

RESEARCH PAPER

Methionine down-regulates TLR4/MyD88/NF- κ B signalling in osteoclast precursors to reduce bone loss during osteoporosis

V Vijayan¹, M Khandelwal², K Manglani¹, S Gupta¹ and A Surolia²

¹Molecular Sciences Laboratory, National Institute of Immunology, New Delhi, India, and

²Molecular Biophysics Unit, Indian Institute of Science, Bangalore, Karnataka, India

Correspondence

A Surolia, Molecular Biophysics Unit, Indian Institute of Science, Bangalore, Karnataka 560 012, India. E-mail:

surolia@mbu.iisc.ernet.in;

S Gupta, Molecular Sciences

Laboratory, National Institute of Immunology, Aruna Asaf Ali

Marg, New Delhi 110 067, India.

E-mail: sarika@nii.ac.in

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BACKGROUND AND PURPOSE

Studies have demonstrated that a moderate intake of amino acids is associated with development of bone health. Methionine, a sulphur-containing essential amino acid, has been largely implicated for improving cartilage formation, however its physiological significance on bone integrity and functionality have not been elucidated. We investigated whether methionine can prevent osteoporotic bone loss.

EXPERIMENTAL APPROACH

The anti-resorptive effect of methionine, (250 mg kg⁻¹ body wt administered in drinking water for 10 weeks), was evaluated in ovariectomized (OVX) rats by monitoring changes in bone turnover, formation of osteoclasts from blood-derived mononuclear cells and changes in the synthesis of pro-osteoclastogenic cytokines.

KEY RESULTS

Methionine improved bone density and significantly decreased the degree of osteoclast development from blood mononuclear cells in OVX rats, as indicated by decreased production of osteoclast markers tartrate resistant acid phosphatase b (TRAP5b) and MIP-1 α . siRNA-mediated knockdown of myeloid differentiation primary response 88 [MyD88], a signalling molecule in the toll-like receptor (TLR) signalling cascade, abolished the synthesis of both TRAP5b and MIP-1 α in developing osteoclasts. Methionine supplementation disrupted osteoclast development by inhibiting TLR-4/MyD88/NF- κ B pathway.

CONCLUSIONS AND IMPLICATIONS

TLR-4/MyD88/NF- κ B signalling pathway is integral for osteoclast development and this is down-regulated in osteoporotic system on methionine treatment. Methionine treatment could be beneficial for the treatment of postmenopausal osteoporosis.

Abbreviations

CTX, C-terminal telopeptides; MIP-1 α , macrophage inhibitory protein-1 α ; MyD88, myeloid differentiation primary response gene 88; NFATc1, nuclear factor of activated T-cells; OVX, ovariectomized; TNFSF11, receptor activator of NF- κ B ligand (also known as RANKL); TLR, toll-like receptor; TRAP5b, tartrate-resistant acid phosphatase 5b

Introduction

Osteoporosis is a progressive and systemic bone disorder prevalent in the ageing population of both developed and developing countries (Feng and McDonald, 2011). It is characterized by a significant decline in bone mineral density and microarchitectural deterioration of the bone tissue (Nancy, 2006). Clinically this disease is classified as primary or secondary (WHO study group, 1994). Primary osteoporosis consists of two subtypes, type I and type II. Type I progresses due to the decline in oestrogen or testosterone levels in the body; which in women is referred to as postmenopausal osteoporosis (Jakob, 2005; Fink *et al.*, 2006). Type II or senile osteoporosis arises as a result of bone ageing and progresses after the age of 70. A secondary form of the disease, secondary osteoporosis, occurs as a result of long-term intake of glucocorticoids, insulin-dependent diabetes mellitus, hyperthyroidism or hypercortisolism (Wright, 1991; Jakob, 2005).

The prevailing view is that osteoporosis develops because of an imbalance between bone formation and bone resorption (Nancy, 2006). For example, in postmenopausal women, oestrogen deficiency-mediated receptor activator of NF- κ B ligand (TNFSF11, also known as RANKL) production by osteoblasts triggers changes in osteoclasts, activating new bone remodelling units within the bone tissue. This accelerated bone remodelling without a commensurate rise in bone formation alters trabecular structure and increases bone fragility (WHO study group, 1994; Seeman, 2003). Osteoporosis can be treated with hormone replacement therapy, bisphosphonates, steroids or monoclonal antibodies (e.g. denosumab). However ulcers, renal toxicity, cancer, eczema, stroke and cardiovascular complications associated with the intake of such medications have restricted their continued usage (Ernesto, 2000; Riggs *et al.*, 2002; Rossouw *et al.*, 2002; Beral and Million Women Study Collaborators, 2003; Handa, 2004; Bridgeman and Pathak, 2011). The development of safer treatment strategies for osteoporosis is therefore warranted.

Therapeutic interventions using amino acids have received much attention in the last few years because of their unique ability to serve as signalling factors in regulatory pathways (Brandy, 1993; Kerstetter *et al.*, 2003; Kimball and Jefferson, 2004, 2006; Ahmed and Hamza, 2009). These studies have shown that arginine, lysine, glutamine, tryptophan and taurine exert diverse pharmacological effects on the bone like increasing NO, type I collagen, GSH and 5-HT synthesis in bone cells and improving calcium and vitamin D absorption (Fini *et al.*, 2001; Ahmed and Hamza, 2009; Pernow *et al.*, 2010; Jiang and Cai, 2011). Wang *et al.* (2006) demonstrated that the conditionally essential amino acid glutamine improves calcium uptake by accelerating GSH synthesis and activating calcium-sensing receptors in the bone. Given the ample evidence that amino acids restore bone health by well-characterized physiological mechanisms, we explored the possibility that methionine has beneficial effects, as little attention has been paid in evaluating the pharmacological effects of methionine on bone remodelling mediated by osteoblasts and osteoclasts.

Methionine ($\text{HO}_2\text{CCH}(\text{NH}_2)\text{CH}_2\text{CH}_2\text{SCH}_3$) is a sulphur-containing essential amino acid critical for the synthesis of vital molecules such as cysteine, carnitine, taurine, lecithin, phosphatidyl choline and other phospholipids. It improves

cartilage synthesis and is therefore used for the treatment of arthritis (Ursini and Pipicelli, 2009). Methionine has also been found to be effective against the progression of Parkinson's disease and schizophrenia (Antun *et al.*, 1971; Meininger *et al.*, 1982). A recent report by Cordomi *et al.* (2013) revealed methionine to be a molecular gear for pharmacology and function. Our preliminary data showed that methionine protects against bone loss *in vivo*, but has no direct effects on differentiated osteoclasts or osteoblasts *in vitro*. Since blood-borne osteoclast precursors are involved in replenishing osteoclast population during bone-loss pathology, we speculated whether methionine modulates the development of osteoclasts cells from their blood-borne precursors. Based on this hypothesis, we examined the modulatory effect of methionine in an experimentally induced osteoporotic model. By knocking down myeloid differentiation primary response gene 88 (MyD88) in developing osteoclasts, a potential mechanism accounting for osteoclastogenesis during osteoporosis and how methionine regulates this pathway is described here. Drug/molecular target nomenclature throughout this manuscript conforms to BJP's Concise Guide to PHARMACOLOGY (Alexander *et al.*, 2013).

Methods

Animals

All the animal experiments were approved by the Institute animal ethics committee (IAEC No. 192/08) in accordance with the *International Guiding Principles for Biomedical Research Involving Animals* issued by the Council for the International Organizations of Medical Sciences. Female Sprague-Dawley rats bred and reared in the institute animal house facility were used in this study. The animals were given standard laboratory chow and water *ad libitum*, and kept in an ambient room with the following conditions: temperature ($22 \pm 4^\circ\text{C}$), humidity 60%–65% and light (7:00–19:00). A total of 90 rats were used in this study.

Skeletally mature 5-month-old rats weighing 320 ± 10 g were used to generate the ovariectomized (OVX) rat model. Treatments were initiated 1 week from the day of ovariectomy. This period of rest allowed the rats to recover from the stress associated with the surgery.

Rats were divided into the following groups: normal rats; sham rats (in these rats, the ovaries were exposed and not removed); OVX rats; OVX rats fed standard chow and supplemented with methionine in their drinking water (100, 250 and 500 mg·kg⁻¹ body wt); OVX rats administered Fosamax (alendronate sodium; Merck & Co., Whitehouse Station, NJ, USA) at a dose of 100 or 200 $\mu\text{g}\cdot\text{kg}^{-1}$ body wt, p.o. alone or in combination with methionine (250 mg·kg⁻¹ body wt). Both treatments were performed 6 h apart. Alendronate (100 or 200 $\mu\text{g}\cdot\text{kg}^{-1}$ body wt) was administered p.o. to OVX rats at an interval of 5 days for 1 month. Rats that received methionine were caged separately to ensure that each animal received the appropriate dose of methionine daily. The duration of methionine therapy was 10 weeks. Cancellous osteopenia (bone loss) requires at least 4 weeks to develop post-ovariectomy and hence this was a prophylactic regime. All studies involving animals are reported in accordance with the ARRIVE

guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Ovariectomy

This surgical procedure was performed by the same person under aseptic conditions. Before surgery, the rats were anaesthetized with a combination of xylazine (10 mg kg⁻¹) and ketamine (75 mg kg⁻¹) administered i.p. Toe pinch, heart rate and body temperature were consistently checked to measure the depth of anaesthesia. Following anaesthesia, an incision was made in the abdomen to gently exteriorize the ovaries. The fallopian tubes were ligated, ovaries excised and then the peritoneal cavities were closed with absorbable sutures. After surgery, rats were administered Diclofenac sodium injection (20 mg kg⁻¹ body weight) to reduce the pain associated with the surgery. Care was taken to ensure that the animals did not suffer from hypothermia.

Bone imaging and histomorphological analysis

Bone imaging was performed using an *In Vivo* Imaging System FX PRO (Kodak, Rochester, NY, USA) employing the bone density software module, Carestream Molecular Imaging software version 5.0.7 (Carestream Health Inc., Rochester, NY, USA). Sectioning of bone was done by standard methods as detailed in Supporting Information Appendix S1. Trabecular thickness and trabecular separation were analysed using Bone J and Image J, NIH software (National Institute of Health, Bethesda, MD, USA). For analysis of adipogenesis, four fields were randomly selected from each section at 40× magnification and two sections were collected from each femur. A total of 16 fields were selected from four sections of each rat followed by quantification of adipocytes in the bone marrow. The area of adipocytes in bone marrow were determined and analysed using Image J.

Biochemical analysis

Tartrate-resistant acid phosphatase 5b (TRAP5b) activity and C-terminal telopeptides (CTX) levels were measured using ELISA kits from Immuno Diagnostic Systems (Fountain Hills, AZ, USA). Cytokines, osteocalcin and osteopontin levels in serum were measured by multiplex kits from Millipore on Bioplex 200™ (Bio-Rad, Hercules, CA, USA). Osteocalcin in cell culture supernatants was assayed by ELISA kit from Biomedical Technologies (Stoughton, MA, USA). Homocysteine levels in serum were measured using a kit from Cusa Biotech, China (Wuhan, China). Soluble (s) TNFSF11, M-CSF and macrophage inhibitory protein-1 α (MIP-1 α also known as CCL3) levels were assayed by indirect ELISA employing 3, 3', 5, 5'-tetramethyl benzidine as substrate.

Isolation and culture of blood mononuclear cells

Blood was collected from rats by retro-orbital puncture into heparin-containing tubes. Briefly, blood was layered on Histopaque 1083 (Sigma-Aldrich, St Louis, MO, USA) and centrifuged at 400x g for 30 min to obtain a buffy coat layer. The mononuclear cells in the buffy coat were enriched by washing with α -minimal essential medium (MEM) (supple-

mented with 10% fetal calf serum and 1% antibiotic-antifungal solution) and seeding on to type I collagen-coated plates (50 μ g·mL⁻¹). Cell viability was ensured to be greater than 90% by trypan blue assay. After 4 h, the culture media was changed and the cells were allowed to proliferate. To initiate differentiation, the mononuclear cells were cultured in the presence of 25 μ g·L⁻¹ recombinant mouse M-CSF (days 1–11) and 20 μ g·L⁻¹ TNFSF11 (days 6–20). Osteoclastogenic media was replaced every 3 days. The formation of osteoclasts was quantified by measuring the level of TRAP5b (a specific marker of osteoclast activity independent of renal dysfunction that indicates the number of osteoclasts) by immunoassay.

Transient siRNA-mediated knockdown

siRNA transfections were performed with Lipofectamine RNAiMAX (Invitrogen™, Life Technologies, Carlsbad, CA, USA). After stimulating with TNFSF11, cells were transferred to either MyD88 siRNA or scrambled siRNA (40 nmol·mL⁻¹). Knockdown efficiency was measured by Western blot. Before the siRNA transfection, the TRAP5b level in the cell culture supernatant was measured. Only those cultures that showed TRAP5b activity between 0.5 and 2 U·L⁻¹ were used for siRNA transfection.

Western blot

Cells were lysed in radioimmunoprecipitation assay lysis buffer containing complete protease inhibitors and sodium orthovanadate. Equal amounts of samples were electrophoresed on either 12 or 15% SDS-PAGE and then blotted to nitrocellulose transfer membrane. Subsequently, the membrane was incubated at room temperature with Western blocker for 1 h. For probing total protein and the phosphorylated forms, membranes were probed with primary and secondary antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence system in LAS4000 with ImageQuant LAS4000 software (GE Healthcare Life Science, Buckinghamshire, UK) and quantified using Image J.

Osteoblast culture

MC3T3-E1 sub-clone 4 (ATCC CRL2593), a pre-osteoblastic cell line derived from new born mouse calvaria, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in α -MEM supplemented with 10% fetal calf serum and 1% antibiotics. For cell culture experiments 2 × 10⁶ cells were seeded in 60 mm Petri plates. After attaining 70% confluence, cells were incubated with methionine (1–500 μ g) before treatment with LPS (1 μ g·mL⁻¹). Alternatively to study the effect of methionine on osteoblast differentiation, cells were treated with methionine with or without 10 mM β -glycerophosphate and 50 μ g·mL⁻¹ ascorbic acid for 30 days.

Statistics

The results were analysed using a statistical program Statistical Package for the Social Sciences (SPSS)/PC+, version 11.0 (SPSS Inc., Chicago, IL, USA). All values are expressed as means \pm SEM. One-way ANOVA was used to evaluate differences between samples of interest and the corresponding controls. *P* value less than 0.05 was considered statistically significant.

Results

Methionine inhibits bone loss in OVX rats

As a first step towards asserting the anti-resorptive effect of methionine, three different doses of methionine (100, 250 and 500 mg·kg⁻¹ body wt) were chosen. The results showed that administration of methionine at doses 100, 250 and 500 mg·kg⁻¹ body wt decreased OVX-induced body wt gain (Figure 1). Serum CTX assay indicated that the level of collagen degradation products was high in OVX rats and low in OVX rats administered 250 mg·kg⁻¹ body wt of methionine (Figure 2). CTX level remained high in rats administered 100 and 500 mg·kg⁻¹ body wt of methionine even at the end of the experimental period (Figure 2). TRAP5b assay showed that methionine at doses 100 and 250 mg·kg⁻¹ body wt decreased TRAP5b activity in serum (Figure 3). While 250 mg·kg⁻¹ body wt of methionine inhibited TRAP5b activity as early as four weeks, 100 mg·kg⁻¹ body wt of methionine imparted its effect only after 10 weeks of administration.

To evaluate whether methionine intake at the studied doses raised the level of its toxic metabolite *viz.* homocysteine, we estimated homocysteine in serum by ELISA. It was observed that ovariectomy itself induced a fourfold rise in serum homocysteine (Figure 4). Administration of methionine at doses 100 and 250 mg·kg⁻¹ body wt significantly inhibited OVX-induced homocysteine production. At a higher dose of 500 mg kg⁻¹, a rise in serum homocysteine level to 15 μmol·L⁻¹ was observed (Figure 4) which we presumed may be the cumulative effect of homocysteine

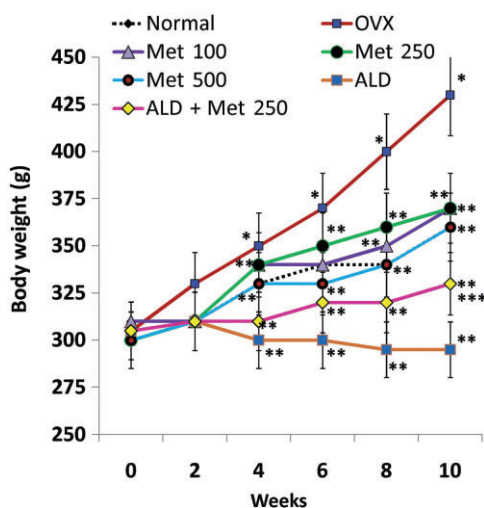


Figure 1

Changes in the body wt of normal, OVX and treatment groups during the experimental period. Methionine at doses 100, 250 and 500 mg·kg⁻¹ body wt was administered in drinking water for 10 weeks. Alendronate (200 μg·kg⁻¹ body wt) was administered p.o. to OVX rats at an interval of 5 days for 1 month. For combination therapy, rats were administered alendronate (100 μg·kg⁻¹ body wt) and methionine (250 mg·kg⁻¹ body wt). Both treatments were performed 6 h apart. Values are mean ± SEM; *n* = 4. **P* < 0.05 compared with normal rat. ***P* < 0.05 compared with OVX rat. ****P* < 0.05 compared with alendronate (200 μg·kg⁻¹ body wt)-treated OVX rats.

augmented upon ovariectomy and that produced by administration of high dose of methionine.

To define the basis of the anti-resorptive effect exerted by methionine at the dose of 250 mg·kg⁻¹ body wt, radiograph analysis and bone density measurements were carried out. Femur bone densitometry measurements showed that methionine (250 mg·kg⁻¹ body wt) significantly improved bone density as evidenced by increased bone density in the proximal and distal regions of femur as compared with OVX rats (Figure 5A, panel c; 5F). Figure 5B shows sections of rat femoral head belonging to different experimental groups. These images acquired and processed by Image J after haematoxylin and eosin staining show changes in bone architecture (growth plate) upon ovariectomy and on methionine (250 mg·kg⁻¹ body wt) and alendronate treatments (200 μg·kg⁻¹ body wt). Figure 5B, panel c shows preservation of trabecular structure in OVX rats on methionine treatment. We observed that OVX rats possessed high bone marrow adiposity with increased trabecular separation (Figure 5B, panel b; 5C, panel b) and low collagen content (Figure 5). After 10 weeks of methionine (250 mg·kg⁻¹ body wt) administration, trabecular separation was reduced (Figure 5B, panel c; 5C, panel c; 5H) while collagen content (Figure 5E, panel c) and trabecular thickness (Figure 5G) were preserved in methionine-administered rats compared with OVX rats. Quantification of trabecular separation, trabecular thickness and adipocyte area showed that bone physiology of methionine-administered rats was comparable with alendronate (200 μg·kg⁻¹ body wt) administered rats

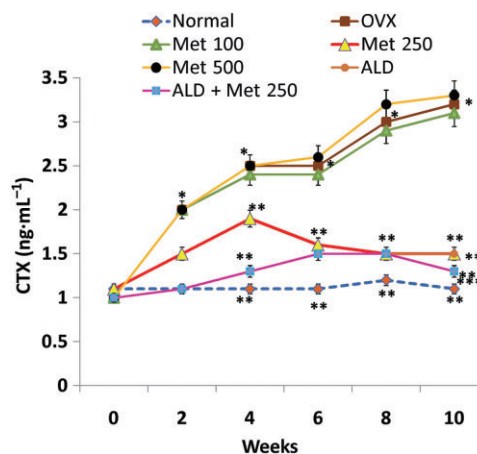


Figure 2

Changes in the level of CTX in serum samples from normal, OVX and treatment groups during the experimental period. Methionine at doses 100, 250 and 500 mg·kg⁻¹ body wt was administered in drinking water for 10 weeks. Alendronate (200 μg·kg⁻¹ body wt) was administered p.o. to OVX rats at an interval of 5 days for 1 month. For combination therapy, rats were administered alendronate (100 μg·kg⁻¹ body wt) and methionine (250 mg·kg⁻¹ body wt). Both treatments were performed 6 h apart. CTX levels in serum were measured by immunoassay. Values are mean ± SEM; *n* = 4. **P* < 0.05 compared with normal rat. ***P* < 0.05 compared with OVX rat. ****P* < 0.05 compared with alendronate (200 μg·kg⁻¹ body wt)-treated OVX rats.

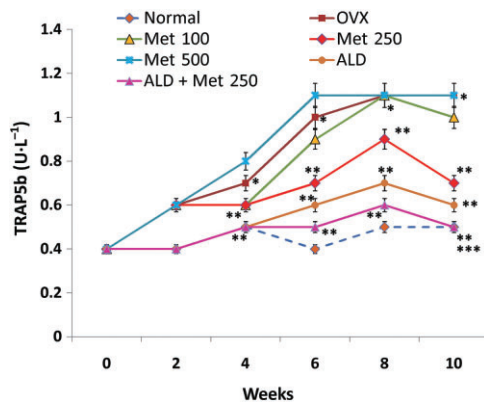


Figure 3

Changes in the activity of TRAP5b in serum of normal, OVX and treatment groups. Methionine at doses 100, 250 and 500 mg·kg⁻¹ body wt was administered in drinking water for 10 weeks. Alendronate (200 µg·kg⁻¹ body wt) was administered p.o. to OVX rats at an interval of 5 days for 1 month. For combination therapy, rats were administered alendronate (100 µg·kg⁻¹ body wt) and methionine (250 mg·kg⁻¹ body wt). Both treatments were performed 6 h apart. TRAP5b activity in serum was measured by immunoassay. Values are mean ± SEM; *n* = 4. **P* < 0.05 compared with normal rat. ***P* < 0.05 compared with OVX rat. ****P* < 0.05 compared with alendronate (200 µg·kg⁻¹ body wt)-treated OVX rats.

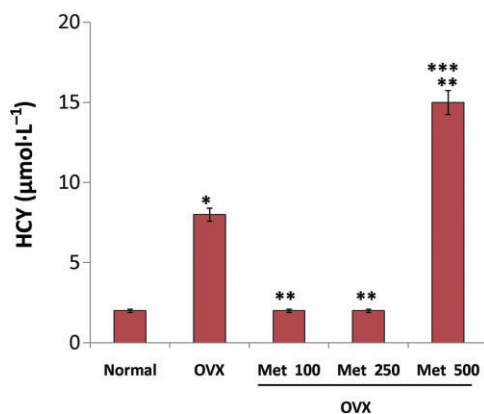


Figure 4

Changes in the level of serum homocysteine (HCY) in OVX rats fed 100, 250 and 500 mg·kg⁻¹ body wt of methionine. Homocysteine in serum was measured by ELISA. Values are mean ± SEM; *n* = 4. **P* < 0.05 compared with normal rat. ***P* < 0.05 compared with OVX rat. ****P* < 0.05 compared with methionine (100 and 250 mg·kg⁻¹ body wt)-treated OVX rat.

(Figure 5B, panel d; 5C, panel d; 5D, panel d; 5E, panel d; 5F–I). Analysis of bone-specific proteins like osteocalcin and osteopontin in serum showed that methionine administration maintained adequate levels of these bone-specific proteins in serum as compared with that of OVX rats (Figure 6). Collectively, the macroscopic and biochemical evaluation revealed that methionine (250 mg·kg⁻¹ body wt) restrains

bone loss and confers protection against development of osteoporosis.

Methionine reduces pro-osteoclastogenic and pro-inflammatory cytokines in OVX rats

Pro-inflammatory and pro-osteoclastogenic cytokines possess potential roles in precipitating bone loss (Fuller *et al.*, 1993; Gao *et al.*, 2007; Fine *et al.*, 2009). At the end of the experimental period, OVX rats showed high levels of Th1-derived cytokines (pro-inflammatory) such as IL-1α, IL-1β, G-CSF, GM-CSF, TNF-α, IL-8 and INF-γ in serum and low levels of Th2 cytokines (with anti-inflammatory role) like IL-4 and IL-10 (Figure 7). Administration of methionine effectively decreased the level of Th1 cytokines and increased the level of Th2 cytokines such as IL-4 and IL-10 in serum (Figure 7). We also observed that cytokines integral for osteoclastogenesis such as MIP-1α, M-CSF and RANKL were significantly lower in methionine-administered rats as compared with OVX rats (Figure 8).

Effect of methionine on osteoblasts

To determine whether methionine modulates TNFSF11 production (a prime factor that regulates osteoclast activity) by osteoblasts, LPS-stimulated osteoblasts were used as model system. Pre-osteoblastic MC3T3E1 cells differentiated using ascorbic acid (50 µg·mL⁻¹) and β-glycerophosphate (10 mM) for 5 days were incubated with methionine (1–500 µg·mL⁻¹; 24 h) prior to LPS (1 µg·mL⁻¹; 3 h) treatment and soluble TNFSF11 (sTNFSF11) release in cell culture supernatants was analysed by ELISA. It was found that methionine inhibited LPS-induced sTNFSF11 release in osteoblast cultures at a dose of 50 µg·mL⁻¹ (Figure 9A).

To evaluate whether methionine affected osteoblast differentiation, pre-osteoblasts were treated with methionine (5, 10 and 50 µg·mL⁻¹) for 30 days. Ascorbic acid and β-glycerophosphate induced cell differentiation was evident on day 5 by the presence of osteocalcin, a non-collagenous bone matrix protein in culture supernatants. Methionine treatment neither induced cell differentiation (not even after 30 days) nor enhanced cell differentiation potential of ascorbic acid and β-glycerophosphate (Figure 9B).

Methionine suppresses the formation of osteoclasts from blood-derived mononuclear cells

To assess the anti-osteoclastogenic potential of methionine, blood mononuclear cells (derived from normal rats) were cultured with TNFSF11 (20 µg·L⁻¹) and M-CSF (25 µg·L⁻¹) for 25 days. Methionine (10, 50 and 100 µg·mL⁻¹) was added to cell culture from day 0 and replaced with fresh cell culture media every other 3 days. M-CSF/ TNFSF11-induced TRAP5b release was evident from day 15 onwards. Methionine at doses 10 and 50 µg·mL⁻¹ inhibited osteoclast activity as evidenced by reduced TRAP5b release in cell culture supernatants (Figure 10A, B). This inhibition of TRAP5b synthesis was not observed at a concentration of 100 µg·mL⁻¹. Interestingly methionine had no inhibitory effect on differentiated osteoclasts *in vitro* (Figure 10C).

To evaluate whether administration of methionine modulated the intrinsic ability of blood mononuclear cells

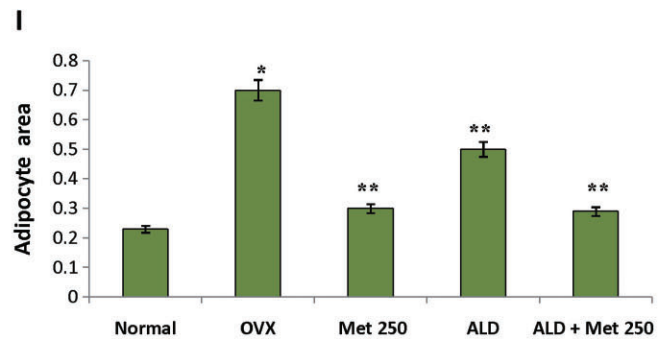
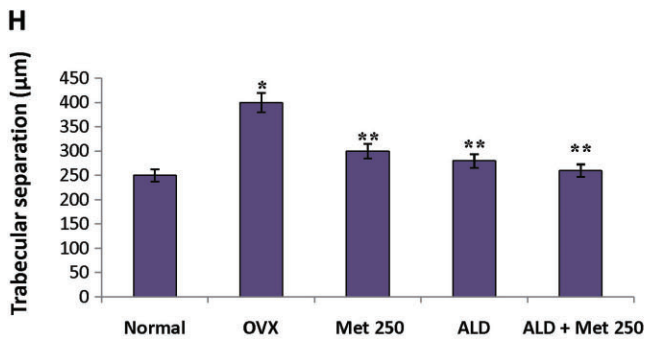
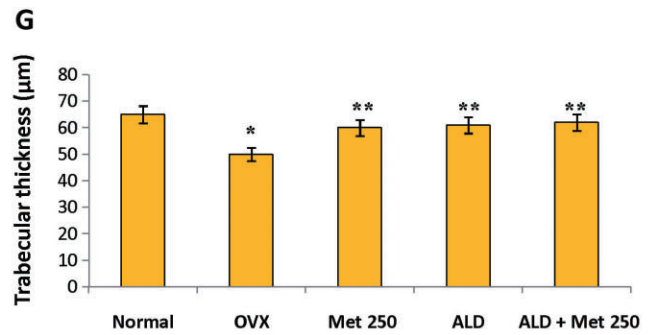
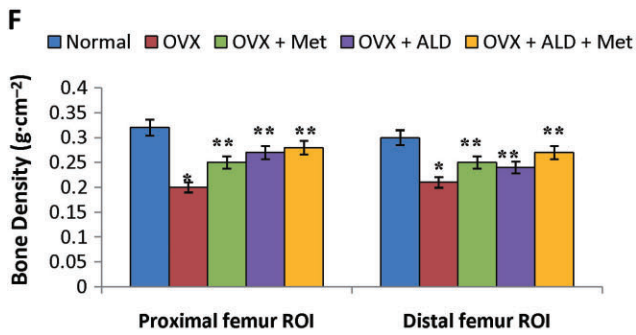
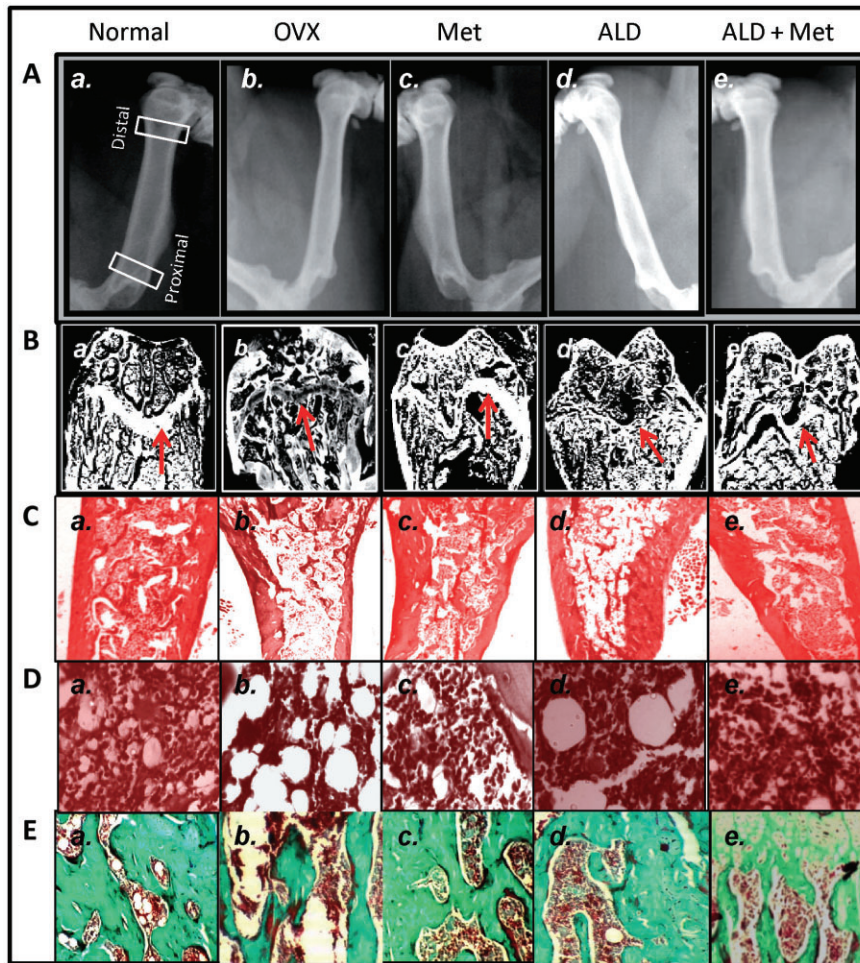


Figure 5

Bone physiology of normal, OVX and treatment groups. Methionine at a dose of $250 \text{ mg}\cdot\text{kg}^{-1}$ body wt was administered in drinking water for 10 weeks. Alendronate ($200 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ body wt) was administered p.o. to OVX rats at an interval of 5 days for 1 month. For combination therapy, rats were administered alendronate ($100 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ body wt) and methionine ($250 \text{ mg}\cdot\text{kg}^{-1}$ body wt). Both treatments were performed 6 h apart. (A) Radiographs of femur were acquired on Kodak FX Pro in the X-ray mode. Each animal received an approximate radiation dose of 1.4 Rad during imaging. (B) Images of femoral bone head of normal and experimental rats acquired using Image J after haematoxylin and eosin (H & E) staining. Red arrows indicate changes in growth plate. (C) H & E staining of femur showing changes in trabecular structure between normal and experimental rats. (D) H & E staining of femur showing changes in adipogenesis between normal and experimental rats. (E) Masson Trichome staining of femur showing changes in collagen level between normal and experimental rats. (F) Bone density measurements of femoral bone samples from normal and experimental rats. Bone density was measured and analysed by bone density software module employing a Carestream Molecular Imaging software version 5.0.7 (G–I) Changes in trabecular thickness, trabecular separations and adipogenesis in normal and experimental rats analysed using Bone J and Image J, Adipocyte area refers to area of bone marrow adipocytes in units of bone trabecula. Values are mean \pm SEM; $n = 4$. * $P < 0.05$ compared with normal rat. ** $P < 0.05$ compared with OVX rat.

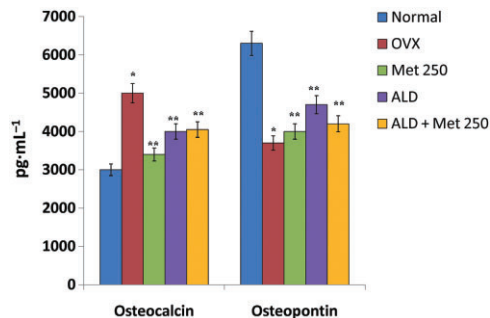


Figure 6

Changes in the levels of osteocalcin and osteopontin in serum of normal, OVX and treatment groups. Methionine at a dose of $250 \text{ mg}\cdot\text{kg}^{-1}$ body wt was administered in drinking water for 10 weeks. Alendronate ($200 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ body wt) was administered p.o. to OVX rats at an interval of five days for one month. For combination therapy, rats were administered alendronate ($100 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ body wt) and methionine ($250 \text{ mg}\cdot\text{kg}^{-1}$ body wt). Both treatments were performed 6 h apart. Osteocalcin and osteopontin levels in serum were measured by multiplex kits from Millipore on Bioplex 200™. Values are mean \pm SEM; $n = 4$. * $P < 0.05$ compared with normal rat. ** $P < 0.05$ compared with OVX rat.

to form TRAP5b-secreting osteoclasts, mononuclear cells were subjected to an osteoclastogenic environment *ex vivo*. Blood mononuclear cells from normal, OVX and methionine-administered rats were enriched and cultured *ex vivo* in the presence of $25 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ M-CSF and $20 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ sTNFSF11 for 25 days. Our results show that mononuclear cells from OVX rats had greater propensity to secrete TRAP5b (Figure 11) while cell cultures derived from blood mononuclear cells of methionine- and alendronate-treated rats showed much lower predisposition to form TRAP5b-secreting osteoclasts. We also observed that the cell cultures derived from methionine-treated OVX rats showed reduced levels of IL-1 α , IL-1 β , TNF- α and MIP-1 α whereas these were significantly high in osteoclast cultures derived from OVX rats (Figure 12). The results therefore indicate that the anti-resorptive nature of methionine was more due to its ability to restrain formation of osteoclasts from blood-borne precursors than its direct effects on functional osteoclasts.

Methionine inhibits TLR-4/MyD88/NF- κ B signalling to reduce osteoclast formation from blood mononuclear cells

The responsiveness of mononuclear cells to differentiate to osteoclast lineage in response to a pathophysiological stimulus varies in terms of pro-inflammatory cytokine production (Itoh *et al.*, 2003). Hence we closely evaluated changes in the developing osteoclasts at days 15–17, a time-point when mononuclear cells shifted to the osteoclast phenotype as evident from the expression of TRAP5b. During this time frame, we observed that the level of MIP-1 α , a chemokine essential for osteoclast development from mononuclear cells, was significantly reduced in cell culture isolated from methionine-treated rats as compared with OVX-derived cell cultures. To elucidate the underlying molecular mechanism for this observed effect, we checked whether methionine administration modulated NF of activated T-cells (NFATc1) expression in developing osteoclasts. It was observed that methionine treatment did not induce any change in the expression status of this master regulator of osteoclastogenesis (Figure 13). Since osteoclast survival during pathological conditions also involves toll-like receptor (TLR) signalling, we monitored the changes in TLR-4 expression in developing osteoclast cultures and found that ovariectomy increased the expression of TLR4 in developing osteoclasts, a change that was not observed in sham-operated rats (Figure 14). Quantification of Western blots showed that TLR4 expression was low in developing osteoclast cultures isolated from methionine-treated rats (Figure 14). Because TLR signalling involves the recruitment of MyD88 adapter protein and finally activation of NF- κ B, we checked the expression of these associated downstream signalling molecules in cell cultures by Western blot. It was observed that ovariectomy not only increased the expression of TLR4 and MyD88, but also elevated the level of nuclear p65 in the developing osteoclasts (Figure 14). Ovariectomy reduced the expression of I κ B in the cytosol (Figure 14). Methionine treatment effectively down-regulated OVX-induced up-regulation of TLR4, MyD88 and nuclear localization of p65 subunit of NF- κ B and restored the level of I κ B in cytosol (Figure 14). Taken together, the results indicate that methionine modulated TLR4/MyD88/NF- κ B signalling during osteoclastogenesis.

Since MyD88 signalling was down-regulated following methionine administration, we questioned whether MyD88 has any role in the synthesis of TRAP5b or development of

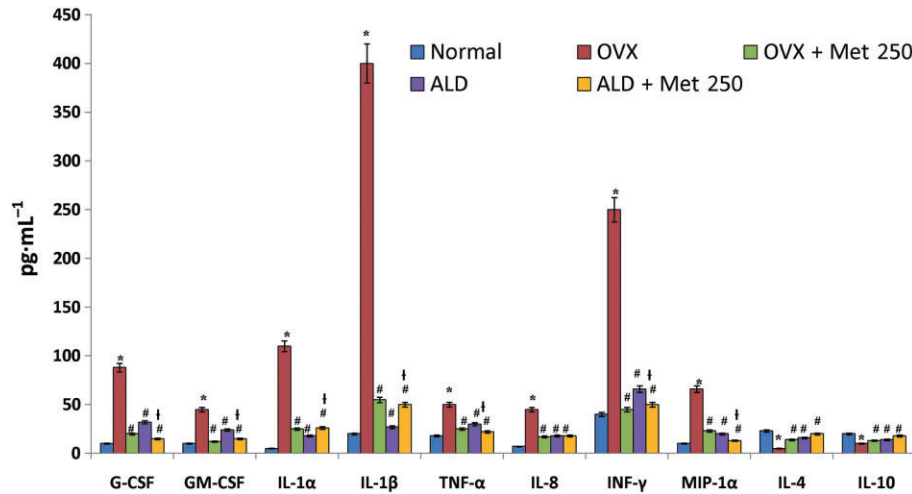


Figure 7

Changes in the levels of cytokine/chemokines in serum of normal, OVX and treatment groups. Methionine at a dose of 250 mg·kg⁻¹ body wt was administered in drinking water for 10 weeks. Alendronate (200 µg·kg⁻¹ body wt) was administered p.o. to OVX rats at an interval of 5 days for 1 month. For combination therapy, rats were administered alendronate (100 µg·kg⁻¹ body wt) and methionine (250 mg·kg⁻¹ body wt). Both the treatments were performed 6 h apart. Cytokines and chemokines in serum were measured using a multiplex kit from Millipore on Bioplex 200™. Values are mean ± SEM; n = 4. *P < 0.05 compared with normal rat. #P < 0.05 compared with OVX rat. †P < 0.05 compared with alendronate (200 µg·kg⁻¹ body wt) administered rat.

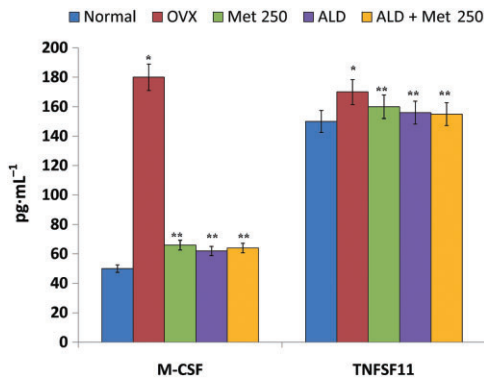


Figure 8

Changes in the level of M-CSF and TNFSF11 in the serum of normal, OVX and treatment groups. Methionine at a dose of 250 mg·kg⁻¹ body wt was administered in drinking water for 10 weeks. Alendronate (200 µg·kg⁻¹ body wt) was administered p.o. to OVX rats at an interval of 5 days for 1 month. For combination therapy, rats were administered alendronate (100 µg·kg⁻¹ body wt) and methionine (250 mg·kg⁻¹ body wt). Both the treatments were performed 6 h apart. M-CSF and soluble TNFSF11 levels in serum were measured by ELISA. Values are mean ± SEM; n = 4. *P < 0.05 compared with normal rat. **P < 0.05 compared with OVX rat.

osteoclasts. We therefore knocked down MyD88 in developing osteoclasts (isolated from experimental rats and stimulated with TNFSF11/M-CSF) by siRNA-mediated transfection and checked osteoclast functionality by monitoring the level of TRAP5b and MIP-1α in the cell culture supernatants. As seen in Figure 15A, MyD88 siRNA transfection significantly down-regulated MyD88 expression, which was not observed in scrambled siRNA knockout cells (Figure 15A). Figure 15B

shows that siRNA-mediated knockdown of MyD88 abolished TRAP5b and MIP-1α levels synthesis in culture supernatants, a phenomenon not observed in scrambled siRNA transfected cells. These results show that MyD88 is essential for the synthesis of TRAP5b and MIP-1α in developing osteoclastogenesis. To summarize: (i) OVX (oestrogen deficit condition) induces TLR4 signalling involving MyD88 in developing osteoclasts; (ii) TLR4/MyD88 signalling in developing osteoclasts is critical for secretion of factors like TRAP5b and MIP-1α; and (iii) methionine down-regulates osteoclast function by inhibiting TLR4/MyD88/NF-κB/MIP-1α signalling in developing osteoclasts.

Co-administration of methionine with alendronate is a superior therapeutic regime for treatment of ovariectomy-induced bone loss

Although amino acid interventions alone have the potential to inhibit the progression of bone loss, it is imperative to demonstrate the potency of a combination therapy consisting of methionine together with a known anti-osteoporotic drug. We therefore investigated the potency of a combination therapy of methionine with alendronate, a nitrogen-containing bisphosphonate. Alendronate (100 µg·kg⁻¹ body wt) administration alongside methionine (250 mg·kg⁻¹ body wt) for 1 month significantly improved bone density and bone architecture as compared with alendronate (200 µg·kg⁻¹ body wt) alone (Figure 5F–I). The body wt (Figure 1), serum bone protein levels (Figure 6), serum pro-osteoclastogenic cytokine levels (Figure 7) and the intrinsic ability of blood mononuclear cells to form osteoclasts (Figures 11 and 12) were observed to be near normal values in alendronate + methionine-treated rats.

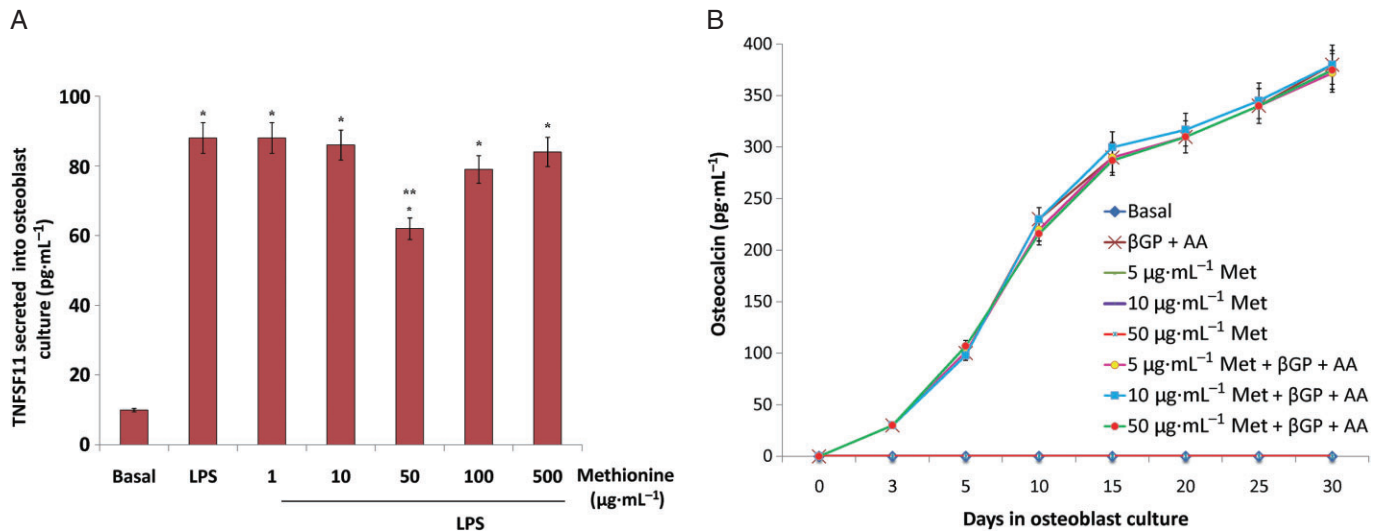


Figure 9

Effect of methionine on osteoblastogenesis *in vitro*. (A) An *in vitro* study showing changes in the level of soluble TNFSF11 in methionine pretreated ($1\text{--}500 \mu\text{g}\cdot\text{mL}^{-1}$) osteoblast cultures stimulated with LPS endotoxin ($1 \mu\text{g}\cdot\text{mL}^{-1}$). Osteoblast differentiation was induced by adding osteogenic media containing β -glycerophosphate (10 mM) and ascorbic acid ($50 \mu\text{g}\cdot\text{mL}^{-1}$) to pre-osteoblast cultures (cell line passage no. 9). Soluble TNFSF11 in cell culture supernatants was assayed by ELISA. (B) Effect of methionine on osteoblast differentiation with or without β -glycerophosphate (10 mM) and ascorbic acid ($50 \mu\text{g}\cdot\text{mL}^{-1}$). Osteoblast differentiation was evaluated by measuring the level of osteocalcin, a bone matrix protein by ELISA. Values are mean \pm SEM of four independent experiments. * $P < 0.05$ compared with basal. ** $P < 0.05$ compared with LPS control.

Discussion

The results of our study demonstrated that administration of methionine at a dose of 250 mg kg^{-1} body wt effectively inhibits bone loss induced by ovariectomy. To define the cellular basis of this protection rendered by methionine, we first evaluated the effect of this amino acid on differentiated osteoclasts and osteoblasts *in vitro* and found that methionine posed no direct effects on mature bone cells. This apparent discrepancy prompted us to assess whether the amino acid modulated precursor forms of these cell types. The influence of methionine on osteoclast maturation was analysed by isolating blood mononuclear cells from OVX- and methionine-administered OVX rats and subjecting them to an osteoclastogenic environment, TNFSF11 and M-CSF *ex vivo*. Although such an *ex vivo* condition is quite the reverse of an *in vivo* scenario (e.g. presence of other cell types in the vicinity and presence of cytokines apart from TNFSF11 and M-CSF), this process enabled us to evaluate whether methionine administration during the progression of bone loss influenced the intrinsic ability of blood mononuclear cells to form osteoclasts. Blood mononuclear cells normally differentiate to macrophages or dendritic cells and form osteoclasts only in response to reparative (bone remodelling or fracture) and pathological processes (Roato *et al.*, 2005; D'Amelio *et al.*, 2008; Ritchlin, 2009). By employing an ELISA that specifically detected only TRAP5b (not TRAP5a by macrophages), we were able to determine the functionality of osteoclasts during the 25 days of culture (Alatalo *et al.*, 2003). It was found that methionine treatment down-regulated the ability of blood-derived osteoclast precursors to form functional osteoclasts,

as indicated by the reduced activity of TRAP5b, a protein synthesized and secreted only by matured and functional osteoclasts (Alatalo *et al.*, 2003).

We presumed that methionine hindered a specific signalling cascade in the osteoclastogenesis process associated with TRAP5b synthesis. Osteoclastogenesis is a multistep process dependent on the cellular interaction of myeloid pre-osteoclast precursors with either osteoblasts or stromal cells under the influence of a wide range of local autocrine and/or paracrine factors such as M-CSF and TNFSF11 and several intracellular signalling molecules and transcription factors like NFATc1 and NF- κ B (Yoshida *et al.*, 1990; Lacey *et al.*, 1998; Novack, 2011). Previous studies showed that TNFSF11 elicited by a deficiency in oestrogen epigenetically regulates osteoclast differentiation by modulating H3K27 demethylase and demethylating histone3-K27me3 in NFATc1 gene (Yasui *et al.*, 2011). Although methionine regulated excessive TNFSF11 production, it was unable to modulate the expression of NFATc1, the master regulator of osteoclastogenesis. It, therefore, became apparent that methionine does not affect the overall function of osteoclasts (as NFATc1 is unchanged), but modulates its activity through a unique signalling mechanism. Interestingly, we found that methionine administration down-regulated ovariectomy-induced TLR4 expression in osteoclast precursors. TLR4 is a pathogen recognition receptor with roles not only in inflammation, insulin resistance and wt gain, but also in osteoclastogenesis (Shi *et al.*, 2006). Previously it was reported that surgically-induced menopause augments TLR4 expression in the frontal cortex of the brain (Sarvari *et al.*, 2012). In this study, an up-regulation in TLR4 expression was observed in developing

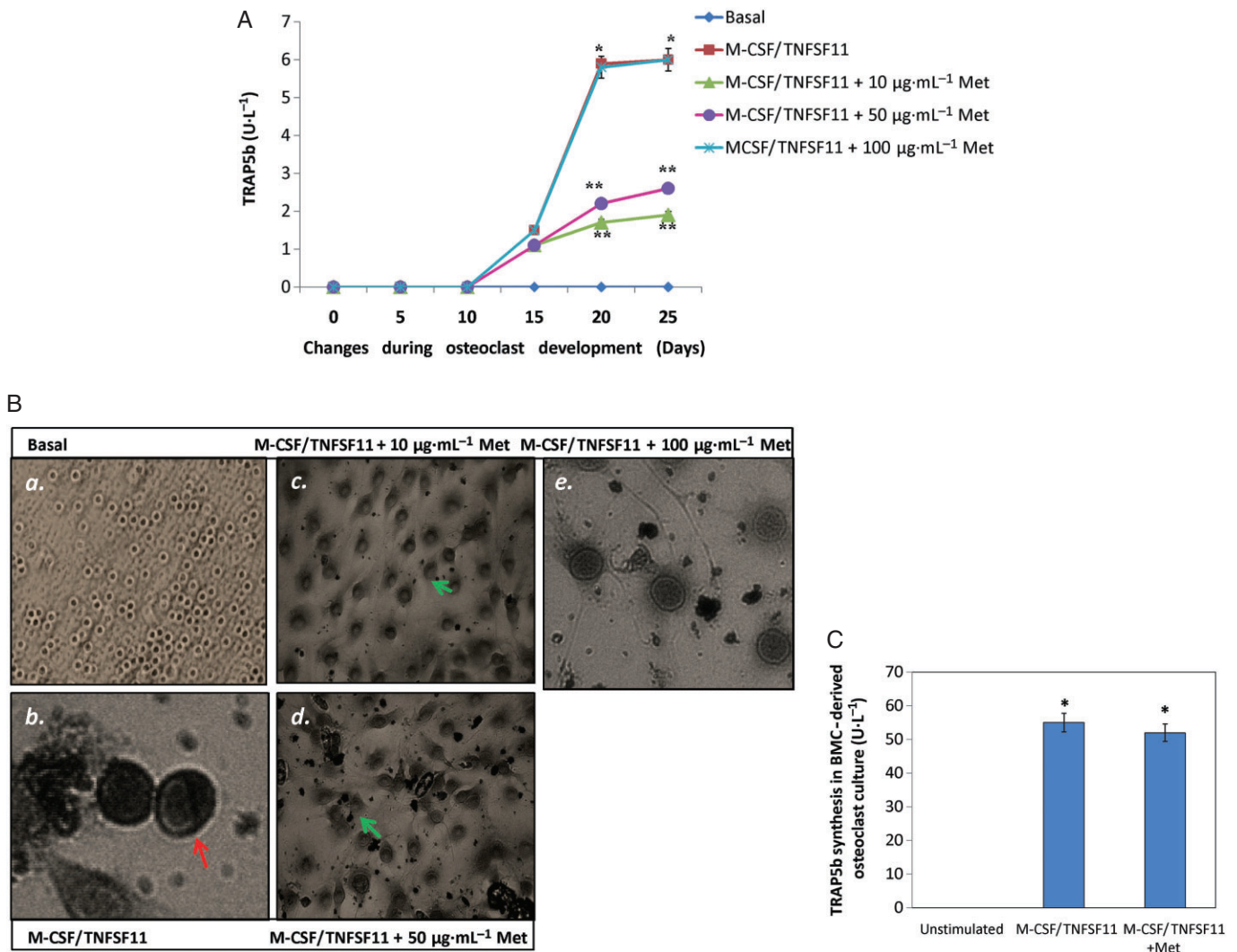


Figure 10

Effect of methionine on osteoclastogenesis *in vitro*. (A) Changes in the level of TRAP5b in blood borne-derived osteoclast cultures grown (developing osteoclasts) in osteoclastogenic media (M-CSF: 25 µg·L⁻¹, days 1–11 and TNFSF11: 20 µg·L⁻¹, days 6–20) in the presence and absence of methionine (10, 50 and 100 µg·mL⁻¹). (B, panels a–e) Photographs showing changes in developing osteoclasts grown in osteoclastogenic media (M-CSF 25 µg·L⁻¹, days 1–11 and TNFSF11 20 µg·L⁻¹, days 6–20), in the presence and absence of methionine. Red arrow indicates developing osteoclast. Green arrows show absence of osteoclast morphology in methionine (10 and 50 µg·mL⁻¹) treated cell culture. (C) TRAP5b level in matured osteoclast cultures (developed osteoclasts) in the presence and absence of methionine. Osteoclasts were grown in (M-CSF, 25 µg L⁻¹ for days 1–11 and TNFSF11, 20 µg L⁻¹ for days 6–20) for 25 days and then incubated with methionine (50 µg·mL⁻¹) for 5 days. Values are mean ± SEM of four independent experiments. **P* < 0.05 compared with basal. ***P* < 0.05 compared with M-CSF/TNFSF11 control. BMC, blood mononuclear cell.

osteoclasts from OVX rats. The up-regulation of this receptor may be the end-result of oestrogen deficiency alone (induced upon ovariectomy) as it was absent in sham-operated rats. Interestingly methionine treatment suppressed this augmentation in TLR4 expression. To confirm that methionine inhibited osteoclast development through TLR4 signalling, a downstream adapter protein that interacts with the conserved cytoplasmic regions of TLR4 was silenced. Knockdown of MyD88 abolished synthesis of both TRAP5b and MIP-1α, a cytokine imperative for osteoclastogenesis and confirmed the crucial role of MyD88 signalling in osteoclast development. Indeed, an earlier work of Sato *et al.* showed that both bone

formation and resorption are reduced in MyD88^{-/-} mice (Sato *et al.*, 2004). Further, downstream, methionine inhibited TNFSF11-induced nuclear localization of NF-κB. NF-κB is a crucial transcription factor essential for osteoclastogenesis after the interaction of TNFSF11 with its receptor TNFRSF11A (RANK; Novack, 2011). Studies have shown that mice defective in p50 and p52 subunits of NF-κB suffer extreme osteopetrosis due to impairments in osteoclast differentiation (Iotsova *et al.*, 1997). Our results demonstrate that methionine inhibits TRAP5b synthesis in the osteoclast by affecting TLR4/MyD88/NF-κB signalling without modulating the expression of NFATc1. Since methionine is also a proven

modulator of DNA methylation (Rowan, 2006), we cannot exclude the possibility that methionine may also epigenetically regulate osteoclast development. Nevertheless further experiments are required to confirm this.

Several lines of research have shown that the maturation of pre-osteoclasts from its haematopoietic stem cells to its functional resorptive stage is regulated by an array of pro-resorptive cytokines in circulation (Fuller *et al.*, 1993; Gao *et al.*, 2007; Fine *et al.*, 2009). We found that methionine induced inhibition of transdifferentiation to osteoclasts (during oestrogen deficiency) may also involve down-regulation of synthesis of pro-inflammatory cytokines such

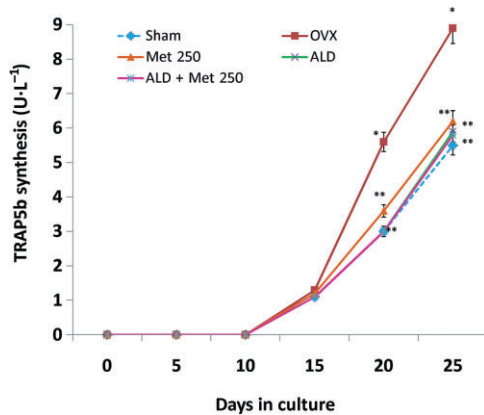


Figure 11

Effect of methionine on osteoclastogenesis *in vivo*. Methionine at a dose of 250 mg·kg⁻¹ body wt was administered in drinking water for 10 weeks. Alendronate (200 µg·kg⁻¹ body wt) was administered p.o. to OVX rats at an interval of 5 days for 1 month. For combination therapy, rats were administered alendronate (100 µg·kg⁻¹ body wt) and methionine (250 mg·kg⁻¹ body wt). Both treatments were performed 6 h apart. Blood mononuclear cells isolated from these rats were cultured in the presence of M-CSF (25 µg·L⁻¹, days 1–11) and TNFSF11 (20 µg·L⁻¹, days 6–20) *ex vivo*. Values are mean ± SEM; *n* = 4. **P* < 0.05 compared with normal rat. ***P* < 0.05 compared with OVX rat.

as IL-1α, IL-1β, TNF-α and MIP-1α. Among these pro-osteoclastogenic cytokines up-regulated by ovariectomy, MIP-1α is a proven stimulator of bone resorption (Choi *et al.*, 2000; 2001; Okamoto *et al.*, 2004). MIP-1α, a chemotactic pro-inflammatory cytokine regulated by NF-κB possesses the ability to stimulate the formation of osteoclast-like multinucleated cells (Choi *et al.*, 2001; Brueckmann *et al.*, 2004). Oh *et al.* (2010) demonstrated that MIP-1 expression stimulated by TNFSF11 facilitates osteoclast survival by activating NF-κB. Our results showed that methionine administration inhibited TNFSF11-induced MIP-1α secretion by modulating

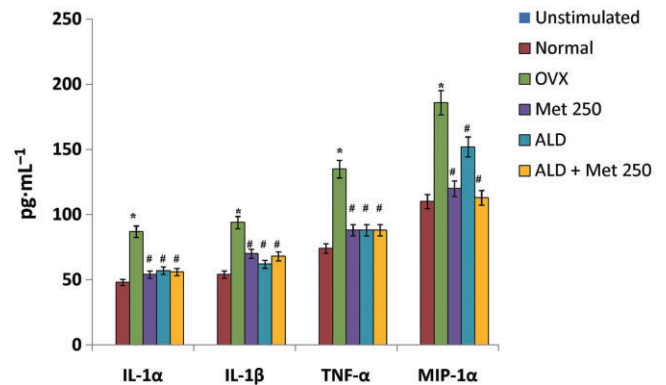


Figure 12

Effect of methionine on pro-inflammatory cytokine production in blood-derived osteoclast cultures *ex vivo*. Methionine at a dose of 250 mg·kg⁻¹ body wt was administered in drinking water for 10 weeks. Alendronate (200 µg·kg⁻¹ body wt) was administered p.o. to OVX rats at an interval of 5 days for 1 month. For combination therapy, rats were administered alendronate (100 µg·kg⁻¹ body wt) and methionine (250 mg·kg⁻¹ body wt). Both treatments were performed 6 h apart. Blood mononuclear cells isolated from rats were cultured in the presence of M-CSF (25 µg L⁻¹, days 1–11) and TNFSF11 (20 µg L⁻¹, days 6–20) *ex vivo*. Pro-inflammatory cytokines in developing blood-derived osteoclast cultures were assayed by multiplex kits from Millipore on Bioplex 200™. Values are mean ± SEM; *n* = 4. **P* < 0.05 compared with normal rat. #*P* < 0.05 compared with OVX rat.

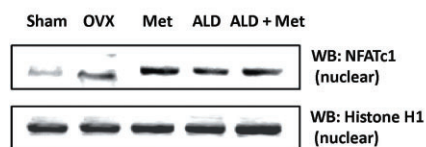
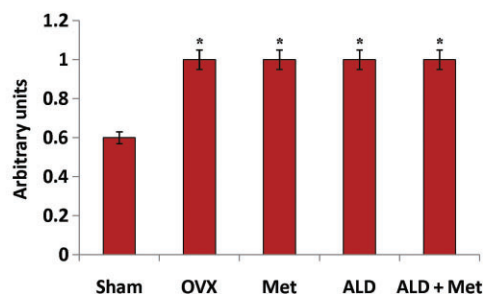


Figure 13

Changes in the expression of NFATc1 transcription factor in developing osteoclasts following ovariectomy and methionine treatment. Methionine at a dose of 250 mg·kg⁻¹ body wt was administered in drinking water for 10 weeks. Alendronate (200 µg·kg⁻¹ body wt) was administered p.o. to OVX rats at an interval of 5 days for 1 month. For combination therapy alendronate was administered at a dose of 100 µg·kg⁻¹ body wt. Both treatments for combination therapy were performed 6 h apart. NFATc1 expression in nuclear extract of blood-derived osteoclast cultures from different experimental rats was analysed by Western blot. One of the four representative Western blots is given. Values are mean ± SEM of four independent experiments. **P* < 0.05 compared with basal.



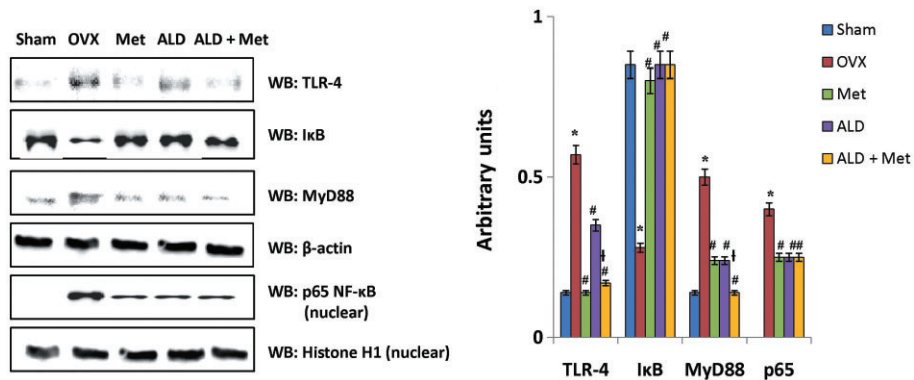


Figure 14

Changes in the expression of TLR4, MyD88 and NF-κB signalling factors in developing osteoclasts following ovariectomy and methionine treatment. Methionine at a dose of 250 mg·kg⁻¹ body wt was administered in drinking water for 10 weeks. Alendronate (200 µg·kg⁻¹ body wt) was administered p.o. to OVX rats at an interval of 5 days for 1 month. For combination therapy alendronate was administered at a dose of 100 µg·kg⁻¹ body wt. Both treatments for combination therapy were performed 6 h apart. The expressions of TLR4, MyD88, IκB and NF-κB signalling proteins were analysed by Western blot. One of the four representative Western blots is given. **P* < 0.05 compared with basal. Values are mean ± SEM; *n* = 4. #*P* < 0.05 compared with OVX rat. †*P* < 0.05 compared with alendronate (200 µg·mL⁻¹)-treated rat.

MyD88 signalling, as knockdown of this adapter protein eliminated synthesis of MIP-1α in developing osteoclast cultures. These *ex vivo* studies indicate that pro-inflammatory cytokines also have a major role in determining the fate of the osteoclast. Accordingly, it is possible that the reduced synthesis of pro-resorptive Th1 cytokines in methionine-administered OVX rats and increased synthesis of Th2 anti-inflammatory cytokines are reasons for suppressed osteoclastogenesis.

The levels of anti-inflammatory cytokines also play vital roles in preventing disease development. It was shown earlier that IL-4 promotes the production of osteoprotegerin, a decoy receptor for TNFSF11 in osteoblasts, in addition to directly inhibiting the differentiation of osteoclast precursors towards diminished osteoclastogenesis (Yamada *et al.*, 2007). Therefore the ability of methionine to improve anti-inflammatory cytokines such as IL-4 in circulation is another mechanism for its anti-resorptive effect.

In addition, our results showed that although methionine could unlikely have any positive effect on osteoblast differentiation, it can prevent untoward TNFSF11 induced by inflammatory agents and therefore render protection during pathological conditions. Accordingly the decreased level of TNFSF11 in the serum of methionine-treated OVX rats may be attributed to this beneficial property of methionine.

In this study, the preservation of trabecular architecture achieved with methionine treatment was comparable with that obtained with alendronate. Alendronate, a nitrogen-containing bisphosphonate with high affinity for bone, acts directly on the osteoclast and inhibits geranylgeranyl diphosphate, the substrate for prenylation of GTP-binding proteins that control cytoskeletal reorganization and vesicular fusion, processes involved in osteoclast activation (Fisher *et al.*, 1999). The main advantage of the combination treatment (methionine + alendronate) over that of alendronate alone was that the former provided the same degree of protection as the latter, but at a lower dose. We deduce that the superior

effect induced by combination therapy must be due to the direct osteoclast inhibitory effect of alendronate and an additive effect exerted by alendronate and methionine on the regulation of osteoclast formation from blood-borne precursors.

To summarize, the findings reported in this study are in concurrence with a clinical study by Pernow *et al.* (2010) which demonstrated that the maintenance of the correct essential/non-essential amino acid ratio is critical for preservation of bone density. Although the dose of 250 mg·kg⁻¹ body wt used in rodents is higher than the recommended dietary supplement for humans, this dose of methionine does not induce hyperhomocysteinaemia but interestingly inhibits hyperhomocysteinaemia induced upon ovariectomy. This may be due to: (i) the antioxidant nature of methionine that nullifies the oxidative effect of homocysteine or (ii) the adequate folate (cofactor of methyl-folate-homocysteine-methyl-transferase in methionine metabolism that converts homocysteine back to methionine) available in the rodent diet (4 ppm). Beyond this dose (lower or higher), no protective effect is seen. Methionine therefore appeared to exert a biphasic effect as after a deflection point, it turned from an anti-resorptive pharmacological agent to a hyperhomocysteinemia inducing substance. Although this is an anticipated risk associated with methionine therapy, the potent anti-resorptive pharmacological effect it possesses cannot be ignored. Methionine is an essential amino acid required for the biosynthesis of many vital molecules in the body and can be acquired only through diet. Over the years, the consumption of rich sources of methionine like beans, lentils, yoghurt, onions, eggs and fish have only enriched human health. Therefore appropriate consumption of methionine-containing foods (in adequate amounts) can never pose any untoward effects but may promote bone health and serve as a prophylaxis against the development of osteoporosis. However, for therapeutic purpose in humans, the dose of methionine has to be standardized by independent human clinical trials for optimization

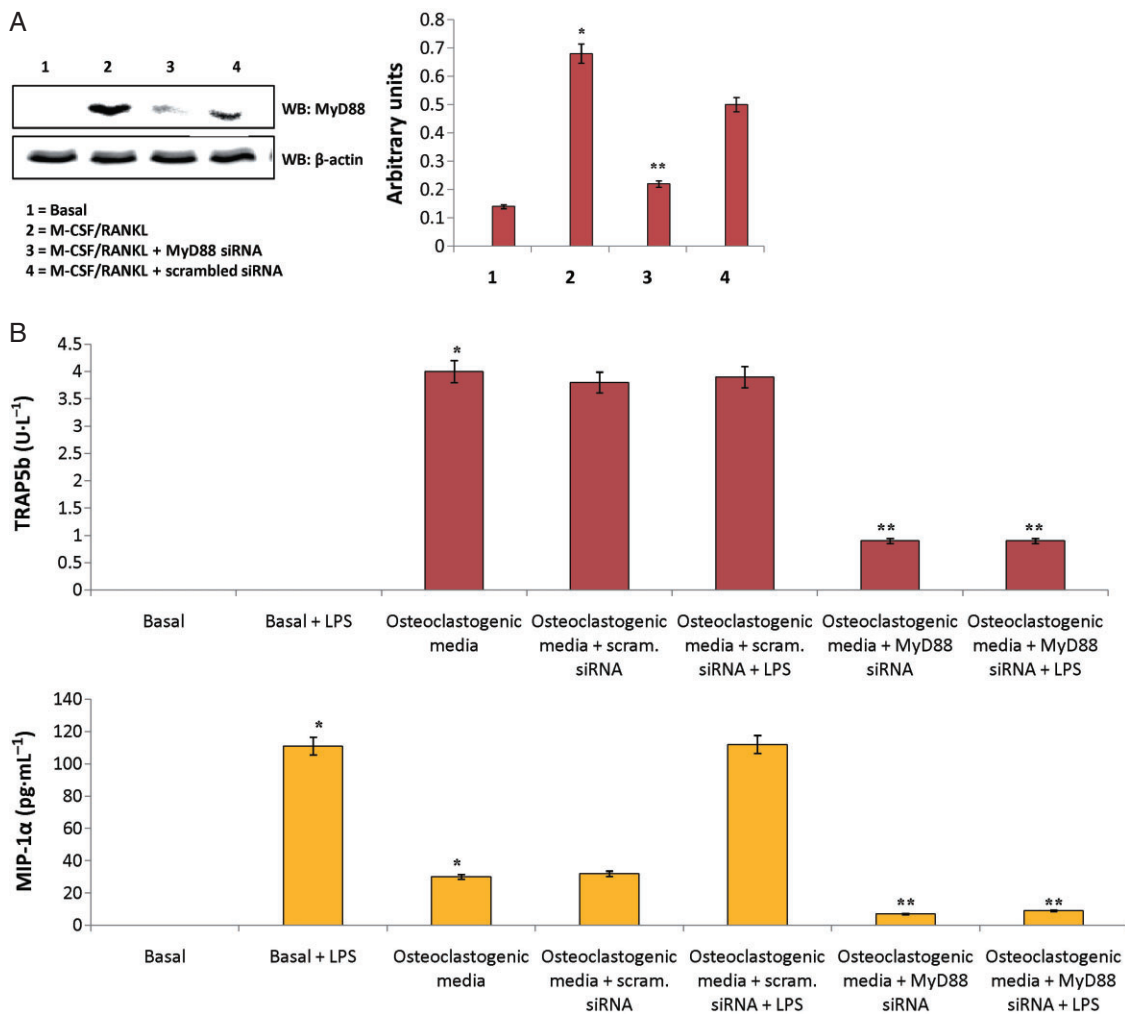


Figure 15

Effect of siRNA mediated knockdown of MyD88 on TRAP5b and MIP-1 α synthesis in developing osteoclast culture. (A) Western blot showing changes in the expression of MyD88 before and after siRNA transfection. siRNA transfections were performed with Lipofectamine RNAiMAX. Cells after stimulation with M-CSF (25 $\mu\text{g}\cdot\text{L}^{-1}$, days 1–11) and TNFSF11 (20 $\mu\text{g}\cdot\text{L}^{-1}$, days 6–20) were transfected either with MyD88 siRNA or scrambled siRNA at concentration of 40 nmol·mL⁻¹. Values are mean \pm SEM of four independent experiments. One of the four representative Western blots is given. (B) Changes in the levels of TRAP5b and MIP-1 α in response to MyD88 siRNA interference in developing osteoclasts stimulated with M-CSF (25 $\mu\text{g}\cdot\text{L}^{-1}$, days 1–11) and TNFSF11 (20 $\mu\text{g}\cdot\text{L}^{-1}$, days 6–20) *ex vivo*. Values are mean \pm SEM of four independent experiments. * $P < 0.05$ compared with basal. ** $P < 0.05$ compared with scrambled siRNA.

of a dose that has anti-resorptive effects without inducing hyperhomocysteinaemia. Although a clinical study by Delrieu *et al.* (1988) showed that methionine does not produce any adverse effect in arthritic patients at doses 5–10 g·day⁻¹, an effective prophylactic anti-resorptive dose of methionine in humans, remains to be established.

Conclusion

Methionine has an anti-resorptive effect by inhibiting the propensity of mononuclear cells to develop into functional osteoclasts via down-regulation of TLR4/MyD88/NF- κ B signalling (Figure 16), a novel therapeutic target that can be useful for the development of treatment regimens against bone disorders.

Author contributions

VV, AS and SG developed the concept and designed the experiment.

VV performed animal experiments, X-ray imaging, ELISA, cell culture experiments and interpreted the data.

MK performed animal experiments and immunoblot analysis.

KM performed animal experiments and multiplex analysis.

The article was drafted by VV, SG and AS.

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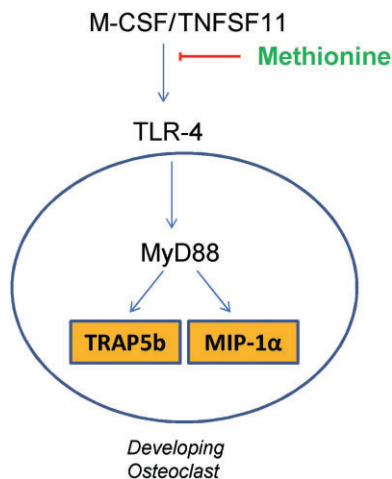


Figure 16

Graphical representation showing the effect of methionine on osteoclast development. Methionine inhibits the propensity of blood mononuclear cells to develop into functional osteoclasts in osteoporotic animals by down-regulation of the TLR4/MyD88/NF- κ B signalling pathway.

photographs of osteoclasts and Samit Chatterjee for help with multiplex analysis. AS is a JC Bose Fellow of the Department of Science and Technology, Government of India. VV and KM were supported with DBT-Postdoctoral Fellowship, Department of Biotechnology and Council of Scientific and Industrial Research, University Grants Commission, India, respectively. The work was financially supported by funding from National Institute of Immunology Core.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Appendix S1 Supplementary material.