potential following transient muscle paralysis, we observed greater numbers of giant osteoclasts (>20 nuclei). This finding ostensibly contradict our previous in vivo observation of increased osteoclast numbers in the proximal tibia following BTxA-induced paralysis of the calf (2). However, as histologic quantification of osteoclast numbers in adjacent cross-sections relies upon an assumption of equivalent cell size, a significant increase in osteoclast size (as observed in vitro) would likely manifest as an increased osteoclast number in stereological assessments. Additionally, we did not determine the resorptive potential of the giant osteoclasts observed in cell culture. The literature suggests that osteoclast size does not always correlate with increased bone resorption. For example, giant osteoclasts observed following long-term bisphosphonate therapy are generally not thought to resorb bone at all, but rather are an artifact caused by prolonged apoptosis (22, 51). In contrast, however, giant osteoclasts associated with inflammatory pathologies such as Paget’s are generally thought to be capable of resorbing large volumes of bone mineral (16, 21, 29, 38). If so, it is reasonable to speculate that a
relatively few giant osteoclasts could account for a sizeable proportion of bone resorption observed following muscle paralysis. As confirmation of the *in vivo* presence of giant osteoclasts via histology may prove technically challenging, given our observations of enhanced cell fusion, it is possible that pharmaceutical strategies to transiently inhibit cell fusion could be implemented to explore this question *in vivo* (18).

Despite these limitations, our results are consistent with the upregulation of inflammatory cells and inflammatory genes prior to the initiation of bone resorption, and our *in vivo* RT-PCR data are also consistent with this altered marrow environment facilitating the formation of giant osteoclasts observed *in vitro*.

Following muscle paralysis, several genes implicated in osteoclast fusion and giant osteoclast formation were upregulated, including the essential protein for osteoclast fusion, DC-STAMP (35, 43, 54), and a second osteoclast fusion related protein, OC-STAMP (36). Inhibition studies have indicated that these genes are essential for normal osteoclast activity, while overexpression studies having resulted in elevated osteoclast formation/bone resorption (11, 28, 52, 55). IL-4 has been shown to promote the formation of multinucleated giant cells from macrophage precursors through the overexpression of E-cadherin (37). Other recent reports identified that upregulation of TNF in the absence of CD44 results in the generation of highly resorbing giant osteoclast (21). While we did not detect a statistical decrease in CD44 gene expression on day 3 (p=0.10), the 87% decrease in comparison to naïve bone marrow and elevated TNF in our data are also consistent with this thesis.

Ultimately, understanding how muscle paralysis of the calf induces inflammatory alterations in tibia bone marrow may reveal unexpected targets to mitigate the catabolic effects of neuromuscular dysfunction, regardless of initiating pathology. One possible explanation is that muscle paralysis induces neurogenic inflammation, which is characterized by the local release of inflammatory mediators in response to trauma or noxious stimuli (10, 23, 30). Classically, antidromic depolarization causes sensory neurons to rapidly release neuropeptides (primarily SP and CGRP; (10, 14)) that act on a variety of cell populations to rapidly orchestrate a focal inflammatory response (7). While direct evidence of neuropeptide release following muscle paralysis has not yet been reported, even brief periods of weightlessness have been associated with afferent sensory nerve depolarization (26).
Taken together, our data suggest that transient calf muscle paralysis induces acute inflammation within the marrow of the adjacent tibia that is temporally consistent with an active role in mediating bone resorption induced by this neuromuscular dysfunction. Our data additionally suggest that the inflammatory cascade swiftly triggered by transient muscle paralysis predisposes bone marrow to the formation of giant osteoclasts, which may account for the rapid and severe destruction of trabecular bone observed in this model. The identification of an acute inflammatory cascade in bone marrow leading to the formation of giant osteoclasts has potential to reveal novel therapeutic strategies for mitigating paralysis-induced bone loss following neuromuscular trauma.
**Figure Legends**

**Figure 1:** Acute CD4+ T cell upregulation in bone marrow following muscle paralysis. Bone marrow was extracted from the proximal metaphysis of the right tibia of naïve mice and mice that had received an IM injection of BTxA the previous day (2U/100g body weight in right calf muscle group). Samples were lysed of red blood cells and stained with TCRβ, CD4 and CD8 antibodies, and FACS analysis performed. Prior to determination of T cell populations within bone marrow, cellular debris was excluded from analysis using traditional forward side-scatter gating (A). Percentages of CD4+ (B) and CD8+ (C) T-cells are shown, and the ratio of CD4+:CD8+ cells determined (D). Additionally, total percentages of T-cells in the marrow are graphed (E). Values represent mean + SE (n=4-5 per group), *p<0.05 vs naïve and day 2, #p<0.05 vs day 1.

**Figure 2:** Marrow culture conditions were permissive to formation of osteoclasts. Whole bone marrow was collected from the right tibia of experimental mice. Samples were lysed of red blood cells, seeded in culture medium supplemented with M-CSF (50 ng/ml) and RANKL (10ng/ml), incubated at 37°C, and stained for TRAP on day 8 of culture. Representative image of bone marrow harvested 3 days after transient muscle paralysis. Giant osteoclasts (blue arrows) and smaller osteoclasts (green arrows) can be seen.

**Figure 3:** Transient muscle paralysis augmented osteoclastic potential of tibia marrow through enhanced osteoclast fusion, but not increased osteoclast number. Whole bone marrow was collected from the right tibia of naïve mice and from mice that had received an IM injection of BTxA injection in the right calf 1, 3 and 7 days previously. Cells were cultured for 8 days, and stained for TRAP. Normal osteoclast (TRAP + w/3+ nuclei) and giant osteoclast (a subset of normal osteoclasts w/20+ nuclei) were quantified in each group on a per well basis using bright field microscopy (10 to 40x magnification). Fold changes of osteoclasts (A) and giant osteoclasts (B) are shown at day 1, 3 and 7 post injection normalized to the naïve controls. Values represent mean + SE (n=4 per group), **p<0.01 vs naïve and day 1.
Figure 4: Size of osteoclasts generated from marrow harvested 3 days after muscle paralysis was consistently larger than that of Naïve marrow cultures. An automated quantification of osteoclast area was performed on two subsamples of the naïve and day 3 cultures (10 mm² per subsample using a composite of eighty light microscopy 20x images) using custom software as described in the Materials and Methods. A Kolmogorov-Smirnov (KS) analysis indicated the area distributions from naïve and day three BTxA marrow were significantly different (p<0.001). The figure represents the distribution of cell areas in each culture with separate power law models (dashed and dotted lines) fit to simulate the in vitro data. Values represent mean + SE.

Figure 5: Osteoclast Fusion Gene Expression Following Muscle Paralysis. Whole bone marrow was flushed from the right tibia of naïve mice and mice that had received an IM injection of BTxA in the right calf 1, 3 and 7 days previously. After preparation of total RNA and synthesis of CDNA, quantitative RT-PCR was performed using DC-STAMP and OC-STAMP gene specific primers. Gene expression levels were quantified relative to the housekeeping gene HPRT. Values represent mean + SE (n=6 per group), *p<0.05 vs naïve.


35. Mensah KA, Ritchlin CT, and Schwarz EM. RANKL induces heterogeneous DC-STAMP(lo) and DC-STAMP(hi) osteoclast precursors of which the DC-STAMP(lo) precursors are the master fusogens. J Cell Physiol 223: 76-83, 2010.


Table 1: Primer sequences for RT-PCR analysis

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<tr>
<th>Target</th>
<th>Forward (5’→3’)</th>
<th>Reverse (5’→3’)</th>
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<td>HPRT</td>
<td>AGTGTGTTGGATACAGGGCCAGAC</td>
<td>CGTGATTTCAAAATCCCTGAAGT</td>
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<td>TNF</td>
<td>AGCCCCCAGTCTGTATCCTT</td>
<td>CTCCCTTTTCGAGAACTCAGG</td>
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<td>GCCGCGGTCTGGGGCTATAC</td>
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<td>OC-STAMP</td>
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Table 2: Relative mRNA expression in whole tibia following calf paralysis [mean ± s.e]

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<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
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<tr>
<td>TNF</td>
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<td>0.91 ± 0.28</td>
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<tr>
<td>CD81</td>
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<td>IL1b</td>
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<tr>
<td>IL11</td>
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<td>0.16 ± 0.05</td>
<td>0.26 ± 0.07</td>
<td>0.21 ± 0.08</td>
</tr>
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 Significant increase (♦) or decrease (♩) vs Naïve (all p < 0.05)
 Undetectable = Unquantifiable expression in at least half the samples