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Interleukin-6 signaling in osteoblasts regulates bone remodeling during exercise

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ABSTRACT

Aerobic exercise has many beneficial effects on human health. One of them, is to influence positively bone remodeling through, however, incompletely understood mechanisms. Given its recently demonstrated role as a mediator of the bone to muscle to bone crosstalk during exercise, we hypothesized that interleukin-6 (IL-6) signaling in bone may contribute to the beneficial effect that exercise has on bone homeostasis. In this study, we first show that aerobic exercise increases the expression of *Il6r* in bones of WT mice. Then, we analyzed a mutant mouse strain that lacks the IL-6 receptor alpha specifically in osteoblasts ($Il6r_{osb}^{-/-}$). As it has been reported in the case of $Il6^{-/-}$ mice, in sedentary conditions, bone mass and remodeling were normal in adult $Il6r_{osb}^{-/-}$ mice when compared to controls. In contrast, $Il6r_{osb}^{-/-}$ mice that were subjected to aerobic exercise did not show the increase in bone mass and remodeling parameters that control littermates demonstrated. Moreover, $Il6r_{osb}^{-/-}$ mice undergoing aerobic exercise showed a severe impairment in bone formation, indicating that activation of bone-forming cells is defective when IL-6 signaling in osteoblasts is disrupted. In sum, this study provides evidence that a function of IL-6 signaling in osteoblasts is to promote high bone turnover during aerobic exercise.

1. Introduction

Endurance or aerobic exercise is a physiological function needed for survival in the wild that has been conserved throughout evolution [1]. Aerobic exercise has many beneficial effects on health, one them being to promote bone remodeling [2–6]. Accordingly, a decrease in physical activity in humans often leads to a decline of bone mass and an increase in the incidence of fractures. This explains why exercise is recommended as a non-pharmacological therapy to prevent osteoporosis [6–10]. Even though clinical studies overwhelmingly support the notion that exercise improves bone quality in children [11] and reduces the risk of fractures in adults [12], the cellular and molecular mechanisms by which exercise may favor bone remodeling are still incompletely understood.

Interleukin-6 (IL-6) is a cytokine that exerts multiple functions in various tissues and organs. In bone IL-6 stimulates osteoclast formation in conditions like estrogen deficiency [13] and in severe skeletal diseases [14–16]. IL-6 is also the first myokine (cytokine produced by the muscle) ever described [17–22]. IL-6 circulating levels increase following aerobic exercise and IL-6 promotes exercise capacity [23]. Recently, cell-specific deletion experiments performed in the mouse showed that the increase in circulating IL-6 levels during exercise is triggered by signaling of the bone-derived hormone osteocalcin (OCN) into myofibers [24,25]. Moreover, once released from muscle, IL-6 goes back to bone, where it binds to osteoblasts through IL-6 receptor (IL-6R) to promote *Rankl* expression, bone resorption, and thereby the decarboxylation and activation of OCN [25].

These observations, along with the fact that, unchallenged $Il6^{-/-}$ mice do not exhibit any overt bone phenotype at rest [26], suggest the possibility that any function IL-6 signaling in bone cells may have could only be detected when circulating levels of this cytokine are high such as for instance during aerobic exercise. To test this hypothesis, we analyzed bone mass and bone remodeling parameters in mice lacking the IL-6R specifically in osteoblasts before or after aerobic exercise. Our analysis provides evidence that IL-6 signaling in osteoblasts may be specifically required in vivo for the regulation of bone turnover during aerobic exercise.

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Table 1
Sequence of primers used for genotyping and qPCR.

Mouse genotyping	
Strain	Sequence 5′ - 3′
Ocn-Cre	F: CAAATAGCCCTGGCAGATTC R: TGATACAAGGGACATCTTCC
Il6r flox	F: CCGCGGGCGATCGCCTAGG R: CCAGAGGAGCCCAAGCTCTC R: TAGGGCCCAGTTCCTTTAT

qPCR	
	Sequence 5′ - 3′
Actb For	CATTGCTGACAGGATGCAGAAGG
Actb Rev	TGCTGGAAGGTGGACAGTGAGG
Alpl For	CCAGAAAGACACCTTGACTGTGG
Alpl Rev	TCTTGTCCGTGTCGCTCACCAT
Bglap For	GCAATAAGGTAGTGAACAGACTCC
Bglap Rev	CCATAGATGCGTTTGTAGGCGG
Col1a1 For	CAGGGTATTGCTGGACAACG
Col1a1 Rev	TTGTTTGCCAGGTTCACCAGA
Gapdh For	CATCACTGCCACCCAGAAGACTG
Gapdh Rev	ATGCCAGTGAGCTTCCCGTTCAG
Il6 For	TACCACTTCACAAGTCGGAGGC
Il6 Rev	CTGCAAGTGCATCATCGTTGTTC
Il6ra For	TGCAGTTCCAGCTTCGATACCG
Il6ra Rev	TGCTTCACTCCTCGCAAGGCAT
Runx2 For	CCTGAACTCTGCACCAAGTCCT
Runx2 Rev	TCATCTGGCTCAGATAGGAGGG
Sp7 For	GGCTTTTCTGCGGCAAGAGGTT
Sp7 Rev	CGCTGATGTTTGCTCAAGTGGTC

2. Methods

2.1. Mouse studies

Mice harboring a floxed allele of *Il6ra* (*Il6r*$^{f/f}$ mice) [27] were kindly donated by Jens Brüning (Max Planck Institute for Metabolism Research, Cologne, Germany). To generate mice with osteoblast-specific deletion of *Il6ra* (hereinafter *Il6r*), *Il6r*$^{f/f}$ mice were crossed with *Ocn-cre* mice (#019509, The Jackson Laboratory) to obtain *Ocn-cre;Il6r*$^{f/f}$ (*Il6r*$^{-/-}_{osb}$). Mouse genotyping was performed using oligonucleotide sequences reported in Table 1. Floxed littermates without Cre transgene (*Il6r*$^{f/f}$) were used as controls.

Six-month-old C57Bl/6 (WT, The Jackson Laboratory, USA), *Il6r*$^{-/-}_{osb}$ *and Il6r*$^{f/f}$ (control) female mice were familiarized and trained to run on a treadmill apparatus for 4 days (17 min/day, increasing speed from 10 to 30 cm/s). On experimental day 5, mice were acclimated to the treadmill for 5 min, and then exercised for 10 min by running at a constant speed of 17 cm/s, followed by a gradual speed increase to 30 cm/s until exhaustion (Fig. 1A).

2.2. Biochemical analysis

Immediately after running, the mice were sacrificed by carbon dioxide inhalation, and whole blood was collected for analysis. Blood was left clotting for 30 min and centrifuged for 15 min at 4 °C for serum separation. Circulating IL-6 levels were determined using a specific ELISA kit (#ab100712, Abcam, Cambridge, UK) following the manufacturer's instructions.

2.3. Gene expression analysis by quantitative PCR

Mice were euthanized immediately after the end of the last run and muscles and bone segments were snap-frozen in liquid nitrogen and kept at −80° until use. Quadriceps and whole femurs (that contained bone marrow) were homogenized by Mikro-Dismembrator U (Gottingen, Germany) and total RNA was isolated using the TRI Reagent® (ThermoFisher Scientific, Waltham, USA) protocol. Reverse transcription was performed by using PrimeScript RT Reagent Kit (Takara, Japan). cDNA samples were used as templates for quantitative PCR (qPCR) analysis on a 7500 Fast Real-Time PCR System (Applied Biosystem, Waltham, MA, USA), performed using PowerUP Sybr Green (ThermoFisher Scientific) and specific primers (Table 1). Gene expression levels of each gene were normalized to *Gapdh* or *Actb* expression.

2.4. Histology

Mice were injected with 30 mg/kg of calcein (Sigma-Aldrich, Saint Louis, MO, USA) one day and 4 days after the last run and euthanized 6 days after the last run (Fig. 4A). Following sacrifice, skeletal segments were dissected and processed for methyl methacrylate (MMA) embedding as described previously [28]. Briefly, bone samples were fixed in 4 % formaldehyde for 24 h and dehydrated through a series of increasing ethanol concentrations. Bones were then infiltrated for 3 days with a plastic embedding mixture containing MMA, butylmethacrylate, methylbenzoate, polyethylene glycol 400 and dry benzoyl peroxide. The polymerization mixture was prepared by adding *N*,*N*-dimethyl-p-toluidine to the infiltrating solution. Sections of 4–7 μm in thickness were cut from MMA blocks, dried at 60 °C and deplasticized with 2-methoxyethylacetate before staining.

2.5. Bone histomorphometry

Quantitative bone histomorphometry was conducted on the 3rd and 4th lumbar vertebrae and tibiae. Experiments were performed in a blinded fashion by a single experimentalist, using Osteomeasure software (OsteoMetrics, Decatur, GA, USA) and following standard procedure and nomenclature [29]. The region of interest was drawn in the secondary spongiosa of proximal tibia, 300 μm below the growth plate and between the two growth plates in lumbar vertebrae. Von Kossa/Van Gieson-stained sections were used to measure trabecular bone volume per tissue volume (BV/TV), while toluidine blue staining was performed to measure osteoblast number per bone surface (N.Ob/BS) and osteoblast surface per bone surface (Ob.S/BS). TRAP-stained sections were used to measure osteoclast number per bone surface (N.Oc/BS) and osteoclast surface per bone surface (Oc.S/BS).

For dynamic bone histomorphometry, evaluation was performed on lumbar vertebra sections, where length of calcein fluorescent single (sLS) and double labeling (dLS) were measured, together with inter label thickness (Ir.L.Th). dLS occurs when a site of bone formation incorporates both calcein injections. sLS may instead reflect one of two situations: 1) sites with scarce bone formation activity that incorporate the first injection of calcein but in which the second injection is hidden or not incorporated at all because mineralization speed is low; 2) bone surfaces that activate new bone formation within the interval between the two calcein injections, so that only the second injection of calcein is incorporated in the mineralizing surface [30]. sLS and dLS were reported as normalized on bone surface (BS) and together with Ir.L.Th were used to obtain derived kinetic indices such as mineralizing surface [MS/BS, calculated as dLS+(0.5 × sLS)/BS and indicating the proportion of bone surface upon which new mineralized bone is deposited during the period of calcein labeling], mineral apposition rate (MAR, calculated as Ir.L.Th/Ir.L.t and showing the speed at which the osteoblast makes bone) and bone formation rate [BFR/BS, calculated as MAR × (MS/BS) and indicating the activity of osteoblasts in making bone in the unit of time].

Pictures were acquired with an optical microscope (Zeiss Axiophot, Jena, Germany) through a digital camera (Jenoptik ProgrRes C5, Jena, Germany).

2.6. Cell culture

Calvaria osteoblasts were isolated from five 3-day-old C57Bl/6 mice.

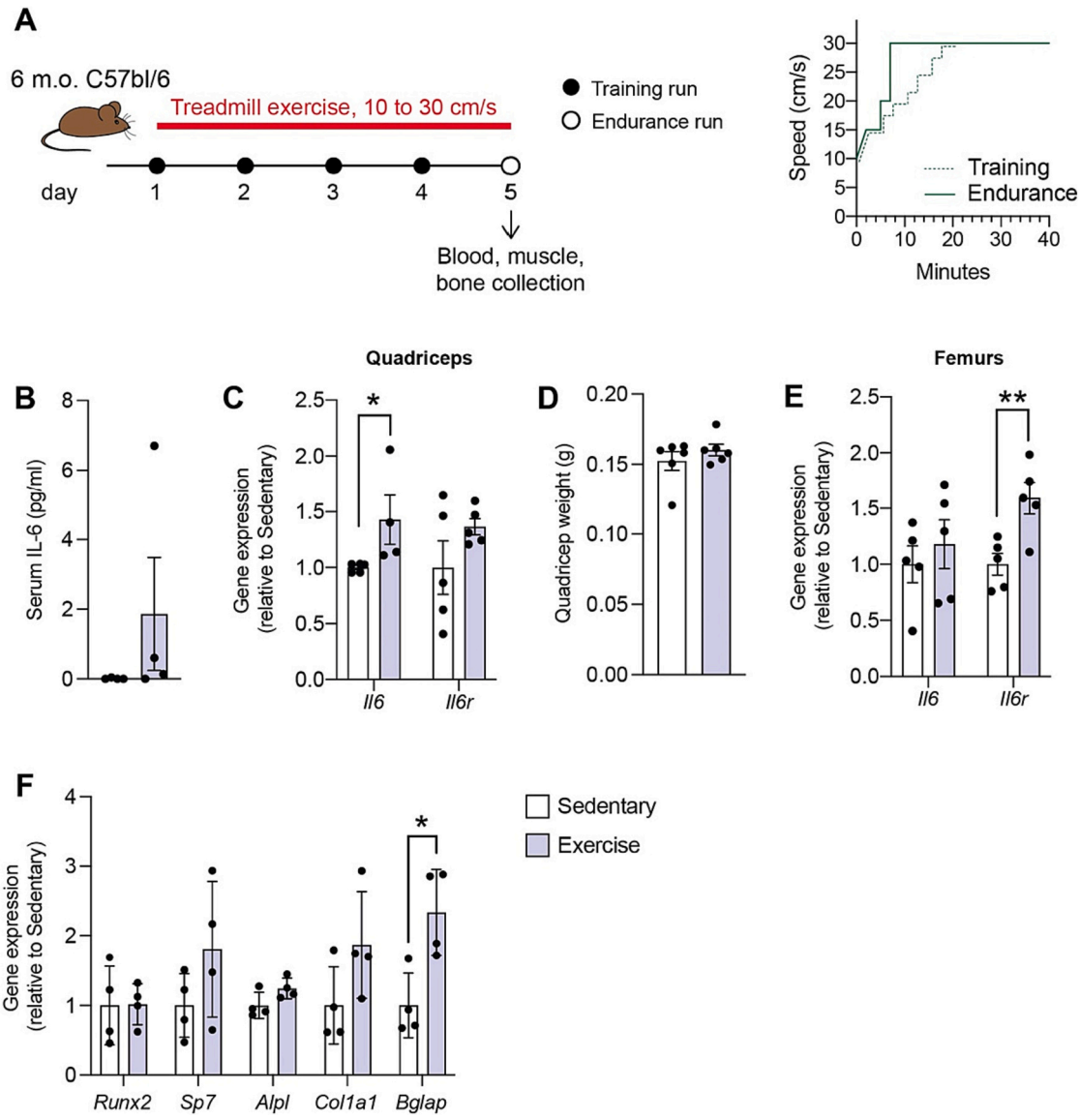

Fig. 1. Experimental design, circulating IL-6 levels and gene expression analysis in sedentary or exercising 6-month-old WT C57Bl/6 mice. A) Experimental scheme and regimen of the treadmill exercise. Mice were trained 20 min per day for 4 days at increasing speed; the 5th day, mice performed 40-minute endurance run, they were euthanized immediately afterward. B) Circulating IL-6 levels measured in WT mice. C) *Il6* and *Il6r* expression in quadriceps. D) Weight of quadricep muscle. E) *Il6* and *Il6r* expression in femurs of WT mice. F) Expression of osteoblast-specific genes in femurs of WT mice. Data are presented as mean ± SEM. Statistical analysis was performed using Student *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$.

Briefly, calvariae were dissected after euthanasia and kept in cold sterile PBS. Soft tissues were carefully removed, and bones were incubated for 20 min in a digestion solution containing 0,64 mg/ml of Collagenase II (240 U/mg, Gibco) and 0,05 % Trypsin-EDTA (Sigma-Aldrich) in HBSS. Cellular suspension from the first digestion was discarded and bone pieces were digested three additional times. Cellular suspension from the second digestion was kept and used for osteogenic differentiation studies. Cellular suspensions from third and fourth digestions were used for MTT assay.

For osteogenic differentiation, osteoblasts were first expanded for 4 days in αMEM (Sigma-Aldrich) supplemented with 20 % FBS (Gibco), 1 % L-glutamine (L-gln, Sigma-Aldrich) and 1 % penicillin/streptomycin (P/S, Sigma-Aldrich). Cells were then plated in 12-well plates at the density of 5×10^4 cells/well and incubated for 14 days with DMEM (Gibco) supplemented with 10 % FBS, 1 % L-gln, 1 % P/S, 4 mM β-glycerophosphate (Sigma-Aldrich) and 50 μg/ml of Ascorbic Acid (Sigma-Aldrich). For gene expression analyses, osteoblasts were treated at day 7 with 50 ng/ml recombinant mouse IL-6 (rIL-6, Sigma-Aldrich) alone or in combination with 100 ng/ml recombinant mouse IL-6R (rIL-6R, R&D biosystems) for 6 h in complete culture medium without FBS. For von Kossa stain, 50 ng/ml recombinant mouse IL-6 (rIL-6, Sigma-Aldrich) alone or in combination with 100 ng/ml recombinant mouse IL-6R (rIL-6R, R&D biosystems) were added to complete osteogenic medium at day 10 and the cells were assayed at day 14. Cells were fixed for 10 min with 4 % formaldehyde solution, stained with 1 % Silver Nitrate solution for 20 min under ultraviolet light and washed with distilled H_2O and 5 % sodium thiosulfate for 5 min. Pictures of mineralized nodules were taken with an inverted microscope and analyzed with Adobe Photoshop.

Proliferation assay was performed using Vybrant MTT Cell Proliferation Assay kit (ThermoFisher Scientific). Osteoblasts were plated in 96-well plates at the density of 5×10^3 cells/well. Twenty-four hours later, cells were treated either with 0,5 ng/ml rIL-6 or 1 ng/ml rIL-6R alone, or a combination of the two. After 48 h, 12 mM of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution was added to each well and incubated for further 4 h. Finally, a sodium dodecyl

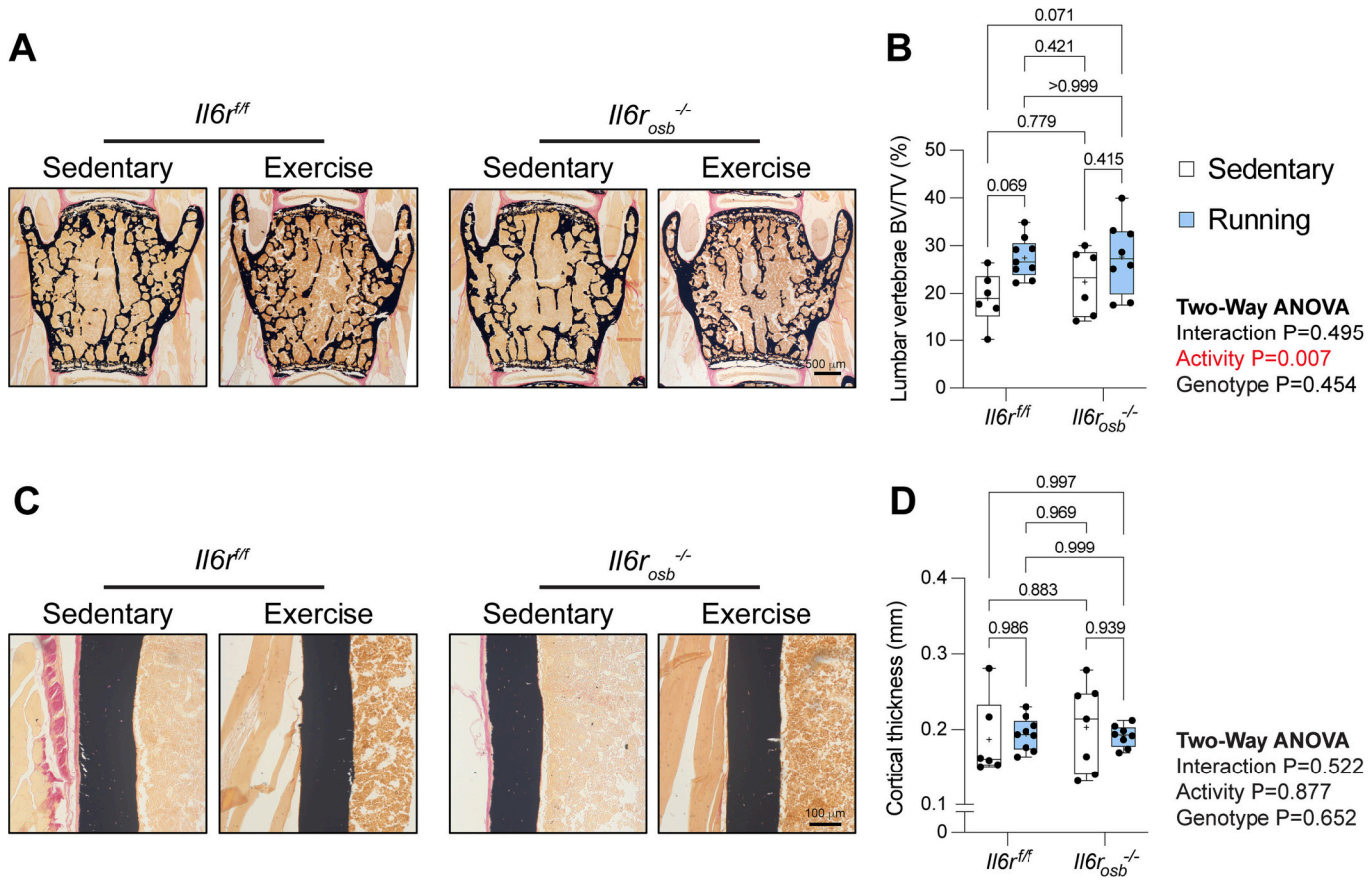

Fig. 2. Histologic and histomorphometric evaluation of bone mass performed in 6-month-old female $Il6r^{f/f}$ and $Il6r^{-/-}_{osb}$ mice in either sedentary or running conditions. Representative histological pictures from Von Kossa/Van Gieson-stained sections of lumbar vertebrae (A) and tibiae (C) and histomorphometric analysis of trabecular bone of lumbar vertebrae (B) and cortical bone of tibial midshaft (D). Data are presented as box plots showing all the experimental points. Horizontal line within the box indicates the median, while the mean is showed as '+'. Statistical analysis was performed using Two-Way ANOVA with multiple comparison test. ANOVA results are reported next to the corresponding graph. Exact *p* values from the comparisons are reported above the box plots.

sulfate-hydrochloric acid (SDS-HCl) solution was added to each well, incubated for 18 h, and absorbance read at 570 nm.

2.7. Statistical test

Student's *t*-test was used to compare two groups. Kruskal-Wallis test was used to compare more than two groups in MTT proliferation assay, gene expression and Von Kossa staining. Two-way ANOVA with Tukey's multiple comparison test was used to detect statistical differences between sedentary and running state in $Il6r^{f/f}$ and $Il6r^{-/-}_{osb}$ mice. In all experiments a p-value <0.05 was considered statistically significant. All graphs and statistical analyses were performed using GraphPad Prism version 9 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Aerobic exercise promotes Il6r expression in bones

The purpose of the first experiment we preformed was to ascertain that circulating IL-6 levels do indeed increase during aerobic exercise in WT mice (Fig. 1A). To that end we measured circulating IL-6 levels in C57Bl/6 6-month-old mice after aerobic exercise. We observed that while IL-6 was undetectable in the serum of sedentary WT mice, its circulating levels increased in mice subjected to an aerobic exercise (Fig. 1B).

Since the main source of circulating IL-6 during physical activity is skeletal muscle [18,25], we analyzed *Il6 and Il6r* gene expression in muscles from sedentary and running mice. This analysis showed the expected increase of *Il6* expression in the quadricep which is a mixed, glycolytic and oxidative, muscle (Fig. 1C). In contrast, *Il6r* expression in the quadriceps was unaffected by exercise (Fig. 1C). Likewise, quadricep weight was similar in sedentary and running WT mice (Fig. 1D). This latter data indicates that the beneficial effect of IL-6 on exercise activity does not require changes in muscle mass. When studying expression of these two genes in bone, we observed that their pattern of expression was exactly opposite to the one seen in muscle of exercising mice. Indeed, *Il6* expression in long bones of adult WT mice was not affected by aerobic exercise but *Il6r* expression was significantly increased by this challenge (Fig. 1E). Endurance exercise was also affecting osteoblast function in long bones, as demonstrated by the upregulation of expression of several genes expressed in osteoblasts, including, as it should, *Osteocalcin* (*Bglap*) (Fig. 1F) [24].

The increase of *Il6r* in bone during aerobic exercise suggested the testable hypothesis that a function of IL-6 signaling in bone in physiological conditions might be to mediate the increase in bone remodeling parameters during aerobic exercise.

3.2. Il6r expression in osteoblasts is needed to observe an increase in bone mass during exercise

To test the aforementioned hypothesis, $Il6r^{-/-}_{osb}$ mice were generated by crossing $Il6r^{f/f}$ mice with *Ocn-Cre* mice. The specificity and efficiency of recombination of the transgenic *Ocn-Cre* mouse model have been previously established [25]. We analyzed bone histological parameters

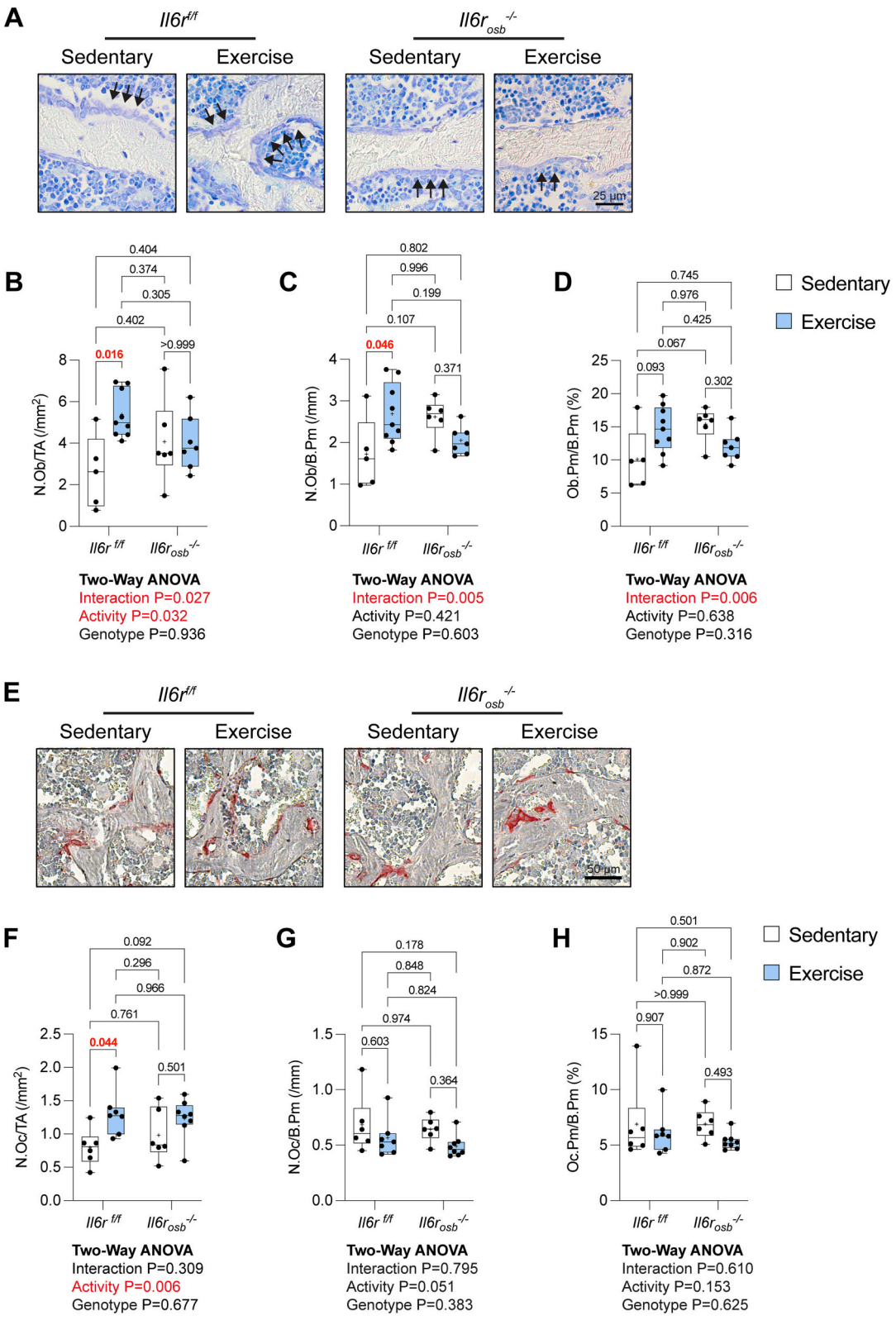

Fig. 3. Static histomorphometry analysis performed in 6-month-old female *Il6r*$^{f/f}$ and *Il6r*$^{-/-}_{osb}$ mice at either sedentary or running conditions. A-D) Representative images from Toluidine blue-stained sections showing osteoblasts (A, arrows) and histomorphometry of osteoblast parameters (B–D). N.Ob/TA: number of osteoblasts per tissue area. N.Ob/B.Pm: number of osteoblasts per bone surface. Ob.Pm/B.Pm: osteoblast surface per bone surface. E–H) TRAP histochemistry highlighting cells of the osteoclastic lineage (E, red stains) and histomorphometry of osteoclast parameters (F–H). N.Oc/TA: number of osteoclasts per tissue area. N.Oc/B.Pm: number of osteoclasts per bone surface. Oc.Pm/B.Pm: osteoclast surface per bone surface. Data are presented as box plots showing all the experimental points. Horizontal line within the box indicates the median, while the mean is showed as '+'. Statistical analysis was performed using Two-Way ANOVA with multiple comparison test. ANOVA results are reported below the corresponding graph. Exact *p* values from the comparisons are reported above the box plots. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

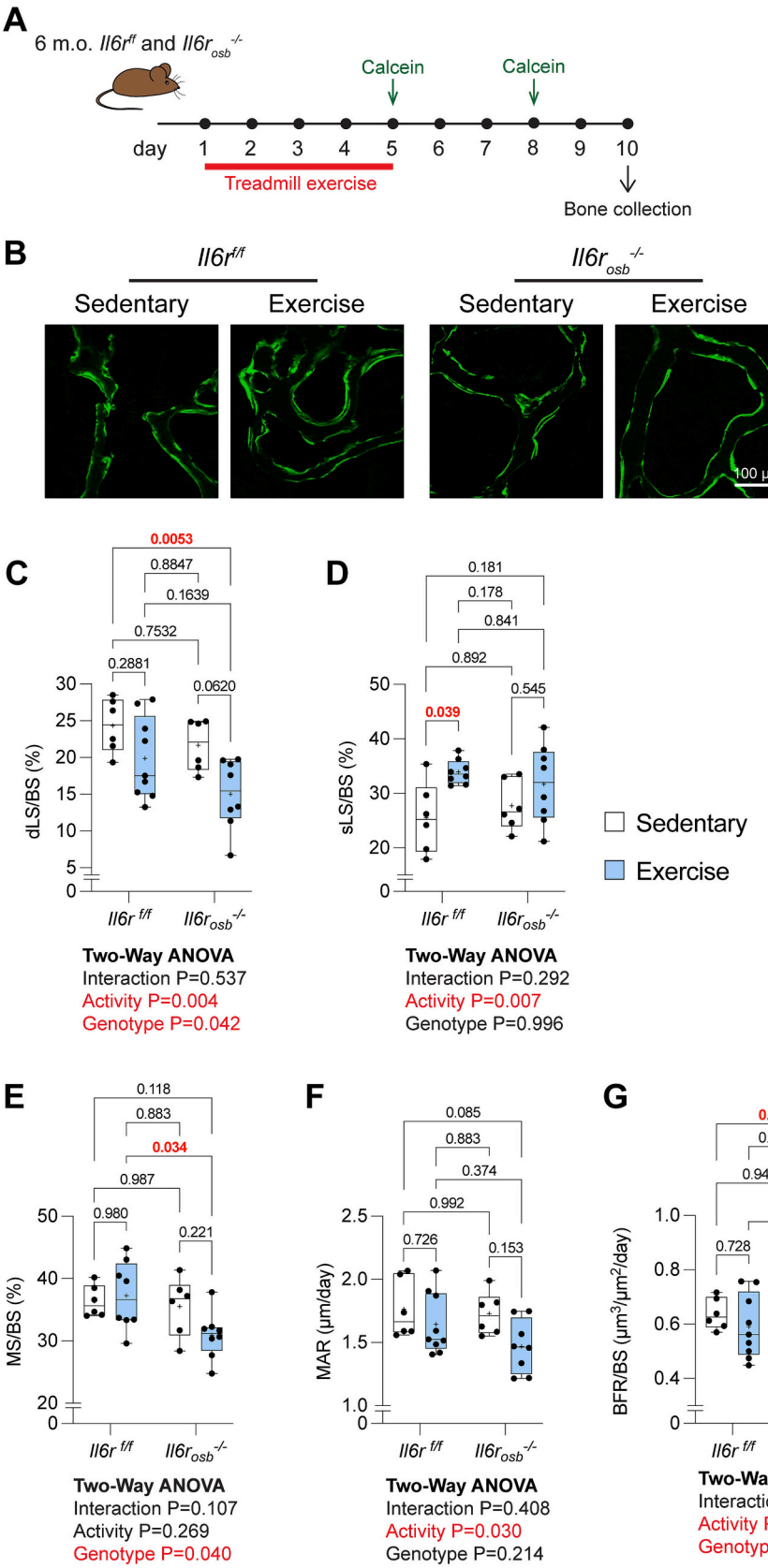

Fig. 4. Dynamic histomorphometric analysis performed in 6-month-old female *Il6r*$^{f/f}$ and *Il6r*$_{osb}^{-/-}$ mice at either sedentary or running conditions. A) Scheme of the treadmill exercise followed by Calcein injections. B–G) Representative pictures of calcein labeling in trabecular bone of 6-month-old female mice (B) and dynamic histomorphometry for bone formation parameters (C–G). dLS/BS: Double labeled surfaces per bone surface. sLS/BS: Single labeled surfaces per bone surface. MS/BS: mineralizing surface per bone surface. MAR: mineral apposition rate. BFR/BS: bone formation rate. Data are presented as box plots showing all the experimental points. Horizontal line within the box indicates the median, while the mean is showed as '+'. Statistical analysis was performed using Two-Way ANOVA with multiple comparison test. ANOVA results are reported below the corresponding graph. Exact *p* values from the comparisons are reported above the box plots.

in 6-month-old female mutant and control mice, at rest or after aerobic exercise (Fig. 2A, C). In terms of exercise capacity, there was no difference in the ability of *Il6r*$^{-/-}_{osb}$ mice to complete a daily exercise, however when performing a 40-min endurance run, these mutant mice ran less than control ones (Fig. S1), confirming what we previously demonstrated [25].

A two-way ANOVA was performed to analyze the effect of sedentary and running activity and of genotypes (*Il6r*$^{f/f}$ *and* *Il6r*$^{-/-}_{osb}$) on lumbar vertebrae trabecular volume. No statistically significant interaction between the effects of physical activity and genotypes was found ($F(1, 25) = 0.479$, $p = 0.495$, Fig. 2B). Simple main effects analysis showed that only physical activity had a statistically significant effect on trabecular bone volume ($p = 0.007$ and $p = 0.454$ respectively, Fig. 2B). Histomorphometric analyses revealed that bone volume over total volume was increased in exercising *Il6r*$^{f/f}$ mice compared to sedentary mice. This increase did not reach statistical significance possibly because of the small sample size available for this study ($p = 0.0692$ two-way ANOVA Tukey's comparison test, Fig. 2B). In contrast, bone volume over total volume did not increase in exercising *Il6r*$_{osb}^{-/-}$ mice compared to sedentary *Il6r*$_{osb}^{-/-}$ mice, although there was a high variability in *Il6r*-deficient groups (Fig. 2B). These results are consistent with the hypothesis that signaling through IL-6R in osteoblasts is necessary to trigger the increase in trabecular bone mass that occurs after aerobic exercise. We did not record, in these experimental conditions, any differences in tibial trabecular bone volume and cortical thickness between sedentary and running mice in both *Il6r*$^{f/f}$, *Il6r*$^{f/f}$ *and* *Il6r*$_{osb}^{-/-}$ mice (Figs. 2C, D and S2A, B).

3.3. The exercise-induced increase of osteoblastogenesis requires the expression of Il6r in osteoblasts

With the goal of explaining what were the cellular events why *Il6r*$^{-/-}_{osb}$ mice failed to increase their bone mass after physical exercise, we quantified osteoblast and osteoclast cell populations in control and mutant mice maintained in sedentary conditions or after aerobic exercise (Fig. 3A).

Two-way ANOVA analysis revealed a significant interaction between the effects of physical activity and the genotype of the mice on osteoblast numbers (N.Ob/TA: $F(1, 23) = 5.603$, $p = 0.027$, Fig. 3B; N.Ob/B.Pm: $F(1, 23) = 9.933$, $p = 0.005$, Fig. 3C; Ob.Pm/B.Pm: $F(1, 22) = 9.059$, $p = 0.006$, Fig. 3D).

In line with the increase in the trabecular bone mass observed in *Il6r*$^{f/f}$ mice that exercised, the osteoblast number calculated on tissue area was significantly increased in exercising adult *Il6r*$^{f/f}$ mice compared to what was observed in sedentary *Il6r*$^{f/f}$ mice, as revealed by Tukey's multiple comparison test (Fig. 3B). This result indicated that aerobic exercise favors osteoblastogenesis in *Il6r*$^{f/f}$ control mice. When normalized per bone surfaces, osteoblast numbers and osteoblasts surfaces were also higher in control mice that had been exercising compared to what they were in sedentary control mice, confirming that there is a recruitment of osteoblast on bone surfaces in running mice (Fig. 3C, D). In contrast, in mice lacking *Il6r* expression in osteoblasts, the number of osteoblasts failed to increase after running indicating that signaling through *Il6r* is necessary for the exercise-induced osteoblastogenesis (Fig. 3B-D). Results of this analysis provide a cellular explanation for the fact that *Il6r*$_{osb}^{-/-}$ mice do not exhibit an increase in bone mass after aerobic exercise.

We also analyzed the number of osteoclasts (Fig. 3E). No significant interaction between the influence of activity and the genotype of the mice on osteoclast parameters was revealed by a Two-way ANOVA analysis (Fig. 3F–H). Simple main effects analysis showed that physical activity had a statistically significant effect on osteoclasts number per tissue area ($F(1, 23) = 9.092$, $p = 0.006$, Fig. 3F). While the total osteoclast number normalized on tissue area was significantly higher in *Il6r*$^{f/f}$ mice that had been exercising compared to sedentary ones (Fig. 3F), this difference was lost when the count was normalized on bone surfaces (Fig. 3G, H). Likewise, osteoclast number and surface did not increase during running in *Il6r*$^{-/-}_{osb}$ mice (Fig. 3F–H), and no differences with the other groups were observed in osteoclast number and surface normalized on bone surfaces (Fig. 3G, H). Taken together, the results of this analysis suggest that aerobic exercise stimulates bone remodeling in part in an IL-6 signaling-dependent manner. Indeed IL-6 signaling in osteoblasts is needed for increase in osteoblastogenesis that occurs during exercise.

3.4. Il6r expression in osteoblasts is needed for bone formation during exercise

Next, we asked whether the absence of *Il6r* expression in osteoblasts during aerobic exercise hampers osteoblast activity and their mineralizing capacity. To that end, we performed dynamic histomorphometry analysis of lumbar vertebrae of sedentary and exercising control and mutant mice after two injections, 4 days apart, of calcein fluorochrome (Fig. 4A).

Calcein deposits at the sites of bone formation, i.e., it binds to hydroxyapatite at the mineralization front and is revealed by green fluorescence. The quantification of double labeled surfaces (dLS/BS), reflecting sites of bone formation that were active in the entire labeling period, revealed a tendency toward a reduction in *Il6r*$^{f/f}$ mice that had been exercising compared to sedentary ones (Fig. 4B, C). Of note, the length of calcein-labeled surfaces with a single line (sLS/BS), which can be an indication of the appearance of sites of new bone formation, tended to increase significantly in exercising compared to resting *Il6r*$^{f/f}$ mice (Fig. 4D). This observation suggested that exercise stimulates the de novo recruitment of bone-forming cells on bone surfaces. In *Il6r*$^{-/-}_{osb}$ mice on the other hand, not only there were no differences in single labeled surfaces between sedentary mice and mice that had been exercising (Fig. 4D) but we observed a marked decrease in double labeled surfaces between sedentary mice and mice that had been exercising (Figs. 4C and S2C). These observations suggest that there is a lack of activation of new bone formation sites and a defective bone formation activity during exercise. Taken together these results establish that aerobic exercise stimulates new bone formation at least in part through an IL-6 signaling in osteoblasts.

We did not detect any differences in the mineralizing surface (MS/BS) between sedentary and exercising *Il6r*$^{f/f}$ mice (Fig. 4E), whereas there was a marked reduction in MS/BS in exercising *Il6r*$^{-/-}_{osb}$ mice compared to what was observed in sedentary control or mutant mice, even though this difference did not reach statistical significance in tibiae (Figs. 4E and S2E). The impairment in the mineralizing bone surfaces of running *Il6r*$^{-/-}_{osb}$ mice, was even more evident when compared to running *Il6r*$^{f/f}$ mice (Fig. 4E and S2E).

Analysis of the mineral apposition rate (MAR), which measures the speed at which a single osteoblast makes bone, revealed no statistical differences between sedentary and exercising *Il6r*$^{f/f}$ mice but a 15 % decrease in *Il6r*$^{-/-}_{osb}$ mice that had been subjected to exercise when compared to sedentary ones (Figs. 4F and S2F).

Bone formation rate (BFR) with which the amount of bone formed per unit of time is evaluated, showed no differences between sedentary and exercising *Il6r*$^{f/f}$ mice whereas there was a drastic reduction in BFR in *Il6r*$^{-/-}_{osb}$ mice that had been exercising compared to sedentary *Il6r*$^{f/f}$ and *Il6r*$^{-/-}_{osb}$ mice (Figs. 4G and S2G). Altogether, these results indicate that the absence of *Il6* signaling in osteoblasts disrupts osteoblast activity and bone formation during aerobic exercise.

3.5. IL-6R is needed for a proper osteogenesis in cell culture

In vivo experiments suggested that the presence of IL-6R in bone is required to increase bone formation during exercise when IL-6 signaling increases. In contrast, IL-6 signaling through its cognate receptor had only a modest role in the skeleton at sedentary conditions (Figs. 4B–G, S2C–G). To add credence to these data, we cultured osteoblast from WT

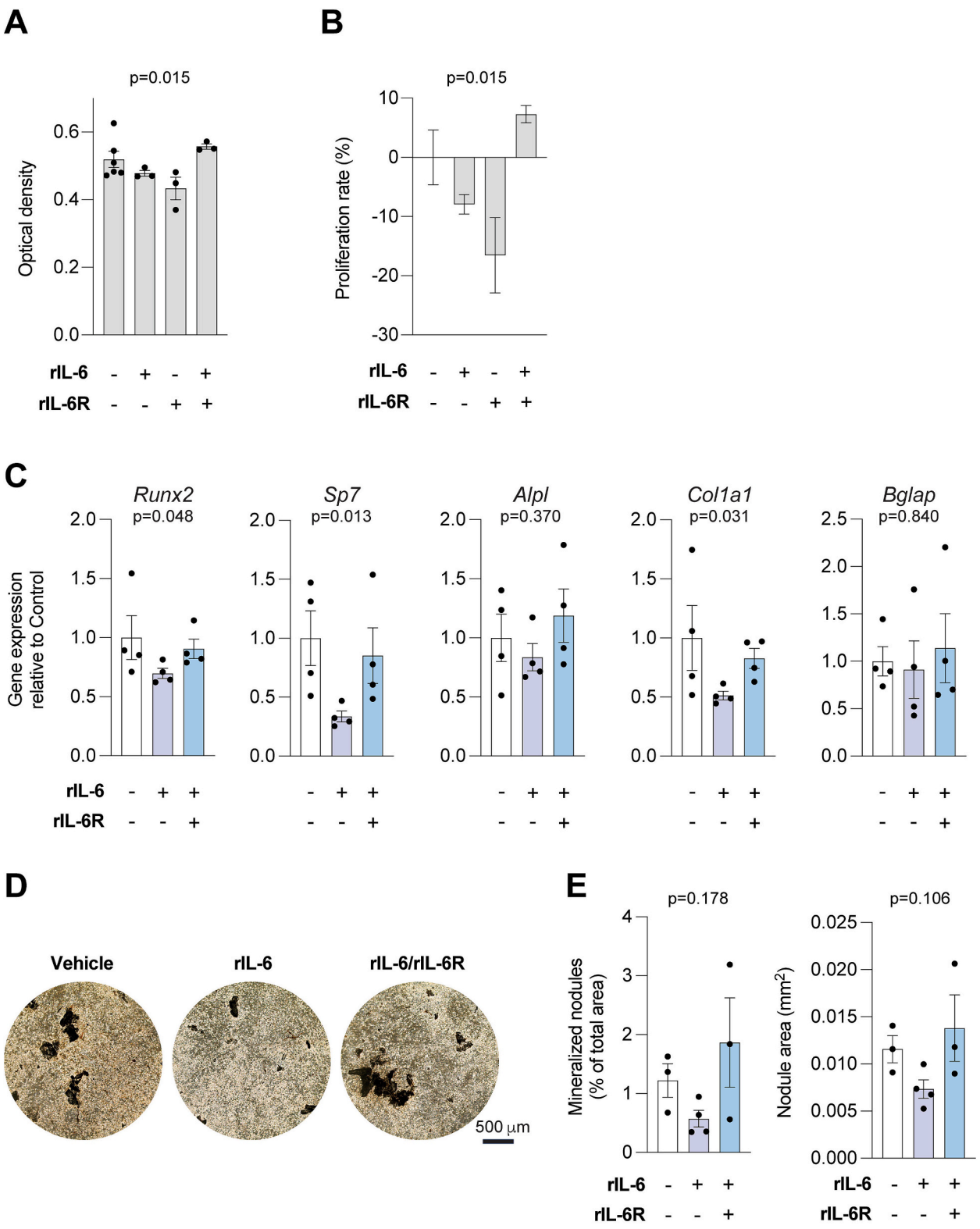

Fig. 5. Cell based assays. A, B) MTT assay results, showing optical density of cells upon different treatments (A) and percentage of proliferation rate relative to untreated cells (B). C) Gene expression analysis performed on osteoblasts after 7 days in osteogenic medium. D) Representative images from Von Kossa-stained cells cultured for 14 days in osteogenic medium. E) Quantification of the area covered by Von Kossa-stained mineralized nodules. Statistical analysis was performed using Kruskal-Wallis test and exact *p* values are reported above each graph.

mice and assessed their proliferation and osteogenic differentiation potential when treated with rIL-6 alone or in combination with rIL-R.

The proliferation capacity of the cells was evaluated by MTT assay. We observed that when cultures were incubated with either rIL-6 or rIL-6R alone, osteoblast proliferation was markedly inhibited whereas the combination of rIL-6 and rIL-6R induced an increase in osteoblast proliferation compared to control conditions (Fig. 5A, B), This result indicated that only when IL-6 and its soluble receptor are present together,

osteoblast proliferation can be enhanced by this cytokine.

After a brief expansion, cells were induced to osteogenic differentiation and at day 7 were treated for 6 h with rIL-6 alone or in combination with rIL-6R. Gene expression analysis revealed that when cells were exposed to rIL-6 alone, the expression of molecular markers of osteoblast differentiation such as *Runx2, Sp7* and *Col1a1* was reduced (Fig. 5C). The addition of rIL-6R to the cultures restored the transcript levels to control condition, again indicating that both the cytokine and its receptor are needed to ensure proper osteogenesis (Fig. 5C). Likewise, Von Kossa staining performed after 14 days of culture in osteogenic medium, showed an inhibition of mineralized nodule formation in cells treated with rIL-6 alone and an increase in the size of mineralized nodules in osteoblast cultures treated with both rIL-6 and rIL-6R (Fig. 5D, E). These experiments indicate that, as it is the case in vivo, the presence of IL-6R in osteoblasts is needed for proper osteoblast differentiation.

4. Discussion

This study provides in vivo evidence based on the analysis of a cell-specific loss of function model in the mouse, that the beneficial changes in bone remodeling caused by aerobic exercise requires IL-6 signaling in osteoblasts.

Circulating IL-6 levels rise after aerobic exercise in both humans and experimental animal models [18,21,24,25,31]. Here, we confirm that *Il6* expression in quadriceps and serum IL-6 levels are increased after 5 days of treadmill exercise in mice, and that this increase occurs in the absence of any gain in muscle weight [24,25]. While the expression of the IL-6 receptor, *Il6r*, did not increase in muscle of exercising mice, it significantly increased in the femurs of these mice. This represents the first in vivo evidence that the expression of IL-6R can be regulated in bone during exercise. The mechanism by which osteoblasts increase their expression of IL-6R remain to be elucidated.

We have shown previously that in mice, signaling through IL-6R in osteoblast is necessary for adaptation to aerobic exercise [25]. As an extension of this initial work, we investigated here the putative role that the exercise-induced increase in circulating IL-6 may have on bone homeostasis. This is an important question in view of the fact that no function in bone could be ascribed to IL-6 unless bone homeostasis is challenged by pathological conditions such as estrogen deficiency, inflammation tumors and other bone diseases [13–16,32]. Is exercise a more physiological challenge that altogether increases circulating IL-6 levels and allows to define a function for this cytokine in bone? To be able to thoroughly dissect the effects of IL-6 signaling in this organ during aerobic exercise, we addressed this question in a mouse model that lacks IL-6R specifically in osteoblasts.

This question was prompted not only by the exercise-induced increase of muscle IL-6 and bone IL-6R, but also by the fact that even though IL-6 has been described, based on gain of function and mostly cell-based assays, as an osteoclastogenic factor produced by osteogenic cells, this function is dispensable in physiological conditions. Indeed, IL-6-deficient mice have a normal number of osteoclasts and normally active bone resorption, with normal bone mass [26]. The fact that mice lacking IL-6 have no phenotype in unchallenged conditions has long raised the question of the physiological role of IL-6 signaling in bone and in particular in osteoblasts.

Our study shows that an osteoblast-specific deletion of *Il6r* in the mouse does not affect the number of osteoclasts or bone resorption when mice are maintained in sedentary conditions. In contrast, when subjected to repeated bouts of aerobic exercise, $Il6r_{osb}{}^{-/-}$ mice do not increase their osteoclast and osteoblast number, as $Il6r^{f/f}$ control mice do. This observation is in line with what it was shown previously in ovariectomized IL-6 deficient mice [26], further confirming our hypothesis that IL-6 signaling becomes important in bone homeostasis when the bone turnover is challenged by physiological conditions, like exercise or pathological ones like ovariectomy. More importantly, kinetic indices of bone formation revealed a marked reduction in the bone-forming

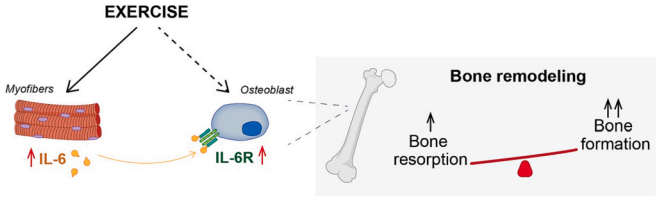

Fig. 6. Schematic representation of how IL-6 signaling in osteoblasts during aerobic exercise affects bone remodeling parameters.

activity of running $Il6r_{osb}^{-/-}$ mice compared to what was observed in sedentary $Il6r_{osb}^{-/-}$ mice, as revealed by the drastic reduction in bone formation rate. This observation revealed a lack of actively functioning osteoblasts in the absence of IL-6 signaling in osteoblasts during aerobic exercise. As a result, the exercise-induced gain in trabecular bone mass seen in $Il6r^{f/f}$ control mice does not occur $Il6r_{osb}^{-/-}$ mice. Altogether these results provide in vivo and genetic-based evidence that a function of IL-6 signaling in osteoblasts is to increase bone turnover in exercising animals. As $Il6r_{osb}{}^{-/-}$ mice have reduced levels of *Rankl* in bones when exercised on a treadmill [25], the possibility exists that the signaling cascade leading to activation of bone turnover during exercise requires first RANKL release by the osteoblasts after IL-6/IL-6R binding, with subsequent stimulation of bone resorption, and then a balanced increase of osteoblastogenesis and bone formation (Fig. 6).

Lastly, our analysis of osteoblast culture confirmed our in vivo results since only a combination of IL-6 and its receptor induced osteoblast proliferation and enhanced expression of osteogenic markers. These results are congruent with previous studies showing the inhibitory effect of rIL-6 on proliferation, collagen synthesis and alkaline phosphatase activity in rat calvarial osteoblasts or mouse osteoblast cell lines [33–35]. Other studies also have demonstrated the requirement of IL-6R in vitro to increase alkaline phosphatase activity and osteogenic capacity of the cells [36,37].

Overall, the inhibition of osteogenesis in cultures where rIL-6 is added but rIL-6R is not present, recapitulates the in vivo scenario where $Il6r_{osb}^{-/-}$ are exercised, and defect of bone formation is observed.

5. Conclusions

Our data further illustrate, this time in the context of bone formation, the importance of the crosstalk between bone and muscle during exercise and the role played by IL-6 in this interplay [24,25]. They also demonstrate that IL-6 signaling in exercising mice is required to generate changes in bone metabolism which lead to higher turnover during aerobic exercise.

CRediT authorship contribution statement

Biagio Palmisano: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Mara Riminucci:** Writing – review & editing. **Gerard Karsenty:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest to declare.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2023.116870.

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