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The role of the P2X7 receptor on Bone Loss in a mouse model of Inflammation-Mediated Osteoporosis

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Abstract (250 words)

In inflammatory autoimmune diseases, bone loss is frequent. In most cases, secondary osteoporosis is caused by treatment with systemic glucocorticoid. However, the pathogenesis behind the bone loss is presumed multifactorial.

We aimed to elucidate the role of the P2X7 receptor on bone mineral density (BMD), microarchitecture, and bone strength in a standardized mouse model of Inflammation-Mediated Osteoporosis (IMO).

In total 146 mice completed our protocol, 70 wild type (WT) mice and 76 P2X7-/- (knockout, KO). BMD at the femur and spine decreased significantly from baseline to day 20 in the WT IMO mice ($p<0.01$). In the WT vehicle, KO vehicle and KO IMO, no significant BMD changes were found.

Bone strength showed a lower mid-shaft max strength ($p=0.038$) and also a non-significant trend towards lower strength at the femoral neck of the WT IMO group. Trabecular bone volume fraction (BV/TV) and connectivity density (CD) after 20 days were significantly decreased in the WT IMO group ($p=0.001$). In contrast, the WT vehicle and KO vehicle, BV/TV and CD did no change at 20 days. Cortical bone revealed no significant microarchitectural changes after 20 days in the WT IMO group, whereas the total cortical area increased significantly in WT vehicle and KO IMO after 20 days (5.2% and 8.8%, respectively).

In conclusion, the P2X7 receptor KO mice did not respond to inflammation with loss of BMD whereas the WT mice had a significant loss of BMD, bone strength and trabecular microarchitecture, demonstrating a role for the P2X7 receptor in inflammatory bone loss.

Key words: P2X7, BMD, IMO, inflammation, osteoporosis, microarchitecture

Introduction

Autoimmune diseases and other chronic inflammatory diseases are often complicated by osteoporosis; 10% of rheumatoid arthritis patients are osteoporotic and 25% are osteopenic [1, 2]. Several factors are known to influence the risk of bone loss and several of those are often present in patients suffering inflammatory diseases i.e. medications, physical inactivity and endocrine factors. Glucocorticoids are widely used in the treatment of inflammatory autoimmune diseases, and it is well known that the use of glucocorticoids is associated with osteoporosis [3]. Glucocorticoid-induced osteoporosis (GIO) is the second most common form of osteoporosis and the most common iatrogenic form of the disease [3]. However, a meta-analysis has shown an increased incidence of osteoporosis in rheumatoid arthritis patients even after correcting for the use of glucocorticoids [4].

Patients suffering from active inflammatory diseases may also have chronically impaired physical activity This relative immobility can further contribute to the bone loss in patients with inflammatory diseases. Also, endocrine factors affecting the calcium and bone homeostasis might influence the bone remodelling leading to bone loss i.e. low estrogen level, high plasma PTH and low plasma 25-OH-vitamin D. However, the inflammatory process itself may induce changes in the regulation of bone turnover that consequently may lead to systemic bone loss.The inflammatory autoimmune diseases include a group of disorders that share the presence of inflammatory and destructive changes that affect the structure and function of articular and periarticular tissues. Several proinflammatory cytokines (such as Tumor Necrosis factor alpha (TNFα), interleukin (IL)-1 and IL-17) are major triggers for osteoclast activation explaining the enhanced bone loss during inflammation. Other, such as IL-12, IL-18, IL-33 and interferon alpha2 (IFN), are strong suppressors of osteoclast differentiation and inhibit bone loss. Thus the cytokine composition of an inflammatory tissue is decisive whether inflammation triggers bone loss or not [5].

In recent years, the role of adenosine triphosphate (ATP) and its cognate receptors in the inflammatory process has been recognized. In particular, the P2X7 receptor which is expressed on the immune cells and bone cells [6] is thought to play an important role in macrophage/microglial and granulocyte function by regulating cytokine production and apoptosis [7, 8]. Moreover, as the P2X7 receptor is known to be up-regulated during inflammation, antagonists of this receptor may serve as novel anti-inflammatory agents [9, 10]. Lister et al have summarised recent advances in the understanding of the role of the P2X7 receptor in inflammatory processes and highlight the potential of P2X7 receptor ligands for the treatment of chronic inflammatory diseases [10]. In the immune system, this receptor has been implicated in the processing and release of cytokines such as IL-1β, and in the initiation of cell death via both apoptotic and necrotic pathways [10-13]. As such, it has been proposed to function as a major regulator of inflammation. Experiments performed in vitro and in vivo in P2X7 receptor null mice demonstrated this receptor to be responsible for ATP-dependent IL-1β release [5, 14]. Furthermore, the P2X7 receptor seems also implicated in the release of many other cytokines [10]. In bone, the P2X7 receptor has multiple actions. It is expressed in both osteoblasts [15, 16] and osteoclasts [17, 18]. It is involved in intercellular communication among osteoblasts [19, 20]. In mouse osteoblasts, P2X7 receptor activation induces blebbing of the plasma membrane [21]. Moreover, P2X7 receptors have been shown to mediate extra cellular signal-regulated kinase (ERK) 1/2 activation by fluid shear stress [22]. Thus, P2X7 receptors in osteoblastic cells couple to multiple signaling pathways, some of which are important in skeletal mechanotransduction.

Osteoclasts seem to consistently express P2X7 receptors. In osteoclasts, the P2X7 receptor has a more complex role where it has been implicated in cell fusion [14], apoptosis[23], the translocation and activation of NF_KB (nuclear factor kappa-light-chain-enhancer of activated B cells)[24] and protein kinase C (PKC) [25] and intercellular communication [26].

Consistent with the above, inactivation of the receptor in mice modulates disease pathogenesis in several animal models of inflammatory and autoimmune diseases [11]. The aim of this study was therefore to investigate the role of the P2X7 receptor in inflammatory mediated bone loss in a mouse model of generalized inflammation.

Material and Methods

Animals:

The study protocol was in compliance with the bioethical guidelines for animal research and was approved by the Danish Animal Experiments Inspectorate (Protocol: 2010/561-1907).

In total 146 mice finalized the study protocol, 70 WT mice and 76 P2X7-/- (KO). Initially eight weeks old mice breeding pairs P2X7-/- on a C57bl/6 background from Jackson Laboratories (Bar Harbor, ME) were obtained and the intended breeding goal was 75 male P2X7-/- mice. Seventy male C57bl/6fBomTac wild type (WT) mice 14 weeks of age from BOM (Taconic, Lille Skensved, Denmark) were included as control animals. The animals were kept in a 12 hour light/dark cycle with light on at 8 am. The animals were fed ad libitum with standard diet (1319F Altromin) and had free access to drinking water (domestic quality tap water). The flow and study groups are presented in figure 1.

The Inflammation-Mediated Osteoporosis model (IMO).

We induced chronic inflammation by subcutaneous implantation/of a non-specific irritant, TALC (Appendix 1) according to the method first published Minne et al [27] and further standardized by Amour and Amour [28].

The animals were weighed and anaesthetised using Hypnorm/dormicum doses 3 µl/g. Using a 21 23 G needle, TALC suspension 800 mg/mL or vehicle was injected subcutaneously (s.c.) to a total dose of 16 mg/g body weight. One injection of TALC was injected in each side of the animal lateral to the spine spreading out the suspension in the length of the needle. Hereafter Calcein (0.8 mg/ml) (Appendix 1) was injected intraperitoneally (20 mg/kg) 10 and 2 days before sacrifice. The mice were kept fasting over night before sacrifice. On the day of sacrifice the mice were anaesthetized and euthanized by cervical dislocation. Blood was drawn by cardiac puncture before cervical

dislocation. The blood samples were coagulated for about 10 min before centrifuged and serum stored at -20°C. The body weight was measured, BMD was measured by DXA-scanning on a PIXImus DXA apparatus (Lunar Corp., Madison, WI, USA) in duplicate measurements and spleen was removed and weighted. The spine and hind legs were isolated and were cleansed of soft tissue. Right femur was stored wrapped in saline gaze and frozen to -20°C. The spleen and right tibia together with fibula were stored in ethanol. Entire left leg and spine were stored in 10% formalin and weight of the spleen was measured.

Analysis

Bone mineral density (BMD) by Duel X-ray absorptiometry (DXA)

BMD, bone mineral content (BMC) and bone area measurements were performed using a Lunar Piximus densitometer (Lunar Corp., Madison, WI). The total spine was chosen as Region of Interest (ROI) spanning from just above os sacrum. The femur was also chosen as a ROI spanning from caput to proximal to the condyles above the knee joint. Left femur was chosen, but if TALC interfered with the ROI the right femur was used. The spine was re-scanned in duplicate (4-5 lowest vertebral bodies), having been stored in 10% formalin. Data were analysed using the manufacturer's software (version 2.0).

Biomechanical testing (Bone strength):

Prior to mechanical testing the bones were thawed and rehydrated to approximately 20 °C in saline 1 day before testing. Bone strength measurements were made using the Lloyd LR 50k (Lloyd Instruments, Fareham, UK). They were performed as a 3-point bending test at the femoral mid-shaft and as a vertical compression test on the femoral head [29].

Load-deformation curves were generated, and maximal load was recorded at a speed of 2 mm per minute with a 100 N load cell. Ultimate load is the maximal load (N) the bone can resist before it breaks.

Micro CT scanning and Microarchitectural analysis

The femurs and tibiae of mice were dissected free of soft tissue, and stored in 10 % formalin. The mid-shaft and the proximal part of left tibia were scanned using a high-resolution micro computed tomography (micro-CT) system (vivaCT 40, Scanco Medical AG, Brüttisellen, Switzerland). The baseline and the 20 day groups of WT and KO were scanned. The scan resulted in a 3-dimensional (3D) reconstruction of cubic voxel sizes of 10.5 * 10.5 * 10.5 μm^3 (2048*2048*2048 pixels) with 32-bit gray levels. Each 3D image dataset consisted of approximately 210 CT slide images. For proximal tibial cancellous bone, 100 slide images (1050 μm) beneath the growth plate were used for analysis of cancellous bone microarchitecture. For mid-shaft cortical bone, all 210 slice images were used for analysis of cortical microarchitecture [30]. The right distal femur were also scanned but not analysed.

Using direct 3D methods the following measures were performed: Bone volume fraction (BV/TV, %), trabecular number (Tb.N, per mm), trabecular thickness (TbTh, mm), trabecular separation (Tb.Sp, mm), connectivity density (Conn D, mm^{-3}) and structural model index (SMI, -) were calculated. From the cortical data bone porosity (%), cortical thickness (mm), BS/TV (mm^{-1}), pore size (mm), total cross sectional area (Tt.ar, mm^2), cortical area (Ct.Ar, mm^2) and cortical area fraction (%) were measured or calculated [30, 31].

Cytokine measurements

The concentrations (pg/mL) of cytokines were measured in serum in duplicates using a commercial kit: Mouse ProInflammatory 7-Plex Ultra-Sensitive Kit (Meso scale Discovery: Rockville). The measurements were performed at Center of Inflammation and Metabolism, Copenhagen University Hospital, Rigshospitalet in accordance with manufacturer's instructions. The coefficient of variation was at low, medium and high concentrations) IL1b: 8.4%; 1.8% and 3.2% (highest at lowest

concentration), IL-12: 8.1%; 2.3% and 4.4% , IFN-y: 7.3%; 1.0% and 2.9%, IL-6: 2.8%; 6.8% and 4.0%; KC/GRO: 7.8%; 5.7% and 2.8%; IL-10: 4.3%; 1.1% and 5.8% and TNFα: 9.0%; 3.4% and 2.6%

The lower limits of detection (LLOD) were: IL1b: 0.75 pg/mL; IL-12: 35 pg/mL ; IFN-y: 0.38 pg/mL; IL-6: 4.5 pg/mL ; KC/GRO: 3.3 pg/mL; IL-10: 11 pg/mL and TNFα: 0.85 pg/mL

Markers of Bone Turnover

In order to investigate the effect of generalized inflammation and P2X7 receptor expression on bone turnover, serum markers of bone formation (total pro-collagen type 1 N-terminal propeptide (P1NP) and (C-terminal telopeptide of type 1 collagen (CTX-1)) were measured. P1NP was measured in serum samples in duplicate using the Rat/mouse P1NP assay from IDS (Immunodiagnostic systems PLC, Boldon, OK) following the protocol supplied. P1NP: precision: intra assay: 5-7.4 %. Inter-assay: 9.2-8 %. CTX-1was measured in serum samples in duplicate using the RATlaps EIA assay from IDS (Immunodiagnostic systems PLC) using the suppliers instructions. Intra-assay variation: CV was 9.2-5.8%, Inter-assay variation: CV: 14.8-10.7 %.

Statistics:

SPSS software version 19 was used. Standard parametric tests, T-test, ANCOVA and ANOVA were used as appropriate. On data unlikely to be normally distributed or with a lower sample size non parametric Mann-Whitney tests were used. Differences were considered statistically significant when $p < 0.05$. Simple descriptives were presented as means ± standard deviation (SD) or standard error of the mean (SEM).

Results

The study included in total 146 mice (Figure 1).

At baseline, the bodyweight differed slightly between WT animals and KO mice, (28.3 ± 1.77 g vs. 31.1 ± 2.4 g, $p<0.002$). We observed no significant difference between WT vehicle animals compared to WT IMO mice and KO vehicle animals compared to KO IMO animals. Furthermore, we observed no significant change in body weight during the 10 and 20 days of the experiment; and no difference between the vehicle and IMO groups in both WT and KO animals.

The spleen weight was 86.4 ± 13.2 mg in the WT animals compared to 104.3 ± 27 mg in the KO animals at baseline. The spleen weight increased significantly in the WT IMO mice to 131 ± 59 mg; $p<0.005$) at day 10 and to 130.8 ± 44.4; $p<0.001$ at day 20, whereas we observed no significant change in spleen weight in the WT vehicle, KO vehicle and KO IMO mice at 10 and 20 days.

Bone mineral density (BMD)

BMD at the femur and spine decreased significantly from baseline to day 20 in the WT IMO mice ($p<0.01$) (Table 1). After correction for the lower weight of the WT mice compared to KO mice the reduction was still significant ($p=0.032$). In the WT vehicle, KO vehicle and KO IMO no significant BMD changes were found from baseline to day 20 (Table 1).

Bone strength

In table 2 the data on bone strength measurements are presented. The data showed increased mid-shaft max strength in WT vehicle ($p=0.028$) and also a non-significant trend towards higher mid-shaft strength of the KO vehicle group at 20 days. In contrast no increase was observed in WT IMO (Table 2).

Bone microarchitecture

The Micro-CT data of trabecular bone showed that bone volume fraction (BV/TV) after 20 days was significantly decreased in the WT IMO group (28.1%, p=0.001). The trabecular space was significantly higher (28.0%, p<0.001) and the trabecular number lower (13.1%, p=0.017). In contrast, the WT vehicle and KO vehicle BV/TV, trabecular space and trabecular number did not change at 20 days. The KO IMO did as well not change in BV/TV, but trabecular number significantly decreased (8.0 %, p=0.019) and trabecular space increased at 20 days (8.7%, p=0.041) to a less extend compared to WT IMO (Table 3A). Representative changes are also shown in figure 2.

The Micro-CT data of cortical bone showed no significant changes in cortical bone area after 20 days in the WT IMO group, whereas the total bone area increased significantly in WT vehicle in KO IMO group after 20 days (5.2% and 8.8%, respectively). The mean 2d cortical bone area fraction was also lower in the 20 days WT IMO group compared to baseline (p=0.005), (Table 3B).

Cytokine measurements

Serum samples were available in sufficient amount enabling cytokine measurements from 117 of the 146 animals. However, 40 samples were lost due to technical error leaving only between 7-15 samples in each group. A substantial part of the cytokine measurements were below *Lower limit of detection or below curve fit*. On IL-1β, TNF-α and IL-12 measurements data were generally lower than the measurements limits (data not shown), e.g. only 11 of the IL-12 measurements were not lower than the limit of detection (LLOD) or below curve fit (being lower than the lowest measurement for the standard curve and therefore measurements cannot be estimated.).

In WT vehicle none of the measured cytokines changes significantly after 10 or 20 days compared to WT baseline. In KO vehicle only IL-10 was significantly increased at 20 days compared to KO baseline. In WT IMO - IL-10 was significantly higher at 20 days and IL-6 was significantly higher at 10 days compared to baseline. In KO IMO - IL-6 was significantly higher at 10 and 20 days

(Table 4A) compared to baseline. Comparing WT and KO in their corresponding groups, IMO 10 days, IMO 20 days, vehicle 10 days and vehicle 20 days: IL 10 was lower at baseline; at 10 days vehicle both Cxcl1 and IL-10 was different; at 10 days IMO IL-10 was different; at 20 days no differences were found between the cytokines comparing WT and KO in both IMO and vehicle (Table 4A). In the WT Comparing IMO and vehicle at 10 and 20 days only 20 days IL-10 was significantly higher in IMO WT ($p=0.006$).

Bone turnover markers

In the WT vehicle CTX-I was significantly higher at 20 days and P1NP was significantly lower at 10 and 20 days ($p<0.001$) compared to baseline. No differences were found in the WT IMO comparing 10 and 20 days to baseline. In KO vehicle P1NP was significantly lower at 10 days compared to baseline and in the KO IMO CTX-I was significantly lower at 20 days compared to baseline. Comparing WT and KO in their corresponding groups found both CTX-I and P1NP significant lower at baseline. At 20 days vehicle CTX-I were significant lower and P1NP higher in the KO compared to the WT. At 20 days IMO the CTX-I and P1NP was lower than in the KO. In the 10 day groups only P1NP were different in the WT vehicle group comparing to 10 days KO vehicle (Table 4B). Comparing between IMO and vehicle P1NP was significantly lower in WT IMO at 20 days ($P=0.002$). P1NP was lower in 10 days KO vehicle compared to IMO ($p=0.04$) but was borderline higher at 20 days ($p=0.05$). No differences were found comparing CTX-I vehicle and IMO groups in WT or KO.

Discussion

The P2X7 receptor is a key factor in inflammatory responses and as the receptor is also important in the regulation of bone formation and resorption, we hypothesized that the receptor plays an important role in the inflammatory bone loss. Our results showed that the P2X7 receptor might play a significant role in the bone loss associated with the chronic inflammation in a mouse model of generalized inflammation. The bone loss seen in WT IMO animals was completely abolished in P2X7 KO animals; this was true for both BMD and for the trabecular microarchitectural parameters. This implies that the role of the receptor is different in bone that is not exposed to chronic inflammation and bone in inflammatory diseases. In previous studies on P2X7 receptor effects in bone, P2X7 KOs have decreased bone mass compared to WT – in our study there is a bone-protective effect of not expressing the P2X7 receptor. The explanation for this might be the role of the P2X7 receptor in the inflammatory response where P2X7 receptor activation has been shown to up-regulate expression of and activate the NALP3 inflammasome. Moreover, P2X7 KO mice have reduced cytokine release and attenuated inflammatory response compared to WT animals [5]. The cytokines TNFα, IL-1, IL-6 and IL-17 are known major triggers of osteoclasts and IL-12, IL-18, IL-33 and IFN are strong suppressors of osteoclast differentiation and inhibit bone loss [32]. In contrast, regulation of "normal" bone metabolism happens in an environment without low grade inflammation and with low levels of cytokines, and the effect of the P2X7 receptor in bone cells might therefore prevail compared to the effect of the receptor in the immune system.

A few published studies on experimentally induced inflammation have shown to induce osteoporosis, though no model completely mimics autoimmune disease in humans. The IMO model was chosen as it gives a robust inflammatory response and as it has been shown to develop osteoporosis [28] . However, the exact mechanisms of the inflammatory response are relatively

unknown though they might involve activation of the inflammasome complex in which the P2X7 receptor is a key factor. In our study, we found a decrease in BMD and in BV/TV in the WT IMO mice while the WT vehicle treated animals maintained their bone mass and structure. Also, spleen weights in the WT IMO animals were increased after 20 days, indicating a clear activation of the immune system. These findings are in line with previous findings from studies using this model and document the reproducibility of this model and the validity of our study. Amour et al. found that TALC significantly increased the NO production (2.5-fold, $p < 0.005$) in WT mice with IMO, but observed no change in iNOS KO mice. In contrast, iNOS KO mice with IMO showed less inhibition of bone formation than WT mice and showed no significant increase in osteoblast apoptosis. The authors concluded that inducible NOS-mediated osteoblast apoptosis and depressed bone formation might play important roles in the pathogenesis of their model [33].

A number of the P2X7-coupled pathways are important in both the immune response and in the control of bone resorption, as bone resorption is increased significantly in inflammatory-induced bone loss. During the inflammatory process large amounts of ATP are released; ATP is a natural ligand for most P2 receptors and an inflammation-induced ATP release would therefore activate a range of P2 receptors, including the P2X7 receptor which requires high concentrations of ATP to be activated. As ATP is rapidly degraded extracellularly, systemically increased levels of ATP are unlikely. Skeletal effects of ATP-mediated purinergic signaling through P2X7 receptors may therefore occur via three mechanisms. The first is the general skeletal bone loss, which might be induced through local activation of P2X7 receptors on immune cells inducing IL-1β and TNF-α production, processing and release to the circulation where it induces a generalized increase in bone resorption through activation of osteoclasts in all parts of the skeleton. Secondly, ATP release as part of the inflammatory process could activate bone cells locally as the immune system and bone are in close contact both in the bone marrow and in affected joints. Here, activation of P2X7

receptors in bone cells could activate osteoclasts both through a direct activation of osteoclast precursors to form mature multinucleated bone-resorbing osteoclasts and through an indirect activation via stimulation of osteoblastic P2X7 receptors and upregulation of RANKL on osteoblasts, thereby inducing osteoclast formation and activity. Finally, due to reduced mobility of patients due to their primary inflammatory diseases, a reduced activation of P2X7 receptors in osteoblasts, usually mediating mechanotransduction and anabolic effects, results in reduced bone formation, which further aggravates the effects of the disease on the skeleton.

The question then arises whether altered P2X7 receptor function in humans affect the risk of inflammatory-induced bone loss. This has been has been addressed in a recent study by Portales-Cervantes [34], where the authors examined the association between polymorphisms in the P2X7 receptor and the risk of rheumatoid arthritis or systemic lupus erythematosus. Even though they were not able to demonstrate differences in the distribution of genotypes between patients and healthy controls, they found affected receptor function and cytokine production in cells from both rheumatoid arthritis patients and systemic lupus erythematosus patients, suggesting a possible involvement of the receptor in the pathogenesis in autoinflammatory diseases [34]. However, the study did not address the role of the receptor in the inflammatory bone loss, neither did it include a full genotyping for all known functional P2X7 receptor polymorphisms. Based on our in vivo findings, loss-of-function polymorphisms might be expected to protect against autoimmune diseases and the associated bone loss. This is in contrast to what has been shown in normal, healthy subjects, where we have demonstrated that loss-of-function of the receptor is associated with increased bone loss and increased fracture risk in humans while gain-of-function polymorphisms in the receptor were associated with increased bone mass and reduced fracture risk [35-37]. However, no studies have so far examined the association between chronic inflammatory diseases and the risk or severity of bone loss in humans and such studies are highly warranted.

We believe that our study provides evidence for the effects of the P2X7 receptor in inflammatory bone loss, as the findings are consistent over a range of evaluation methods. Both BMD, bone microstructure, and bone strength all points to the same conclusion.

Furthermore, the cytokine data on IL 6 support that inflammation is induced in the model used. Thus, the suppressed bone loss in KO mouse demonstrates that the P2X7 receptor is responsible for inflammatory-mediated bone loss in this model. However, our study has also some limitations, especially that the data on serum bone markers in total are inconclusive and thus does not support neither increased nor suppressed bone loss. Our data on IL-1b and bone turnover markers were expected to support the observations on BMD, bone microstructure, bone strength and IL-6. However, measuring cytokines are well known for their low half-life, and changes in cytokine levels are expected throughout the experiment. Our results showed IL-1b below the level of detection in all situations which might be a limitation of our study. We are limited by the low blood volume in mice, and cardiac puncture was therefore chosen to maximize the available serum. Cardiac puncture is however difficult. It is likely that stress and tissue damage has some impact depending on the duration and skill of our technique in cardiac puncture. Cardiac puncture as well as hypnorm dormicum injection could also affect especially cytokine.

In conclusion, our study is the first to demonstrate the effects of the P2X7 receptor on bone in chronic inflammation. We found that ablation of the P2X7 receptor KO in a mouse model of IMO protected against inflammatory bone loss and maintained bone strength in the animals. The potential implications for patients with inflammatory diseases are evident. However, studies in humans are needed to further clarify the association between P2X7 receptor function and autoimmune bone loss.

Legends

Figure 1.

Flow diagram and group design of 146 mice included in the study

Figure 2.

Representative 3D bone architecture WT and KO mice at baseline, vehicle 20 days and IMO 20 days.

Table 1.

Bone mineral density measured by DXA in WT and KO mice at baseline, vehicle and IMO at 10 and 20 days. Statistical comparison was made between baseline and day 10 as well as baseline and 371 day 20. * $p<0.001$.

Table 2.

Biomechanical bone strength measurements in WT and KO mice at baseline, vehicle and IMO at 10 and 20 days.

Table 3.

Comparison of 3-D microarchitectural parameters of trabecular bone (A) and comparison of 3-D microarchitectural parameters and cross-sectional areas of cortical bone (B) WT and KO mice at baseline, vehicle and IMO at 20 days.

Table 4.

Table A: Cytokine measurements, * $p<0.05$, compared to baseline. # $p<0.05$ comparing between KO/WT in corresponding groups. £ $p<0.05$ comparing IMO and vehicle in corresponding groups, WT or KO. 3 measurements or lower resulted in data coded as missing, -. LLOD denotes Lower limit of detection at 50% or more.

Table B: Bone turnover markers, *$p<0.05$, 10 and 20 days vs. baseline. # $p<0.05$ comparing between KO/WT in corresponding groups. £ $p<0.05$ comparing between IMO and vehicle in corresponding groups KO or WT.

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Table 1: Bone Mineral Density

BMD	g/cm^2	Baseline Mean (SD)	10 days Mean (SD)	20 days Mean (SD)
WT Vehicle	**Total**	0.0515 (0.0018)	0.0519 (0.0019)	0.0534 (0.0015)
	Femur	0.0736 (0.0047)	0.0739 (0.0040)	0.0739 (0.0035)
	Spine	0.0488 (0.0034)	0.0532 (0.003)	0.0508 (0.0025)
WT IMO	**Total**	0.0515 (0.0018)	0.0579 (0.0035)	0.0545 (0.0044)
	Femur	0.0736 (0.0047)	0.0726 (0.0049)	**0.0695*** (0.0050)
	Spine	0,0488 (0.0034)	0.0490 (0.0037)	**0.0460*** (0.0057)
KO Vehicle	**Total**	0.0521 (0.0020)	0.0531 (0.0017)	0.0539 (0.0016)
	Femur	0,0707 (0.0052)	0.0733 (0.0039)	0.0747 (0.0033)
	Spine	0.0520 (0.0041)	0.0516 (0.0037)	0.0513 (0.0034)
KO IMO	**Total**	0.0521 (0.0020)	0.0560 (0.0027)	0.0530 (0.0023)
	Femur	0.0707 (0.0052)	0.0739 (0.0040)	0.732 (0.0030)
	Spine	0.0520 (0.0041)	0.0539 (0.0014)	0.0520 (0.0043)

Table 2: Biomechanical bone strength

	Max bone strength (MPa)	Baseline Mean (SD)	20 days Mean (SD)	P-value
WT Vehicle	**Midt-shaft**	19.1 (2.0)	20.9 (2.0)	0.028
	Femoral neck	17.5 (2.7)	17.1 (1.3)	ns
WT IMO	**Midt-shaft**	19.1 (2.0)	19.0 (2.4)	ns
	Femoral neck	17.5 (2.7)	16.3 (2.4)	ns
KO Vehicle	**Midt-shaft**	19.1 (2.6)	19.8 (1.3)	ns
	Femoral neck	16.4 (2.7)	17.3 (2.7)	ns
KO IMO	**Midt-shaft**	19.1 (2.6)	20.0 (1.5)	ns
	Femoral neck	16.4 (2.7)	18.5 (2.2)	ns

Table 3A. Comparison of 3-D microarchitectural parameters of trabecular bone in 4 groups

	3-D Microarchitecture	Baseline Mean (SD)	20 days Mean (SD)	P-Value
WT Vehicle	**BV/TV (%)**	23.5 (4.0)	23.4 (3.2)	ns
	Tb.N (per mm)	4.64 (0.59)	4.60 (0.54)	ns
	Tb.Th (mm)	0.06 (0.01)	0.070 (0.01)	0.052
	Tb.Sp (mm)	0.19 (0.06)	0.21 (0.03)	ns
	Conn.D (mm^{-3})	158.4 (23.1)	139.5 (25.4)	0.06
	SMI (-)	1.57 (0.31)	1.48 (0.26)	ns
WT IMO	**BV/TV (%)**	23.5 (4.0)	16.9 (5.1)	0.001
	Tb.N (per mm)	4.64 (0.59)	4.03 (0.59)	0.017
	Tb.Th (mm)	0.06 (0.01)	0.061 (0.01)	ns
	Tb.Sp (mm)	0.19 (0.03)	0.25(0.04)	<0.001
	Conn.D (mm^{-3})	158.4 (23.1)	106.7 (31.3)	<0.001
	SMI (-)	1.57 (0.31)	1.87 (0.39)	0.042
KO Vehicle	**BV/TV (%)**	23.3 (4.0)	23.2 (3.4)	ns
	Tb.N (per mm)	5.28 (0.33)	4.99 (0.49)	ns
	Tb.Th (mm)	0.06 (0.004)	0.06 (0.005)	ns
	Tb.Sp (mm)	0.18 (0.01)	0.18 (0.03)	ns
	Conn.D (mm^{-3})	167.0 (23.9)	157.9 (26.8)	ns
	SMI (-)	1.48 (0.18)	1.38 (0.33)	ns
KO IMO	**BV/TV (%)**	23.3 (4.0)	22.4 (4.0)	ns
	Tb.N (per mm)	5.28 (0.33)	4.86 (0.47)	0.019
	Tb.Th (mm)	0.06 (0.004)	0.06 (0.004)	ns
	Tb.Sp (mm)	0.18 (0.01)	0.20 (0.02)	0.041
	Conn.D (mm^{-3})	167.0 (23.9)	140.5 (19.9)	0.006
	SMI (-)	1.48 (0.33)	1.38 (0.33)	ns

Table 3B: Comparison of 3-D microarchitectural parameters and cross-sectional areas of cortical bone in 4 groups

		Baseline Mean (SD)	20 days Mean (SD)	P-value
WT Vehicle	**3-D Microarchitecture**			
	Porosity (%)	2.40 (0.14)	1.92 (0.09)	0.009
	Cort.Th (mm)	0.24 (0.01)	0.25 (0.00)	ns
	BS/TV (mm^{-1})	3.50 (0.16)	4.31 (0.13)	ns
	Pore size (mm)	0.04 (0.01)	0.04 (0.01)	ns
	2-D cross-sectional area			
	Tt.Ar (mm^2)	2.28 (0.05)	2.40 (0.04)	ns
	Ct.Ar (mm^2)	1.17 (0.02)	1.24 (0.01)	0.008
	Ct.Ar/ Tt.Ar (%)	51.46 (0.7)	52.21 (1.2)	ns
WT IMO	**3-D Microarchitecture**			
	Porosity (%)	2.40 (0.14)	2.53 (0.20)	ns
	Cort.Th (mm)	0.24 (0.01)	0.21 (0.14)	ns
	BS/TV (mm^{-1})	3.50 (0.16)	4.16 (0.28)	ns
	Pore size (mm)	0.04 (0.01)	0.06 (0.03)	ns
	2-D cross-sectional area			
	Tt.Ar (mm^2)	2.28 (0.05)	2.32 (0.07)	ns
	Ct.Ar (mm^2)	1.17 (0.02)	1.13 (0.04)	ns
	Ct.Ar/ Tt.Ar (%)	51.46 (0.7)	48.45 (0.7)	0.005
KO Vehicle	**3-D Microarchitecture**			
	Porosity (%)	2.00 (0.10)	1.93 (0.16)	ns
	Cort.Th (mm)	0.23 (0.00)	0.23 (0.01)	ns
	BS/TV (mm^{-1})	4.30 (0.13)	4.28 (0.10)	ns
	Pore size (mm)	0.02 (0.01)	0.02 (0.01)	ns
	2-D cross-sectional area			
	Tt.Ar (mm^2)	2.11 (0.06)	2.39 (0.04)	0.004
	Ct.Ar (mm^2)	1.06 (0.03)	1.143 (0.01)	0.025
	Ct.Ar/ Tt.Ar (%)	50.47 (0.8)	47.97 (1.1)	ns
KO IMO	**3-D Microarchitecture**			
	Porosity (%)	2.00 (0.10)	2.20 (0.15)	ns
	Cort.Th (mm)	0.23 (0.00)	0.23 (0.00)	ns
	BS/TV (mm^{-1})	4.30 (0.13)	4.00 (0.20)	ns

	Pore size (mm)	0.02 (0.01)	0.03 (0.01)	ns
	2-D cross-sectional area			
	Tt.Ar (mm^2)	2.11 (0.06)	2.30 (0.04)	0.012
	Ct.Ar (mm^2)	1.06 (0.03)	1.12 (0.01)	ns
	Ct.Ar/ Tt.Ar (%)	50.47 (0.8)	48.97 (0.8)	ns

Table 4A: Cytokine measurements

	Cytokines pg/mL	Baseline Mean (±SEM)	10 days Mean (±SEM)	20 days Mean (±SEM)
WT vehicle	**Cxcl1**	47.6 (4.7)	47.9 (13.6)#	46.1 (4.8)
	IFN	2.3 (0.6)	2.7 (1.1)	2.1 (0.4)
	IL10	21.0 (3.0)#	20.0 (2.3)#	18.2 (1.7)£
	IL6	38.2 (14.1)	82.6 (31.0)	71.3 (11.2)
WT IMO	**Cxcl1**	47.6 (17.1)	54.3 (8.2)	54.9 (6.9)
	IFN	2.3 (0.6)	2.0 (0.5)	1.7 (0.1)
	IL10	21.0 (3.0)#	23.0 (3.5)	28.1 (2.3)£
	IL6	38.2 (14.1)	96.5 (20.9)*	57.2 (11.6)
KO vehicle	**Cxcl1**	67.3 (11.2)	63.5 (6.4)#	56.9 (5.5)
	IFN	2.0 (1.0)	5.2 (1.6)	-
	IL10	10.2 (1.0)#	14.0 (2.0)#	20.1 (2.6)*
	IL6	20.9 (10,2)	43.6 (15,4)	46.0 (12,5)
KO IMO	**cxcl1**	67.3 (11)	66.4 (13.6)	-
	IFN	2.0 (1.0)	-	-
	IL10	10.2 (1.0)#	14.8 (4.6)	-
	IL6	20.9 (10.2)	100.3 (51.4)*	90.8 (35.0)*

Table 4B: Bone turnover markers

	Bone markers (serum)	**Baseline** Mean (±SEM)	**10 days** Mean (±SEM)	**20 days** Mean (±SEM)
WT vehicle	CTX ng/ml	62.4 (8.1)#	50.6 (7.6)	88.5 (8.2)*/#
	P1NP pg/ml	67.1 (6.7)	53.2 (3.8)#	36.7 (2.8)*/#/£
WT IMO	CTX ng/ml	62.4 (8,1)#	56.4 (7.0)	76,7 (8.1)#
	P1NP pg/ml	67.1 (6,7)	51.2 (6.3)	54.2 (3.7)#/£
KO vehicle	CTX ng/ml	29.4 (4.4)#	46.1 (8.3)	19.4 (2.2)#
	P1NP pg/ml	49.1 (5.1)	32.7 (3.8)*/#/£	54.9 (4.5)#/£
KO IMO	CTX ng/ml	29.4 (4.4)#	59.2 (12.3)	18.1 (2.2)*/#
	P1NP pg/ml	49.1 (5.1)	42.9 (4.7)£	40.4 (1.2)#/£

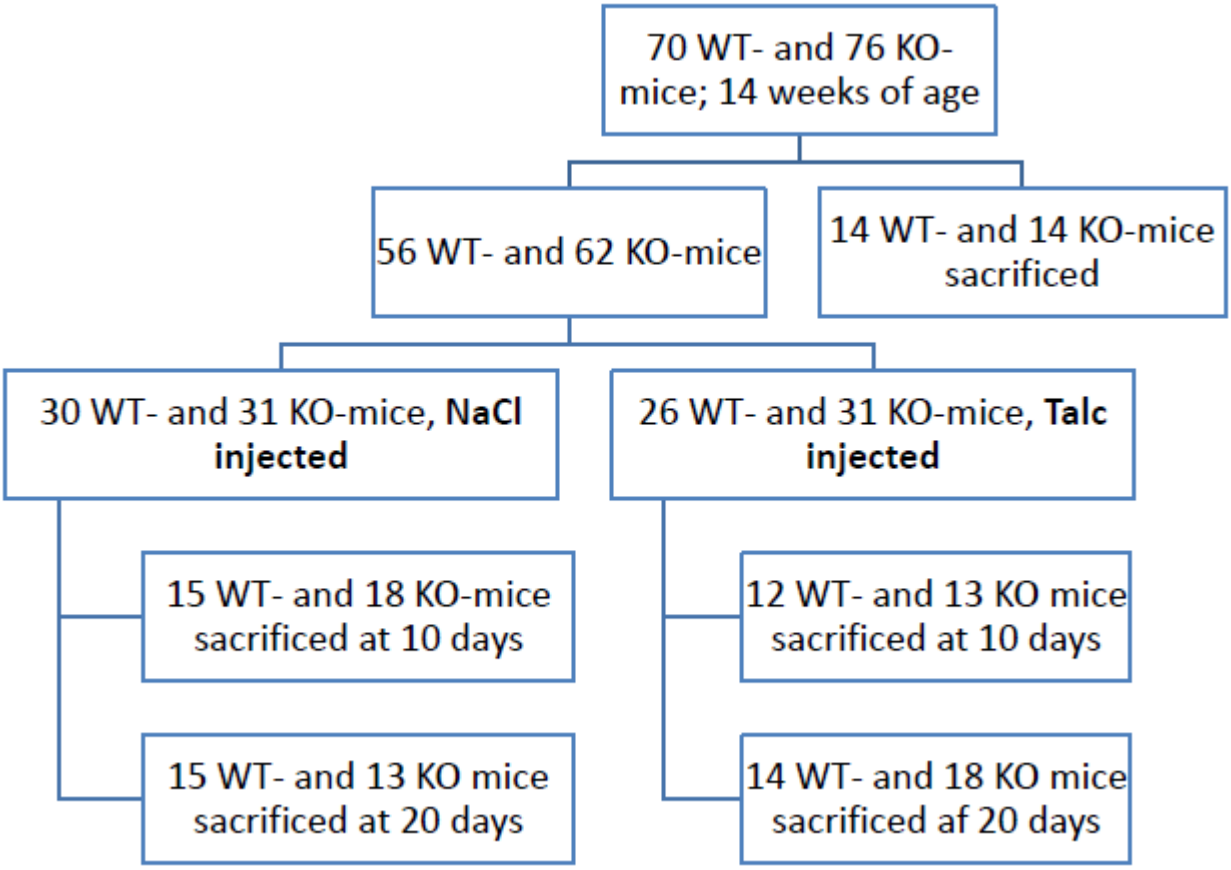
70 WT- and 76 KO-mice; 14 weeks of age
56 WT- and 62 KO-mice
14 WT- and 14 KO-mice sacrificed
30 WT- and 31 KO-mice, NaCl injected
26 WT- and 31 KO-mice, Talc injected
15 WT- and 18 KO-mice sacrificed at 10 days
15 WT- and 13 KO mice sacrificed at 20 days
12 WT- and 13 KO mice sacrificed at 10 days
14 WT- and 18 KO mice sacrificed af 20 days

Figure 1

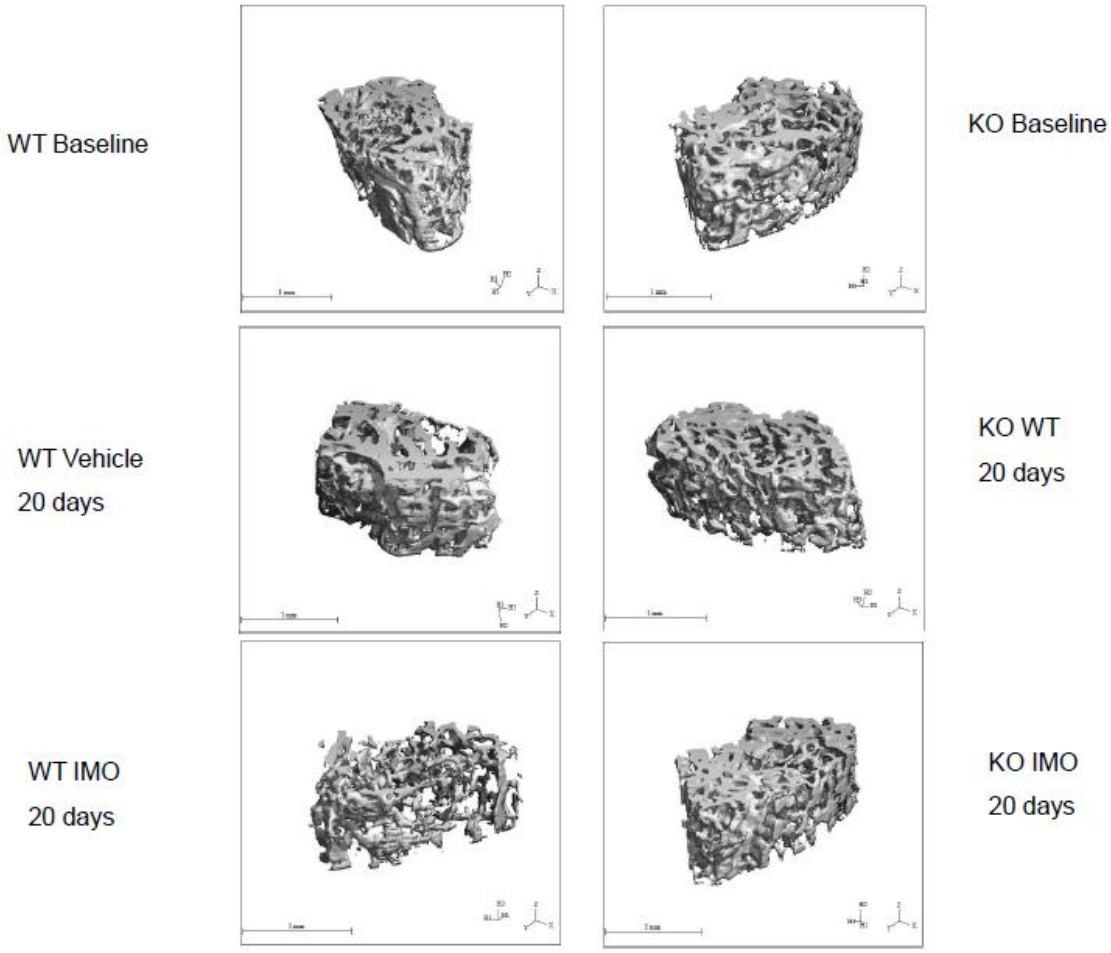
WT Baseline
KO Baseline
WT Vehicle
20 days
KO WT
20 days
WT IMO
20 days
KO IMO
20 days

Figure 2

Highlights

We aimed to elucidate the role of the P2X7 receptor on bone mineral density (BMD), microarchitecture, and bone strength in a standardized mouse model of Inflammation-Mediated Osteoporosis (IMO).

Seventy wild type (WT) mice and 76 P2X7-/- (knockout, KO) were investigated. BMD at the femur and spine decreased significantly from baseline to day 20 in the WT IMO mice ($p<0.01$). In the WT vehicle, KO vehicle and KO IMO, no significant BMD changes were found.

Bone strength showed a lower mid-shaft max strength ($p=0.038$). Trabecular bone volume fraction (BV/TV) and connectivity density (CD) after 20 days were significantly decreased in the WT IMO group ($p=0.001$). In contrast, the WT vehicle and KO vehicle, BV/TV and CD did no change at 20 days.

The P2X7 receptor KO mice did not respond to inflammation with loss of BMD whereas the WT mice had a significant loss of BMD, bone strength and trabecular microarchitecture, demonstrating a role for the P2X7 receptor in inflammatory bone loss.