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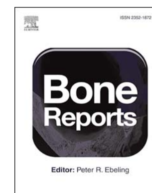

Lactobacillus acidophilus inhibits bone loss and increases bone heterogeneity in osteoporotic mice via modulating Treg-Th17 cell balance

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ABSTRACT

Osteoporosis is one of the most important but often neglected bone disease associated with aging and post-menopausal condition leading to bone loss and fragility. Probiotics have been associated with various immunomodulatory properties and have the potential to ameliorate several inflammatory conditions including osteoporosis. *Lactobacillus acidophilus* (LA) was selected as probiotic of choice in our present study due its common availability and established immunomodulatory properties. In the present study, we report for the first time that administration of LA in ovariectomized (ovx) mice enhances both trabecular and cortical bone microarchitecture along with increasing the mineral density and heterogeneity of bones. This effect of LA administration is due to its immunomodulatory effect on host immune system. LA thus skews the Treg-Th17 cell balance by inhibiting osteoclastogenic Th17 cells and promoting anti-osteoclastogenic Treg cells in ovx mice. LA administration also suppressed expression of osteoclastogenic factors (IL-6, IL-17, TNF-α and RANKL) and increased expression of anti-osteoclastogenic factors (IL-10, IFN-γ). Taken together the present study for the first time clearly demonstrates the therapeutic potential of LA as an osteo-protective agent in enhancing bone health (via tweaking Treg-Th17 cell balance) in postmenopausal osteoporosis.

1. Introduction

Osteoporosis is an increasingly common chronic condition of bones with > 200 million affected individuals worldwide (Laird et al., 2017) Osteoporosis is associated with reduced density and quality of bone leading to weakened skeleton thereby increasing the risk of fractures responsible for increased morbidity and mortality (Haczynski and Jakimiuk, 2001). In addition, osteoporosis will take a heavy toll on the economy with an estimated burden of USD 131.5 billion worldwide by 2050 (Haczynski and Jakimiuk, 2001). Postmenopausal osteoporosis is one of the commonly occurring skeletal diseases promoting bone resorption, enhancing bone loss and fragility in women over 45 years of age (Haczynski and Jakimiuk, 2001). Reduction in ovarian production of estrogen in postmenopausal phase is the main cause for initial phase of rapid bone loss in women, with an annual bone loss rate of 3–5% (Manolagas, 2010). The postmenopausal osteoporosis prevalence and severity are modulated by nutrition, smoking, body mass index, genetic factors and aging (Manolagas, 2010). At the cellular level, the central mechanism by which sex steroid deficiency induces bone loss is via an increase in osteoclast formation and osteoclast lifespan (Manolagas, 2010; D'Amelio et al., 2008). In humans, estrogen deficiency is associated with an expansion of RANKL and TNF-α expressing T cells (Zhao et al., 2016). Estrogen deficiency have been related for setting up of a chronic inflammatory state which in turn leads to bone loss, and this breaking up of the inflammatory cascade at any point can prove to be effective in alleviating bone loss in different animal models (Gao et al., 2007). Despite all the current treatments available for promoting bone health, the number of osteoporotic patients is on the rise worldwide. In addition, conventional bone loss treatments have unwanted side effects and are not always effective. Therefore, new approaches to enhance bone health are needed.

Recently the importance of probiotics as a driver of health had

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emerged as a novel approach for treating various inflammatory diseases including bone health. Probiotics are live microbial feed supplements that when administered in adequate amounts confer various health benefits to the host (Yousf et al., 2015). Several strains of *Lactobacillus* have been reported with therapeutic effect in experimental models of rheumatoid arthritis (RA), inflammatory bowel disease, atopic dermatitis etc. (Petersen et al., 2012). Various species of *Lactobacillus* such as *L. acidophilus*-NCFM and *L. salivarius* Ls-33 completely protected SCID mice from colitis (Narva et al., 2004). Several strains of *Lactobacillus* are now being employed as effective therapeutics for treatment of various diseases including osteoporosis, as administration of certain probiotics blunts bone loss that usually follows ovariectomy (Chiang and Pan, 2011; Ohlsson et al., 2014; Britton et al., 2014). This approach can be highly invaluable for prevention of increased bone joint destruction in patients where inflammation and bone loss cannot be prevented by established older methods. The mechanism of probiotics effecting bone health had been a topic of research in the last few years, surprisingly very few studies till date have fully dissected this nexus. Nevertheless, the direct clinical approach of administering probiotics can be a novel approach for treating bone loss in osteoporosis via modulating host immunity (Yousf et al., 2015; Britton et al., 2014; Li et al., 2016). This mechanism of probiotics induced blunting of inflammation can thus prove to be an effective and cheap alternative for future therapeutics in the field (Yousf et al., 2015; Britton et al., 2014; Li et al., 2016).

Regulatory T cells (Treg cells) inhibit osteoclastogenesis and are thus known protectors of bone health. Whereas Th17 cells induce osteoclastogenesis and are involved in enhanced bone loss in osteoporosis. Also, Treg cells inhibit the differentiation of Th17 cells and vice-versa (McGovern et al., 2012). This delicate homeostatic balance between Treg-Th17 cells is of utmost importance in various inflammatory conditions. The fact that intestinal microenvironment favours Treg cell generation (Omenetti and Pizarro, 2015) is the focus of our interest in the present study as to how probiotics induced Treg cells would regulate bone health. Surprisingly, very few studies till date have analyzed this connection. Also, the magnitude of Treg cell induction by different probiotics strains has still not been well characterized. A broader interpretation of this question would reveal many possible mechanisms whereby the probiotics may affect bone health. Thus, use of probiotic strains may afford opportunities for new interventions to improve bone health and reduce the risk for osteoporosis and fracture. We selected *Lactobacillus acidophilus* (LA), as probiotic of our choice in the present study, since no study has been done till date to delineate its role in mediating bone health. Also, the effect of LA on Treg-Th17 equilibrium is still not defined. LA is one of the most common probiotic strains available in our homes (curd, yoghurt, kefir). In our present study, we report for the first time that administration of LA due to its immunomodulatory effect on Treg-Th17 cell balance (enhanced Treg and decreased Th17 cells) in vivo, suppresses expression of osteoclastogenic factors (IL-6, IL-17, RANKL and TNF-α) and induces expression of anti-osteoclastogenic factors (IL-10 and IFN-γ) leading to enhanced bone mass. The present study highlights this interesting and novel field of bone biology (termed now as “Osteomicrobiology”) and will have immense clinical implications in near future (Dar et al., 2018; Jones et al., 2017). Administration of various probiotic strains can thus open new avenues in treatment of various inflammatory bone conditions such as osteoporosis and RA.

2. Methods

2.1. Animals

Thirty female mice (BALB/c) of 8–10 weeks with an average body weight of 25 g were selected. Mice were housed in standard cages, maintained under specific pathogen-free conditions and fed sterilized food and autoclaved water ad libitum. Mice were either ovariectomized (ovx) or sham operated with intra-peritoneal injection of Ketamine (100–150 mg/kg) and Xylazine (5–16 mg/kg) through standard protocol and were grouped under three categories with 10 mice in each group (Group A: No Probiotic + sham operated, Group B: ovx and Group C: ovx + LA). Ovx + LA group received daily oral dose of 200 µl (of 10^9 cfu/ml *Lactobacillus acidophilus* suspension) constituted in drinking water for a period of 6 weeks post-ovariectomy as per standardized protocols (Ohlsson et al., 2014; Britton et al., 2014), while control mice received normal water. At the end of experiment (6 week), animals were sacrificed for further analysis. All the procedures involving animals were conducted according to the requirements and with the approval of the Institutional Animal Ethics Committee of SIPS.

2.2. Lactobacillus acidophilus (LA) culture

Lactobacillus acidophilus (LA) ATCC 4356 was provided by Dr. Rajesh K. Mondal from Department of Microbiology, Dr. Harisingh Gour Central University, Sagar (MP)-470003, India. Briefly, LA were inoculated in deMan, Rogosa, and Sharpe broth (MRS; Difco) and grown at 37 °C for 20 h, then resuspended in phosphate-buffered saline (PBS) before administering to the mice with 200 µl suspension (10^9 cfu/ml *Lactobacillus acidophilus*).

2.3. Antibodies and reagents

The following antibodies/kits were bought from BD Biosciences (USA): PerCP-Cy-5.5 Rat Anti-Mouse CD4-(RM4-5) (550954), APC-Rat-Anti-Mouse-CD8a-(53-6.7) (561093), PE-Rat-Anti-Mouse-CD45R/B220-(RA3-6B2) (553089) and mouse Cytometric Bead Array-(CBA) kit. The reagents Anti-Human/Mouse Rorγt-PE-(AFKJS-9) (12-6988), Anti-Mouse/Rat Foxp3-APC-(FJK-16s) (17-5773), Foxp3/Transcription factor staining buffer (0-5523-00), RBC lysis buffer (00-4300-54) were obtained from eBioscience (USA). Anti-Mouse-CD254-(TRANCE-RANKL)-PE (IK22/5) (510005), Anti-Mouse-CD8-PE/Cy7 (53-6.7) (100721), Anti-Mouse-IFN-γ-FITC (XMG 1.2) (505805) and Anti-Mouse-IL-17A-PE (TC11-8H10.1) (506903) were obtained from BioLegend (USA). Mouse/Rat Estradiol ELISA Kit (Calbiotech, Spring Valley, CA, USA) and all procedures were followed according to the manufacturer's instruction.

2.4. Scanning electron microscopy (SEM)

For SEM analysis, femur cortical bone samples were kept in 1% Triton for 48–72 h and later transferred to 1 × PBS solution till final analysis is done. Bone slices were made and dried under incandescent bulb before SEM analysis and scanned in NOVA NANOSEM 450 microscope equipped with a tungsten filament gun operating at WD 10.6 mm and 20 kV. SEM images were digitally photographed at low (15 ×), intermediate (1000 ×), and high magnifications (10,000 ×) to picture the best cortical structure. Images were later processed and analyzed using Adobe Photoshop 7.0. The data was further analyzed using MATLAB software (Mathwork, USA).

2.5. Atomic force microscopy (AFM)

Femur cortical bone samples were dried in dust free environment with 60 W lamps for 6 h followed by high vacuum drying and subsequently analyzed by Atomic Force Microscope (INNOVA, ICON, Bruker), operating under the Acoustic AC mode (AAC or Tapping mode). This was assisted with cantilever (NSC 12(c) from MikroMasch, Silicon Nitride Tip) by NanoDrive™ version 8 software at a force constant of 0.6 N/m having resonant frequency at 94–136 kHz. The images were taken in air at room temperature at the scan speed of 1.5–2.2 lines/s. The data analysis was done using of Nanoscope analysis software. The data was further analyzed using MATLAB software (Mathwork, USA).

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