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**Increased bone resorption and osteopenia are a part of the lymphoproliferative phenotype of mice with systemic over-expression of interleukin-7 gene driven by MHC class II promoter**

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## 1. Summary

Mice with interleukin (IL)-7 transgene under the control of E<sub>α</sub> promoter over-express IL-7 in MHC class II-positive cells and develop specific immune phenotype, marked by an increase in CD45R<sup>+</sup> cells in both the bone marrow and peripheral blood. We show that IL-7 transgenic mice have a bone phenotype characterized by an age-related loss of trabecular bone in both axial and long bones. Osteopenia was the result of increased number of active osteoclasts on the surface of trabecular bone. Furthermore, IL-7 transgenic mice showed increased osteoclastic but unchanged osteoblastic potential of the bone marrow *in vitro*. IL-7 over-expression also created osteoclastogenic microenvironment within the bone marrow which promoted the commitment of precursors towards the osteoclast lineage. These findings are important for immunological disturbances where IL-7 is involved and where alterations in the immune system are accompanied by changes in bone metabolism, such as multiple myeloma, rheumatoid arthritis and postmenopausal osteoporosis.

## 2. Introduction

Interleukin-7 (IL-7) is produced by thymic and bone marrow (BM) stromal cells and mediates critical steps during early T and B lymphocyte development in mice [1]. Transgenic mice over-expressing IL-7 under different promoters develop lymphoproliferative phenotype [2,3]. IL-7 has been implicated in bone homeostasis, but its effects on bone cells are contradictory [4-6]. *In vitro*, IL-7 had inhibitory effect on both osteoclasts (OCL) [4] and osteoblasts (OBL) [6]. *In vivo*, young female transgenic mice expressing human IL-7 gene selectively in OBLs showed increased numbers of early B lymphocytes in the BM and increased trabecular bone mass [7,8]. Mice expressing an IL-7 transgene driven by MHC class II (E<sub>α</sub>) promoter [3] develop enlarged marrow cavity and focal osteolysis related to the lymphoproliferative phenotype [9]. Within the immune system, MHC class II molecules are constitutively expressed by stromal cells and antigen-presenting cells, and can be induced in other cells during immune response [10]. As the control of IL-7 production by MHC class II promoter mimics pathological states marked by prolonged stimulation of lymphocytes [10], such as in autoimmune diseases, we assessed bone morphology and function in mice over-expressing IL-7 transgene driven by the MHC class II (E<sub>α</sub>) promoter.

## 3. Materials and Methods

### *IL-7 transgenic mice*

Male and female mice hemizygous for IL-7 transgene in C57BL/6 background (IL-7 TG mice) [3,9] and wild-type (Wt) littermates were analyzed at 8 weeks, and 6 or 12 months of age. IL-7 transgene carriers were identified by PCR amplification [9]. Immune phenotype was confirmed by flow cytometry [11].

### *Bone histomorphometry*

After fixation in 4% paraformaldehyde and demineralization in EDTA, vertebrae or tibiae were dehydrated and embedded in paraffin. Serial sections (5-8  $\mu\text{m}$  thick) were stained with Goldner's trichrome stain for the measurement of static bone morphometric parameters. The measurements were performed in the trabecular bone area of the proximal metaphysis, 200  $\mu\text{m}$  from the growth plate, and equidistant from endocortical bone. Histomorphometrical analysis of the bone in IL-7 transgenic mice included trabecular bone volume (BV/TV), trabecular number (Tb.No), trabecular separation (Tb.Sp), trabecular width (Tb.Wi) and epiphyseal plate thickness (Ep.Th) [12], measured using Osteomeasure software (Osteometrics Inc, Decatur, Georgia, USA) on Axio Imager A1. OCL-like cells were identified as tartrate-resistant acid phosphatase (TRAP)-stained cells on bone surfaces, with  $\geq 3$  nuclei and counted separately on the epiphyseal plate, trabecular bone and endosteal surfaces. The number of OCLs was expressed per millimeter of bone length.

#### *Bone cell cultures*

BM cells from long bones were cultured in conditions stimulating either OCL [13] or OBL differentiation [14]. Briefly, BM cells were flushed out from long bones, washed, passed through a 40  $\mu\text{m}$  pore size nylon cell strainer and resuspended in  $\alpha$ -MEM supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA).

For OBL cultures [14], BM cells were seeded into 6-well plates and cultured at a concentration of  $7 \times 10^6$  cells in 3 ml per well in  $\alpha$ -MEM/10%FBS. On day 4 of culture medium was changed. On day 7 of culture, the medium was changed and supplemented with 8 mM  $\beta$ -glycerophosphate,  $10^{-8}$ M dexamethasone and 50  $\mu\text{g/ml}$  ascorbic acid. On day 11, cultures were fixed in 2% paraformaldehyde in PBS for 10 minutes and stained for alkaline phosphatase (AP) using a commercial kit (Sigma-Aldrich), and for mineralizing (Von Kossa

staining) or total fibroblast colonies (methylene blue staining). AP positive, mineralising or fibroblast colonies were counted in 3 wells per group for each experiment [14].

For OCLic cultures [13] BM cells were incubated overnight with 5 ng/mL recombinant murine macrophage colony stimulating factor (rmM-CSF, R&D Systems, Abingdon, UK) in  $\alpha$ -MEM/10%FBS to stimulate monocyte-macrophage lineage, followed by harvesting of non-adherent cells, enriched in noncommitted hematopoietic progenitors [13]. Non-adherent cells were seeded into a 48-well plate and cultured in  $\alpha$ -MEM/10%FBS with 20 ng/mL of recombinant murine receptor activator of NF- $\kappa$ B ligand (rmRANKL) and 10 ng/mL of rmM-CSF, in the density of  $0.5 \times 10^6$  cells in 0.5 mL. Medium was changed on day 3 of culture, and on day 6, the cultures were fixed with 2.5% glutaraldehyde in PBS for 30 minutes at room temperature and stained for TRAP, using a commercial kit (Sigma-Aldrich). TRAP-positive cells with  $\geq 3$  nuclei were considered OCLs [13]; their functional activity was confirmed by their ability to resorb calcium phosphate films (BD Biosciences, San Jose, California, USA). For co-culture experiments, the supernatants from Wt and IL-7 TG OCL cultures were collected at the end of the culture period (day 6) and frozen. Supernatants were added in a 25% final volume to new OCL cultures, together with  $\alpha$ -MEM/10%FBS, rmRANKL and rmM-CSF, at day 1, and again at day 3 of culture.

Neutralization of IL-7 was performed by following a protocol for neutralization of soluble cytokines [13]. Briefly, supernatant from IL-7 TG OCL cultures was first incubated with neutralizing IL-7 antibody (R&D Systems) for 1 h at 37 °C, and then added to Wt OCL cultures in a final volume ratio of 25%. The final concentration of anti-IL-7 antibody in the OCL culture was 2  $\mu$ g/mL [4].

For peripheral blood mononuclear cell (PBMC) differentiation, mononuclear cells from orbital plexus blood were cultured at density of  $2 \times 10^6$  cells/mL in osteoclastogenic conditions (20 ng/mL RANKL and 10 ng/mL M-CSF) in  $\alpha$ -MEM/10% FBS for 8 days. CD45R<sup>+</sup> cells were isolated from full BM by magnetic separation using Dynabeads sheep anti-Rat IgG (DynaL Biotech, Oslo, Norway) and FITC-conjugated rat anti-mouse CD45R antibody. The separation was repeated until there were <1% of CD45R<sup>+</sup> cells in the supernatant, as confirmed by flow cytometry. BM cells with or without CD45R<sup>+</sup> population were then cultured in osteoclastogenic culture conditions with 20 ng/mL RANKL and 10 ng/mL M-CSF in  $\alpha$ -MEM/10% FBS.

#### *Gene expression analysis*

Total RNA was extracted from cultured cells using a commercial kit (TriPure; Roche, Basel Switzerland). For PCR amplification, 2  $\mu$ g of total RNA was converted to cDNA by reverse transcriptase (Applied Biosystems, Foster City, CA). The amount of cDNA corresponding to 20 ng of reversely transcribed RNA was amplified by qPCR, using specific amplicon sets and SYBR Green chemistry for  $\beta$ -actin, and TaqMan Assays and TaqMan chemistry for IL-7 and IL-7R (Applied Biosystems). Quantitative PCR was conducted using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Each reaction was performed in duplicate or triplicate in a 25  $\mu$ L reaction volume. The generated data were analyzed by plotting the fluorescence signal  $\Delta R_n$  vs. the cycle number. An arbitrary threshold was set on the linear phase midpoint of the log  $\Delta R_n$  vs. cycle number plot. The cycle threshold (Ct) value was defined as the cycle number at which  $\Delta R_n$  crossed this threshold. The expression of specific genes was calculated according to the standard curve of gene expression in the calibrator



sample (cDNA from control osteoclastogenic culture) and normalized to the expression of the gene for  $\beta$ -actin (“endogenous” control) [14].

#### *Statistical analysis*

All values are expressed as mean  $\pm$  standard error of the mean, and the groups were compared using ANOVA with Student–Newman–Keuls post hoc test. The  $\alpha$  level was set at 0.05. All experiments were repeated at least three times and the data are from a representative experiment.

## **4. Results**

### *Lymphoproliferative phenotype of IL-7 TG mice*

Lymphoproliferative phenotype of IL-7TG mice [9] was visible at 8 weeks of age (48.5 $\pm$ 3.8% CD45R<sup>+</sup> cells in IL-7 TG vs. 28.9 $\pm$ 0.7% in Wt female mice) and was fully developed at 6 months (59.8 $\pm$ 10% CD45R<sup>+</sup> cells in IL-7 TG vs. 16.8 $\pm$ 5.1% Wt female mice) and 12 months (63.8 $\pm$ 0.3% CD45R<sup>+</sup> cells vs. 16.2 $\pm$ 3.2% in WT female mice, and 69.2 $\pm$ 4.8% CD45R<sup>+</sup> cells vs. 15.9 $\pm$ 4.2% in WT male mice, respectively).

### *IL-7 TG mice have increased bone resorption in vivo and develop osteopenia*

IL-7 TG mice developed marked osteopenia with age (Fig. 1). Decrease in the number and thickness of trabecular bone in long bones at 12 months (Fig. 1 and 2) was preceded by vertebral trabecular bone loss already at 6 months of age (7.1 $\pm$ 1.3% in IL-7 TG vs. 20.2 $\pm$ 1.1% in Wt female mice and 12.0 $\pm$ 1.8% in IL-7 TG vs. 20.0 $\pm$ 5.8% in Wt male mice;  $p < 0.05$ , ANOVA and Student-Newman-Keuls post hoc test). Bone growth, assessed as the epiphyseal plate thickness, was not affected (Fig. 1). Trabecular bone loss in IL-7 TG mice

was preceded by an increase in active OCL on metaphyseal and trabecular surfaces at 6 months, followed by increased OCLs at endosteal surfaces at 12 months (Table 1).

*IL-7 TG mice have increased osteoclastic and unchanged osteoblastogenic potential of BM cells*

As bone loss *in vivo* can result not only from increased number or activity of OCLs but also from decreased number or activity of OBLs or disturbed balance in the activity of both cell types [6,15], we tested osteoclastogenic and osteoblastogenic potential of IL-7 TG BM. Only female mice were used for subsequent experiments because bone phenotype was similar in both sexes. At 8 weeks of age, non-adherent BM cells from both IL-7 TG and Wt mice generated similar numbers of OCLs in *ex vivo* cultures (Table 2). At 6 and 12 months of age there were significantly more OCLs in cultures of IL-7 TG BM (Table 2). OCL generation in PBMC cultures was also increased in 12-month-old IL-7 TG mice ( $262.0 \pm 17.1$  vs.  $141.1 \pm 21.2$  OCL/well in Wt cultures,  $p < 0.05$ ). There was no difference between IL-7 TG and Wt mice in BM osteoblastogenic potential assessed as the number of AP-positive colonies (Table 2), nor in number of mineralizing or total fibroblast colonies (data not shown).

*BM microenvironment for increased osteoclastogenesis in IL-7 TG mice*

Immune phenotype of IL-7 TG mice includes up to four-fold increase in CD45R<sup>+</sup> BM cells [9], which may also have OCL differentiation potential *in vitro* [19,20]. To assess possible contribution of CD45R<sup>+</sup> cells to increased osteoclastogenic potential of IL-7 TG BM, we cultured separated CD45R<sup>+</sup> BM population. CD45R<sup>+</sup> BM cells from IL-7 TG and Wt 12 month-old mice had similar osteoclastogenic potential ( $74.5 \pm 4.1$  OCL/well in vs.  $86.5 \pm 7.5$ ,

respectively,  $p=0.1919$ ), indicating that they had the same differentiation potential. As the  $CD45R^+$  population was increased up to 3-fold in IL-7 TG BM, it indicates that it is the enlarged progenitor pool, and not their differentiation potential per cell, which either gives rise to more OCLs or provides a supportive microenvironment for OCL differentiation. To further test the role of  $CD45R^+$  BM population in OCL differentiation in IL-7 TG mice, we cultured whole BM and BM depleted of  $CD45R^+$  population using the same osteoclastogenic culture conditions. Whereas depletion of  $CD45R^+$  cells did not affect OCL differentiation in Wt mice, OCL numbers decreased up to 60% in IL-7 TG mice (Fig. 3), confirming that increased  $CD45R^+$  population and its interactions with  $CD45R^-$  microenvironment are responsible for increased osteoclastogenesis in IL-7 TG BM. To assess possible humoral factors in the environment over-expressing IL-7 [18], we cultured Wt OCL cultures with supernatants from IL-7 TG cultures. IL-7 TG culture supernatants stimulated OCL formation in Wt mice ( $309.0 \pm 13.8$  OCL/well in Wt cultures with IL-7 TG supernatants vs.  $167.4 \pm 12.0$  OCL/well in Wt cultures without addition of supernatant;  $p < 0.05$ ). This demonstrated that the stimulation of osteoclastogenesis could also be mediated by a soluble factor from IL-7 TG OCL cultures. Although IL-7 mRNA expression generally increased with the OCL differentiation in IL-7 TG OCLs (relative mRNA quantity day 0:  $0.99 \pm 0.02$ , day 2.5:  $1.61 \pm 0.07$ , day 5:  $4.53 \pm 0.06$ ), the addition of IL-7 antibody in concentrations shown to fully inhibit IL-7 activity [4] could not abrogate the effect of supernatant transfer ( $230.3 \pm 5.2$  OCL/well in Wt cultures,  $387.3 \pm 5.3$  OCL/well in Wt cultures + 25% IL-7 TG culture supernatant, and  $386.3 \pm 27.2$  OCL/well in Wt cultures + 25% IL-7 TG culture supernatant + 2  $\mu\text{g/mL}$  IL-7 antibody;  $p < 0.05$ ). On the other hand, the expression of IL-7R mRNA, as assessed by quantitative PCR, was increased early in OCL

cultures from IL-7 TG but not in with Wt mice (day 0 IL-7R RNA relative quantity  $23.0 \pm 1.3$  in IL-7 TG and  $8.8 \pm 0.9$  in Wt OCL cultures,  $p < 0.05$ ).

## 5. Discussion

Our study showed that systemic IL-7 over-expression linked to MHC class II expression induces not only the accumulation of B cells and its progenitors, but also OCL differentiation and subsequent trabecular bone loss *in vivo*. Bone loss was specific for trabecular bone, as the morphology of the epiphyseal growth plate was not affected. Bone trabeculae were decreased in number and thickness, enlarging the BM cavity.

The loss of bone in IL-7 TG mice was the consequence of increased number of OCL on trabecular and endosteal surfaces. These findings demonstrated that the primary target of IL-7 over-expression *in vivo* was bone resorption, regardless of the underlying cause of over-expression, because findings in the IL-7 over-expression in MHC class II cells was similar to that in other *in vivo* models, such as treatment of intact mice with IL-7 or in IL-7 receptor (IL-7R) knockout mice [5]. Increased osteoclastogenesis as direct cause of bone loss was confirmed by demonstrating that BM cells from IL-7 transgenic mice formed more functional OCLs *in vitro* than BM of age- and sex-matched Wt mice. Alterations in the osteoclastogenic potential were at least in part age-related because increased osteoclastogenesis was not evident in IL-7 TG mice before 6 months of age, and only 12 month old TG animals presented a fully developed bone phenotype.

Although bone loss is often caused by disturbances in both bone formation and resorption, IL-7 over-expression by MHC class II cells did not affect OBL differentiation, contrasting reports from other IL-7 over-expression models, such as inhibition of new bone formation by

IL-7 in neonatal calvarial organ cultures [6]. Unchanged osteoblastogenic lineage cell differentiation in IL-7 TG mice indicated that the target of IL-7 over-expression in MHC class II-positive cells *in vivo* were OCL precursors, which is in line with the *in vitro* evidence that IL-7 increases myeloid colony progenitors when combined with other colony stimulating factors [16]. Our preliminary results show that the expression of IL-7R mRNA was increased early in OCL cultures from IL-7 TG compared with Wt mice, indicating that OCL precursors from IL-7 TG BM cells had greater sensitivity to IL-7 and thus possibly a greater capacity for differentiation along OCL differentiation pathway. Support for this pathogenetic mechanism comes from studies showing that IL-7 stimulates *in vitro* murine OCL differentiation from cells attached tightly to the bone surfaces by expanding the pool of OCL precursors [17] as well as human OCL differentiation from peripheral blood stem cells by upregulating T lymphocyte production of osteoclastogenic factors [17,18]. BM population responsible for the increase in OCL differentiation could be CD45R<sup>+</sup>, as we demonstrated that removal of CD45R<sup>+</sup> cells resulted in normal osteoclastogenesis *in vitro*. Although some reports assign osteoclastogenic properties of CD45R<sup>+</sup> BM cells to contaminating CD45R<sup>-</sup> cells [21], our study clearly demonstrated that this cell population is important in osteoclastogenesis in conditions with IL-7 over-expression.

Increase in OCL progenitor pool was not the only mechanism mediating the effect of IL-7 over-expression in the BM, as the differentiation stimulus could be transferred *in vitro* by humoral factors from transgenic OCL cultures. Furthermore, the finding that IL-7 specific antibody could not fully abrogate the effect of supernatant transfer demonstrated that IL-7 is not the sole mediator of increased osteoclastogenesis in IL-7 TG mice, but rather its

interactions with the whole BM microenvironment. This is in line with the proposal that IL-7 effects on bone cells depend on whether IL-7 is delivered systematically or locally [17]. BM microenvironment plays a fundamental role in haematopoiesis, especially of B lymphocytes [22]. IL-7 over-expression may change BM microenvironment to promote differentiation of CD45R<sup>+</sup> cells along OCL pathway, as well as to secrete yet unidentified soluble osteoclastogenic factors in CD45R<sup>-</sup> BM populations. IL-7 has been shown to stimulate cytokine secretion from IL-7R-expressing BM stromal cells [22]. Another downstream cytokine in IL-7 over-expressing environment could be RANKL, the key osteoclastogenic factor that binds to its RANK receptor on OCL precursors [15] and is able to induce CD45R<sup>+</sup> cell proliferation in combination with IL-7 [23]. RANKL, shown to increase with IL-7 stimulation of T lymphocytes [15], mediates bone resorption together with tumor necrosis factor- $\alpha$  in IL-7 treated mice [18]. As we added soluble RANKL and M-CSF in culture, the contribution of increased RANKL expression in stromal cells or T lymphocytes could not be assessed. Also, we could not use the experimental models with exogenously added IL-7 to test cell responses to excess IL-7 as IL-7 protein is already over-expressed and secreted *in vivo* and *in vitro* in IL-7 TG mice [3]. Our future research focuses on the identification of factors in the BM microenvironment in IL-7 TG mice and their relationship to CD45R<sup>+</sup> cell population.

In conclusion, systemic over-expression of IL-7 in MHC class II positive cells caused a specific bone phenotype in which CD45R<sup>+</sup> population, expanded by IL-7 over production, could serve as OCL precursors and stimulate production of soluble factors to increase the osteoclastogenic potential of BM cells. These findings are important for immunological disturbances where IL-7 is involved and where alterations in the immune system are

accompanied by changes in bone metabolism, such as multiple myeloma, rheumatoid arthritis and postmenopausal osteoporosis [6,24].

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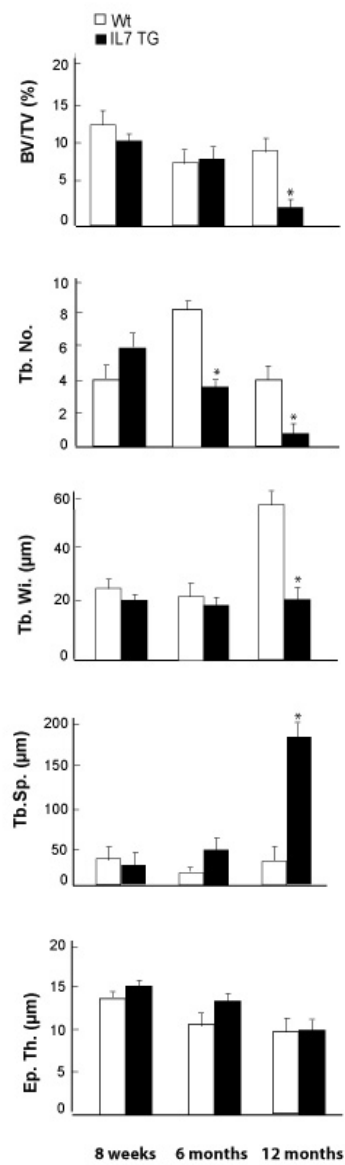
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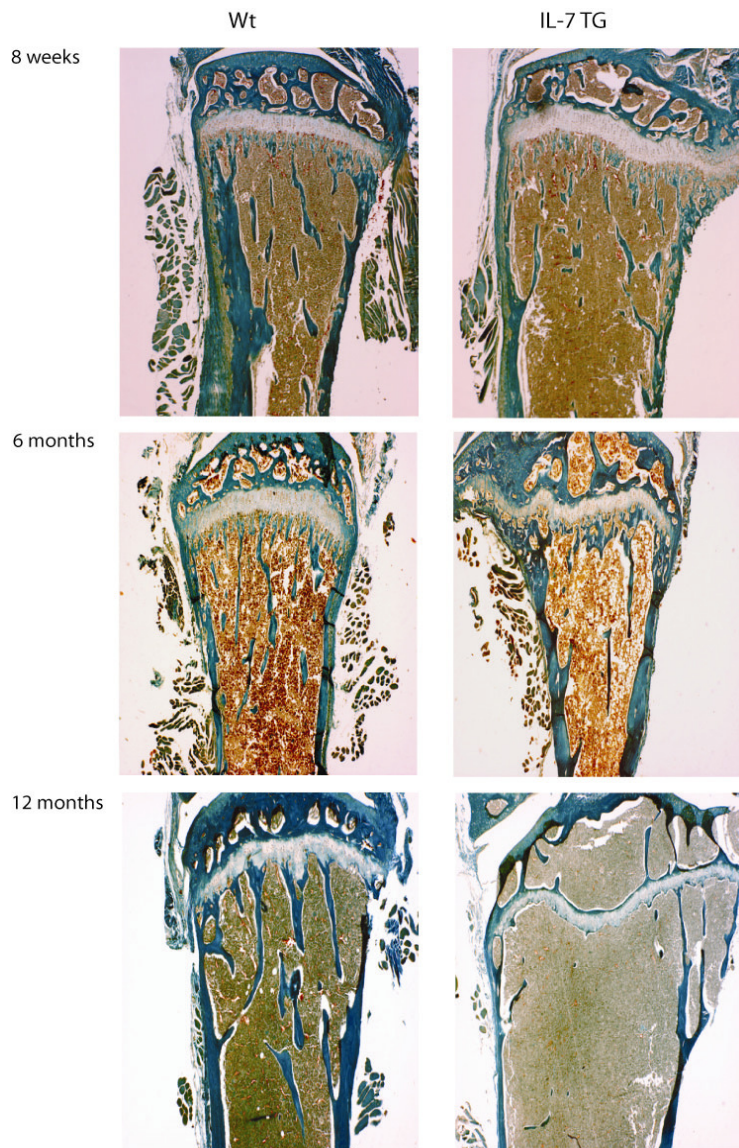
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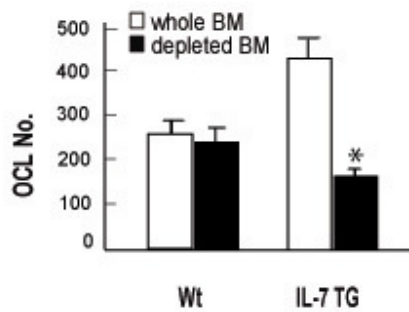
**Figure 1.** Decreased bone mass in IL-7 transgenic (TG) female mice. Tibias from TG mice and wild-type (Wt) littermates (n=6) were assessed for trabecular bone mass (BV/TV), epiphyseal plate thickness (Ep.Th.), trabecular number (Tb.No.), trabecular width (Tb.Wi.), trabecular separation (Tb.Sp.). Asterisk – significant difference vs. Wt mice ( $p < 0.05$ , ANOVA and Student-Newman-Keuls post hoc test).



**Figure 2. Decreased trabecular bone volume in IL-7 transgenic female mice.** Goldner's trichrome staining of tibias from female IL-7 transgenic mice (IL-7 TG) and wild-type (Wt) mice.



**Figure 3.** Decreased osteoclast (OCL) number in cell cultures from bone marrow (BM) depleted of CD45R<sup>+</sup> population. OCL formation in cultures of whole BM or BM depleted of CD45R<sup>+</sup> population from wild-type (Wt) or IL-7 transgenic cultures (IL-7 TG) (n=6 per group), stimulated with 20 ng/ml rm RANKL and 10 ng/ml rm M-CSF. Asterisk – significant difference vs. non-separated culture (p<0.05, ANOVA and Student-Newman-Keuls post hoc test).



**Table 1.** Tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts in tibiae of IL-7 transgenic (TG) and wild-type (Wt) mice\*

<i>Cells</i>	<i>Mice</i>	<i>Number of osteoclasts per mm bone perimeter</i> <i>(mean ± SEM)</i>		
		<i>8 weeks</i>	<i>6 months</i>	<i>12 months</i>
Chondroclasts	Wt	0.0±0.0	2.6±0.7	0.0±0.0
	IL-7 TG	0.0±0.0	2.9±0.4	0.0±0.0
Metaphyseal osteoclasts	Wt	3.3±0.4	10.8±2.1	28.0±1.3
	IL-7 TG	3.7±0.7	19.9±2.7 <sup>†</sup>	70.7±16.2 <sup>†</sup>
Trabecular osteoclasts	Wt	2.2±0.2	1.4±0.4	6.3±3.8
	IL-7 TG	2.4±0.6	6.3±1.8 <sup>†</sup>	16.3±5.3 <sup>†</sup>
Subendostal osteoclasts	Wt	0.3±0.0	0.7±0.4	23.7±6.4
	IL-7 TG	0.2±0.0	2.7±1.0	51.3±12.9 <sup>†</sup>
Total	Wt	5.8±0.6	15.4±3.3	58.0±4.5
	IL-7 TG	6.4±0.1	31.8±4.9 <sup>†</sup>	138.3±32.8 <sup>†</sup>

\*Serial sections from tibiae of IL-7 TG and Wt littermates (4 sections per tibia, 6 mice per group) were stained for TRAP.

<sup>†</sup>p<0.05 vs. Wt, ANOVA and Student-Newman-Keuls post hoc test.

**Table 2.** Osteoclastogenic and osteoblastogenic potential of bone marrow (BM) from IL-7 transgenic (TG) and wild-type (Wt) mice\*

<i>Age</i>	<i>OCL number</i> <i>(mean ± SEM)</i>		<i>AP-positive OBL colonies</i> <i>(mean ± SEM)</i>	
	<i>Wt</i>	<i>IL-7 TG</i>	<i>Wt</i>	<i>IL-7 TG</i>
8 weeks	194.7±25.9	196.3±26.7	49.3±1.2	48.7±2.2
6 months	267.0±11.1	457.0±20.3 <sup>†</sup>	51.3±0.7	57.0±2.5
12 months	199.5±6.2	428.7±19.9 <sup>†</sup>	79.0±2.1	84.0±1.5

\*For OCL differentiation, non-adherent BM cells [13] were cultured at the density of  $10^6$  cells/mL/well of 48-well plates, with 10 ng/ml rmM-CSF and 20 ng/mL rmRANKL. After 6 days, TRAP-positive cells with  $\geq 3$  nuclei were counted (n=6). For OBL differentiation [14], BM cells were cultured at density of  $7 \times 10^6$  cells/3 mL/well of 6-well plates, with 8 mM  $\beta$ -glycerophosphate, 50  $\mu$ g/mL ascorbic acid and  $10^{-8}$ M dexamethasone. Alkaline phosphatase (AP)-positive OBL colonies were counted at day 11.

<sup>†</sup>p<0.05 vs. Wt, ANOVA and Student-Newman-Keuls post hoc test.