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Short communication

Regulation of bone mass by the gut microbiota is dependent on NOD1 and NOD2 signaling

Claes Ohlsson[a], Giulia Nigro[b], Ivo Gomperts Boneca[c,d], Fredrik Bäckhed[e], Philippe Sansonetti[b], Klara Sjögren[a,*]

[a] Centre for Bone and Arthritis Research, Institute of Medicin, Sahlgrenska Academy at University of Gothenburg, SE-413 45 Gothenburg, Sweden
[b] Institut Pasteur, Unité de Pathogénie Microbienne Moléculaire, 75724 Paris, France
[c] Institut Pasteur, Biology & Genetics of the Bacterial Cell Wall Unit, Department of Microbiology, 75724 Paris, France
[d] INSERM, Equipe Avenir, 75015 Paris, France
[e] Center for Cardiovascular and Metabolic Research/Wallenberg Laboratory, Sahlgrenska Academy at University of Gothenburg, SE-413 45 Gothenburg, Sweden

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ABSTRACT

Germ-free (GF) mice have increased bone mass that is normalized by colonization with gut microbiota (GM) from conventionally raised (CONV-R) mice. To determine if innate immune signaling pathways mediated the effect of the GM, we studied the skeleton of GF and CONV-R mice with targeted inactivation of MYD88, NOD1 or NOD2. In contrast to WT and *Myd88*$^{-/-}$ mice, cortical bone thickness in mice lacking *Nod1* or *Nod2* was not increased under GF conditions. The expression of *Tnfα* and the osteoclastogenic factor *Rankl* in bone was reduced in GF compared to CONV-R WT mice but not in *Nod1*$^{-/-}$ or *Nod2*$^{-/-}$ mice indicating that the effect of the GM to increase *Tnfα* and *Rankl* in bone and to reduce bone mass is dependent on both NOD1 and NOD2 signaling.

1. Introduction

We have shown that absence of GM in germ-free (GF) mice results in increased bone mass associated with altered immune status in bone and reduced bone resorption and that colonization of GF mice with a normal GM results in a normalisation of bone mass and immune status in bone [1]. Antibiotic administration increases bone density in young mice supporting a role of the GM in the regulation of bone mass [2]. In addition, probiotics limit bone-loss following ovariectomy (ovx) and GF mice are protected from trabecular bone-loss induced by sex-steroid depletion [3–6]. It has been proposed that the GM influences bone mass via an effect on the immune system but the possible role of different innate immune signaling pathways for the effect of the GM on bone mass is unknown.

In the gut the innate immune system recognizes bacteria and other infectious agents by pattern recognition receptors such as toll-like receptors (TLRs) situated on the cell-surface. There are alternative signaling pathways but most TLR signaling is mediated via the adaptor protein MYD88 to activate MAP kinase and nuclear factor kappa B-driven pro-inflammatory signaling [7]. Bacterial recognition also takes place in the cytoplasm by the Nod-like receptors (NLRs), NOD1 and NOD2. They bind bacterial peptidoglycan and after that a common protein kinase, RIP2, is recruited which in turn activates the NFκB signaling pathway leading to expression of genes such as cytokines and chemokines. Although the NOD1 and NOD2 proteins elicit a similar inflammatory response their location and function differ. *Nod1* is expressed in most cell types and induces pro-inflammatory signaling after detection of diaminopimelic acid-type peptidoglycan found mainly in Gram-negative bacteria [8]. *Nod2* is broadly expressed in cells of myeloid origin and to some extent in lymphoid and non-hematopoietic cells, including intestinal epithelial stem cells [9,10]. NOD2 detects all types of peptidoglycans found in Gram-positive and Gram-negative bacteria. The outcome of the activation of both TLRs and NLRs is an inflammatory response [7].

To determine the possible role of different innate immune signaling pathways for the effect of the GM on levels of the pro-inflammatory cytokine *Tnfα* in bone and on bone mass, we evaluated the skeleton in GF and CONV-R mice with targeted inactivation of *Myd88*, *Nod1* or *Nod2*.

2. Material and methods

2.1. Mice

At the gnotobiotic facility at the University of Gothenburg, female GF C57Bl6/J mice were maintained in flexible plastic film isolators

* Corresponding author at: Institute of Medicine, Centre for Bone and Arthritis Research, Vita Stråket 11, SE-413 45 Gothenburg, Sweden.
E-mail address: Klara.Sjogren@medic.gu.se (K. Sjögren).

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under a strict 12-h-light cycle (lights on at 06:00 h). Sterility was routinely confirmed by culturing and PCR analysis from faeces using universal bacterial primers amplifying the 16S rRNA gene (8F, AGAGTTTGATCCTGGCTCAG; 338R, TGCTGCCTCCCGTAGGAGT). Age-matched female CONV-R C57Bl6/J mice were transferred to identical isolators at weaning. Both groups of mice were fed an autoclaved chow diet (Labdiet, St. Louis, MO) ad libitum. The *Myd88*$^{-/-}$ mice were initially purchased (Jackson Laboratory, Bar Harbor, ME, USA). The *Myd88*$^{-/-}$ were backcrossed at least eight generations to C57Bl6/J and the last two crossings were performed using mice from our colony. GF and CONV-R mice were separated by a maximum of three generations. The study protocols were approved by the University of Gothenburg Animal Studies Committee.

Female GF WT control, *Nod1*$^{-/-}$ and *Nod2*$^{-/-}$ mice on a C57Bl6/J background were maintained at the gnotobiotic facility at Institut Pasteur. *Card4/Nod1*-deficient (*Nod1*$^{-/-}$) mice were initially generated by Millennium Pharmaceuticals Boston, MA, USA. *Card15/Nod2*-deficient C57Bl/6J (*Nod2*$^{-/-}$) mice were provided by J.-P. Hugot (Hôpital Robert Debré, Paris, France) [11]. The *Nod1*$^{-/-}$ and *Nod2*$^{-/-}$ mice were backcrossed at least 8 times to C57Bl6/J. All mice were kept under specific-pathogen-free conditions, and all animal experiments were approved by the committee on animal experimentation of the Institut Pasteur and by the French Ministry of Agriculture.

Blood was collected from the axillary vein under anesthesia with Ketalar®/Domitor® vet and the mice were subsequently killed by cervical dislocation. Tissues for RNA preparation were immediately removed and snap frozen in liquid nitrogen for later analysis. Bones were excised and fixed in 4% paraformaldehyde.

2.2. RNA Isolation and Real Time PCR (RT-PCR)

Total RNA was prepared from bone using RNeasy Mini Kit (Qiagen, CA, USA). The RNA was reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (#4368814, Applied Biosystems, Stockholm, Sweden). RT-PCR analyses were performed using predesigned RT-PCR assays from Applied Biosystems for the analysis of Tumor necrosis factor alpha (*Tnfα*, Mm00443258_m1), receptor activator of nuclear factor-κB ligand (*Rankl*, Mm00441908_m1 and cathepsin K (*Ctsk*, Mm00484036_m1). The mRNA abundance of each gene was adjusted for the expression of *18S* (4308329) ribosomal RNA.

2.3. High-resolution μCT

High-resolution μCT analyses were performed on the distal femur by using an 1172 model μCT (Bruker micro-CT, Aartselaar, Belgium). The femurs were imaged with an X-ray tube voltage of 50 kV and current of 201 μA, with a 0.5-mm aluminium filter. The scanning angular rotation was 180° and the angular increment 0.70°. The voxel size was 4.48 μm isotropically. The NRecon (version 1.6.9) was employed to perform the reconstruction following the scans. Cortical measurements were performed in the diaphyseal region of femur starting at a distance of 3.59 mm from the growth plate and extending a further longitudinal distance of 134.5 μm in the proximal direction.

2.4. Statistical analysis

We used GraphPad Prism for all statistical analysis. Results are presented as the means ± SEM. Between-group differences were calculated using unpaired t-tests, GF vs. CONV-R. A two-tailed $p \leq 0.05$ was considered significant.

3. Results

We have previously shown that 7-week-old C57bl6/J mice raised GF have increased cortical bone area of the femur compared to CONV-R mice [1]. In a study by Li J-Y et al. similar results, with increased cortical area and thickness of GF compared to CONV-R mice were found in the femur of 20-week-old C57bl6/J mice [6]. In the present study, we used 9-week-old C57bl6/J mice from the gnotobiotic facility at the Pasteur Institute, France and 10-week-old C57bl6/J mice from the gnotobiotic facility at the University of Gothenburg, Sweden, to determine the possible role of different innate immune signaling pathways for the effect of the GM on bone mass. Similar to earlier studies, we found that WT mice raised GF in the Pasteur Institute had increased cortical thickness of the femur diaphysis compared to CONV-R mice ($p < 0.01$; Fig. 1A). We hypothesized that the GM might regulate bone mass via TLR signaling and we, therefore, studied mice lacking MYD88, which mediates most TLR signaling in the innate immune system. *Myd88*$^{-/-}$ mice had a pronounced increase in cortical bone mass when they were raised GF compared to CONV-R, demonstrating that the effect of the GM on bone mass is independent of the adaptor protein MYD88 ($p < 0.01$; Fig. 1B). In contrast, mice with a targeted inactivation of *Nod1* or *Nod2* displayed no increase in cortical bone mass in the GF state, demonstrating that the effect of GM on cortical bone mass is dependent on both NOD1 and NOD2 signaling (Fig. 1C, D).

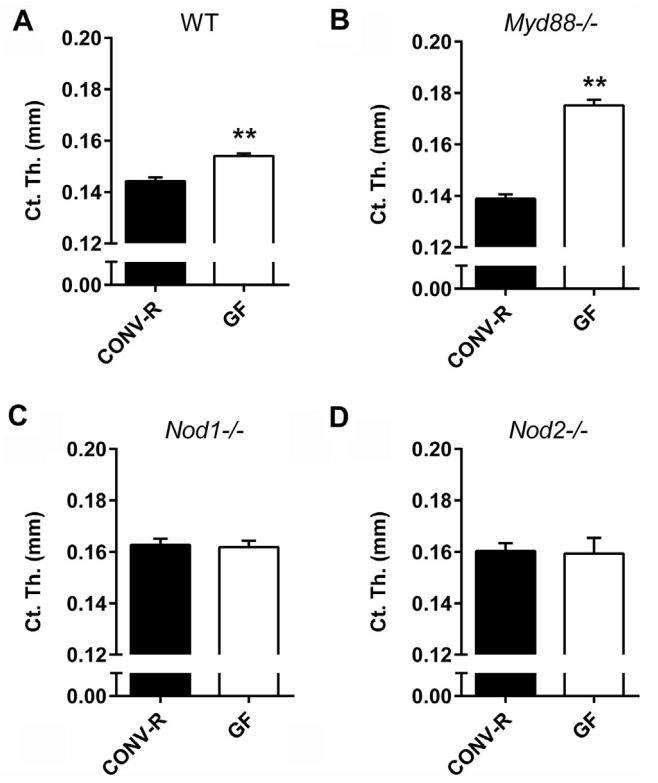

Fig. 1. The increased cortical bone mass in germ-free mice is dependent on NOD1 and NOD2 signaling. Cortical thickness (Ct Th) was measured at 9–10 weeks of age by μCT in the mid-diaphyseal region of femur in mice raised either conventionally (CONV-R) or germ-free (GF); (A) WT mice, (B) *MyD88*$^{-/-}$ mice, (C) *Nod1*$^{-/-}$ mice and (D) *Nod2*$^{-/-}$ mice. Values are given as mean ± SEM, (n = 4–6). ** $p \leq 0.01$. Student's *t* test, GF vs. CONV-R.

We have earlier shown that the increased bone mass in GF compared to CONV-R mice is associated with increased levels of pro-inflammatory cytokines and increased formation of bone resorbing osteoclasts [1]. To investigate the mechanism for the effect of the GF state on cortical bone in the different mouse-models, we measured expression of pro-inflammatory cytokines in bone. The expression of *Tnfα*, an inflammatory cytokine produced by immune cells that promotes osteoclastogenesis, was decreased in femur of GF compared to CONV-R WT mice but not in GF compared to CONV-R mice deficient of NOD1 or NOD2, demonstrating that the stimulatory effect of the GM