



Published in final edited form as:

Open Bone J. 2014 ; 6: 8–17. doi:10.2174/1876525401406010008.

B Cell Production of Both OPG and RANKL is Significantly Increased in Aged Mice

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Abstract

Aging is a risk factor for osteoclastic bone loss and bone fracture. Receptor activator of NF- κ B ligand (RANKL) is the key effector cytokine for osteoclastogenesis and bone resorption, and is moderated by its decoy receptor osteoprotegerin (OPG). The development of an inflammatory environment during aging leads to increased bone resorption and loss of bone mineral density (BMD). Interestingly, animal and clinical studies show that OPG is actually increased in aging but fails to fully compensate for endogenous RANKL. Osteoblast- and B-lineage cells are significant sources of physiological OPG, however osteoblast OPG production declines with age, suggesting that elevated OPG in aging may be a consequence of changes in B cell function. In this study we examined BMD and indices of trabecular bone structure during aging, and B cell production of both RANKL and OPG in young and aged mice. Our data reveal significant loss of BMD and trabecular structure with age commensurate with significantly elevated concentrations of both OPG and RANKL in aged mice, and a decline in B cell populations in aged animals. Taken together our data suggest that B cells may be responsible for the elevated concentrations of OPG during aging and are essential to counteract excessive age-associated bone resorption. Paradoxically, B cells themselves likely contribute RANKL in aging and the loss of B cells with age may further contribute to the imbalance in OPG relative to RANKL that predisposes age-associated bone loss.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

Keywords

Aging; OPG; RANKL; B cells; Osteoporosis; Osteoclast

1. INTRODUCTION

Aging is a pivotal risk factor for bone loss and for the development of osteoporosis [1] a disease of the skeleton associated with increased risk of bone fractures.

Components of the immune and skeletal systems are deeply integrated and as a consequence, changes in immune function are transduced through the immuno-skeletal interface, an association of common cells and cytokine effectors that mediates distinct albeit different functions in the immune and skeletal systems. Alterations in immune function thus lead to significant changes in skeletal metabolism, often causing imbalanced bone turnover with consequent loss of BMD. Prominent cellular components of the immuno-skeletal interface include monocytes (the source of osteoclast precursors) and lymphocytes (both B cells and T cells).

Osteoclasts form in the bone marrow (BM) in the presence of permissive levels of Macrophage colony stimulating factor (M-CSF), and the key osteoclastogenic cytokine RANKL is moderated by its physiological decoy receptor OPG [2]. In conditions of physiological bone remodeling bone-forming osteoblasts have historically been considered to be an important source of OPG in the bone microenvironment. Interestingly, human B cells have also been recognized as a significant source of OPG [3] although the significance of this discovery for bone biology was not immediately appreciated. We recently reported that B cell deficiency in mice leads to elevated basal osteoclastic bone resorption and diminished BMD and volume, as a consequence of a bone marrow deficiency in OPG. Rescue studies involving transplantation of B cells back into B cell knock out (KO) mice restored OPG to wild type (WT) levels and prevented the development of osteoporosis [4]. Using WT mice to quantify B-lineage sources of OPG production we further demonstrated that mature B cells accounted for 45% of total bone marrow OPG production. In addition, B cell precursors, immature B cells and plasma cells further contributed OPG to the bone microenvironment with the entire B cell lineage accounting for up to 64% of total OPG production [4]. Interestingly, as previously reported for human B cells *in vitro* [3] CD40 costimulation by T cells led to enhanced B cell OPG production in mice *in vivo*, further defining an important role for T cell-mediated regulation of B cell OPG. Indeed bone marrow OPG production was significantly compromised by T cell deficiency and/or by genetic ablation of CD40 and CD40L costimulatory molecules leading to elevated bone resorption and reduced bone mass in each of these models [4]. Taken together these data demonstrated that B cell and T cell function is important for maintenance of basal bone homeostasis and the RANK/RANKL/OPG axis is thus a key mechanism by which immune cells regulate or disrupt physiological bone turnover [5–8]. Indeed, we have reported that a decline in B cell expression of OPG and a concomitant increase in B cell RANKL, drives bone loss in a rat model of HIV-infection [9], a malady long associated with osteoporosis in humans [6]. In a recent clinical study we further demonstrated the existence of this B cell

RANKL/OPG imbalance in the context of human HIV-infection, where the B cell RANKL/OPG ratio was significantly correlated with loss of BMD [10].

Numerous studies show that OPG production is also imbalanced in aged humans and rodents, and although osteoblast-specific production of OPG is typically reported to be diminished [11–13] surprisingly, total OPG production is routinely found to be elevated with age in both men and women [11–17]. The source of elevated OPG in aging remains unknown.

It is well established that aging leads to immunosenescence, a progressive decline in immune responsiveness to exogenous antigens and a paradoxical increase in autoimmunity [18]. Immunosenescence strongly impacts B cell lineages and may be a reflection of intrinsic B cell defects as well as altered microenvironment, including helper T cell function [18]. Aging further promotes accumulation of autoreactive T and B cells [19, 20] leading to a chronic inflammatory state that has been referred to as "Inflammaging" [21]. These disruptions in B cell function brought about by aging thus have the capacity to influence the immuno-skeletal interface impacting RANKL and OPG production and hence affecting the process of osteoclastic bone resorption.

In this study we examined changes in BMD and bone structure with aging in mice and quantified total serum RANKL and OPG, and bone marrow B cell RANKL and OPG production. We report that B cell OPG production significantly increase with aging, although B cells themselves contribute significant concentrations of RANKL in aged mice.

2. METHODS

All reagents were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO), unless otherwise indicated.

2.1. Mice

All animal studies were approved by the Emory University Animal Care and Use Committee and were conducted in accordance with the NIH Laboratory Guide for the Care and Use of Laboratory Animals.

Mice were housed under specific pathogen free conditions and were fed gamma-irradiated 5V02 mouse chow (Purina Mills, St. Louis, MO), and autoclaved water *ad libitum*. The animal facility was kept at $23 \pm 1^\circ\text{C}$, with 50% relative humidity and a 12/12 light/dark cycle.

Young (6–12 weeks of age) female C57BL6 WT were from Jackson Labs (Bar Harbor, ME) and aged (20–24 month old) mice were from the National Institute on Aging (NIA) aged mouse colony at Charles River Laboratories (Wilmington, MA).

2.2. Bone Densitometry

BMD (g/cm^2) quantifications were performed in anesthetized mice by Dual energy X-ray absorptiometry (DXA) using a PIXImus 2 bone densitometer (GE Medical Systems) and calibrated daily using a factory-supplied phantom. Anesthetized mice were placed on the

imaging tray in a prostrate position. Total body DXA was performed and region of interest boxes placed to quantify anatomical sites including lumbar spine and femur. The left and right femurs were averaged for each mouse and the mean used for group calculations. Cross sectional BMD measurements at specific ages including 2, 3, 4, 6, 10, 15 and 21 months were repurposed from baseline BMDs from multiple studies previously conducted at these time points. The short-term *in vitro* reproducibility is 1.7% [22].

2.3. Micro-Computed Tomography

Micro-Computed Tomography (μ CT) was performed in L3 vertebrae *ex vivo* to assess trabecular bone microarchitecture using a μ CT40 scanner (Scanco Medical AG, Bruettisellen, Switzerland) calibrated weekly with a factory-supplied phantom. A total of 500 tomographic slices at a voxel size of 6 μ m (70 kVp and 114 mA, and 200 ms integration time) were taken at the L3 vertebra (total area of 3.0 mm) and trabecular bone segmented from the cortical shell. Projection images were reconstructed using the auto-contour function for trabecular bone. Representative vertebral samples based on mean BV/TV were reconstructed in 3D to generate visual representations. Indices and units were standardized per published guidelines [23].

2.4. Biochemical Indices of Bone Resorption and Bone Active Cytokines

CTx was quantified in mice serum using RATlaps (CTx) ELISAs (Immunodiagnostic Systems Inc. Fountain Hills AZ). OPG or RANKL was quantified in serum using mouse specific ELISAs (MOP00 for mouse OPG and MTR00 for RANKL) R & D Systems, Minneapolis, MN.

2.5. Cell purification, and RANKL and OPG ELISAs

Bone marrow was extracted from long bones by insertion into a 0.5 ml centrifuge tube with a hole pierced into the bottom with an 18 gauge needle, and the tube inserted into a 1.5 ml centrifuge tube and spun at $13,500 \times g$ for 60 seconds. Splenocytes were recovered from spleens following disaggregation by gentle scraping with glass slides. Recovered bone marrow and splenocytes were gently resuspended in 15 volumes of red cell lysis buffer (1 mM EDTA; 100 mM KHCO₃, 1.7 M NH₄Cl; pH 7.3) for 10 minutes at room temperature followed by 2 washes with PBS. B cells were purified from bone marrow or spleen using immunomagnetic beads (Miltenyi Biotech, Auburn, CA).

Conditioned media was generated by plating 1×10^7 purified B lineage cells into 48 well plates in 1 ml/well of RPMI 1640 supplemented with 10% FBS and antibiotics. After 48 h media was collected for analysis of OPG or RANKL by mouse specific ELISAs (MOP00 for mouse OPG and MTR00 for RANKL) from R & D Systems.

2.6. Flow Cytometry

To quantify B cell populations in spleen and bone marrow single cell suspensions (10^6 cells/reaction) were prepared and surface stained in FACS buffer (phosphate-buffered saline (PBS) containing 1% Fetal Bovine Serum (FBS) for 30 minutes at 4°C, and washed once in FACS buffer. B cells were stained using CD45R (B220)-APC or IgD-FITC (eBioscience Inc. (San Diego, CA) and gates established using isotype and fluorochrome-matched

controls. Samples were acquired on an Accuri flow cytometer (BD Immunocytometry Systems).

2.7. Statistical Analysis

Statistical significance was determined using GraphPad InStat version 3.0 for Windows (GraphPad Software Inc. La Jolla, CA). Gaussian distribution was assessed using the Kolmogorov and Smirnov test. Simple comparisons were made using unpaired 2-tailed Student's *t* test or Mann-Whitney for non-parametric data. Multiple comparisons were performed using One-way ANOVA with Tukey's Multiple Comparison *post hoc* test. *P* < 0.05 was considered statistically significant. Data are presented as Mean \pm SD unless otherwise indicated.

3. RESULTS

In mice, bone modeling proceeds until peak BMD occurs at around 5 months, at which point bone remodeling begins [24]. The skeleton is further impacted by reproductive senescence at 12–15 months of age with a decline in estradiol production in females [25]. Age-associated immunosenescence begins at around 14 months of age [26] and mice are considered aged by around 24 months of age. Mice thus represent a time-compressed model of human skeletal, immunological and age-associated changes making them ideal for skeletal studies.

3.1. Bone Densitometry

BMD was quantified by DXA in mice at multiple ages including 2, 3, 4, 6, 10, 15 and 21 months. Our data (Fig. 1A) shows a gain in femoral BMD between 2 months of age and 10 months of age followed by a rapid decline in BMD between 10 and 15, and 15 and 21 months of age and declining to the original 2-month baseline by 21 months. By contrast BMD peaked by 4 months at the lumbar spine and then declined progressively thereafter falling below the 2-month baseline by 21 months. By contrast total body measurements were more stable showing a peak between 10 and 15 months with a slow decline after 10 months of age.

3.2. Micro-Computed Tomography

Because DXA underestimates the trabecular compartment we further performed high-resolution (6 μ m) μ CT of isolated vertebral bodies (a trabecular rich region) from mice just prior to immunological and reproductive senescence (12 months of age) and in aged mice (24 months of age). Representative 3D visual reconstructions are shown in Fig. (1B). Quantitative microarchitectural indices of trabecular bone structure were computed for multiple mice (Table 1) and reveal that vertebral trabecular bone volume fraction (BV/TV) was significantly decreased between the ages of 12 and 24 months. Interestingly, the decline in BV/TV was predominantly a consequence of an increase in tissue volume (TV) a reflection of total bone size with only a modest decline in bone volume (BV) that failed to reach statistical significance. Although the data suggest a continued increase in total bone size over the life of the mouse, overall BMD reflected by TV.D was significantly diminished in the aged mice suggesting an overall loss of bone mass. Diminished trabecular structure was reflected by a significant increase in trabecular separation (Tb. Sp.) a reflection of the

distance between trabeculae, while trabecular thickness (Tb. Th.) and trabecular number (Tb. N.) were significantly decreased between 12 and 24 months.

Taken together these data reveal a significant decline in trabecular BMD and volume with deteriorating structural indices following immunological senescence in mice.

3.3. Biochemical Indices of Bone Resorption

C-terminal Telopeptide of type I collagen (CTX), a specific and sensitive biochemical index of global bone resorption was quantified in serum of 12 and 24 month of mice and revealed a significance increase in bone resorption at 24 months (Fig. 1C).

3.4. Global Serum Levels of OPG and RANKL are Elevated During Aging in Mice

Several studies in humans have reported that serum OPG production increases with age in both men and women [11–17]. To validate the mouse as a model for aging studies we quantified OPG and RANKL in the serum of 3, 12 and 24 month old female mice and documented significant increase in both RANKL (Fig. 2A) and OPG (Fig. 2B).

3.5. B Cell OPG and RANKL Production During Aging

The role of B cells in the bone loss associated with aging in mice and humans is presently unclear. As B cells are a significant source of basal OPG, changes in B cell number and/or function, during aging, have the potential to impact osteoclastogenesis and bone resorption. We thus investigated whether aging affects B cell OPG and/or RANKL production. Bone marrow and spleens were harvested from young (2 months old) and mice approaching old age (21 months old) and B cells purified by immunomagnetic selection. B cell conditioned mediums were generated and OPG and RANKL production by B cells was quantified 48 h later by commercial ELISAs. Our data reveal that B cells isolated from the bone marrow of aged mice secrete ~25 fold more OPG than an equivalent number of young B cells (Fig. 3A). OPG production by splenic B cells from old mice (Fig. 3B) was similarly elevated although by a smaller magnitude (~ 12 fold). Interestingly, while no RANKL production was detectable by B cells isolated from the bone marrow or spleen of young mice, B cells from aged mice produced significant levels (Fig. 3C and D).

3.6. B Cell Numbers are Diminished in Aging Mice

In order to determine whether the C57BL6 mouse model used for our bone studies recapitulate the diminution of B cells associated with aging in humans [27] we quantified the B cell compartments of young mice (2 months of age) and old mice (21 months of age). Flow cytometric analysis of spleens and bone marrow from young and old mice did indeed reveal changes in B cell number. The spleens of aged mice contained 26% fewer B cells than in young mice (Fig. 4A). Similarly, B220⁺ cells in the bone marrow were decreased by 46% (Fig. 4B). The B220 marker represents mature B cells (B220^{Bright}) as well as immature B cells and B cell precursor populations (B220^{Dim}) showing up as 2 peaks (Fig. 4B). To further determine changes specifically in mature B cells we further stained bone marrow with IgD a marker of mature B cells, which revealed that mature B cells were diminished by 49% (Fig. 4C).

4. DISCUSSION

Bone loss ensues during aging as a consequence of enhanced osteoclastic bone resorption relative to bone formation. Previous studies have reported that peak bone strength is achieved by 5 months of age in mice [24]. Our studies show that shortly after attainment of peak BMD the mouse skeleton begins to lose bone mass, although the temporal effect was somewhat anatomical site specific with lumbar spine degeneration beginning shortly after peak BMD (around 4 months of age) while femoral BMD peaked later at around 10 months before declining. Interestingly total body BMD was observed to be relatively stable, peaking between 10 and 15 months of age, followed by a slow modest downward trend between 10 and 20 months. Because DXA grossly underestimates trabecular bone, the most metabolically active compartment, the apparent stability of total body BMD is likely a reflection of cortical bone, being lost more slowly. Trabecular bone, more predominant in the spine, is rapidly lost in mice likely accounting for the early decline in total BMD observed in the spine, while cortical-rich femoral bone is observed to be maintained for a longer period of time. μ CT analysis of the trabecular compartment of the lumbar spine revealed a significant decline in BV/TV, but interestingly this was largely a consequence of increased bone size (TV) likely due to ongoing periosteal bone apposition concurrent with endosteal bone resorption, leading to bone growth without significant trabecular bone gain and a net decline in trabecular BV/TV.

Reproductive senescence occurs between 12 and 15 months with a significant decline in estradiol production in female mice [25]. Immunosenescence in mice begins by 14 months [26] and both reproductive and immunosenescence may be key stages at which cortical bone loss begins and B cell RANKL and OPG production begin to change, although a detailed temporal analysis of OPG and RANKL production by B cells remains to be performed.

Surprisingly, animal and clinical studies suggest that OPG is increased with aging in men and women, despite elevated bone resorption. It has further been suggested that this rise in OPG may be a homeostatic mechanism to limit bone loss [14]. Interestingly, osteoblast derived OPG appears to decline with age and the source of elevated OPG in aging has remained unclear. Our data suggest that B cells are a likely source of these elevated OPG concentrations associated with aging.

Interestingly, B cells were not only associated with elevated concentrations of OPG, but also appear to be a significant source of RANKL in aged mice. The lack of RANKL expression in young animals is consistent with our previous study in which no difference in basal RANKL expression was observed between B cell replete and B cell deficient animals [4]. These data are further consistent with a recent study showing that conditional RANKL ablation in B cells fails to impact bone mass in estrogen-replete mice up to 7 months of age [28]. B cells have however been observed to secrete RANKL and may directly contribute to bone loss when activated as occurs in several pathological conditions including estrogen deficiency [28, 29], periodontal infection [30, 31], psoriatic arthritis [32] and HIV infection [9, 10].

Although aged B cells produced both OPG and RANKL, OPG production in the bone marrow outpaced that of RANKL by ~ 3 fold while spleen RANKL and OPG production was similar. Although the stoichiometry of the RANKL/OPG interaction *in vivo* is difficult to define, it is likely that an increase in RANKL would require more than an equivalent increase in OPG to fully compensate. Given that bone loss ensues in aging despite increased OPG (in mice and humans) the overall total increase in OPG from all sources is likely outpaced by the overall total increase in RANKL from all sources.

It should further be pointed out that we quantified soluble RANKL production by B cells only. While osteoblasts are associated predominantly with membrane bound RANKL, B cells are considered to produce mostly secreted RANKL, however, there may also be additional membrane associated RANKL that causes an increase in total RANKL exacerbating bone resorption. Furthermore, OPG is known to have other ligands beside RANKL, including TRAIL. Although we did not quantify TRAIL in our studies, it is possible that TRAIL may decoy some OPG. Thus membrane bound forms of RANKL and other OPG ligands may conspire to diminish the overall concentration of OPG available to neutralize RANKL.

Furthermore, TNF α has long been recognized as a key protagonist of bone loss in inflammatory conditions including rheumatoid arthritis [33], postmenopausal osteoporosis [7] and indeed in the context of aging [34]. TNF α can potentially synergize with RANKL [35] at the level of RANK signal transduction [36] to further increase its osteoclastogenic potency and to activate bone resorption [37]. The double onslaught of increased bioavailable RANKL (free RANKL) and TNF α may push up resorption to levels that cannot be compensated for by the increases in OPG.

Our data suggest that aging leads to an upregulation in OPG by B cells that compensates, in part, for elevated RANKL production, paradoxically also in part from B cells, thus partially mitigating age-associated bone resorption.

In adults, B lymphocytes are derived from hemopoietic stem cells (HSC) in the bone marrow that progress through a series of distinct developmental stages defined by various phenotypic markers and the rearrangement and expression of immunoglobulin genes [38]. Age-related defects in the proliferative potential of Common Lymphoid Progenitors (CLP) and their progeny, the pre-pro-B cells suggest that the ability of CLP to differentiate into pre-pro-B cells is compromised by immunosenescence [38]. It is generally agreed that B cell precursors are diminished with age as are memory B cells in the periphery [27, 39]. Clinical studies further report that both the frequency of circulating memory B cells and the absolute number are reduced in the elderly [40, 41]. Although it has been suggested that homeostatic expansion of antigen-experienced memory B cells may continue to compensate and fill available niches [42], it is also now becoming apparent that available niches for B cells in germinal centers and lymph nodes also declines with age, leading to diminished space for B cell engraftment [43]. Our data in aged mice confirm this reduction on B cell populations in the spleen, which shows a modest decrease, as well as a large decline in bone marrow mature B cells and B cell precursor populations. This decline in B cells may in part, limit the capacity of this population to provide adequate OPG to offset enhanced RANKL-mediated

bone resorption. By contrast, the decline in B cells may also limit the production of pro-resorptive RANKL produced.

In conclusion, our data suggest that age-associated alterations in B cell number and function lead to elevated B cell OPG production that likely protects against bone loss and limits its severity by compensating, in part, for elevated total and B cell RANKL production during aging. Our data suggest that B cells may represent a key target of future anti-osteoporotic therapies to ameliorate senile osteoporosis.

ACKNOWLEDGEMENTS

MNW is supported, in part, by a grant from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development (5I01BX0-00105) and by NIAMS grants (AR053607, AR056090 and AR059364) and NIA grant AG040013. The contents of this manuscript do not represent the views of the Department of Veterans Affairs, the National Institutes of Health or the United States Government.

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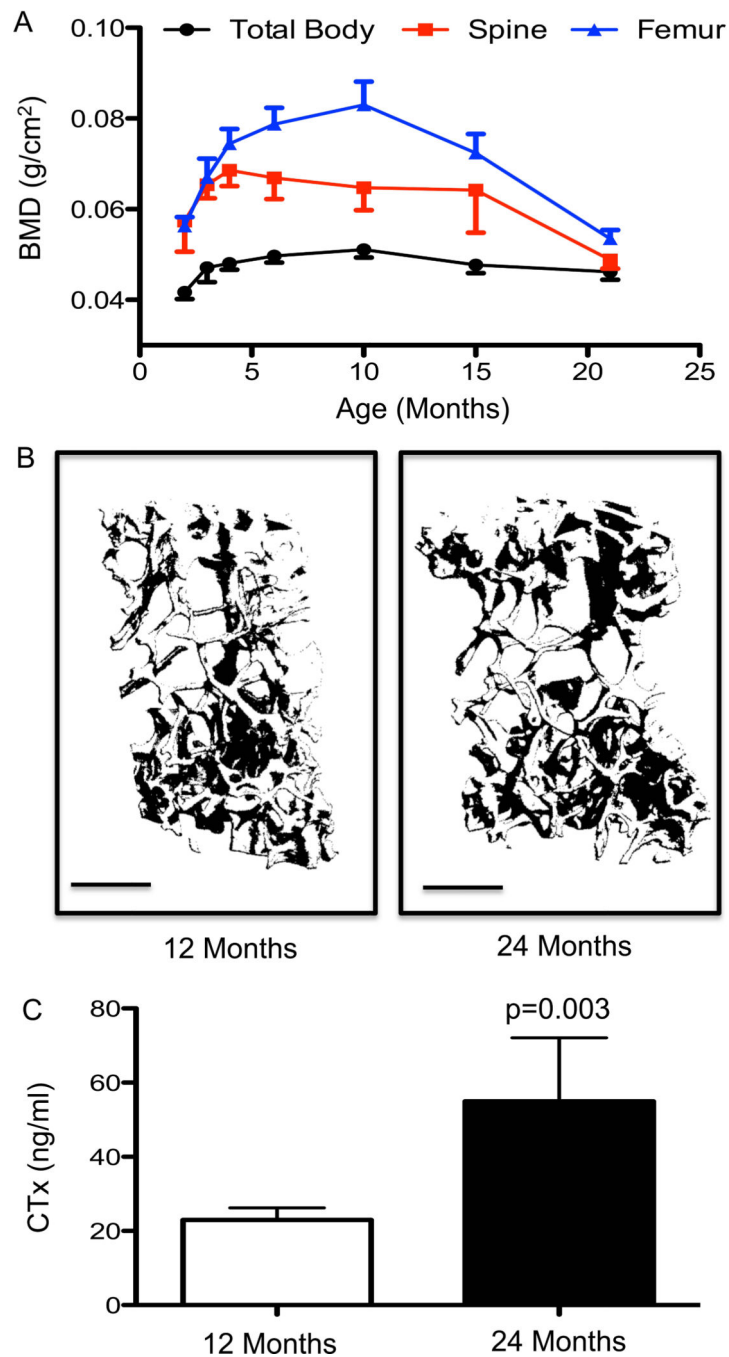


Fig. (1). Changes in BMD, trabecular bone structure and bone resorption in aged mice. **(A)** BMD was quantitated in female C57BL6 mice at 2, 3, 4, 6, 10, 15 and 21 months of age by DXA. Data presented for Lumbar Spine (Red), average of left and right femur for each mouse (Blue) and for total body (Black). N = 6–21 mice/data point. **(B)** Representative 3D μ CT reconstructions for L3 vertebrae from 12 and 24 month old mice. Black scale bar = 500 μ m. **(C)** Biochemical index (CTx) of *in vivo* bone resorption in 12 and 24 month old mice. N = 18 mice 12 months old and 5 mice 24 month old mice; Mann-Whitney test.

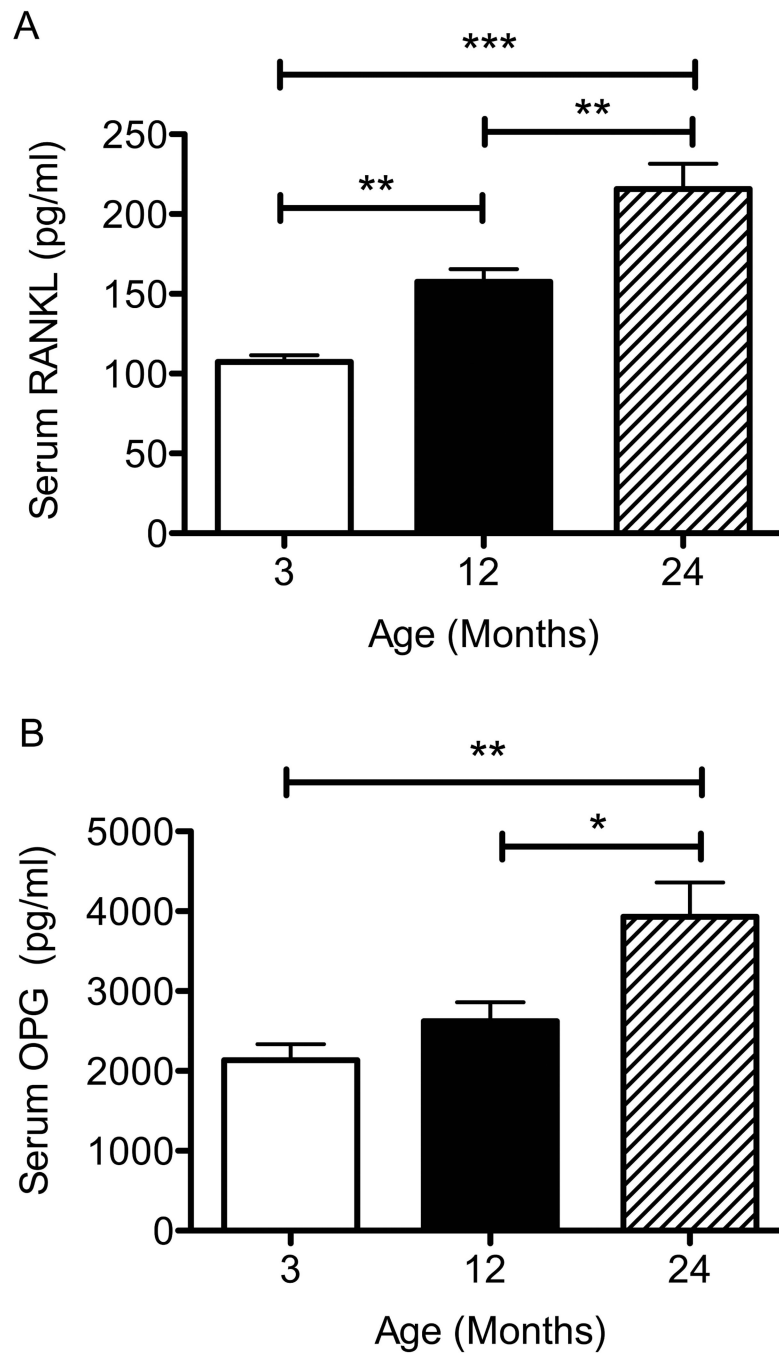


Fig. (2). Changes in serum OPG and RANKL production in young, mature and aged mice. The concentrations of circulating (A) RANKL and (B) OPG, were quantified in the serum of young (3 months), skeletally mature (12 months) and aged (24 month) female C57BL6 mice by ELISA. Data presented as mean \pm SEM, N=7–14 mice/group; *p 0.05; **p 0.01, ***p 0.001 by One-way ANOVA with Tukey's Multiple Comparison Test.

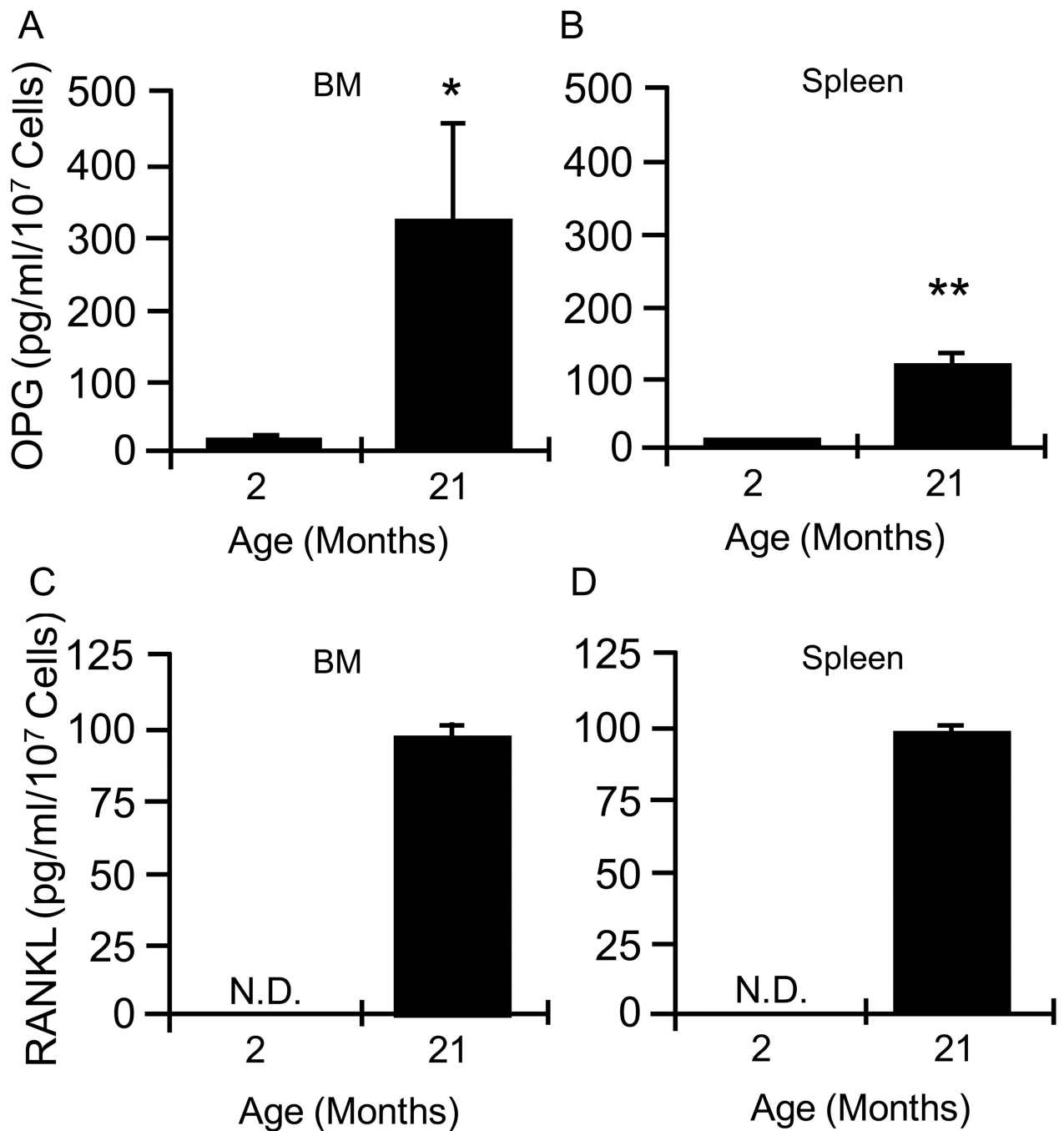


Fig. (3). Changes in OPG and RANKL production by B cells in young and aged mice. Production of OPG by B cells purified from (A) bone marrow (BM) and (B) spleen in young and old female C57BL6 mice. Production of RANKL by B cells purified from (C) bone marrow (BM) and (D) spleen in young and old mice. B cells were immunomagnetically purified from young (2 months old) and old (21 month old) mice. OPG and RANKL production from 1×10^7 cells was quantitated in 48 h conditioned mediums by ELISA. N=6 mice/group; *p 0.05; **p 0.01, Students t test.

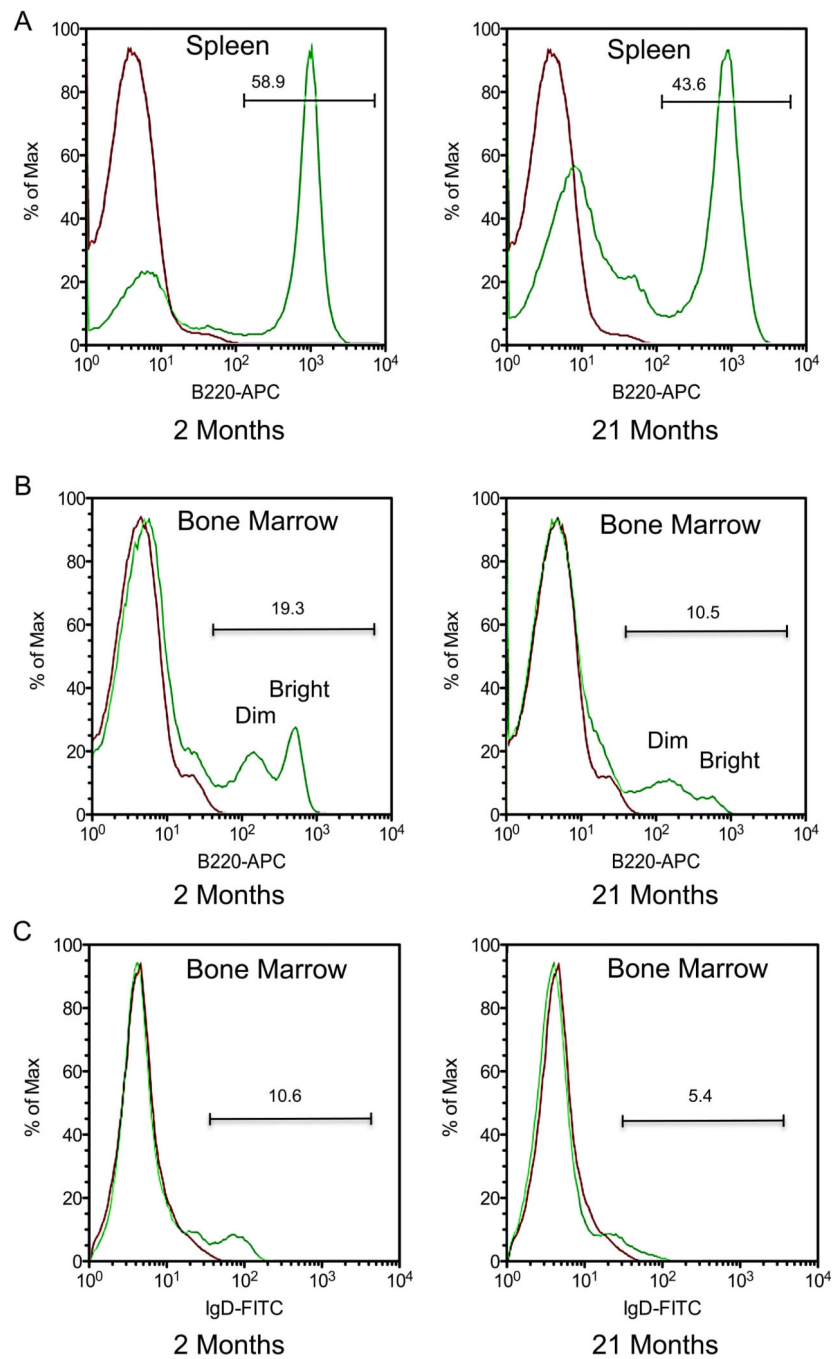


Fig. (4). B lineage cells are diminished in aged mice. Percentage of B220⁺ cells (total B lineage) in the (A) Spleen and (B) bone marrow (BM) of young (2 months of age) and old (21 months of age) female C57BL6 mice. (C) Mature (IgD⁺) B cells in the BM of 2 and 21 month old mice. Cells quantitated by Flow Cytometry. Isotype control shown in red.

Table 1

μ CT trabecular indices for lumbar spine in 12 and 24 month old mice.

Trabecular Indices	12 Month	24 Month	% Change	P
TV [mm ³]:	3.24 ± 0.089	3.87 ± 0.30	+19.5	<0.0001
BV [mm ³]:	0.3852 ± 0.0768	0.3446 ± 0.1002	-10.5	0.3681
BV/TV [%]:	0.1191 ± 0.0247	0.0892 ± 0.0251	-25.1	0.0262
Tb.Th [mm]:	0.0490 ± 0.0045	0.0433 ± 0.0051	-11.5	0.0288
Tb.N [/mm]:	2.81 ± 0.30	2.14 ± 0.38	-24.0	0.0011
Tb. Sp [mm]:	0.3596 ± 0.0386	0.4869 ± 0.0841	+35.4	0.0014
TV. D [mg HA/cm ²]	130.6 ± 31.8	91.0 ± 35.6	-30.4	0.0294

Trabecular indices: tissue volume (TV), trabecular bone volume (BV), trabecular thickness (Tb. Th), trabecular separation (Tb. Sp), trabecular number (Tb. N), trabecular total volumetric BMD (TV. D). Mean ± SD of 8 (12 month old) and 9 (24 month old) mice per group. P values from Student's t test.