Hematopoietic or Osteoclast-Specific Deletion of Syk Leads to Increased Bone Mass in Experimental Mice

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Syk is a non-receptor tyrosine kinase critically involved in signaling by various immunoreceptors including B-cell-receptors and activating Fc-receptors. We have previously shown that Syk also mediates immunoreceptor-like signals required for the in vitro development and function of osteoclasts. However, the perinatal lethality of Syk−/− mice precluded the analysis of the role of Syk in in vivo bone metabolism. To overcome that problem, we generated mice with osteoclast-specific (SykΔOC) or hematopoietic (SykΔHaemo) Syk deficiency by conditional deletion of Syk using Cre recombinase expressed under the control of the Ctsk or Vav1 promoter, respectively. Micro-CT analysis revealed increased bone trabecular density in both SykΔOC and SykΔHaemo mice, although hematopoietic Syk deficiency caused a more severe phenotype than osteoclast-specific Syk deficiency. Osteoclast-specific Syk deficiency reduced, whereas hematopoietic Syk deficiency completely blocked in vitro development of osteoclasts. Both interventions inhibited the resorptive activity of osteoclasts and osteoclast-specific gene expression. Kinetic analysis of Syk protein levels, Cre expression and the genomic deletion of the Syk flox allele revealed complete and early deletion of Syk from SykΔHaemo osteoclasts whereas Syk was incompletely deleted at a later stage of osteoclast development from SykΔOC cultures. Those results provide an explanation for the in vivo and in vitro difference between the SykΔOC and SykΔHaemo mutant strains and suggest late activation of, and incomplete target gene deletion upon, osteoclast-specific Cre expression driven by the Ctsk promoter. Taken together, our results indicate that Syk plays an indispensable role in osteoclast-mediated in vivo bone resorption and suggest that Syk-specific inhibitors may provide therapeutic benefit in inflammatory and other diseases characterized by excessive osteoclast-mediated bone resorption.

Keywords: SYK (spleen tyrosine kinase), tyrosine kinase, osteoclasts, Cre-Lox, in vivo, mice
INTRODUCTION

Osteoclasts are multinuclear giant cells of hematopoietic origin which develop from myeloid progenitors through a unique biochemical maturation program followed by homotypic fusion (1, 2). Osteoclasts are the sole cell types in the mammalian organism capable of actively resoring bone tissue and therefore play a critical role in bone homeostasis. Defective osteoclast development or function leads to increased bone mass (osteopetrosis) (3), whereas excessive (pathological) bone resorption occurs during osteoporosis (4), inflammatory joint diseases (e.g., arthritis-induced bone erosions in rheumatoid arthritis) (5, 6) and cancer-induced bone loss (7, 8).

Osteoclast development and function requires a number of extracellular cues including M-CSF, RANKL, as well as integrin-mediated adhesive processes (9). The importance of those pathways is indicated by the severe bone resorption defects in mice lacking M-CSF (10), RANK (11, 12), RANKL (13, 14), or β3 integrins (15). Culturing myeloid progenitors derived from human blood or mouse bone marrow in the presence of M-CSF and RANKL also leads to formation of osteoclast-like cells with in vitro bone resoring capacity, allowing the analysis of osteoclast development and function in cell culture.

Syk is a non-receptor tyrosine kinase critically involved in various functions of the immune system, as well as certain non-immune-related biological processes (16). Syk is required for B-cell-receptor signaling and therefore the development of B-cells (17, 18). It is a critical component of signaling by a number of activating Fc-receptors such as Fce-receptors and Fcγ-receptors on neutrophils, macrophages, and mast cells (19–22), as well as the Fc-receptor-related collagen receptor GpVI of platelets (23, 24). Syk also mediates signaling by β1, β2, and β3 integrins in neutrophils, monocytes/macrophages, and platelets (25–27). Syk deficiency causes perinatal lethality (17, 18) likely due to the role of Syk in lymphatic vascular development (28). Most, if not all of those functions of Syk is related to its binding to receptor-associated tyrosine-phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) linking immunoreceptors to downstream signaling pathways (16, 29–32). The role of Syk in various immune and inflammatory processes also translates into its role in autoantibody-induced arthritis (24, 33–35) and dermatitis (36, 37) in experimental mice.

We and others have previously shown that the ITAM-containing adapter molecules DAP12 and FcRγ are involved in in vitro osteoclast development and function, and that mice lacking both DAP12 and FcRγ show strongly increased mineralized bone mass (38–43). One of the possible mechanisms for those phenotypes could be that, similar to immune cells (16, 29), the ITAM-containing DAP12 and FcRγ adapters would activate the Syk tyrosine kinase in osteoclasts, thus triggering osteoclast development and function. Indeed, Syk-deficient bone marrow cells failed to develop to mature multilnucleated osteoclasts or to show resorptive activity in in vitro cultures (40, 42, 44, 45), and this in vitro phenotype was linked to ITAM signaling by DAP12 and FcRγ (42–44). Those studies provided an unexpected link between immunoreceptor-like signaling and bone homeostasis and therefore provided one of the foundations of the field of osteoimmunology (46, 47). In addition, Syk-mediated pathways have also been linked to integrin signal transduction and the osteoclast cytoskeleton (16, 26, 42, 44, 48). Unfortunately, however, it is at present unclear whether Syk is also involved in bone homeostasis in live animals, as bone morphology of Syk-deficient animals could not be tested because of the perinatal lethality of Syk−/− mice (17, 18).

To overcome the perinatal lethality of Syk−/− animals, we have generated mice with osteoclast-specific or hematopoietic-specific Syk deletion using the Cre-Lox recombination approach. Analysis of the mice with tissue specific Syk deletion revealed strong increase in bone mass upon osteoclast-specific and, particularly, hematopoietic-specific Syk-deficiency, indicating a critical role for Syk in in vivo bone homeostasis. Further experiments aimed at understanding the different severities of the bone phenotypes in the two strains indicated that the effect of Syk deficiency on osteoclast development strongly depends on the timing and extent of Cre expression and Cre-mediated inactivation of the Syk gene.

MATERIALS AND METHODS

Animals

Mice carrying the Syk<sup>tm1.Tara</sup> (referred to as Syk<sup>flox</sup>) allele of the Syk gene (49) were obtained from Alexander Tarakhovsky (Rockefeller University) and were maintained in homozygous (Syk<sup>flox/flox</sup>) form. Mice carrying the Ctsk<sup>tm1.cre</sup>/Syk<sup>−/−</sup> (referred to as Ctsk<sup>Cre</sup>/Syk<sup>−/−</sup>) knock-in mutation resulting in the osteoclast-specific expression of the Cre recombinase under the control of the endogenous promoter of the Ctsk gene and at the same time inactivating the Ctsk gene (50) were obtained from Shigeaki Kato (University of Tokyo) and were maintained in heterozygous form (referred to as Ctsk<sup>Cre</sup>/Syk<sup>−/−</sup> or Ctsk<sup>Cre</sup>) to avoid homozygous inactivation of the Ctsk gene. Mice carrying the Commd10<sup>Tg(Vav1−/−cre)A2Kio</sup> transgenic insertional mutation expressing the Cre recombinase in the entire haemopoietic lineage from the exogenous Vav1 promoter (51) and at the same time inactivating the Commd10 gene (52) were obtained from the Jackson Laboratory and were maintained in heterozygous form (referred to as Vav-Cre) to avoid homozygous inactivation of the Commd10 gene. Mice carrying the LysM-Cre<sup>Cre−<sub>Cre</sub>Lyz2<sub>tm1(cro)</sub>Lo</sup> (referred to as LysM<sup>C</sup>) knock-in mutation expressing the Cre recombinase in the entire myeloid compartment from the endogenous promoter of lysozyme M (53) were purchased from the Jackson Laboratory and were maintained in homozygous form (referred to as LysM<sup>M-Cre</sup>).

Osteoclast-specific deletion of Syk was achieved by crossing the Ctsk-Cre and Syk<sup>flox/flox</sup> mice to obtain Ctsk<sup>Cre−/+Syk<sup>flox/flox</sup></sup> (referred to as Syk<sup>ΔOCS</sup>) animals. Deletion of Syk in the entire hematopoietic compartment was achieved by crossing the Vav-Cre and Syk<sup>flox/flox</sup> mice to obtain Commd10<sup>Tg(Vav1−/−cre)A2Kio/+Syk<sup>flox/flox</sup></sup> (referred to as Syk<sup>ΔHaem</sup>) animals. Myeloid-specific deletion of Syk was achieved by crossing the LysM-Cre and Syk<sup>flox/flox</sup> mice to obtain LysM<sup>C</sup>/Cre<sup>−/+Syk<sup>flox/flox</sup></sup> (referred to as Syk<sup>ΔMely</sup>) animals. The allele obtained by Cre-mediated deletion of the Syk<sup>flox</sup> allele will be referred to as the Syk<sup>Δ</sup> allele.
Genotyping of the mice was performed by allele-specific PCR. All mice were on the C57BL/6 genetic background. Wild type C57BL/6 animals were obtained from our breeding colony. The mice were kept in individually sterile ventilated cages (Tecniplast) in a specific pathogen-free facility. All animal experiments were approved by the Animal Experimentation Review Board of Semmelweis University.

**Micro-CT Analysis**

Mice were sacrificed at 9 weeks of age and their right femurs were subjected to micro-CT analysis by a SkyScan 1172 micro-CT apparatus as described (54, 55). A 70 kV and 124 μA X-ray source with 0.5 mm aluminum filter and a rotation step of 0.5° was used during image acquisition, followed by reconstruction with the SkyScan NRecon software, resulting in an isometric 5 μm voxel size. Volume of interest was selected according to the manufacturer's instructions. Further analysis was performed using the Skyscan CTAn and CTVol software. The lower threshold of binary images was set to an absolute value of 85 throughout the entire study. Our study design did not allow the calculation of absolute bone hydroxypatite densities.

Quantitative analysis was performed on the trabecular region of the distal femoral metaphysis beginning 50 sections (0.25 mm) from the distal growth plate to an additional 400 sections (2 mm) to the proximal direction, including the entire trabecular area within that range, identified manually by visual inspection. Quantitative parameters included percent bone volume (BV/TV), trabecular number, trabecular thickness and trabecular separation as described (54, 55).

Representative cross sections represent the 200th section (1 mm) from the distal femoral growth plate. 3D images show an axial cylinder of a diameter of 500 μm between sections 150–450 from the distal growth plate.

**Histological Procedures and Immunostaining**

Femurs isolated from mice at 9 weeks of age were fixed in 4% paraformaldehyde (Sigma-Aldrich) followed by decalcification in Osteomoll (Merck) for 3 weeks. The samples were then dehydrated, and embedded in paraffin (Leica) using a Leica EG1150H embedding station. Eight micrometers of thick sections were obtained using a Thermo Scientific HM340E microtome and were processed for hematoxylin and eosin (Leica) staining, or for immunostaining for the calcitonin receptor using anti-Calcitonin Receptor (Abcam AB11042) and anti-rabbit Alexa Fluor 488 (Life Technologies, A1034) antibodies. Microscopic images were taken by a Nikon ECLIPSE Ni-U inverted microscope connected to a Nikon DS-Ri2 camera.

**In vitro Culture and Resorption Assays**

*In vitro* osteoclast cultures were performed essentially as described before (54, 55). Bone marrow cells obtained by flushing the tibia and femur of wild type or mutant mice were cultured in the presence of 10 ng/ml murine M-CSF and 50 ng/ml murine RANKL (Peprotech) with medium changes every 2 days. In parallel macrophage cultures, the cells were cultured under identical conditions except that RANKL was omitted.

Cultures were terminated and osteoclast-specific staining was performed using a commercial tartrate-resistant acid phosphatase (TRAP) staining kit (Sigma-Aldrich) at the indicated times after the first addition of RANKL. Photomicrographs were taken using a Leica DMi6000B inverted microscope. The images were then analyzed either manually or by the ImageJ software. Osteoclasts were defined as TRAP-positive cells with 3 or more nuclei.

For *in vitro* resorption assays, osteoclasts were cultured under similar conditions for 7 days on an artificial hydroxypatite surface (Sigma-Aldrich) followed by washing, imaging by dark field microscopy and further analysis by ImageJ software.

**Biochemical Studies**

For protein content analysis, osteoclast, or macrophage cultures were washed and then lysed in a Triton-based lysis buffer containing 100 mM NaCl, 30 mM Na-HEPES (pH 7.4), 20 mM NaF, 1 mM Na-EGTA, 1% Triton X-100, 1 mM benzamidine, freshly supplemented with 0.1 U/ml Aprotinin, 1:100 Mammalian Protease Inhibitor Cocktail, 1:100 Phosphatase Inhibitor Cocktail 2, 1 mM PMSF, and 1 mM Na3VO4 (all from Sigma-Aldrich). Insoluble material was removed, the lysate supernatants were supplemented with 4× Laemmli’s sample buffer and boiled for 10 min. Whole cell lysates were run on SDS-PAGE, electroblotted to nitrocellulose membranes, and then processed for immunoblotting with antibodies against Syk (N19; Santa Cruz) or β-actin (Clone AC-74; Sigma-Aldrich). After incubation with peroxidase-labeled secondary antibodies (GE Healthcare), the signal was developed using the ECL system (GE Healthcare) and exposed to X-ray film. X-ray films were then scanned and processed with Adobe Photoshop.

**Quantitative RT-PCR Analysis**

To test osteoclast specific and Cre gene expression changes, mouse myeloid progenitors were differentiated into osteoclasts or macrophages in the presence of 50 ng/ml M-CSF with or without 50 ng/ml RANKL for 0–3 days, followed by RNA extraction and reverse transcription as previously described (54–56). For quantitative reverse transcription (RT)-PCR analysis of the osteoclast-specific genes, the following TaqMan assays were used: Acp5 (TRAP; Taqman Mm00475698_m1), Ctsk (cathepsin K; Mm00484039_m1), Calcr (Calcitonin receptor; Mm00432271_m1), Nfatc1 (NFATc1; Mm00479445_m1), and Tm7sf4 (DC-STAMP; Mm04209235_m1) as previously described (54, 55). For assessment of Cre expression, the 5′- TGACGGTGGGAGAATGTTAATC forward and 5′- GCC CGT TCT GTG CCT ACT primer were used. Transcript levels relative to GAPDH were calculated using the comparative Ct method (54, 55).

**Sequencing of the Germine Syk<sup>flox</sup> Allele**

To determine the exact sequence of the Syk<sup>flox</sup> allele, tail DNA was amplified using the 5′- GCC CGT TCT GTG CCT ACT
GG–3′ forward and 5′- TAG CTA ACC AAA CCC ACG GC–3′ reverse primers spanning the 5′-loxP site, or the 5′- CCA AAG CGG AGT CCT CAC AT–3′ forward and 5′– GTG GGT CCC ATC TTT CC–3′ reverse primers spanning the 3′-loxP site. PCR products were then sent to Microsynth for sequencing and the obtained sequences were aligned with the genomic sequence of the wild type Syk gene to obtain the sequence of the Syklox allele.

Genomic PCR Analysis
Osteoclast cultures were washed at the indicated times after the start of RANKL treatment, followed by isolation of genomic DNA and PCR using standard procedures.

Two different PCR assays were performed on the genomic DNA of osteoclast cultures. In PCR 1, the 5′- GCC GGT TCT GTG CCT ACT GG–3′ forward primer (P fwd) was used along with the 5′- TAG CTA ACC AAA CCC ACG GC–3′ reverse primer (P rev1) to separate the Syk+ and Syklox alleles (234 and 349 bp product length, respectively). In PCR 2, the same P fwd forward primer was used with the 5′- GTG GGT CCC ATC TTT CC–3′ reverse primer (P rev2) to separate the Syk+ , Syklox and Syk− alleles (1314, 1560 and 452 bp product length, respectively).

Statistical Analysis
Experiments were performed the indicated number of times. Diagrams show mean and SEM from the indicated number of independent experiments. Micro-CT measurements were analyzed by two-way (factorial) ANOVA with the presence/absence of Cre and the Syk genotype as the independent parameters. Other measurements were analyzed by one-way ANOVA followed by Tukey or Unequal n HSD post hoc test. In case of the kinetic analysis of osteoclast morphology, statistical analysis was performed on the area under the curve (AUC). P-values below 0.05 were considered statistically significant.

RESULTS
The Effect of Osteoclast-Specific Syk Deletion on Trabecular Bone Architecture
The Syk−/− mutation causes perinatal lethality making it technically impossible to analyze the bone morphology of adult Syk−/− mice. We decided to overcome that problem by generating lineage-specific Syk-deficient animals. As a first approach, we crossed mice in which the cDNA of the Cre recombinase has been inserted into the osteoclast-specific Ctsk gene (referred to as CtskCre/+ or Ctsk-Cre mice) (50) with mice carrying a floxed Syk allele (referred to as Syklox/lox mice) (49). The resulting CtskCre/+Syklox/lox (referred to as Syklox/lox) mice are expected to have defective Syk expression in osteoclasts due to Cre-mediated excision and inactivation of the Syk gene.

We then subjected Syklox mice and the appropriate controls to micro-CT analysis of the distal femur. As shown in the longitudinal sections of the femurs of female mice in Figure 1A, the Syklox mutation strongly increased the density of the trabecular area compared to wild type mice, whereas no dramatic difference could be observed in Ctsk-Cre or Syklox/lox animals. Analysis of representative cross-sections of male or female mouse femurs also showed increased trabecular density in Syklox mice but not in Ctsk-Cre or Syklox/lox animals, particularly in the case of female mice (Figure 1B). The increased trabecular density was also evident in three-dimensional reconstruction of an axial cylinder within the trabecular area of the femurs (Figure 1C).

We also processed micro-CT images for quantitative analysis, incorporating data from the entire trabecular space within a defined distance range from the distal femoral growth plate. As shown in Figure 2, the percent bone volume (BV/TV) was strongly increased in Syklox mice, whereas no substantial difference could be observed in Ctsk-Cre or Syklox/lox mice. Male wild type mice had an ∼2.8-fold higher (10.8%) basal percent bone volume (BV/TV) than their female counterparts (3.9%). However, the increase in BV/TV in Syklox mice was more robust in female (4.4-fold) than in male (1.8-fold) animals (Figure 2). We have also performed statistical analysis by two-way (factorial) ANOVA which determines the interaction of the two (Ctsk-Cre and Syklox/lox) mutations, i.e., whether the co-existence of the two mutation in the Syklox resulted in a statistically significant difference beyond an additive effect. That analysis revealed a significant increase of the BV/TV values both in male (p = 0.028) and, especially, in female (p = 0.00005) mice.

Further quantitative (Figure 2) and statistical (two-way ANOVA) analysis of the trabecular bone revealed a higher trabecular number in Syklox mice (p = 0.0069 and 0.00001 for males and females, respectively), whereas no consistent change was observed in the trabecular thickness of the same animals (p = 0.85 and 0.87 for males and females, respectively). In agreement with the increased trabecular number, trabecular separation was reduced in Syklox mice (p = 0.00032 and 0.0011 for males and females, respectively).

Taken together, our results indicate that osteoclast-specific deletion of Syk causes increased bone trabecular mass primarily due to increased bone trabecular number rather than a higher trabecular thickness. However, the phenotype observed in Syklox mice (Figure 2) appeared to be less dramatic than that reported for Tyrobp−/− Fcer1g−/− double knockout mice lacking both the DAP12 and Fcγ ITAM-containing adapter molecules which were previously proposed to signal through Syk (42, 43, 57).

The Effect of Hematopoietic Deletion of Syk on Trabecular Bone Architecture
The apparently less severe bone phenotype of Syklox mice compared to Tyrobp−/− Fcer1g−/− (DAP12/FcγR double knockout) animals (42, 43, 57) could either be due to a less critical role for Syk in in vivo bone homeostasis or the less complete deletion of Syk in Syklox animals. To test this latter possibility, we turned to mice with Syk deficiency in the entire hematopoietic compartment due to deletion by the Vav-Cre transgene which causes Cre expression during the early stages of hematopoiesis (51). Accordingly, we subjected Vav-Cre Syklox/lox (referred to as SykΔHema) mice and appropriate controls to microCT analysis of the distal femur. As shown in Figure 3A, Syk deletion in the entire hematopoietic compartment by the SykΔHema mutation caused a very strong increase in trabecular density in the
longitudinal sections of the femurs of female animals, whereas no substantial changes were observed in Vav-Cre or Syk<sub>flox/flox</sub> mice. An increased trabecular density in Syk<sup>ΔHaemo</sup> mutants could also be observed in cross-sections of the distal femurs of male and, in particular, female mice, whereas no obvious differences could be seen in Vav-Cre or Syk<sub>flox/flox</sub> animals (Figure 3B). Three-dimensional reconstitution of a trabecular area cylinder also showed visible increases in the trabecular density in Syk<sup>ΔHaemo</sup> animals (Figure 3C).

Further quantitative analysis of the microCT data (Figure 4) indicated a strongly increased percent bone volume (BV/TV) in Syk<sup>ΔHaemo</sup> mice in both male and female animals. Importantly, BV/TV values in Syk<sup>ΔHaemo</sup> mice appeared to be substantially higher than corresponding Syk<sup>ΔOC</sup> animals (compare Figures 2, 4). On the other hand, similar to the Syk<sup>ΔOC</sup> results, the BV/TV fold increase in Syk<sup>ΔHaemo</sup> over wild type animals was higher in females (7.9-fold) than in males (4.0-fold), again primarily due to the higher basal values in male wild type mice. Statistical analysis by two-way ANOVA revealed a highly significant interaction between the effects of the Vav-Cre and Syk<sub>flox/flox</sub> mutations (p = 0.00032 and 0.00003 for males and females, respectively), indicating that Cre-mediated deletion of Syk in Syk<sup>ΔHaemo</sup> mice strongly increases trabecular bone mass.
Further quantitative assessment (Figure 4) and statistical analysis (two-way ANOVA) revealed that, similar to the SykΔOC mice, the increased trabecular bone volume was primarily due to an increased trabecular number (p = 0.0010 and 0.00001 for males and females, respectively), rather than significant changes in trabecular thickness (p = 0.31 and 0.61 for males and females, respectively). Trabecular separation was also reduced in SykΔHaemo mice (p = 0.0045 and 0.0071 for males and females, respectively).

Taken together, early deletion of Syk in the entire hematopoietic system results in dramatic increase in the mineralized trabecular bone mass, indicating a critical role for Syk in in vivo bone homeostasis. The bone phenotype seen in SykΔHaemo mice is grossly comparable to that reported for Tyrobp−/−/Fcer1g−/− (DAP12/FcRγ double knockout) animals (42, 43, 57), raising the possibility that the majority of DAP12/FcRγ signals proceeds through Syk in live mice. However, the 30–45% BV/TV values observed in SykΔHaemo mice are substantially higher than the corresponding values (15–20%) in SykΔOC animals, raising the possibility that the lower values in the latter mutants may be due to incomplete deletion of Syk by Cre expression from the Ctsk-Cre mutation.

Bone Histological Analysis
We have also performed histological analysis of the distal femur of wild type, SykΔOC or SykΔHaemo mice. As shown in Figure 5A, a much more dense trabecular network was seen in SykΔOC and, especially, SykΔHaemo mice than in wild type animals. Again, this was more pronounced in female mice because of the lower trabecular density in female than in male mice in the wild type cohorts.

To test the presence of mature osteoclasts on the trabecular bone surface, we have performed immunofluorescence staining of bone sections for calcitonin receptor, an osteoclast-specific differentiation marker. As shown in Figure 5B, calcitonin receptor signals were evident on the lining of trabecular rods (dark areas) in wild type sections. Similar signals were also seen but at substantially lower numbers in SykΔOC sections, whereas no such signals were seen in SykΔHaemo sections (Figure 5B). Those results suggest that the number of calcitonin receptor-positive osteoclasts is reduced in SykΔOC and, especially, in SykΔHaemo mice.

In vitro Osteoclast Development in Lineage-Specific Syk Mutants
We next tested in vitro development of osteoclasts from wild type, SykΔOC or SykΔHaemo bone marrow cells in the presence of recombinant M-CSF and RANKL cytokines. Bone marrow cells were first cultured for 2 days in low (10 ng/ml) M-CSF and non-adherent cells (referred to as myeloid progenitors) were then cultured in the presence of 50 ng/ml M-CSF and 50 ng/ml RANKL. Osteoclast development was then tested by assessing cell morphology and positive histochemical staining for the osteoclast-specific TRAP enzyme.

As shown in Figure 6A, no TRAP-positive multinuclear cells (osteoclasts) were seen 2 days after addition of RANKL to the cultures. However, osteoclasts started to appear in wild type cultures on day 3 and formed very large multinucleated TRAP-positive cells 3.5 days after the initial RANKL treatment. Some osteoclasts also formed in SykΔOC cultures, though they were much smaller in size and failed to fuse into very large cells even by 3.5 days after RANKL treatment (Figure 6A). On the other hand, practically no osteoclasts (multinucleated TRAP-positive cells) could be observed in SykΔHaemo cultures (Figure 6A).

We have also quantitated the extent of in vitro osteoclast formation. To this end, we have counted the number of osteoclasts (defined as TRAP-positive cells with 3 or more
FIGURE 3 | Micro-CT analysis of hematopoietic Syk-deficient mice. Representative micro-CT images of the femurs of 9-week-old mice of wild type (WT) and the indicated mutant mice. (A) Longitudinal sections of the femur of female mice. (B) Cross-sections of the femur of male or female mice. (C) 3D reconstitution of an axial cylinder of the trabecular area of the distal metaphysis of femurs of male or female mice. Images are representative of 5–7 mice per gender and genotype. WT samples are identical to those shown in Figure 1.

nuclei; Figure 6B) and calculated the percent of the culture area covered by the osteoclasts (Figure 6C). Though the two different quantification approaches were related to each other, they also complemented each other, since later stages of osteoclast development may lead to the emergence of very large osteoclasts which occupy large culture areas but are small in numbers (as seen in the last two images in wild type cultures in Figure 6A).

As seen in Figures 6B,C, there were practically no osteoclasts in any of the cultures 2 days after the initial RANKL addition. However, osteoclasts rapidly emerged afterwards in wild type cultures, reaching a maximum number 1 day later. The area covered by wild type osteoclasts increased further in the next 12 h, even though the number of osteoclasts started to decline, indicating the fusion of the cells into a few very large osteoclasts in this final stage of osteoclast development (Figures 6B,C). The number of osteoclasts also increased in Syk^ΔOC cultures and was temporally even comparable to that of wild type osteoclasts (Figure 6B). However, those Syk^ΔOC osteoclasts covered a significantly smaller area than in wild type cultures throughout the experiments (Figure 6C), which was in line with the smaller size of Syk^ΔOC osteoclasts in Figure 6A. On the other hand, again in agreement with the photomicrographs in Figure 6A, practically no osteoclasts could be identified in Syk^ΔHaemo cultures (Figures 6B,C).
We have also performed more detailed statistical analyses (one-way ANOVA) of the area under the curve (AUC) from data presented in Figures 6B,C. In case of the number of osteoclasts (Figure 6B), no statistical difference was seen between the wild type and SykΔOCS cultures (p = 0.12), likely reflecting the fact that the osteoclast numbers only declined on the last day in the SykΔOCS samples (Figure 6B). However, the number of osteoclasts in the SykΔHaemo cultures was statistically highly significantly reduced compared to wild type ones (p = 0.0013). The total area covered by osteoclasts was highly significantly reduced both by the SykΔOCS (p = 0.00058) and the SykΔHaemo (p = 0.00024) mutations.

The above results confirm prior studies indicating a critical role for Syk during in vitro osteoclast development (40, 42, 44). On the other hand, they also indicate an incomplete osteoclast developmental defect in SykΔOCS cultures (as opposed to the complete defect in SykΔHaemo ones), suggesting incomplete deletion of Syk in SykΔOCS mutants.

Analysis of the in vitro Resorptive Activity of Osteoclasts

We also attempted to test the in vitro resorbing capacity of osteoclasts. To this end, myeloid precursors were plated on an artificial hydroxyapatite layer and cultured in the presence of M-CSF and RANKL (50 ng/ml each) for 7 days, followed by assessment of hydroxyapatite resorption by dark field microscopy. It should be noted that this assay measures the combined effect of both osteoclast development and osteoclast-mediated matrix resorption.

As shown in Figure 7A, wild type osteoclast cultures were able to resorb substantial areas of the hydroxyapatite layer (resorbed areas show a dark appearance). In contrast, only small areas of resorption could be observed in SykΔOCS cultures and no resorption was seen in SykΔHaemo cultures (Figure 7A). Quantification of the resorbed area revealed ~40% resorption in wild type cultures, which was strongly reduced by the SykΔOCS and completely eliminated by the SykΔHaemo mutations (Figure 7B). Statistical analysis (one-way ANOVA) revealed highly significant reduction of the resorption activity both by the SykΔOCS (p = 0.00040) and the SykΔHaemo (p = 0.00038) mutations.

These results confirm an important role for Syk in the development and/or function of bone-resorbing osteoclasts (42), and also indicate slight differences between the SykΔOCS and SykΔHaemo mutations.

Analysis of Osteoclast-Specific Gene Expression

We next tested the changes of osteoclast-specific gene expression in osteoclast cultures from the different genotypes. We have also tested additional control macrophage cultures generated under identical conditions except that RANKL treatment was omitted. As shown in Figure 8, the expression of DC-STAMP (encoded by the Tm7sf4 gene), TRAP (Acps5), calcitomin receptor (Calcr), NFATc1 (Nfatc1) and cathepsin K (Ctsk) mRNA strongly increased upon osteoclastic differentiation whereas no such increase could be observed in parallel macrophage cultures. The expression of all those genes were reduced in both the SykΔOCS and SykΔHaemo cultures (Figure 8), though the defect ranged from a moderate (Tm7sf4) to a very strong (Calcr) reduction. It should also be noted that the reduced expression of Ctsk in SykΔOCS samples is likely partially due to the inactivation of one of the two alleles of the Ctsk gene by the Ctsk-Cre (CtskCre/+) mutation present in those cells. Taken together, gene expression data indicate a role for Syk in regulation of osteoclast-specific gene expression.
Analysis of Syk Protein Levels in Osteoclast Cultures

The different severity of the in vivo bone phenotypes (Figures 1–5) and in vitro osteoclast developmental defect (Figure 6) between the Syk\textsuperscript{ΔOC} and Syk\textsuperscript{ΔHaemo} mutants raised the possibility that Syk is incompletely deleted from Syk\textsuperscript{ΔOC} osteoclasts. To test this more specifically, we performed Western Blot analysis of Syk expression during osteoclastic and macrophage differentiation of wild type and mutant bone marrow cells.

As shown in Figure 9A, Syk was present in all wild type cultures and its expression slightly even increased during osteoclast differentiation from wild type myeloid progenitors. Importantly, Syk was also present throughout the assessment period in Syk\textsuperscript{ΔOC} cultures (Figure 9A). On the other hand, Syk was completely absent throughout the entire observation period in Syk\textsuperscript{ΔHaemo} cultures (Figure 9A). Semiquantitative analysis of the Western blot samples (Figure 9B) confirmed the presence of Syk in all wild type and Syk\textsuperscript{ΔOC} but not in Syk\textsuperscript{ΔHaemo} samples. Although there was a tendency of reduced Syk expression in Syk\textsuperscript{ΔOC} osteoclasts as compared to wild type osteoclasts, this difference was not statistically significant, indicating that the Syk\textsuperscript{ΔOC} mutation is not able to reduce Syk expression at the overall cell population level.

The above results provided direct evidence supporting our assumption that Syk is incompletely deleted from Syk\textsuperscript{ΔOC} but it is completely absent from Syk\textsuperscript{ΔHaemo} osteoclast cultures.

Genetic Analysis of Syk Deletion During Osteoclastogenesis

One of the possible explanations for the observed differences between the Syk\textsuperscript{ΔOC} and Syk\textsuperscript{ΔHaemo} mutants is that Cre expression from the Ctsk-Cre mutation occurs at a late stage of osteoclast development which, combined with the potentially long survival of the Syk protein, leads to reduction of Syk protein levels only at a late stage when osteoclast development has already
occurred. The fact that the substantial expression of the Ctsk gene (encoding for cathepsin K) begins at 2 days, and is maximal at 3 days after RANKL treatment (Figure 8) (54, 55) would be in line with that possibility.

As a first approach to address the above issue, we performed qPCR-based analysis of the expression the Cre recombinase in osteoclasts and macrophages from the different genotypes (Figure 10A). As expected, no Cre expression could be observed.
in wild type cultures. Somewhat surprisingly, no Cre mRNA could be detected in Syk$^{ΔHaemo}$ cultures either which, together with the complete absence of Syk protein in those cultures (Figure 9) suggests that the Vav-Cre transgene is activated at an early stage of hematopoiesis but it is silenced at the stage of myeloid differentiation tested in our experiments. On the other hand, Cre expression could be readily observed in Syk$^{ΔOC}$ osteoclast but not macrophage cultures (Figure 10A). Importantly, substantial Cre expression in Syk$^{ΔOC}$ osteoclast was first observed 2 days after the initial RANKL treatment, and continued afterwards. Given that a longer time may be needed to the effective deletion of both Syk alleles, the somewhat partial deletion efficacy of the Ctsk-Cre transgene and that the Syk mRNA and protein likely does not immediately disappear after the Cre-mediated inactivation of the Syk gene, these results are in line with the continued presence of Syk in Syk$^{ΔOC}$ osteoclasts beyond 2 days after the initial RANKL administration (Figure 9).

As a more direct approach to test Cre-mediated deletion of Syk in our osteoclast cultures, we decided to perform PCR-based analysis of the Syk genomic locus from the cells of our various genotypes. To this end, we first amplified and sequenced the genomic DNA around the two loxP insertion sites, which was used along with the publicly available mouse genomic sequence and the original description of the Syk$^{flox}$ mutation (50) to reconstruct the entire sequence of the Syk$^{flox}$ allele (Supplementary Figure 1). The organization of the Syk$^+$ (wild type), Syk$^{flox}$ and Syk$^\Delta$ (result of Cre-mediated deletion) alleles is shown in Supplementary Figure 2, indicating the inserted loxP and other sequences, as well as the sites and results of Cre-mediated recombination. Based on this organization, we have designed two PCR protocols (termed PCR 1 and PCR 2) to amplify specific alleles from genomic DNA (Supplementary Figure 2). PCR 1 (Figure 10B and Supplementary Figure 2) was our standard genotyping PCR protocol using the P fwd and P rev1 primer pair, and was able to distinguish between the Syk$^+$ and the Syk$^{flox}$ allele, based on the increased length of the PCR product caused by the 115 bp insertion during the generation of the Syk$^{flox}$ allele (49). However, PCR 1 was not able to detect the deleted (Syk$^\Delta$) allele because the sequence corresponding to the P rev1 primer was deleted during Cre-mediated excision of the floxed sequences from the Syk$^{flox}$ allele (Supplementary Figure 2). Therefore, we designed a novel PCR protocol (PCR 2; Figure 10C and Supplementary Figure 2) using the same P ffd forward primer along with a new P rev2 reverse primer, spanning the entire floxed sequence, allowing the simultaneous detection of all three (Syk$^+$, Syk$^{flox}$, and Syk$^\Delta$) alleles. We then cultured wild type, Syk$^{ΔOC}$ and Syk$^{ΔHaemo}$ bone marrow cells in the presence of M-CSF and RANKL for different periods of time and analyzed their genomic DNA with both the PCR 1 (Figure 10B) and PCR 2 (Figure 10C) protocols.

Results with PCR 1 are shown in Figure 10B. In line with our expectations, the Syk$^+$ allele was present throughout the assay period in wild type osteoclast cultures and the Syk$^{flox}$ allele was present in all Syk$^{ΔOC}$ samples. Though the latter finding indicated the presence of the non-recombined Syk$^{flox}$ allele throughout osteoclast development, it did not exclude...
Results with PCR 2 (which could detect all three alleles including the Syk\(^+\) allele; see Supplementary Figure 2) is shown in Figure 10C. Those experiments confirmed the expected exclusive presence of the Syk\(^+\) allele throughout the experiment in wild type cultures, as well as the exclusive presence of the Syk\(^\Delta\) allele throughout the Syk\(^{ΔHaemo}\) samples, indicating complete deletion of the Syk\(^{Δlox}\) allele in the Syk\(^{ΔHaemo}\) cultures. In contrast to the static picture in wild type and Syk\(^{ΔHaemo}\) cultures, the Syk\(^{ΔOC}\) cultures showed dynamic changes in the Syk locus (Figure 10C). While only the Syk\(^{Δlox}\) allele was seen 1 day after the initial RANKL treatment, the Syk\(^{Δ}\) allele appeared and its amount gradually increased during the next 3 days, parallel to a proportional decline (but not complete disappearance) of the Syk\(^{Δlox}\) allele (Figure 10C). It should be noted that the appearance of the smaller-size Syk\(^{Δ}\) allele likely had a competitive advantage over the larger-size Syk\(^{Δlox}\) allele in these PCR reactions, leading to a likely underestimation of the amount of the Syk\(^{Δlox}\) allele. Taken together, those results and the time course of the changes indicate that Ctsk-Cre-mediated deletion of the Syk\(^{Δlox}\) allele occurs gradually during 2–4 days after RANKL addition and that only an incomplete genetic deletion of Syk is achieved even until the end of the observation period. The above results indicate slow and gradual deletion of the Syk\(^{Δlox}\) allele in Syk\(^{ΔOC}\) osteoclast cultures, which is in line with the slow activation of the Ctsk gene during in vitro osteoclast development (Figures 8, 10A) (54, 55). These results may also explain the less severe in vivo phenotypes (Figures 1–5) and less pronounced in vitro osteoclast developmental defect (Figure 6), as well as the continuous presence of Syk in osteoclast cultures (Figure 9), in the Syk\(^{ΔOC}\) mutants, as compared with the Syk\(^{ΔHaemo}\) mutants which show early and complete deletion of the Syk\(^{Δlox}\) allele from the beginning of the entire osteoclast developmental process.

**Analysis of Myeloid-Specific Syk Deletion**

Osteoclasts are derived from early myeloid progenitors through a developmental process related to that of macrophages. Therefore, we have also tested certain aspects of osteoclast biology in Syk\(^{ΔMyelo}\) mutants in which Syk is conditionally deleted using the myeloid-specific LysM-Cre knock-in mutation. The Syk\(^{ΔMyelo}\) mutation strongly reduced (but did not completely abrogate) osteoclast development, both in terms of the number of osteoclasts (Supplementary Figure 3A) and the area covered by osteoclasts (Supplementary Figure 3B). As shown in Supplementary Figure 3C, Syk expression was strongly reduced (but did not completely disappear) in both Syk\(^{ΔMyelo}\) osteoclasts and macrophages. The Syk\(^{ΔMyelo}\) mutation also partially reduced osteoclast-specific gene expression, i.e., the upregulation of the mRNA of the Tm7sf4, Acp5, Calcr, Nfatc1, and Ctsk genes (Supplementary Figure 3D). We have also tested Cre expression in wild type and Syk\(^{ΔMyelo}\) cells. As shown in Supplementary Figure 3D, Cre mRNA was absent from wild type cells but it was expressed in all Syk\(^{ΔMyelo}\) samples. Interestingly, Cre expression was especially high in early myeloid progenitors (Day 0 samples) and declined afterwards both in osteoclast and macrophage cultures. Taken together, the Syk\(^{ΔMyelo}\) mutation leads to strong but incomplete deletion of substantial deletion (reduction) of the Syk\(^{Δlox}\) allele given the tendency of PCR to amplify even small amounts of the target templates when no competing templates are present. In contrast, neither the Syk\(^+\) nor the Syk\(^{Δlox}\) allele could be amplified from Syk\(^{ΔHaemo}\) cultures (Figure 10B), suggesting complete deletion of the Syk\(^{Δlox}\) allele from those cells, likely in an earlier stage of hematopoietic development. Unfortunately, the Syk\(^{Δ}\) allele could not be detected with the PCR 1 protocol (Figure 10B and Supplementary Figure 2).
Syk during early myeloid differentiation, leading to strongly reduced but not completely abrogated in vitro development of osteoclasts.

**DISCUSSION**

In this manuscript, we provide direct genetic evidence for the role of the Syk tyrosine kinase in normal bone homeostasis in adult mice. The perinatal lethality of Syk⁻/⁻ mice was overcome by lineage-specific conditional deletion of Syk in osteoclasts (SykΔOC mice) or in the entire hematopoietic system (SykΔHaemo mice). Both osteoclast-specific and hematopoietic Syk deletion led to increased trabecular bone mass and defective in vitro osteoclast development and function. However, hematopoietic Syk deletion caused more robust changes than osteoclast-specific Syk deletion both in vivo and in vitro. Our results suggest that this is due to late and incomplete deletion of Syk in osteoclast-specific Syk mutants, likely caused by late activation and modest activity of Cre expression driven by the Ctsk gene promoter during osteoclast development.

We and others have previously shown that Syk plays an important role in in vitro osteoclast development and osteoclast-mediated resorptive activity (40, 42, 44). However, the role of Syk in bone homeostasis in live mice could not be tested because of the perinatal lethality of Syk⁻/⁻ mice (17, 18), although bone density appeared to be increased in third-trimester Syk⁻/⁻ fetuses (44). Unfortunately, the in vitro osteoclast phenotypes cannot be directly extrapolated to the in vivo situation since a number of mutations even within the same pathway, such as DAP12 (38, 41–43) or PLCγ2 (54, 58, 59) deficiency, provide examples of practically complete in vitro osteoclast defects despite only moderately increased in vivo bone mass. Our in vivo results, especially those with the SykΔHaemo mice, provide the first direct genetic evidence for a major and critical role of Syk in bone homeostasis in live animals.

The two main models used in this study clarify different aspects of the role of Syk in bone metabolism: the SykΔOC mice provide evidence for an osteoclast-specific role of Syk but it only leads to limited defects, while the SykΔHaemo mice have the widest Syk deletion without embryonic lethality and therefore show the maximum extent of bone resorption defects.

Despite the clear in vivo phenotypes of conditional Syk-deficient mice, a number of questions related to the cell type(s) responsible remain open. Our experiments with the SykΔOC mice indicate that the role of Syk in bone metabolism is at least in part mediated by Syk expression in osteoclasts. However, it is at present unclear why SykΔHaemo mice have a more severe phenotype than the SykΔOC animals. A reasonable explanation,
also supported by our in vitro findings, is that the SykΔOC mutation only partially deletes Syk in the osteoclast lineage (see further discussion below). However, we cannot exclude the possibility that changes to (a) hematopoietic lineage(s) other than osteoclasts in the SykΔHaemo mice also contribute to the increased bone mass. In addition, it is also possible that Syk deletion in osteoclasts and/or other hematopoietic cells indirectly promote osteoblast-mediated bone production. It should be mentioned that prior studies (44) showed normal bone production by Syk−/− osteoblasts, therefore it is unlikely that Syk deficiency in osteoblasts (e.g., through a leaky Cre expression) contributes to the observed in vivo bone phenotypes. It should also be noted that our micro-CT studies indicate increased trabecular number rather than a higher trabecular thickness as the main cause of the in vivo bone phenotypes. Unfortunately, different groups have reported different contributions of the changes of trabecular number and trabecular thickness to increased bone mass linked to osteoclast defects (42, 43, 54, 55), making it rather difficult to determine the contribution of osteoclasts and osteoblasts to a bone phenotype based on micro-CT data.

An interesting question arising from this study is why the SykΔOC mutation causes a less severe osteoclast phenotype than the SykΔHaemo mutation. Our results clearly indicate that the SykΔOC mutation is less effective in inactivating the Syk gene in osteoclasts. One possible explanation is the fact that the Ctsk-Cre mutation triggers Cre activation at a relatively later time point (starting at ~2 days after RANKL treatment) which, combined with the likely continued presence of the preexisting Syk mRNA and Syk protein beyond complete deletion of both Syk alleles, may lead to a late disappearance of the Syk protein at a time point where osteoclast development and osteoclast-mediated bone resorption has already occurred. The activation kinetics of the Ctsk gene (Figure 8) and of the Ctsk-Cre mutation (Figure 10A), as well as the late appearance of the SykΔ allele (Figure 10C) all support this explanation. Another possible explanation is that the level of Cre expression from the Ctsk-Cre mutation is too low to provide complete Syk deletion and therefore a significant amount of Syk remains present even after activation of the Ctsk-Cre mutation. In this respect, it is interesting to see that the maximum level of Cre expression in SykΔOC cultures (Figure 10A) is at least an order of magnitude less than that in the SykΔMyelo cultures (Supplementary Figure 3D). Nevertheless, both scenarios and our own results are consistent with prior reports from the literature showing good specificity but incomplete deletion of target genes (incomplete penetrance) by the Ctsk-Cre mutation (54, 55, 60). Those results also point to the fact that the suitability of Cre-expressing mouse strains for the lineage-specific deletion of floxed alleles depends not only on the specificity of the Cre expression but also on its timing, i.e., whether sufficient time is available for nearly complete deletion of the target gene.

Though the main message of our manuscript is the increased in vivo bone mass upon conditional deletion of Syk in live mice, some of our results also address the mechanism of the contribution of Syk to osteoclast development and function. While osteoclast-specific gene expression was reduced in SykΔOC and SykΔMyelo cultures, it was not completely abrogated even in SykΔMyelo cells which practically completely lacked Syk protein expression. Therefore, Syk may not only be involved in osteoclast-specific gene expression but maybe also in later processes such as (pre)osteoclast fusion or the osteoclast-mediated resorption process. It is particularly interesting in this respect that DC-STAMP was only moderately affected by Syk deletion, suggesting that a possible role of Syk in (pre)osteoclast fusion may rely on mechanisms other than DC-STAMP expression. It is also worth noting that practically complete defect of matrix resorption was seen in both SykΔOC and SykΔMyelo cultures (i.e., no substantial difference between the two mutations could be seen in this assay), which, however, is complicated by the fact that this assay measures both osteoclast development and the resorptive activity of the cells, and that the longer culture period could have allowed more complete Syk deletion by the Ctsk-Cre mutation. It is also of interest why the number of osteoclasts are reduced on Day 3.5 in the SykΔOC cultures (Figure 6). This may be simply due to the fusion of the cells reducing the number of individual osteoclasts, apoptotic disappearance of osteoclasts during this late stage of culture, and/or active deletion of Syk toward that time period.

We and others have shown that Syk is required for the development of autoantibody-induced arthritis in experimental mice (24, 33–35) and Syk has been proposed as a therapeutic target in human rheumatoid arthritis (61–63). A possible role for Syk in various immune and other cells such as neutrophils, macrophages, mast cells or even platelets (16, 22–24, 26–29, 31, 64–67) may provide an explanation for this observation. Nevertheless, it is important to note that both murine arthritis models (33) and human rheumatoid arthritis (5) are accompanied with bone erosions. Therefore, the role of Syk in osteoclast-mediated in vivo bone resorption may also provide an additional cell type beyond immune/inflammatory cells in which Syk inhibitors may have a beneficial therapeutic effect. In addition, Syk-mediated bone resorption may also be a therapeutic target in other diseases characterized by osteoclast-mediated bone resorption such as osteoporosis (4) or osteolytic cancer metastases (7, 8).

Taken together, our results provide direct genetic evidence for the role of Syk in in vivo bone metabolism and therefore may contribute to the rationale of developing Syk inhibitors for the treatment of diseases characterized by pathologic bone loss.

ETHICS STATEMENT

All animal experiments were approved by the Animal Experimentation Review Board of the Semmelweis University.

AUTHOR CONTRIBUTIONS

DC, DG, and AM supervised the project. DC, DG, and AM conceived the study, designed the experiments, and performed the qPCR experiments. PA and ZJ performed the histological studies. CD-N conducted the micro-CT scanning. AM supervised the project.
**ACKNOWLEDGMENTS**

We thank Nóra Kiss for expert technical assistance, Bence T. Szabó for help with micro-CT scanning, and Shigeaki Kato and Alexander Tarakhovsky for sharing mutant mouse strains.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00937/full#supplementary-material
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