ADA-deficient SCID is associated with a specific microenvironment and bone phenotype characterized by RANKL/OPG imbalance and osteoblast insufficiency

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Running Title: ADA-SCID bone phenotype

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Nonstandard Abbreviations used:

ADA = Adenosine Deaminase
BMT = Bone Marrow Transplantation
CTX = Fragments of the Type I Collagen
ERT = Enzyme Replacement Therapy
IL-1ra = Interleukin-1 receptor antagonist
GT = Gene Therapy
OB = Osteoblast
OC = Osteoclast
OPG = Osteoprotegerin
PINP = N-terminal propeptide of type I procollagen
pQCT = Peripheral Quantitative Computed Tomography
RANKL = Receptor activator of nuclear factor kappa B ligand
ABSTRACT

Adenosine Deaminase (ADA) deficiency is a disorder of the purine metabolism leading to combined immunodeficiency and systemic alterations, including skeletal abnormalities. We report that ADA deficiency in mice causes a specific bone phenotype characterized by alterations of structural properties and impaired mechanical competence which is distinct from the phenotype observed in Rag2γc-/- immunodeficient mice. These alterations are the combined result of an imbalanced RANKL/OPG axis, causing decreased osteoclastogenesis and an intrinsic defect of osteoblast function leading to osteoblast insufficiency with subsequent low bone formation. In vitro osteoblasts lacking ADA displayed an altered transcriptional profile and growth reduction, while osteoclastogenesis was normal. Furthermore, the bone marrow microenvironment of ADA deficient mice showed a reduced capacity to support in vitro and in vivo hematopoiesis. Treatment of ADA deficient neonatal mice with enzyme replacement therapy, bone marrow transplant, or gene therapy resulted in full recovery of the altered bone parameters. Remarkably, untreated ADA-SCID patients showed a similar misbalance in RANKL/OPG levels alongside severe growth retardation. Gene therapy with ADA-transduced hematopoietic stem cells increased serum RANKL levels and children’s growth. Our results indicate that the ADA metabolism represents a crucial modulatory factor of bone cell activities and remodeling. The trials described herein have been registered with www.ClinicalTrials.gov under identifiers: NCT00598481 and NCT00599781.
INTRODUCTION

Genetic defects in the adenosine deaminase (ADA) gene are among the most common causes for severe combined immunodeficiency (SCID). Lack of ADA causes accumulation of purine metabolites in plasma, lymphoid tissues, and red blood cells. ADA-SCID patients suffer from lymphopenia, absent cellular and humoral immunity, failure to thrive, and recurrent infections. The additional presence of skeletal, hepatic, renal, lung, and neurological abnormalities underline that ADA deficiency is a multiple organ pathology. Approximately 50% of early-onset ADA-deficient patients exhibit radiologically detectable bone defects. Lack of organized cartilage columnar formation, large lacuni containing hypertrophied cells, lack of trabecular formation with uninterrupted areas of calcified cartilage as well as few osteoblasts and osteoclasts with normal mineralizing osteoid have been reported. Nonetheless, the underlying cellular and molecular mechanisms have remained unclear due to the complexity of the skeletal phenotype and the fact that bone abnormalities are observed also in other immunodeficiencies. Increasing evidence underline the importance of an intense crosstalk between immune and bone cells regulating not only bone remodeling but also hematopoiesis. Therefore ADA deficiency represents a important model, to study both, the impact of altered purine metabolism and immunodeficiency on bone and bone marrow (BM) stroma.

Bone is a highly complex organ, which participates in mineral metabolism, provides structural integrity for the body and supports hematopoiesis. Through a well-organized balance of bone resorption and formation in a time and space dependent manner, bone remodeling enables bone mechanical competence and adaptation to varying mechanical demand. This complex process requires interaction between different cell types and is regulated by a variety of mechanical and molecular factors. Osteoclasts (OC) derived from monocyte/macrophage precursor cells differentiate into multinucleated giant cells specialized in bone resorption. Monocyte/macrophage function has been described to be modulated by adenosine receptor activation.
Osteoblasts (OBs) are bone-forming cells derived from mesenchymal origin. They secrete an extracellular matrix consisting mainly of type I collagen, which they later mineralize. Human OBs possess all four Adenosine receptor subtypes and produce extracellular Adenosine, which modulates their secretion of IL-6 and osteoprotegerin (OPG).\(^{15}\) Adenosine exhibits a potent mitogenic effect on murine calvarial OBs\(^{16}\), while ATP is a known inhibitor of bone formation.\(^{17}\)

Besides their established role in bone remodeling, OBs are crucial component of the hematopoietic stem cell (HSCs) niche.\(^{18-20}\) The interaction of HSCs with OBs is critical for maintaining stem cell properties, including self-renewal capacity and the ability to differentiate into multiple lineages.\(^{21}\)

OBs and OCs interact closely to maintain bone homeostasis. Their crosstalk is mediated by the Receptor Activator of NF-kappaB Ligand (RANKL) and its decoy receptor OPG. RANKL is produced by OBs, but also activated T and B lymphocytes. The source of OPG had historically been attributed to OBs, until recently BM B cells have been described as a major source of BM OPG.\(^{22}\) Since the RANKL to OPG ratio regulates OCs activity and formation, it is conceivable that T-B immunodeficiencies such as ADA-SCID are accompanied by a general misbalance of bone homeostasis.

In ADA deficient patients, like in other forms of SCID, bone marrow transplantation (BMT) is an effective treatment.\(^{23}\) Enzyme replacement therapy (ERT) with polyethylene glycol–conjugated bovine ADA (PEG-ADA) provides metabolic detoxification but often insufficient immune reconstitution.\(^{24}\) Recently, gene therapy (GT) with BM CD34+ cells transduced with a gammaretroviral vector has been shown to correct both the immune and metabolic defects of ADA-SCID children pretreated with low intensity conditioning.\(^{25,26}\) However, little information is available on the corrective effects of these treatments on the associated bone defects.\(^{7,27,28}\)

Due to the complexity of the interactive scenario described herein and the limited availability of biological materials obtained from ADA deficient patients, we first focused our study on the bone phenotype of the ADA-/- mouse model. ADA-/- mice retain many features associated
with ADA deficiency in humans, including T- and B-cell lymphopenia and a profound metabolic defect. Elevated Adenosine levels cause abnormal alveolar development, leading ADA-/- mice to die postnatally within 3 weeks.

We hypothesized that the altered purine metabolism in ADA deficiency impairs OBs and OCs genesis and activity through immuno-dependent and -independent processes resulting in a specific bone phenotype. We characterized the in vivo bone phenotype of ADA-/- mice and evaluated possible OB and/or OC defects in vitro. In parallel, we analyzed the ADA-/- BM stromal cell compartment and assessed its capacity to support hematopoiesis. We extended our study to 15 ADA-SCID patients, either naïve for treatment, under ERT or after GT to assess bone parameters. Results were discussed in light of potential correction of the ADA bone phenotype by current treatment regimens.
MATERIALS & METHODS

Mice

ADA deficient mice have been described previously. 29 Breeding pairs for FVB;129-Adatm1MW-TgN(PLADA)4118Rkmb were purchased from Jackson Laboratories (Bar Harbor, USA). Rag2-/-γc-/- mice30 on BALB/c background were obtained from the Central Institute for Experimental Animals (Nogawa, Japan). Matched wildtype controls (BALB/c) were purchased from Jackson Laboratories. All animals were bred and maintained in a specific pathogen-free animal facility. Procedures were performed according to protocols approved by the Committee for Animal Care and Use of San Raffaele Scientific Institute (IACUC 318).

Patients and clinical trials

Patients were enrolled in two subsequent phase I/II clinical protocols approved by San Raffaele Scientific Institute’s Ethical Committee and Italian National Regulatory Authorities (ClinicalTrials.gov: NCT00598481/ NCT00599781). Orphan Drug Status to ADA vector-transduced CD34+ cells was granted by the European Medicines Agency (EMEA/OD/053/05) to the Italian Telethon Foundation. Patients’ parents signed informed consent to experimental treatment. GT treatment was performed as described. 31 Only patients not displaying any other congenic or endocrine diseases were included. In case of healthy donors or patients on ERT not enrolled in either trial, parents or adult subjects signed informed consent in accordance with the Declaration of Helsinki for research studies on peripheral blood.

pQCT

Measurements were performed using a Stratec Research SA+ pQCT scanner (Stratec, Pforzheim, Germany), voxel size of 0.070 mm³, scan speed of 3 mm/s. All images were obtained with 360 projections and section thickness of 500 or 100 micron.

To separate muscle from bone, scans were analyzed twice with pQCT software 6.00B, using contour mode 1 and peel mode 2, threshold of -50 mg/cm³, inner threshold of 40 mg/cm³,
filter 2 F03F04 for calculation of total area, and threshold of 280 mg/cm³, inner threshold of 400 mg/cm³ for the calculation of bone area. Bones alone were analyzed using contour mode 2 and peel mode 2 with a threshold of 350 mg/cm³ for the calculation of trabecular and total bone parameters in metaphysis and with a threshold of 600 mg/cm³ for cortical bone parameters in diaphysis.

Histomorphometry

Femora from 19-days old mice were fixed, dehydrated and embedded in methyl methacrylate without decalcification. Histomorphometric analysis was performed on Toluidine blue and Alizarin Red S stained sections using a light microscope (Nikon Axiophot, Long Island, USA) equipped with an image analysis system (Nikon DS-5Mc Videocamera; NIS Elements AR 2.20 Nikon software).

Calvarial OB cultures and lentiviral transduction

Calvaria from newborn mice were explanted, digested with collagenase and grown until confluence. In absence of stimuli ADA-/- OBs were transduced with pCCLsin.cPPT.hPGK.hADA.Wpre (PGK-ADA) at a multiplicity of infection of 100 as described previously 32. Median ADA Activity: 13460.5 ±1904 nmol/h/mg protein. At days 3, 5, 8, 10, 12 and 15 replated (15.000/well) cells were harvested and counted using an automated cell counter (Coulter Counter ZM, Electronics, Luton, UK).

Human OB-like cultures

Human bone cell cultures were established using trabecular bone samples obtained from waste materials during orthopedic surgery. 33 Cells were tested for Alkaline Phosphatase and Osteocalcin production after 1,25(OH)2D3 10⁻⁸ M to ensure that they were endowed with OB characteristics.
**In vitro osteoclastogenesis**

Flushed total BM was plated in presence of 100ng/ml M-CSF (Peprotech, Rocky Hill, USA) for 3 days. Cells were replated at 5000/well in 96-well-plates and cultured in presence of 25ng/mL M-CSF and 100ng/mL RANKL (Peprotech). Cultures were fixed, TRAP staining was performed according to manufacturer’s instructions (Sigma) and multinucleated (3 or more nuclei) TRAP+ cells were scored.

**Alamar Blue viability assay**

OBs, M-CSF dependent BM macrophages and LTC-IC stromal layers were isolated as described above and plated in 96-well-plates. 10μL Alamar Blue (Biosource, Camarillo, USA) reagent was added per well. After 3 hours, viability was measured (ex. 530 nm, em. 590 nm) using a Victor3 Microplate Reader (Perkin Elmer, Norwalk, USA).

**ADA enzymatic activity**

Intracellular ADA enzyme activity was analyzed by adenosine to inosine conversion followed by high-performance capillary electrophoresis. Red Blood Cell lysis was performed on BM and spleen samples.

**Gene Expression Analyses**

RNA was extracted using EUROzol (Euroclone, Wetherby, UK) and transcribed into cDNA using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real Time PCR reactions were carried out using Assay-on-Demand gene expression arrays (Applied Biosystems). The relative expression of each gene was normalized to HPRT as endogenous control. mRNA levels were quantified using the comparative threshold-cycle (CT) method. Arrays utilized: Mm01187117_m1, Mm00801666_g1, Mm01337566_m1, Mm00485009_m1, Mm00435452_m1, Mm00441908_m1, Mm03003491_m1.
**FACS Analyses**

Staining for AnnexinV and 7-AAD was performed on cultured OBs according to manufacturer’s instructions (both BD Pharmingen, San Diego, USA). Stainings for OC precursors from flushed total BM were performed as described previously. 32 Rat anti-mouse monoclonal antibodies utilized: fluorescein isothiocyanate-conjugated anti-CD117 (2B8; BD Pharmingen); phycoerytrin-conjugated anti-F4/80 (Serotec, Oxford, UK), allophycocyanin-conjugated anti-CD11b (M1/70; BD Pharmingen) and Pacific Blue-conjugated anti-CD48 (HM48-1; Biolegend, San Diego, USA). Samples were analyzed using a BD FACS Canto and DiVa software (BD Pharmingen).

**ELISA**

Murine RANKL, OPG (R&D Systems, Minneapolis, USA) and murine PINP (IDS, Boldon, UK) were assayed on sera from 19-days old male ADA+/+ and ADA-/-, Rag2γc+/+ and Rag2γc-/- or 12-weeks old ADA+/+ and rescued ADA-/- mice. ELISA for CTX (RatLaps ELISA; Nordic Biosciences Diagnostics, Herlev, Denmark) was performed according to manufacturer’s instructions on serum samples from 12-weeks old ADA+/+ or rescued ADA-/- mice starved for 6 hours. Assessment of CTX in untreated ADA-/- mice of 19-days of age was not feasible due to the starvation protocol. Increased sensitivity sRANKL and human OPG ELISA (Biomedica, Vienna, Austria) were performed on plasma from patients and pediatric normal donors according to manufacturer’s instructions.

**Stromal Cultures (CFU-F, LTC-IC)**

CFU-F assays were performed using MesenCult medium (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer’s protocol. Stromal feeder layers for LTC-IC assays were established from flushed BM using MyeloCult medium (StemCell Technologies) according to the manufacturer’s instructions. Within 1 week after irradiation (15Gy), lineage negative cells isolated using StemSep Murine Progenitor Enrichment separation (StemCell Technologies) were added. ADA-/- cells showed comparable numbers
and viability after irradiation at 15Gy and 30Gy excluding a potential bias due to differential irradiation sensitivity (data not shown). After 4 weeks of Co-Culture, adherent and non-adherent from each well were transferred into semisolid MethoCult (StemCell Technologies). CFU were scored after 10 days.

**Proteome Profile**

Supernatants from LTC-IC Cultures established as described above were analyzed using Proteome Profiler Arrays (R&D Systems) according to manufacturer's instructions. Densitometric analysis was carried out using ImageQuant software (Molecular Dynamics).

**5-Fluorouracil treatment**

Neonatal ADA+/+ and ADA-/- mice were transplanted into the temporal vein with 3000 ADA+/+ lineage negative cells, as described. Transplanted cells were purified by StemSep Murine Progenitor Enrichment separation (StemCell Technologies). Starting from 6 weeks of age, surviving mice received weekly i.p. injections with 150mg/kg 5-Fluorouracil (Sigma).

**ERT, BMT and GT treatment**

ADA-/- were rescued by weekly i.p. injections (1000U/kg) with PEG-ADA (Adagen, Enzon, Piscataway, USA), by BMT or GT as described previously. 5x10^6 ADA+/+ or transduced ADA-/- BM cells were infused by injection through the temporal vein of irradiated neonatal ADA-/- mice.

**Statistical Analyses**

For comparisons between groups, an unpaired 2-tailed Student's t test was used. A P-value <0.05 was considered significant. For analyses of LTC-IC assays the difference in scored colonies was evaluated fitting a generalized linear model based on Poisson distribution, taking in account possible differences between experiments and first order interactions. For Proteome Profile analyses a linear model was used to assess differences in protein
expression, taking into account possible difference between experiments. $P$-values were adjusted for multiplicity by means of Benjamini-Hochberg false discovery rate method. All analyses were performed with R software (ver. 2.8.0).
RESULTS

ADA deficient mice display a specific bone phenotype

Since bone defects due to alterations of cross-talk between hematopoietic and bone cells have been described in other immunodeficient mice, we studied the ADA-/- bone phenotype in comparison to double mutant Rag2γc-/- mice. The latter lack T, B and NK cells but do not suffer from the profound metabolic defect typical for ADA-/- mice. Comparing both immunodeficient models allowed us to distinguish the contribution of immunodeficiency from that of the metabolic disease to the ADA bone phenotype. Because of strain differences, ADA-/- and Rag2γc-/- mice were compared to their respective sex- and age-matched wildtype controls.

ADA-/- mice were analyzed at 19-days of age, when they are generally smaller, while Rag2γc-/- mice (X-ray not shown) are comparable to wildtype controls (Supplementary Figure 1A). In order to characterize bone size and structural parameters peripheral quantitative computed tomography (pQCT) was performed on tibiae and femora retrieved from ADA+/+, ADA-/-, Rag2γc+/+ and Rag2γc-/- mice. As shown in Figures 1A-B, a significantly lower total area and medullary canal area was found in both immunodeficient mouse models. This difference was more pronounced in the ADA-/- compared to Rag2γc-/- mice, resulting in significant reduction in the Canal Area to Total Area ratio (Figure 1C). Consequently, cortical thickness (Figure 1D) was found to be significantly larger in Rag2γc-/- mice, while no difference was measured in ADA-/- mice. The Strength-Strain-Index (SSI), direct estimate of bone strength, was significantly reduced only in ADA-/- mice (Figure 1E).

The observed structural differences were not accompanied by changes in cortical bone density (Figure 1G). Interestingly, the trabecular density was significantly reduced in ADA-/- mice, but significantly increased in Rag2γc-/- mice (Figure 1H).

ADA-/- mice suffer from significant weight loss during the last days of life (Supplementary Figure 1B). Since developmental changes in bone strength are secondary to increasing loads imposed by larger muscle forces, the functional muscle-bone unit, was evaluated to distinguish whether any observed bone defect is aspecifically related to insufficient muscle
mass. We found a large reduction in the muscle cross sectional area (MCSA) while bone mineral content (BMC) remained unchanged, resulting in a significantly higher proportion between BMC and MCSA in ADA-/- mice (Supplementary Figure 1C).

**ADA-/- mice display low trabecular bone volume**

The low trabecular density, detected by pQCT, was confirmed at static histomorphometric evaluation. As shown in Figure 2A, longitudinal growth of ADA-/- femora is impaired compared to controls. To assess defects in the trabecular bone network, metaphyseal sections were analyzed by image analysis software (Figures 2B-C). As summarized in Supplementary Table 1, the Trabecular Bone Volume and Trabecular Number in ADA-/- mice were significantly lower compared to wildtype. Consequently, Trabecular Separation was significantly increased, while Trabecular Thickness was not different between both groups. Despite early reports on a selective toxicity of ADA substrates for chondrocytes, we did not detect any gross differences between the growth plates of ADA+/+ and ADA-/- mice (Figures 2D-E).

**Reduced RANKL/OPG ratio and reduced bone formation in ADA-/- mice**

RANKL and OPG, well characterized key players of crosstalk between OBs or stromal cells with hematopoietic OC precursors, synchronize osteoclastogenesis and bone resorption. As shown in figure 3A, both immunodeficient models showed significantly reduced RANKL levels, while serum OPG levels were in the normal range for both groups (Figure 3B). Consequently, the RANKL/OPG ratio, indicator of in vivo bone turnover, was reduced in ADA-/- and Rag2γc-/- mice (Figure 3C). To assess new bone formation we measured the levels of the N-terminal propeptide of type I procollagen-(PINP). As shown in Figure 3D, PINP levels were significantly reduced in sera of ADA-/- mice, while comparable in Rag2γc-/- mice and controls.
ADA activity is bone cells

ADA is ubiquitously expressed, but little information is available on the specific levels of enzymatic activity in bone cells. Remarkably, ADA activity in wildtype OBs was found 3-fold higher compared to mesenchymal progenitor cells, 2-fold higher compared to OC precursors and therefore in the range of other lymphoid organs such as the spleen or BM (Supplementary Table 2)

ADA-/- osteoblast activity is impaired

The reduced periosteal bone apposition in vivo and the high ADA activity detected in wildtype OBs in vitro suggested defects of the osteoblastic compartment in ADA-/- mice. ADA-/- OBs showed a significantly lower proliferation rate compared to wildtype cells in vitro (Figure 4A). This was associated with a significant decrease in viability and a significant increase in the percentage of apoptotic cells (see Figures 4B-C). Importantly, transduction with a lentiviral vector encoding for ADA rescued both the growth defects and increased sensitivity to apoptosis observed in ADA-/- cells (Figures 4C-E). We performed quantitative gene expression arrays designed to assess whether OB differentiation is altered in ADA-/- mice (Figure 4D). Runx2, known to contribute to early osteogenic differentiation, was expressed at comparable levels. While expression levels of Collagen Type1 and Osteocalcin recognized markers of OB activity were significantly decreased in ADA-/- OBs. Alkaline Phosphatase, essential for matrix mineralization, revealed 2-fold increased expression levels. Interestingly, RANKL expression levels were significantly reduced, while OPG levels were 3-fold increased. ADA inhibition with EHNA (100mM) is a commonly used method to mimic ADA deficiency in vitro. For unavailability of sufficient patient’s material, we utilized EHNA to block ADA activity in primary human OBs. As demonstrated in murine OBs (Figure 4E) EHNA efficiently blocked ADA activity reducing OBs proliferation to levels comparable to ADA-/- cells. Proliferation of human OBs was significantly inhibited when cultured with EHNA (Figure 4F), culturing with EHNA and Adenosine completely ablated proliferation.
No intrinsic defect of ADA-/- osteoclasts

Consistently with the small medullary canal area observed in ADA-/- mice, serum RANKL levels were found to be significantly decreased suggesting a block in osteoclastogenesis. To exclude a lack of OC precursors we assessed their percentage in the BM by FACS staining for CD11b, cKit, CD48 and F4/80. The CD11b+/cKit- population was selected as myeloid precursors; within this subpopulation we distinguished committed myeloid precursors from macrophages by CD48 and F4/80 staining. The relative percentages of OC precursors of both the monocyte and macrophage lineages were increased in the BM of ADA-/- mice (Supplementary Figure 2A). This significant difference was maintained when the percentage of OC precursors was normalized for the absolute number of total BM cells and the median size of the BM cavity (Supplementary Figure 2B). No differences in viability of OC precursors and in vitro osteoclastogenesis from M-CSF and RANKL stimulated BM were detected (Supplementary Figure 2C-D).

Reduced hematopoietic support of ADA-/- stroma

Given the importance of OB for the maintenance of the HSC niche, profound defects of the osteoblastic compartment in ADA-/- mice described herein imply a potential impact on the hematopoietic stem cell niche and/or hematopoiesis itself. Using fibroblast colony forming unit (CFU-F) assays we showed that ADA+/+ and ADA-/- mesenchymal progenitor cells grow equally in vitro (Figure 5A). Nonetheless, when co-cultured with ADA+/+ or ADA-/- lineage negative cells, ADA-/- stromal cells supported colony formation less efficiently than wildtype cells (Figure 5B). This difference was significantly different considering wildtype versus ADA-/- feeder layers (p=0.012), but independent from the co-cultured lineage negative cells (N.S.). Proteome Profiling of total cytokine and chemokine production from the supernatant of LTC-IC cultures revealed reduced levels of M-CSF, IL-6, CXCL1/10 and sICAM-1 (Figure 5C). Interestingly, only the IL-1 receptor antagonist (IL-1ra) was expressed at higher levels compared to wildtype. The secreted form of the IL-1ra is an acute-phase protein intervening in the counterregulation of inflammatory processes and is highly expressed in liver and
Gene Expression Arrays for IL1-ra revealed 5.6-6.2-fold increased expression levels in spleen, liver and BM from ADA-/- mice. Normal IL-1ra expression was detected in cultured OBs, stromal and mesenchymal cells (Figure 5D), indicating that hematopoietic cells are likely to be the source of increased IL-1ra levels detected in Proteome Profiles from ADA-/- LTC-IC assays. To assess potential defects of the hematopoietic stem cell niche in situ, we assessed the percentage of CD150+CD48- within the Lineage negative, Sca1+ and cKit+ compartment, which are generally believed to be quiescent HSC with a frequency of 50% repopulating capacity \(^{41}\). As shown in Figure 5E the percentage of LSK CD150+CD48- HSC is significantly lower in ADA-/- mice compared to wildtype. Additionally, we transplanted neonatal ADA+/+ and ADA-/- mice with wildtype lineage negative cells. Hematopoietic reconstitution was assessed by monitoring survival upon weekly injections of the cell-cycle-dependent myelotoxic agent 5-fluorouracil. \(^{42}\) As shown in Figure 5F, due to hematopoietic failure, ADA-/- mice transplanted with wildtype cells died earlier compared to ADA+/+ mice \((P=0.011)\).

**Recovery of altered bone parameters in rescued ADA-/- mice**

To assess the effect of different treatments on the ADA bone phenotype, we rescued newborn ADA-/- mice with ERT, BMT or ex vivo lentiviral-mediated GT. Serum concentrations of RANKL, OPG, PINP and CTX were assessed in surviving mice at 12 weeks of age. As shown in Figure 6, mice rescued by all three different treatments displayed full correction of serum RANKL and consequently RANKL/OPG levels (Figure 6A-C). Serum PINP levels were completely rescued, while CTX levels were comparable to controls (Figure 6D-E). pQCT analyses confirmed that all bone parameters previously observed to be altered in naïve ADA-/- mice recovered to normal (Figure 6F and Supplementary Figure 3).

**ADA-SCID patients show delayed growth and altered bone remodeling**

From the few case reports in ADA-SCID patients it has remained unclear whether their severe growth delay is associated with specific defects in bone remodeling. To assess
whether bone turnover is impaired we measured RANKL and OPG ratio in plasma of 5 ADA
deficient patients naïve for treatment and displaying severe growth retardation (Table 1). In
all patients RANKL levels were severely reduced or undetectable ($P=0.005$). Interestingly,
serum OPG levels were significantly increased ($P=0.003$), resulting in RANKL versus OPG
ratio significantly reduced compared to age-matched normal donors ($P=0.011$).

**Gene Therapy but not Enzyme Replacement normalizes RANKL in ADA-SCID patients**

As reported in Table 2, patients receiving ERT long-term (1-21 years), displayed low or
undetectable serum RANKL levels ($P=0.004$), while OPG was in the normal range ($P=0.504$).
This resulted in a significantly reduced RANKL/OPG ratio ($P=0.033$). All patients displayed
significantly reduced height compared to age-matched standards. We next analyzed RANKL
and OPG in patients treated with HSC-GT combined to reduced intensity conditioning. As
reported in Table 3, in patients treated with GT, RANKL and consequently the RANKL/OPG
ratio significantly increased 1-2 years after treatment ($P=0.005; P=0.031$). Importantly, both
serum RANKL ($P=0.979$) and the RANKL/OPG ratio ($P=0.515$) were not significantly
different from the range observed in pediatric normal donors aged 1-5 years (see Table 1).
Consistently, 8 out of 9 patients displayed an increase in percentile of height at the last
follow-up after treatment. However, the percentile of height and bone age (Greulich/Pyle)
were not fully normalized in all patients.
DISCUSSION

Skeletal defects have been reported to be a common feature of ADA deficiency in humans\textsuperscript{3-5,8,27,28}, nonetheless it has remained unclear whether they are a consequence of SCID or caused by alterations in purine metabolism. The present study has filled this gap by providing evidence that ADA deficiency in mice is associated with a specific bone phenotype, characterized by alterations in structural properties and mechanical competence. However, these structural alterations being only partially superimposable to those observed in TB\textsuperscript{-}Rag2\textgamma c\textsuperscript{-/-} mice, could not be ascribed to immunodeficiency per se. While the small medullary canal area appeared a joint feature in both immunodeficient models, low bone mass and SSI were observed only in ADA\textsuperscript{-/-} mice. The reduced trabecular density, specifically affected by ADA deficiency, was related to a low trabecular number, rather than reduced trabecular thickness. Since fetal bone development predicts bone mass accrual as a consequence of increasing trabecular bone volume in function of growth\textsuperscript{43}, the decreased trabecular number might be the expression of impaired ossification of the cartilaginous template with subsequent deterioration of bone structural properties. However, no mineralization defects were measured in the cortical bone of ADA\textsuperscript{-/-} mice and no osteoid seams were observed in static histomorphometric evaluation, indicating an appropriate acquisition of tissue material properties. Although no gross alteration of the growth plate morphology could be observed, the contribution of reduced growth plate activity for the determination of the bone phenotype is likely since both ADA deficient mice and patients display significantly reduced longitudinal growth. Contrarily, the contribution of muscle dysfunction can be considered minimal, since the higher Muscle-Bone-Unit here observed, was related to the faster loss of muscle than bone mass due to metabolic toxification at a later stage of survival.

The described ADA bone phenotype is consistent with the hypothesis that the altered purine metabolism impairs OCs and OBs genesis, proliferation and activity through immunodependent and -independent processes.\textsuperscript{14-17} Due to reduced RANKL levels, the serum RANKL/OPG ratio was significantly reduced in both ADA\textsuperscript{-/-} and Rag2\textgamma c\textsuperscript{-/-} mice. The small
medullary canal area fits this observation suggesting a shift of the bone remodeling sequence towards reduced osteoclastogenesis. Interestingly proteome profiling of cytokine and chemokine production from LTC-IC cultures revealed reduced levels of M-CSF and IL-6, factors involved in OC activity and generation. 9 It is unlikely that this shift is due to an intrinsic defect of OC precursors since in the presence of M-CSF and RANKL they equally form TRAP+ cells in vitro.

The high ADA activity measured in wildtype OBs indicates a strong dependency of this cell type on the ADA metabolic pathway. Interestingly, enzymatic activity measured in mesenchymal progenitor cells was 3-fold lower, indicating that differentiating OBs must upregulate ADA expression considerably. It is therefore conceivable that, with increasing dependency on the ADA enzyme during proliferation or differentiation, OB function and viability becomes severely affected.

The reduced outward displacement of the thin cortex as well as the lower serum PINP levels in ADA-/- mice are in agreement with the hypothesis that OBs are the major casualty of ADA substrates' toxicity. The lower proliferation rate of ADA-/- OBs observed in vitro, associated with decreased viability and increased apoptosis, confirms this hypothesis and underlines the importance of purine metabolism in OB function. Importantly, correction of ADA expression by lentiviral vector transduction leads to full correction of ADA-/- OB growth defects and apoptosis, providing direct evidence that the OBs insufficiency state is cell intrinsic.

Proliferation of primary murine and human OBs was similarly reduced, when mimicking ADA deficiency using the ADA inhibitor EHNA. Thus, low OB numbers may contribute to the bone phenotype observed in both ADA deficient mice and patients. In accordance with impaired bone formation, the expression profile of murine ADA-/- OBs is characterized by low Collagen Type 1 and Osteocalcin levels. However, it is likely that OBs compensate for their low numbers expressing elevated levels of Alkaline Phosphatase, so that no differences of bone material properties were detected. Similarly to the low RANKL/OPG ratio observed in ADA-/- sera, their respective RNA expression levels were altered also in ADA-/- OBs. Being the dominant mediators of osteoclastogenesis, their misbalance confirms the central role of
OBs determining the ADA bone phenotype and suggests the activation of a compensatory mechanism for bone mass preservation.\textsuperscript{9}

Since RANKL ligand is also produced by B and T cells, its reduced serum levels can be partially attributed to the lymphopenia in both ADA-/\(-\) and Rag2cy-c-/\(-\) mice. Nonetheless, lymphocytes are severely reduced but not completely absent in BM and thymus of both immunodeficient models, which probably accounts for the residual serum RANKL levels. Additionally, RANKL expression in contrast to OPG is downregulated in ADA-/\(-\) OBs. Its reduced expression might be induced by the increased levels of IL-1ra, detected in ADA-/\(-\) mice, which may act by preventing IL-1 signaling, a potent stimulator of bone resorption. IL-1 modulates OCs activity directly\textsuperscript{44} or indirectly through its ability to stimulate RANKL production by OBs\textsuperscript{45}; therefore IL-1ra secreted at elevated levels in ADA-/\(-\) mice likely restricts IL-1 induced bone resorption and RANKL expression. It can be speculated that the reduced endosteal resorption might counterbalance the low periosteal bone apposition in order to fulfill the physical requisite of bone strength for loading and lightness for mobility.\textsuperscript{46}

Considering that BM B cells are a major producer of OPG\textsuperscript{22}, it was unexpected to observe normal serum OPG levels in ADA-/\(-\) mice. Nonetheless OPG may be produced by other cell sources and indeed we found a 3-fold upregulation of OPG mRNA levels in ADA-/\(-\) OBs.

These data suggest an OB insufficiency state as consequence of ADA deficiency that, given the interplay between osteogenesis and hematopoiesis, implies an impact on the BM microenvironment and the HSC niche.\textsuperscript{18-20} In vivo depletion of OBs caused loss of HSC, followed by a marked decrease in BM cellularity.\textsuperscript{20} Consistently, the OB insufficiency in ADA-/\(-\) mice is associated with a reduced BM cellularity (not shown). The capacity of ADA-/\(-\) stromal cells to support colony formation from lineage negative cells was significantly lower compared to wildtype. This reduced capacity might be associated with the low production of IL-6, crucial survival factor for HSC and is in agreement with the defective hematopoietic support reported in IL-6 deficient mice.\textsuperscript{47} The hypothesis that the HSC niche in ADA deficiency is specifically affected was further supported by the reduced percentage of LSK CD150+CD48- HSC in ADA-/\(-\) BM and by the hematopoietic failure and premature death of
ADA-/ mice transplanted with wildtype HSC when challenged with 5-Fluorouracil. Our data may provide a possible explanation for the higher toxicity of pre-transplant conditioning and transplant failures observed in ADA-SCID patients treated with BMT\textsuperscript{24,48} as well as for frequent findings of hematopoietic abnormalities; \textsuperscript{1} and A. Aiuti (unpublished results). Future studies will be needed to assess whether current therapeutic approaches may be improved by simultaneous targeting of the niche. In this regard transplant of normal or ADA engineered mesenchymal stem cells may facilitate HSC engraftment or contribute to the reconstitution of the bone cell compartment.

Comparative analyses of adult ADA-/ mice treated with ERT, GT or BMT showed full correction of RANKL, RANKL/OPG and PINP as well as recovery of bone growth. We observed a significant increase of serum OPG levels in mice treated with GT or BMT, a phenomenon which has also been reported in allotransplanted patients. \textsuperscript{49,50} This is likely related to pre-transplant conditioning rather than the transplant procedure itself, consistently non-irradiated PEG-ADA treated mice displayed normal OPG levels.

The results obtained in 15 ADA-SCID patients, either naïve or under different treatments have confirmed that bone defects are a common feature associated with ADA deficiency in humans and extended the relevance of our findings to the human disease phenotype. RANKL and OPG levels in naïve ADA deficient patients, displaying serious growth retardation, were significantly lower compared to healthy controls. GT with autologous gene corrected stem cells, but not ERT, resulted in a significant increase in serum RANKL and the RANKL/OPG ratio. The less efficient correction of bone parameters in ADA-SCID patients as compared to mice might be due to the fact that all mice received treatment within the first 5 days of life, while patients were treated at variable ages. Moreover, ERT could be more efficient to correct the murine ADA bone phenotype, because to rescue ADA-/ mice up to 50 times higher doses of PEG-ADA are required as compared to standard doses in humans. Since OBs are not derived from HSC, they are not expected to be corrected by either treatment. It is possible that endogenous ADA production by resident hematopoietic cells is more efficient to cross-correct OB function compared to circulating PEG-ADA. Moreover
patients treated with GT show superior immune reconstitution and lymphocyte counts with respect to patients treated with ERT\textsuperscript{31}, representing an important source of RANKL.

In summary, we report an ADA specific bone phenotype, characterized by low bone mass accrual, size acquisition and impaired mechanical competence, which is due to an OB insufficiency with subsequent impact on the HSC niche. Our results emphasize the role of the ADA metabolism in modulating bone cell activities, and add a stromal component to the series of immunological defects described in ADA deficiency. The presented data show that correction of the ADA bone phenotype is feasible with current treatment options, but longer follow-up in these patients will be needed to assess whether bone defects are resolvable over a longer period of time.
ACKNOWLEDGMENTS

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AUTHORSHIP

A.V.S. designed and performed most research, analyzed data, and wrote the paper; E.M. designed and performed in vitro experiments and analyzed data; R.J.H. performed animal experiments; E.Z. performed pQCT analyses; F.C. performed histomorphometric analyses; M.C. collected patients data; E.G., C.M.R. and M.C.C. followed patients, A.A. performed the statistical analysis; F.C. conducted the biochemical studies; M.G.R. and A.V. contributed to the study design; A.R. designed the research and wrote the paper; A.A. designed the research, analyzed data and revised the paper; all authors checked the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest related to this article.
REFERENCES


Table 1: ADA-SCID patients naïve for therapy display significantly reduced growth. $P<0.0005 (***)$, $P=0.0005-0.005 (**)$, $P=0.005-0.05 (*)$.

<table>
<thead>
<tr>
<th>Pt</th>
<th>Age [yrs]</th>
<th>Percentile Height [Z-Score]</th>
<th>RANKL [pg/mL]</th>
<th>OPG [pg/mL]</th>
<th>RANKL/OPG [x10³]</th>
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ND aged 1-5 years, n=10 (SEM): 11.49 (3) 104.1 (7) 13.4 (5)

$P$-Value: 0.005** 0.003*** 0.011*
Table 2: ADA-SCID patients on ERT with PEG-ADA display reduced growth. $P < 0.0005 (***)$, $P = 0.0005-0.005 (**)$, $P = 0.005-0.05 (*)$.

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<tr>
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<th>OPG [pg/mL]</th>
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ND aged 1-15 years, n=14 (SEM): 8.06 (1.6) 106.9 (5.6) 8.4 (1.7)

$P$ Value: 0.004** 0.504 0.033*
Table 3: ADA-SCID patients treated with Hematopoietic Stem Cell Gene Therapy display a significant increase in RANKL. \( P = 0.0005-0.005 \) (**), \( P = 0.005-0.05 \) (*), N.S. = Not Significant, N.D. = Not Done.

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<th>Post</th>
<th>OPG [pg/mL] Pre</th>
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\( P \)-Value: 0.005** 0.698 N.S. 0.031*
FIGURE LEGENDS

Figure 1: Ex vivo femoral pQCT analyses of ADA+/+ and ADA−/- compared to Rag2γc+/+ and Rag2γc-/- mice. (A) Total Area [mg/cm³]. (B) Medullary Canal Area [mm²]. (C) Cortical Thickness [mm]. (D) Canal area/Total Area Ratio [AU]. (E) Strength Strain Index [mm³]. (F) Schematic view representing cross-sectional bone sections in diaphysis of all four groups of mice analyzed. (G) Cortical Density [mm/cm³] (H) Trabecular Density [mm/cm³]; Box & whiskers graphs; \( P < 0.0005 \) (***), \( P = 0.0005-0.005 \) (**), \( P = 0.005-0.05 \) (*); ADA+/+ (n=22), ADA−/- (n=18), Rag2γc+/+ (n=10), Rag2γc-/- (n=14).

Figure 2: Reduced longitudinal growth and trabecular bone in ADA−/- mice. ADA−/- femora and tibiae (not shown) are significantly shorter compared to wildtype [mm], \( P < 0.0005 \) (***)). Metapyseal bone sections of ADA+/+ (B) and ADA−/- (C) femora; 2.5x, Scale bar= 600µm and 20x enlargement of ADA+/+ (D) and ADA−/- (E), Scale bar= 50µm indicate defects in ossification but not in cartilage formation. Histomorphological analyses were performed on ADA+/+ (n=5) and ADA−/- (n=5) femora; BV TV= Trabecular Bone Volume [%], Tb Th= Trabecular Thickness [µ], Tb N= Trabecular Number [1/mm], Tb S= Trabecular Separation [mm].

Figure 3: Reduced RANKL/OPG ratio and PINP levels in ADA deficient mice. (A) Murine serum RANKL [ng/mL] in ADA+/+ (n=22) and ADA−/- (n=18), Rag2γc+/+ (n=10) and Rag2γc-/- (n=14) mice. (B) Murine OPG [ng/mL]. (C) murine RANKL/OPG ratio [AU]; \( P < 0.0005 \) (***), \( P = 0.0005-0.005 \) (**), \( P = 0.005-0.05 \) (*), N.S.= not significant. (D) In vivo bone formation rate as assessed by PINP levels are significantly lower in ADA−/- mice compared to wildtype controls; Serum PINP levels [ng/mL]; ADA+/+ (n=9), ADA−/- (n=9), Rag2γc+/+ (n=15) and Rag2γc-/- (n=13).

Figure 4: Defects in ADA−/- osteoblast activity. (A) In vitro ADA−/- OBs grow significantly less than wildtype cells; \( P = 0.005-0.05 \) (*). (B) Viability as assessed by the Alamar Blue
staining is significantly lower in cultured ADA-/ OBs. (C) The percentage of apoptotic cells as assessed by Annexin V+/7AAD- staining is significantly higher in ADA-/ OBs, but normalizes in cells transduced with a lentiviral vector encoding for ADA (ADA-/ TR); \( P=0.0005-0.005 \) (**). (D) Taqman Gene Expression analyses for osteoblastic differentiation markers Runx2, Type 1 Collagen, Alkaline Phosphatase and Osteocalcin as well as RANKL and OPG; fold expression versus wildtype (represented by dashed line), normalized for HPRT endogenous control; Average of three independent experiments +SD. (E) Growth of murine ADA+/+ OB after 12 days in vitro is reduced when cultured with EHNA (100µM) and abolished when cultured with EHNA and Adenosine (1mM); ADA-/ OBs regain growth after lentiviral vector transduction; Growth as percentage of initially plated OBs +SD. (F) Growth of human OB after 13 days of in vitro is reduced when cultured with EHNA (100µM); \( P<0.01 \) (**).

**Figure 5: Stromal insufficiency to support hematopoiesis.** (A) CFU-F Assays show comparable frequency of mesenchymal progenitor cells in ADA+/+ or ADA-/ total BM. (B) LTC-IC Assays of ADA+/+ or ADA-/ feeder layers co-cultured with ADA+/+ or ADA-/ lin-cells. ADA+/+ or ADA-/ LTC-IC grow significantly less when co-cultured with ADA-/ feeder layers \( P=0.012 \). (C) Proteome Profile from supernatants of ADA+/+ and ADA-/ Co-Cultures, performed in triplicate; Average +SD; \( P=0.0005-0.005 \) (**), \( P=0.005-0.05 \) (*). (D) Taqman Gene Expression analyses for IL-1ra; Spleen, Liver, BM and bone in vivo; Stromal cells as used for LTC-IC, mesenchymal progenitor cells as cultured for CFU-F assays, OBs in vitro; fold expression versus wildtype (represented by dashed line), normalized for HPRT endogenous control, Average of three experiments +SD. (E) The percentage of quiescent hematopoietic stem cells, as assessed by FACS staining for Lineage negative, Sca1+, cKit+, CD150+, CD48- cells is significantly lower in ADA-/ (n=8) compared to ADA+/+ mice (n=8); \( P<0.0001 \). (F) Survival curves of ADA+/+ and ADA-/ mice transplanted with ADA+/+ lineage negative cells after multiple 5-FU injections at days 7, 14, 21 and 28; \( P=0.011 \) (*).
Figure 6: Rescue of the ADA bone phenotype by enzyme replacement therapy, gene therapy or bone marrow transplantation. Comparable serum levels of (A) RANKL [ng/mL] (B) OPG [ng/mL] (C) RANKL/OPG ratio [AU] (D) PINP [ng/mL] and (E) CTX [ng/mL] at 12 weeks of age. Scatter Plots +Average; PEG-ADA (n=7), GT (n>4), BMT (n=10), wildtype controls (n=7). (F) Strength Strain Index [mm$^{3}$]; Average +SD; PEG-ADA (n=4), GT (n=5), BMT (n=5) wildtype controls (n=4).
Figure 3

(A) mRANKL [ng/mL] vs ADA+/+ ADA-/- Rag2yc+/+ Rag2yc-/-

(B) mOPG [ng/mL] vs ADA+/+ ADA-/- Rag2yc+/+ Rag2yc-/-

(C) RANKL/OPG vs ADA+/+ ADA-/- Rag2yc+/+ Rag2yc-/-

(D) PINP [ng/mL] vs ADA+/+ ADA-/- Rag2yc+/+ Rag2yc-/-

** N.S. ***
Figure 6

A. RANKL

B. OPG

C. RANKL/OPG

D. PINP

E. CTX

F. SSI
ADA-deficient SCID is associated with a specific microenvironment and bone phenotype characterized by RANKL/OPG imbalance and osteoblast insufficiency

Aisha V. Sauer, Emanuela Mrak, Raisa Jofra Hernandez, Elena Zacchi, Francesco Cavani, Miriam Casiraghi, Eyal Grunebaum, Chaim M. Roifman, Maria C. Cervi, Alessandro Ambrosi, Filippo Carlucci, Maria Grazia Roncarolo, Anna Villa, Alessandro Rubinacci and Alessandro Aiuti