Association between periodontal disease and inflammatory arthritis reveals modulatory functions by melanocortin receptor type 3

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Abstract

Background. Since there is clinical evidence for an association between periodontal disease and rheumatoid arthritis, it is important to develop suitable experimental models to explore pathogenic mechanisms and therapeutic opportunities.

Methodology. The K/BxN serum model of inflammatory arthritis was applied using distinct protocols, and modulation of joint disruption afforded by dexamethasone and calcitonin, in comparison to the melanocortin (MC) receptor agonist $DTrp^{8}$-γMSH (DTrp), established. Wild type and MC receptor type 3 (MC_3) null mice of different ages were also used.

Results. There was significant association between severity of joint disease, induced with distinct protocols and volumes of the arthritogenic K/BxN serum, and periodontal bone damage. Therapeutic treatment with dexamethasone (10µg/mouse), elcatonin (30ng/mouse) and DTrp (20µg/mouse) revealed unique and distinctive pharmacological properties, with only DTrp protecting both joint and periodontal tissue. Further analyses in non-arthritic animals revealed higher susceptibility to periodontal bone loss in *Mc3r*-/- compared to wild type mice, with significant exacerbation at 14 weeks of age.

Significance. These data reveal novel protective properties of endogenous MC_3 on periodontal status in health and disease and indicate that MC_3 activation could lead to the development of a new genus of anti-arthritic bone-sparing therapeutics.

Keywords: Dexamethasone, Inflammatory Arthritis, K/BxN arthritis model, Melanocortins, Rheumatoid arthritis,

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease associated with progressive disability, early death, increased risk of cardiovascular events and other extra-articular manifestations that have a major impact in the quality of life of sufferers [1,2]. The current clinical approach is to start early, after diagnosis, with aggressive therapy, followed by treatment adjustments according to changes in disease activity. However, despite the important progress in RA therapies during the last decade several needs are still unmet. The introduction of biologics in the early '90s revolutionized the treatment of RA and other chronic diseases such as inflammatory bowel disease. However, although highly effective and generally faster acting than disease-modifying anti-rheumatic drugs (DMARDs) a significant proportion of patients are not responsive, and may also experience an increased risk of opportunistic infections and treatments are costly [3]. Thus there is justification for exploiting novel therapies. Additionally, it is also desirable to produce new therapeutics with efficacy not only on pain and inflammation in the joint, but also able to temper systemic complications of RA affecting the heart, lungs, muscles and bone [1].

Targeting the melanocortin (MC) system [4] to treat RA may represent an alternative opportunity to drug discovery [5]. Indeed, one of the melanocortin agonists ACTH (adrenocorticotropin hormone) was shown to be effective in human RA over 60 years ago [6], yet it is truly experiencing a renewed interest [7]. This is prompted by the fact that ACTH may afford biological actions beyond the endogenous production of cortisol [8,9], provoking activation of peripheral MC receptors, including the melanocortin receptor 3 (MC_3). This peripheral mechanism of action of ACTH, hence independent from adrenal release of glucocorticoids, might also underlie efficacy in conditions such as proteinuric nephropathies [10] and multiple sclerosis [11].

Surmounting evidence indicates an important counter-regulatory role for the melanocortin pathway during inflammation, including in the osteo-articular system, where melanocortin receptors are expressed by osteoblasts, osteoclasts, chondrocytes, fibroblasts and immune cells. Pharmacological targeting with MC peptides leads to a variety of protective actions including increased matrix deposition, reduced fibroblast activation, osteoblast and chondrocyte proliferation [12,13,14,15,16,17]. *In vivo*, the synthetic peptide DTrp^{8}-γMSH (DTrp) reduces clinical signs of disease in models of inflammatory arthritis [18] and urate crystal peritonitis [19] by a mechanism involving MC_3. In addition, the pan-MC agonist peptide AP214 also displays anti-arthritic properties [20]. Recent work by Gomez-SanMiguel *et al*, reported that the MC agonist alpha-melanocyte stimulating hormone (αMSH) can reduce joint inflammation together with an improvement of extra-articular signs associated with systemic arthritis, by increasing body weight and reducing levels of muscle wasting markers [21].

An important clinical manifestation associated with arthritis is periodontal disease. There is epidemiological evidence associating inflammation of the gum to incidence of RA [22] and, conversely, there is higher incidence of periodontitis in RA patients [23]. Intriguingly, recent reports demonstrated presence of alveolar bone loss, an important feature of periodontitis, in rodents during the time course of experimental models of arthritis, namely collagen-induced and adjuvant-induced arthritis [24,25,26]. Herein, we investigated the presence of alveolar bone loss in a different model of experimental arthritis; one induced by the arthritogenic K/BxN serum which is much faster in its kinetics, and it is characterized by leukocyte infiltration, synoviocyte proliferation, cartilage and bone erosion, thus resembling many features of human RA in its active flares [27,28], In addition, we established the involvement of the melanocortin system in the development of alveolar bone loss by using a combination of genetically engineered mice and pharmacological approaches.

2. Materials and Methods

2.1. Animals.

Male mice (7-8 weeks old) were maintained on standard chow pellet diet and had free access to water with a 12-hour light-dark cycle. C57BL/6J wild-type (WT) were purchased from Charles River (Kent, UK). *Mc3r*-/- mice were a generous gift of Dr Chen (Merck Laboratories). All animal studies were approved and performed under the guidelines of the Ethical Committee for the Use of Animals, Barts and The London School of Medicine and Home Office Regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986).

2.2. Production of K/BxN serum

K/BxN mice were produced by crossing the C57B1/6 mice (carrying the KRN homozygously) and the NOD/Lt mice (carrying the A^{g7} allele homozygously) [27]. The offspring develop spontaneous arthritis, evident at 6 weeks, with 100% incidence. At 9 weeks of age (when the titers of anti-glucose-6-phosphate isomerase (GPI) antibodies are maximal implying potent arthritogenic properties of the serum) mice were exsanguinated by cardiac puncture under anaesthesia (isofluorane). Blood was allowed to clot overnight at 4°C. Serum was recovered with a Pasteur pipette and centrifuged 10 min at 500xg at 4°C. The serum from different mice obtained on a given day was pooled, aliquoted and stored at -80°C until use.

2.3. K/BxN serum transfer arthritis model.

Arthritis was induced by the i.p. injection of serum from K/BxN arthritic mice. Three different protocols were studied: a) protocol 50+50, where mice received two injections of 50µl of serum on days 0 and 2; b) protocol 100+100, where mice received two injections of 100µl of serum on days 0 and 2; c) protocol 200, consisting of one single injection of 200µl of serum on day 0. The protocol 100+100 was then

selected for subsequent experiments. The development of the disease was monitored daily by assessing the paw volume using a plethysmometer (Ugo Basile, Comerio, Italy), body weight, clinical score (score per paw: 0= no signs of inflammation, 1=subtle inflammation, localized, 2=easily identified inflammation but localized, 3=evident inflammation, not localized; max score=12 per mouse) and disease incidence (mice showing overt signs of joint inflammation, i.e. a clinical score of 1 or above) [29]. Severe arthritis (number of paws per mouse that reached a maximum score of 3) was also recorded.

2.4. Pharmacological treatments.

Mice (n=5) were treated i.p. once daily, starting from day 2 (1h after the second K/BxN injection), with 10µg/mouse dexamethasone (SIGMA, Poole, UK), 20µg/mouse DTrp^{8}-γMSH (DTrp; American Peptide, Sunnyvale, CA, USA), 30ng/mouse elcatonin (Bachem, Bubendorf, Switzerland) or vehicle (PBS). Doses were selected from previous studies in this or similar rodent models [18,30,31].

2.5. Measurement of alveolar bone loss.

Alveolar bone loss was evaluated as previously described [32]. Mice were euthanized and maxillae were hemisected, exposed overnight in 3% hydrogen peroxide, mechanically defleshed, and stained with 0.3% methylene blue. The palatal faces of the molars were photographed using a stereomicroscope and a digital camera (Kodak EasyShare C743; Rochester, USA). Quantitative analyses included the measurement of the area between the cement enamel junction and the alveolar bone crest in the first molar, using Image J software (Maryland, USA).

2.6. Myeloperoxydase activity assay.

MPO activity was measured as an index of granulocyte infiltration. Briefly, maxillae tissue samples were homogenized in 0.5% hexadecyltrimethylammonium bromide

dissolved in phosphate buffer solution (pH = 6) using Precellys®24 homogeniser in Precellys lysing CK14 tubes (Bertin Technologies). The homogenized tissues were centrifuged at 13 000xg for 5 minutes (at 4°C) and the supernatants were placed on 96 well plates. Buffer, supplemented with 1% hydrogen peroxide/O-dianisidine dihydrocholoride, was added to each well. Optical density readings were taken for 3 minutes at 30 seconds intervals at 450 nm using a microplate reader NOVOstar™ (BMG Labtech, Aylesbury, UK). Activity was normalized to the sample protein concentration determined with a BCA kit® (Pierce, Cramlington, UK) and expressed as mU/mg protein.

2.7. Histological analyses.

Maxillae tissues were fixed in 10% buffered formalin (pH 7.4) for 24 h at room temperature. The specimens were demineralized in 14% ethylenediamine tetracetic acid (EDTA) for 2 weeks, dehydrated in graded ethanol and embedded in paraffin. Serial sections (5 µm) were stained for tartrate-resistant acid phosphatase (TRAP, Sigma–Aldrich, Saint Louis, MO, USA). Histological osteoclast counting was performed in the coronal two thirds of the distal alveolar bone adjacent to the first molar in five consecutive microscopic fields (40x)/section. Samples were analyzed using an Axioskop 40 microscope (Carl Zeiss, Gottingen, Germany), attached to a digital camera (PowerShot A620; Canon, Tokyo, Japan). For each animal (n=4), three maxillae sections were analyzed. All the slides were counted in a blinded manner by a single examiner. For neutrophil staining tissue specimens were blocked by incubation in 1.5% H_2O_2 in methanol solution for 30 min. Primary antibody against neutrophil elastase (Santa Cruz Biotechnology, Heidelberg, Germany) was used with Vectastain ABC kit anti-rabbit (Vector Laboratories, Burlingame, USA). The sections were counterstained using hematoxylin. Slides were developed using Peroxidase substrate kit DAB Kit (Vector Laboratories, Burlingame, USA).

178 *2.8 Statistical analysis*

179 Data were analyzed by Student's *t*-test, one or two-way ANOVA followed by
180 Bonferroni or multiple comparison test, two-way ANOVA followed Newman-Keuls
181 multiple comparison test or Pearson correlation test, as appropriate. In all cases data
182 are presented as mean ± SEM of *n* independent observations and were considered
183 statistically significant when $p<0.05$.

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3. Results

3.1. Arthritis severity in the K/BxN serum transfer model correlates with alveolar bone loss.

Recent reports have indicated that experimental models of collagen and antigen-induced arthritis are associated with development of alveolar bone loss. We evaluated whether development of aggressive joint inflammation using the K/BxN serum transfer model of arthritis was also associated with this extra-articular manifestation. We therefore utilized three dosing strategies to manipulate the severity of arthritis: mice received two injections of either 50µl or 100µl of serum on days 0 and 2 (*protocol 50+50* or *protocol 100+100* respectively) or a single injection of 200µl of serum on day 0; *protocol 200*. All three regimens induced overt signs of arthritis. As expected, the arthritic response produced with protocol 50+50 was milder, evidenced by the low clinical score (Figure 1A), inconsistent increase in paw volume (Figure 1B) and reduced severity (Figure 1C) compared with the other two protocols. Comparatively, although mice received the same total amount of arthritogenic serum using protocols 100+100 and 200, administration in two separate injections (day 0 and 2) resulted in higher clinical scores, more gradual and consistent increase in paw volume and, importantly, 100% disease incidence (Figure 1D). We next analysed alveolar bone loss in the maxillae of these mice subjected to serum transfer-induced arthritis. Interestingly, bone loss in the maxillae was highly correlated with the severity of localized inflammation in the joints (Figure 2A), suggesting that extra-articular manifestations of relevance for RA also occur in this model. An overall comparison between non-arthritic and arthritic mice indicated a significant (21%) increase in alveolar bone loss (Figure 2B). This could also be seen macroscopically by an increased corono-apical area between the cement enamel junction (CEJ) and the alveolar bone crest (ABC) on the palatal side of the first molar (Figure 2C*).*

3.2. Melanocortin treatment reduces arthritis and prevents alveolar bone loss.

The melanocortin system is widely associated with joint disease and inflammation both mechanistically and therapeutically [5,8,18,19]. We studied if pharmacological intervention with melanocortin-based compounds could have an impact, not only in joint inflammation or other arthritis-related manifestations like cachexia, as recently described [21], but also in alveolar bone loss. We therefore assessed whether the peptide DTrp could prevent the development of alveolar bone loss we observed using this passive transfer model of arthritis. Dexamethasone (Dex), very potent as an anti-inflammatory drug but with detrimental bone effects, and elcatonin (ECT), a bone protective molecule but with mild anti-inflammatory effects, were used for comparison. As expected Dex afforded a potent anti-inflammatory effect, reducing clinical score (-40% at day 8; Figure 3A), paw volume (-59%; Figure 3B), and incidence of severe arthritis (-93%; Figure 3C). The effect of ECT was less pronounced with a significant statistical difference only in reducing paw volume. DTrp presented a moderate and significant attenuation in all parameters measured (clinical score -23%, paw volume -44%, severity -57%).

Determination of myeloperoxidase (MPO) activity in maxillae tissues (analyzed at day 8) showed a significant reduction in the groups treated with either Dex or DTrp, but not with ECT (Figure 3E). Although the degree of anti-inflammatory activity attained by treatment of animals with Dex and DTrp in the periodontal tissue appears to be similar, assessment of alveolar bone loss revealed interesting differences: the anti-arthritic effect of DTrp is associated with bone protection, as evident from the positive correlation (R=0.87) between bone loss and clinical score (Figure 3F). In contrast, the anti-arthritic effect of Dex was inversely correlated with alveolar bone loss (R=-0.87), in accordance with the well-known effects of Dex on bone metabolism. These findings suggest that melanocortin therapy, in addition to potent modulation of

inflammation and arthritis, could also have the advantage over corticoids therapy in preserving bone integrity.

3.4. MC_3 deficient mice display increased alveolar bone loss.

Our next approach consisted of the study of the role of the melanocortin receptor 3 (MC_3) using genetically modified mice lacking this receptor, as it has been reported that MC_3 is as pivotal target for the anti-inflammatory and anti-arthritic actions of melanocortin drugs, including ACTH [8,9]. Arthritis was induced using the 100+100 protocol, as in previous experiments, although in this case 12-week old mice were used (due to stock availability). As shown in Figure 4, arthritis developed similarly to previous experiments with younger mice. The clinical score and disease severity was identical in WT and *Mc3r*-/- mice although some differences were found in the paw volume (Figure 4). As expected, when alveolar bone loss was assessed in maxillae (day 8), WT arthritic mice presented an increase in bone loss corroborating our previous findings. However, the basal values of alveolar bone loss obtained in *Mc3r*-/- mice were elevated compared with WT mice, and no further alveolar bone loss was obtained following arthritis induction (Figure 4D). The fact that the mice used in this experiment were older made us hypothesise that there might be an association between MC_3 deficiency and physiological bone metabolism.

3.5. Aging-associated alveolar bone loss is accelerated in MC_3 deficient mice.

The evidence obtained in our previous experiments suggests that activation of MC_3 (the main receptor that mediates DTrp actions [19]) might protect from alveolar bone loss. Since there is evidence in the literature furthering an association between the melanocortin system and bone metabolism [12], as well as linking normal bone loss

associated with aging [33,34], next we sought to investigate if MC_3 played any role in these phenomena.

Maxillae from WT and *Mc3r*-/- mice harvested at different ages (1.5, 3.5 and 4.5 months old) were analyzed for alveolar bone loss. Interestingly, periodontal bone loss was accelerated in *Mc3r*-/- mice, showing a 39% increase at 3.5 months of age compared to younger mice (1.5 months), while only 15% increase was observed in same age WT mice (Figure 5).

There is some indication for a functional association between MC_3 and osteoclastogenesis, as the number of osteoclasts is increased in *Mc3r*-/- arthritic mice, compared to WT [18]. We then studied if osteoclasts numbers were also different in the maxillae of the two genotypes by quantifying the $Trap^+$ cells in the cervical area of the first molar of the left lower maxillae (n=4 mice). Although basal levels (1.5 months/old) were different, we quantified a 40% increase in $Trap^+$ cells in older mice in *Mc3r*-/- but not in WT mice, where values remained stable (Figure 6A). We also observed a significant increase in the number of neutrophils in the junctional epithelium of *Mc3r*-/- mice compared to WT (Figure 6B). However, no differences were found in the rest of epithelial and connective tissue suggesting that neutrophils might not be playing a crucial role in the alveolar bone loss as measured in our experimental conditions.

4. Discussion

There is clinical evidence that periodontal disease is associated with RA, although it is unclear whether the link is causal or casual. Bone resorption in the maxillae (associated with a high degree of immune cell infiltration) is reminiscent of the RA joint where large numbers of blood-borne cells can be found in the exudate during the active phases of the disease. To gain information on pathogenesis to subsequently inform on therapeutic opportunities, it is important to develop animal models where disease development could be monitored at the two sites in parallel.

The recent appreciation that mice subjected to the gold standard model of RA, collagen induced arthritis, develop alveolar bone loss that parallel ankle joint damage [25] represents an important conceptual and experimental advance. Park *et al* reported severe periodontal bone damage 16 weeks after induction of arthritis using type-II collagen: this was associated with increased osteoclastic activity and impaired repair ability due to reduced bone formation by osteoblasts [25]. Here we used the K/BxN serum model of inflammatory arthritis, an aggressive model that mimics the active phases of RA and it is much faster in its onset. Injection of the serum rich in anti-glucose-6-phosphate isomerase immunoglobulins fixes complement onto cartilage with initiation of an inflammatory reaction, highly reliant on cytokines and eicosanoids [27,29]. Herein we first determined whether this rapid model (~7-8 days against >30 days for the collagen-induced arthritis) also led to periodontal disease. Thus, administration of the arthritogenic serum along three different protocols induced evident joint inflammation, with more consistent and reproducible data with the 100+100 µl protocol and truly mild when using the 50+50 µl protocol. A good association was observed between serum dosage and protocol of administration, which resulted in distinct severity of arthritis, and the corresponding degree of

alveolar bone loss measured in maxillae. Collectively these data, coupled to the two recent studies [24,25] indicate that experimental polyarthritis in rodents is indeed associated with periodontal disease features.

The melanocortin receptor agonist ACTH is an anti-arthritic drug indicated for the treatment of acute inflammatory episodes of gout [35] as well as RA, as shown by the seminal work of Hench and colleagues of the Mayo Clinic taught us [36,37]. The equally old observations of Gutman [6] have been repeated in more rigorous clinical studies confirming ACTH efficacy in human gouty arthritis [38,39,40]. All these studies indicate that ACTH is effective and safe for the treatment of acute gout and presents as a good alternative in patients with comorbidities in which steroids and colchicine are not recommended. Why is ACTH so effective in arthritides? Ritter and colleagues indicated the possible existence of mechanisms aside adrenal stimulation and glucocorticoid release [40], inciting us to identify peripheral modulation of MC_3 as a very important contributor of the anti-inflammatory actions of the peptide [8]. However, ACTH is a pan-MC receptor agonist. Herein we used peptide $DTrp^8$-γMSH (abbreviated DTrp), which although not totally selective in *in vitro* expression cell systems, retains functional selectivity in the mouse as demonstrated by its lack of efficacy in *Mc3r*-/- animals. Importantly, DTrp displays anti-arthritic effects in the K/BxN animal model of arthritis [18,19,41].

The pharmacological experiments suggested that MC-based therapy may yield a unique opportunity. Whilst the glucocorticoid dexamethasone afforded the expected therapeutic effect on the arthritic joint [18], measured in terms of score, swelling, disease severity and MPO activity, it did not affect – and rather worsened – alveolar bone loss. This effect can be chiefly due to the osteoclast activating property of

glucocorticoids [42]. Calcitonin, on the other hand, was selected because representing an opposite therapeutic, with very little modulation of inflammatory arthritis [43] yet its daily delivery to mice from day 2 significantly protected from alveolar bone loss associated with this model of experimental arthritis. This was predicted in view of the potent action of calcitonin in stopping bone resorption [44], being able for instance to override the activating effect of glucocorticoids [43].

DTrp revealed unique properties since was able to inhibit arthritis, albeit to an intermediate level between dexamethasone and calcitonin, and also attenuate bone loss associated with periodontal disease. In more detail, the DTrp group showed a positive high correlation between clinical score and bone loss (i.e. reduced bone loss associated with the anti-arthritic effect, R=0.87), and this was the exact opposite of that calculated for Dex-treated mice (R=-0.87). This finding is of relevance as prolonged steroid therapy is associated with bone density loss, osteoporosis and fractures. These results indicate that MC receptor agonists, possibly better if selective for MC_3, represent a novel class of anti-arthritic therapeutics able to target joint disease without aggravating unwanted effects on distant organs and tissues. This notion is further substantiated by a recent study where the beneficial effect of melanocortin treatment on joint inflammation and against systemic muscle wasting (cachexia) was demonstrated [21].

The bone-protective effect of DTrp is likely due a direct osteoclast effect that it is additive to modulation of local inflammation, as shown by the MPO activity measurements in maxillae samples. In agreement with these pharmacological data, we have reported a higher degree of osteoclastogenesis in *Mc3r*-/- mice, measured both *in vivo* in arthritic joints and *in vitro*, using bone marrow derived osteoclasts [18].

Furthermore, in wild type osteoclasts, application of DTrp reduced cell activation and resorptive activity. In these experiments, *Mc3r*-/- mice did not presented more pronounced arthritis, at variance from what we reported previously [18], likely due to differences in protocol and animal age. On the same token though, this new result allows to separate periodontal bone loss and joint arthritis, indicating that the former occurs independently from modulation of the latter. Collectively, these data prompt us to identify MC_3 as a modulatory receptor on osteoclast differentiation and activation.

The use of genetically engineered mice can shed new insights into the biological functions of genes of interest. The involvement of MC_3 in bone metabolism emerges from pharmacological evidence or from the use of *Mc3r*-/- in settings of experimental pathology. But if MC_3 plays important non-redundant role in bone physiology, then its absence might produce a phenotype in 'healthy' mice. Indeed, these mice present decreased linear growth and femur length as well as reduced bone mineral density [45,46]. To this end we monitored the degree of bone erosion in the maxillae of mice at different ages, comparing wild type and *Mc3r*-/- animals. These experiments confirmed a higher susceptibility to alveolar bone loss in the transgenic lacking the MC_3 receptor, with presence of significant bone loss as early as 14 weeks of age, whilst wild type mice displayed similar degrees of damage at 18 weeks. Thus, endogenous MC_3, possibly activated by circulating ACTH or αMSH, exerts a tonic inhibitory role on bone metabolism in the maxillae hence in its absence there is a higher susceptibility to bone loss hence disease. Though congruent with the data presented above, this hypothesis requires corroboration by future studies. With ageing *Mc3r*-/- mice become obese, an effect evident at 12 weeks of age [47]. Since it is reported that obese mice have a different microbiota compared to lean animals [48], one could not exclude that a different microbiota predisposes to higher susceptibility to periodontal disease. Again, focused and systematic analyses on the

periodontal compartment of MC_3 deficient mice can shed light onto this novel biology of the MC system we have unveiled here.

In summary, we describe a novel experimental association between periodontal disease and inflammatory arthritis with two distinct outcomes: first, the modulatory function of MC_3 on periodontal status in health and disease; second, the distinct pharmacology of DTrp as compared to other anti-arthritic or bone-protective compounds, suggesting the potential development of a new genus of anti-arthritic therapeutics, centred on MC_3 activation and able to spare or correct alveolar bone damage.

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List of abbreviations

ACTH (adrenocorticotropic hormone), Dex (dexamethasone), DMARDs (disease-modifying anti-rheumatic drugs), $DTrp^{8}$-γMSH (DTrp), ECT (elcatonin), MC (melanocortin), MC_3 (melanocortin receptor 3), myeloperoxydase (MPO), rheumatoid arthritis (RA), Trap (tartrate-resistant acid phosphatase), wild type (WT)

Competing interests

Authors declare no competing interests.

Authors contributions

TMM designed study, performed experiments, analyzed and interpreted data and wrote manuscript; MFMM performed experiments, analyzed and interpreted data and revised manuscript; LVN performed experiments and revised manuscript; AA performed experiments and revised manuscript; MAC interpreted data and revised manuscript; TAS interpreted data and revised manuscript; MP designed study, wrote manuscript and provided funding.

574 **Figure Legends**

575 **Figure 1.** *Comparison of three protocols for the K/BxN serum transfer arthritis model.*
576 Arthritis was induced by the i.p. injection of serum from K/BxN arthritic mice using
577 three different protocols: 50+50 (two injections of 50µl on days 0 and 2); 100+100
578 (two injections of 100µl on days 0 and 2); 200 (one single injection on day 0). Clinical
579 score (A), paw volume (B) and disease incidence (C) were recorded for 7 days.
580 Panel (D) shows the number of paws per mouse that reached the maximum score
581 (3). Representative images of ankle, wrist and digits swelling are shown in panel (E).
582 Data are the mean±SEM of 4-6 mice per group. *p<0.05 two-way ANOVA followed
583 by Bonferroni multiple comparison test.

584

585 **Figure 2.** *Correlation of arthritis with alveolar bone loss in the K/BxN serum transfer*
586 *model*. Alveolar bone loss was evaluated at day 7 in the palatal aspect of the first
587 upper molar of the right hemi-maxillae. (A) Correlation between alveolar bone loss
588 and clinical score on mice studied in the protocol comparison experiment (see Figure
589 1) analyzed by Pearson correlation test (n=14). (B) Overall increase in alveolar bone
590 loss in arthritic mice (pooled data from all mice, mean±SEM, n=14) compared to
591 control mice, analyzed by t-test (*p<0.05). (C) Representative photographs of the
592 maxillae showing evidence of alveolar bone loss (white arrows).

593

594 **Figure 3.** *Effect of dexametasone, DTrp and elcatonin in arthritis and alveolar bone*
595 *loss.* Arthritis was induced using the 100+100 protocol (100µl of serum on days 0 and
596 2) and monitored by daily recording the clinical score (A), paw volume (B), severity
597 (number of paws reaching the maximum score) (C) and disease incidence (D).
598 Myeloperoxidase activity was measured in the left hemi-maxillae at day 8 (E).
599 Alveolar bone loss was analyzed in the right hemi-maxillae at day 8 and correlated

600 with clinical score recorded that day (F). Drugs were administered i.p. once daily:
601 dexamethasone (Dex) 10µg/mouse; DTrp^{8}-γMSH (DTrp) 20µg/mouse; elcatonin
602 (ECT) 30ng/mouse; Vehicle PBS (Veh). Non-arthritic mice were included as controls
603 (Ctrl). Data are the mean±SEM of 5-6 mice per group. Data were analyzed by two-
604 way ANOVA followed by Bonferroni multiple comparison test (A-C), one-way ANOVA
605 followed by Bonferroni multiple comparison test (E) and Pearson correlation test (F).
606 In all cases *p<0.05.

607

608 **Figure 4.** *Arthritis and alveolar bone loss in MC_3 deficient mice.* Arthritis was induced
609 in C57BL/6J wild type mice (WT) and melanocortin receptor 3 deficient mice (*Mc3r*-/-)
610 using the 100+100 protocol (100µl of serum on days 0 and 2). Disease was
611 monitored by daily recording of the clinical score (A), paw volume (B), and disease
612 severity (number of paws reaching the maximum score) (C). Alveolar bone loss was
613 analyzed in the right hemi-maxillae on the last day of the experiment (day 8). Data
614 are the mean±SEM of 5 mice per group. Statistical analyses were carried out by two-
615 way ANOVA followed by Bonferroni multiple comparison test (A-C) and one-way
616 ANOVA followed by Newman-Keuls multiple comparison test *vs.* WT-Ctrl (D). In all
617 cases *p<0.05.

618

619 **Figure 5.** *Impact of aging on alveolar bone loss in MC_3 deficient mice.* (A) Alveolar
620 bone loss was evaluated in the right hemi-maxillae in mice from different ages (1.5,
621 3.5 and 4.5 months old) in both C57BL/6J wild type mice (WT) and melanocortin
622 receptor 3 deficient mice (*Mc3r*-/-). Data are the mean±SEM of 6-17 mice. Data were
623 analyzed by two-way ANOVA followed by Bonferroni multiple comparison test,
624 *p<0.05 vs. 1.5months, $^{\dagger}p$<0.05 WT *vs. Mc3r*-/-.

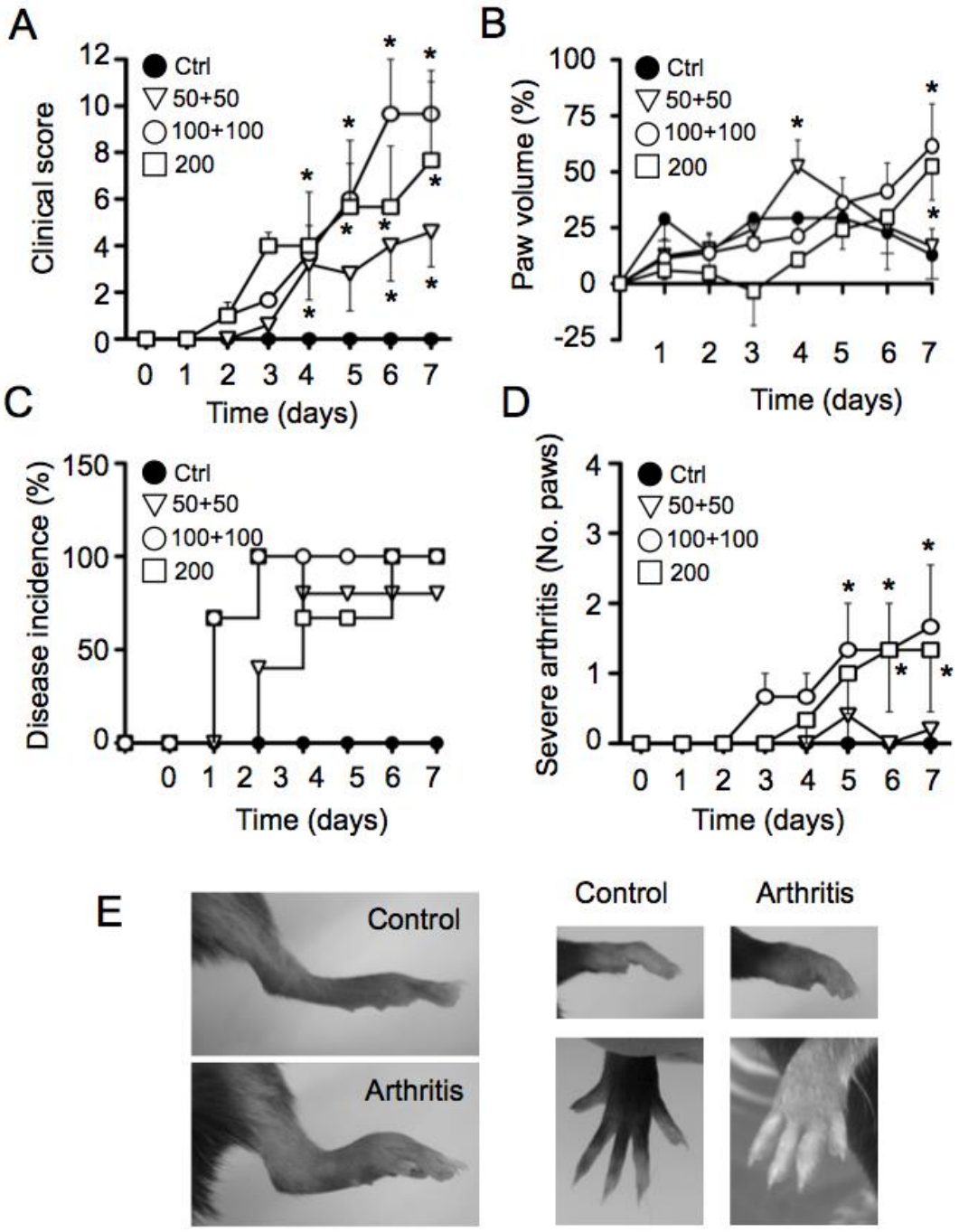
625

626 **Figure 6.** *Analysis of osteoclasts and neutrophils in gingival tissues.* The left hemi-
627 maxillae were used for histological evaluation of osteoclast activity by Trap staining
628 and neutrophil infiltration. (A) The number of osteoclasts on the cervical area of the
629 first molar was counted. Representative images of $Trap^+$ cells are shown. (B)
630 Sections were stained for neutrophil elastase as a marker of neutrophils.
631 Representative images of 1.5 months old mice are shown. Data are the mean±SEM
632 of 2-4 mice. Data were analyzed by one-way ANOVA followed by Bonferroni multiple
633 comparison test, *p<0.05.

634

635 Figure 1

636



637

638 Figure 2

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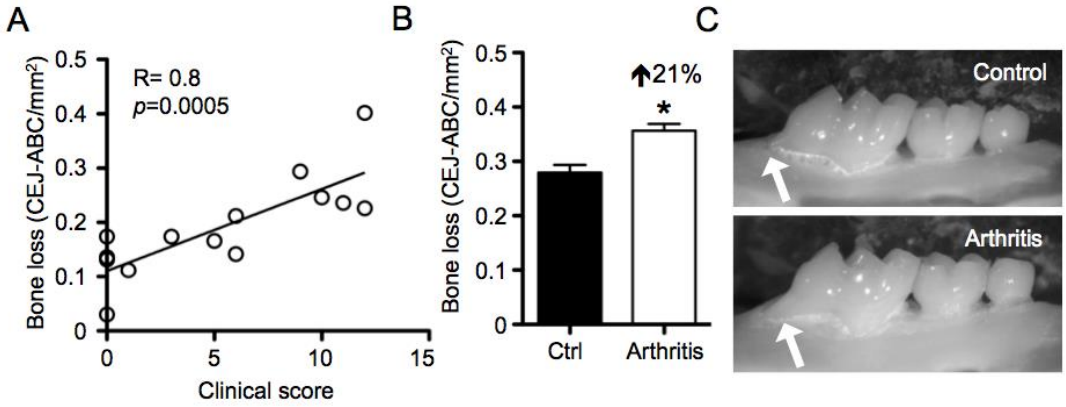

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641 Figure 3

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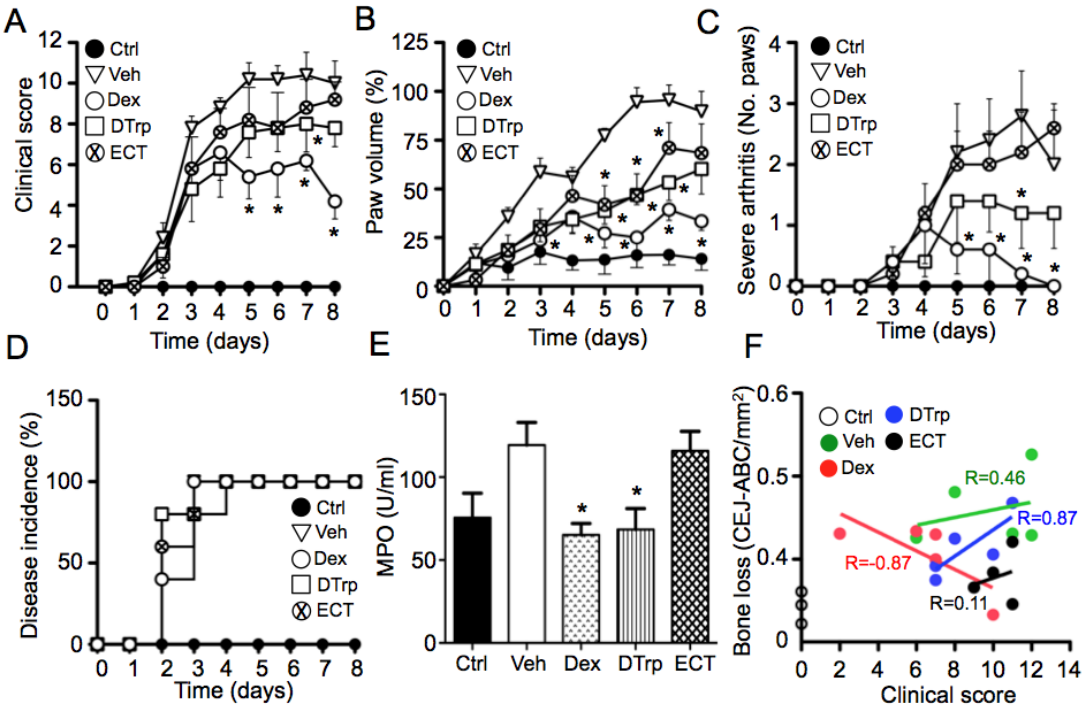

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644 Figure 4

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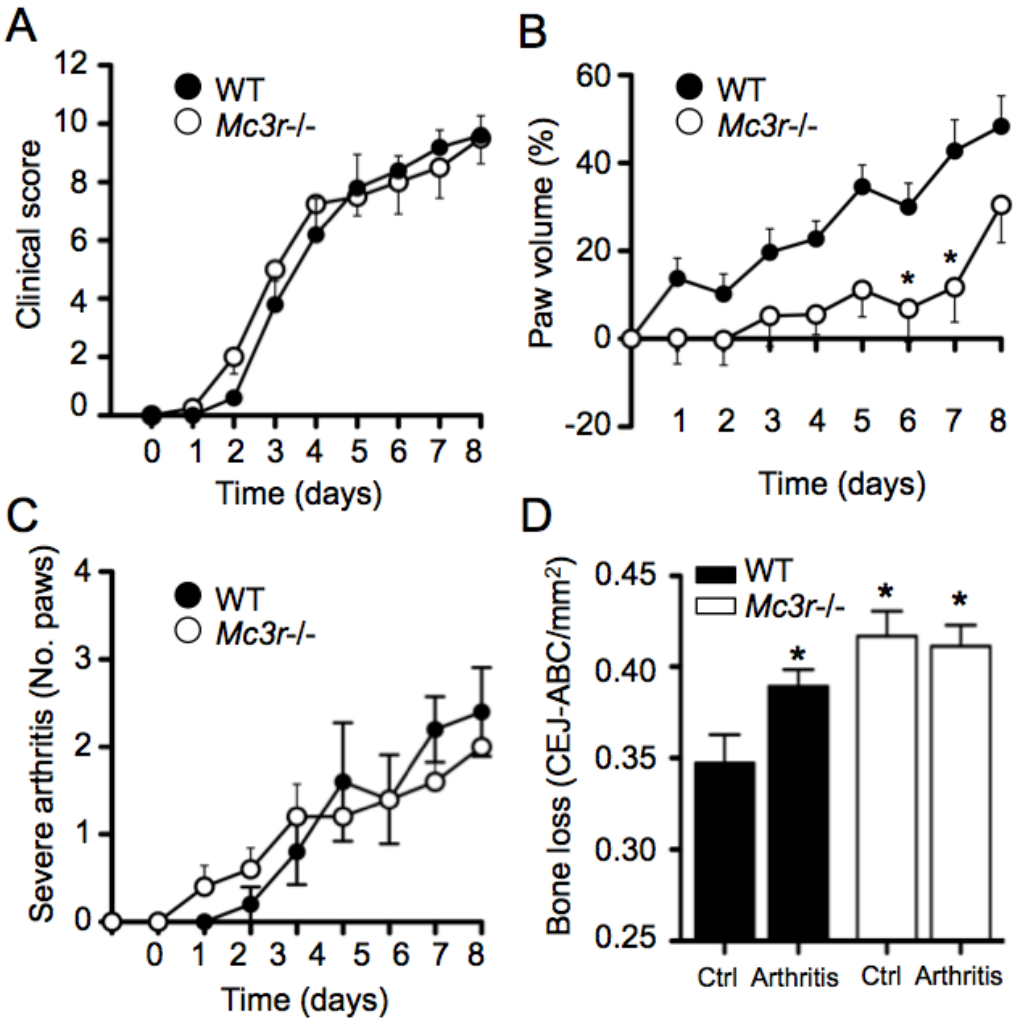

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647 Figure 5

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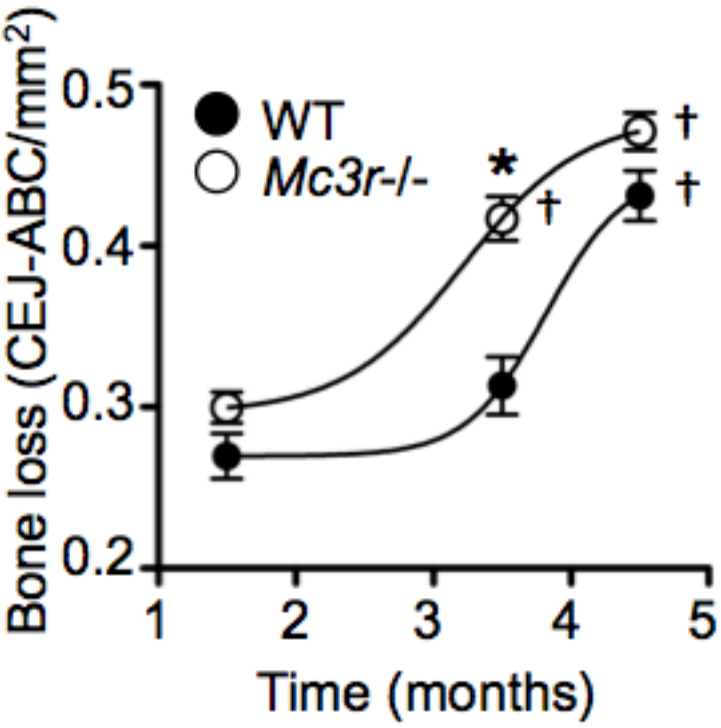

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652 Figure 6

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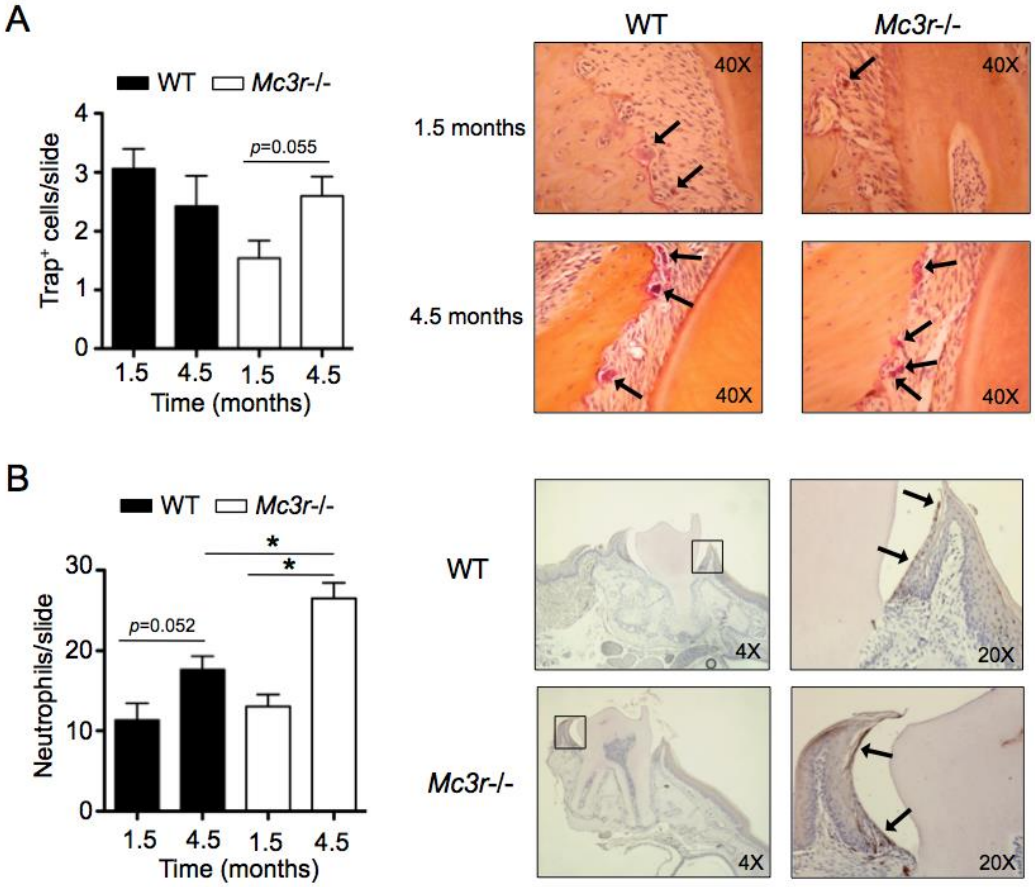

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