Functional inhibition of osteoblastic cells in an in vivo mouse model of myeloid leukemia

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Running Head: in vivo osteoblastic inhibition in leukemia

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ABSTRACT

Pancytopenia is a major cause of morbidity in acute myeloid leukemia (AML), yet its cause is unclear. Normal osteoblastic cells have been shown to support hematopoiesis. To define effects of leukemia on osteoblastic cells, we employed an immunocompetent murine model of AML. Leukemic mice had inhibition of osteoblastic cells, with decreased serum levels of the bone formation marker osteocalcin. Osteoprogenitor cells and endosteal-lining osteopontin+ cells were reduced, and osteocalcin mRNA in CD45- marrow cells was diminished. This resulted in severe loss of mineralized bone. Osteoclasts were only transiently increased without significant increases in bone resorption, and their inhibition only partially rescued leukemia-induced bone loss. In vitro data suggested a leukemia-derived secreted factor inhibited osteoblastic cells. Since the chemokine CCL-3 was recently reported to inhibit osteoblastic function in myeloma, we tested its expression in our model, and in AML patients. Consistent with its potential novel role in leukemic-dependent bone loss, CCL-3 mRNA was significantly increased in malignant marrow cells from leukemic mice and from samples from AML patients. Based on these results, we propose that therapeutic mitigation of leukemia-induced uncoupling of osteoblastic and osteoclastic cells may represent a novel approach to promote normal hematopoiesis in patients with myeloid neoplasms.
Introduction

Efficacy of treatment for AML, the most common adult acute leukemia, is limited and recurrence is common. Therefore, the identification of additional therapeutic targets is needed. One of the major causes of morbidity and mortality of acute leukemia is the disruption of normal hematopoiesis, causing neutropenia, anemia, and thrombocytopenia. Hematopoietic damage often occurs prior to overt systemic leukemia, which suggests that leukemic cells play an active role in the inhibition of normal hematopoiesis. The mechanisms by which AML inhibits normal hematopoiesis are poorly understood and it is unclear whether this is a direct effect of the leukemic cells on the normal hematopoietic cells in the marrow, or whether the microenvironment mediates leukemia-dependent hematopoietic damage.1

Cells of the mesenchymal/osteoblastic lineage play an essential role in the regulation of normal hematopoietic stem cells (HSCs)2–4. In addition to data suggesting that activation of osteoblastic cells expands HSCs and that osteoblastic injury results in myeloablation2,4, specific disruptions of the osteoblastic compartment without genetic manipulation of the hematopoietic system results in a myeloproliferative disorder, demonstrating the important role osteoblasts play in hematopoietic stem and progenitor cell regulation5. Osteoclasts as well as endothelial cells have also been shown to play a role in normal hematopoiesis and regulation of HSCs, particularly in their mobilization from the marrow6–8.
In xenograft models, human acute myeloid leukemia (AML) cells reside at the endosteal surface of bone\textsuperscript{9,10}, where they are found in close proximity to osteoblastic and osteoclastic cells, however the interactions between leukemia and these microenvironmental cells have not been clearly defined. Moreover, xenograft models, while beginning to elucidate in vivo intercellular relationships, juxtapose hematopoietic and non-hematopoietic cells from different species, and may not recapitulate normal leukemia-microenvironment regulatory interactions. Nonetheless, a number of xenograft studies have suggested that leukemia disrupts molecular mechanisms used by normal HSCs to home to the endosteal niche, including CD44\textsuperscript{11,12} and the well-established interaction between CXCR4 and its ligand CXCL12\textsuperscript{13-15}.

We hypothesized that leukemic cells alter osteoblastic and osteoclastic cell function resulting in measurable skeletal changes, which may impair support of normal hematopoiesis. To study interactions between leukemia and the marrow microenvironment, we utilized a well-characterized murine model of myelogenous leukemia\textsuperscript{16,17}. In this model, leukemia is initiated by immature hematopoietic cells that have been engineered to co-express the BCR/ABL and Nup98/HoxA9 fusion products (Fig. 1a-c), both of which have been documented in human leukemias, providing relevance of this model to human disease\textsuperscript{18}. This model recapitulates blast-crisis Chronic Myelogenous Leukemia (bcCML)\textsuperscript{16} with very rapid disease progression as well as a lack of chronic disease. Therefore it may closely represent the effects of AML on the normal marrow.
microenvironment. We employed this in vivo model to define the progressive effects of AML on bone forming and bone resorbing cells.

**Materials and Methods**

**Mice**

The Institutional Animal Care and Use Committee at the University of Rochester School of Medicine and Dentistry approved all animal studies.

**Patient Samples**

Peripheral blood and marrow aspirates were collected from both patients with AML and healthy volunteer donors. Marrow aspirates were obtained from the posterior iliac crest. Blood and marrow plasma was isolated by centrifugation and analyzed for protein levels by ELISA. Bone marrow and peripheral blood mononuclear cells were isolated and CD34+CD38- normal and leukemic cells were isolated as previously described\(^1\). Relative gene expression by real time quantitative PCR. All patients and volunteers provided written, informed consent in accordance with the Declaration of Helsinki on protocols approved by the Research Subjects Review Board of the University of Rochester.

**Model of AML**

The MSCV-BCR/ABL-IRES-GFP, and MSCV-Nup98/HoxA9-YFP vectors (Fig. 1a, b) were previously described\(^1\). Marrow cells from 6-8 week old male CD45.1 mice were enriched for hematopoietic stem and progenitor cells (HSPCs)
by FACS sorting to purify the population of lineage negative, sca-1⁺, c-kit⁺ (LSK) cells. LSK cells were infected with both viral vectors simultaneously as previously described ¹⁶. 6-8 week old male C57bl/6 primary recipients were sub-lethally irradiated (6Gy) using a ¹³⁷Cs source of radiation (GAMMACELL-40). Immediately following irradiation the mice were injected by tail vein with 2x10⁴ leukemic cells in 0.1 mLs of phosphate buffered saline (PBS) containing 2% heat inactivated fetal bovine serum (FACS buffer). After 15 days the spleens of primary recipient mice were harvested and crushed using the plunger of a 3 mL syringe. The resulting cell suspension was strained using a 40-μM pore size cell strainer, resuspended in Cryostor CS10 (Biolife Solutions) at a concentration of 2x10⁷ cells/mL and cryogenically stored in liquid nitrogen. To induce leukemia in animals used for all described experiments cells were thawed and 2x10⁵ cells in 0.1 mLs FACS buffer were injected by tail vein into non-irradiated 6-8 week old male C57bl/6 mice. These are referred to as leukemic mice, and were sacrificed at the times after secondary transplantation as described for each experiment. Normal controls were always age and sex-matched to the leukemic mice in the same experiment.

**Murine marrow, spleen, and peripheral blood cell collection**

For hematopoietic analysis, marrow cells were flushed from the long bones of the hind limbs of mice using a 25-gauge needle. Spleen cells were collected by crushing the spleen inside of a 40-μm pore size cell strainer using the plunger from a 3ml syringe. Peripheral blood cells were collected by sub-mandibular
bleeds followed by incubation for 20 minutes at room temperature in 2% 500,000 molecular weight dextran to precipitate the red blood cells.

**Flow cytometric analysis and fluorescent activated sorting**

Using cells collected as previously described red blood cells (RBCs) were lysed in 1 ml RBC lysis buffer (156 mM NH₄Cl, 127 μM EDTA and 12 mM NaHCO₃) for 5 minutes at room temperature, 1x10⁷ cells were suspended in 100μL FACS buffer and stained with appropriate antibodies. The cells were washed and data was collected on a LSR-II (Beckton Dickson). The data was analyzed using FlowJo software (Tree Star). For sorting, cells were prepared as described for flow cytometric analysis and GFP⁺/YFP⁺ cells were sorted using a FACS Aria cell sorter (Beckton Dickson) into FACS buffer.

**Histology and Immunohistochemistry**

Hind limbs were collected, cleaned of soft tissue, fixed in 10% neutral buffered formalin for 48 hours, and decalcified in 14% EDTA for 10 days. Tissues were then processed and embedded in paraffin, 5μm thick sections were cut and used for hematoxylin and eosin staining (H&Es), immunohistochemistry or TRAP staining. Immunohistochemical staining for GFP and OPN utilized the monoclonal JL-8 antibody (Clontech, 632380) and the AKm2A1 antibody (Santa Cruz biotechnology, inc. sc-21742) respectively. Both immunohistochemical stains were performed using the M.O.M. kit (Vector Laboratories, inc. PK-2200)
and were counterstained with hematoxylin. TRAP staining was performed as previously reported \(^{20}\) and counterstained with fast-green.

**Osteoblastic Cell Collection from long bones**

Osteoblastic cells were collected from the long bones of the hind limbs according to a previously described protocol\(^{21}\). In brief, the long bones of the hindlimbs were cleaned of soft tissue, and the bone marrow was flushed with a 25-gauge needle and discarded. The resulting cleaned and flushed bones were cut into <1mm fragments and digested twice in collagenase (Stem Cell Technologies, 7902) for 30 and 60 minutes sequentially. Remaining fragments were removed from the collected digest by passing through a 40\(\mu\)m pore size cell strainer. The cells obtained were seeded in 6-well cell culture dishes at \(1 \times 10^6\) cells per well for cell culture and bone nodule formation, or magnetically separated according to CD45 expression using the IMagnet system (BD biosciences, 552311) and a biotinylated CD45 antibody (ebioscience, 13-0451-82).

**Bone Nodule Assay**

Following 4 days in culture, media was changed to mineralizing media and 50 \(\mu\)g/ml L-Ascorbic acid 2-phosphate (Sigma), and 10mM Glycerol 2-phosphate disodium salt hydrate (Sigma) were added. At specified times following the addition of osteogenic media (see figure legends), cells were fixed with 10% neutral buffered formalin for 30 minutes followed by detection of alkaline
phosphatase activity using a staining buffer containing 100 mM Tris-HCL (Sigma), 0.005% w/v Naphthol AS MX-PO₄ (Sigma), and 0.03% w/v Red Violet LB salt (Sigma), with a final pH of 8.3 for 45 minutes. Following alkaline phosphatase staining, cells were von Kossa stained (2.5% w/v AgNO₃ (Sigma) for 30 minutes).

**Osteoprogenitor Cultures from Marrow**

Whole marrow flushed from the long bones of normal or leukemic mice was sorted as described for GFP⁻/YFP⁻ cells and seeded in 6-well tissue culture dishes at 4x10⁶ cells per well in α-MEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) and then used for bone nodule assays.

**MicroCT analysis**

The right hindlimb of each mouse was fixed in 10% Formalin for 48 hours, and then stored in 70% ethanol at 4°C. MicroCT analysis was performed as previously described

**ELISAs**

All ELISAs were performed as indicated by manufacturer’s instructions. CTX measurements were performed using the RatLaps ELISA (AC-06F1, Immunodiagnostic Systems Inc.). TRACP 5b protein measurement performed using Mouse TRAP ELISA (SB-TR103, Immunodiagnostic Systems Inc.). Osteocalcin measurement performed using the Mouse Osteocalcin EIA kit (BT-
Bovine CTX from bone wafer culture media was measured using the Crosslaps® for Culture ELISA (AC-07F1, Immunodiagnostic Systems Inc.). Murine CCL3 protein measurement was performed using the Mouse CCL3/MIP-1 alpha Quantikine ELISA Kit (MMA00, R&D systems). Human CCL3 protein measurement was performed using the Human CCL3/MIP-1 alpha Quantikine ELISA Kit (DMA00, R&D systems).

**Zoledronic Acid Treatment**

Mice were given a 0.25 mg/kg intraperitoneal injection of zoledronic acid bi-weekly for 2 weeks prior to induction of disease and throughout the course of the disease.

**Osteoclastogenic cultures**

Spleen cells were collected and red blood cells were lysed as previously described. The remaining cells were cultured at a concentration of 8.75x10⁵ cells/ml in alpha Minimum Essential Medium (α-MEM) containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco) (complete α-MEM), and 30 ng/ml M-CSF (R&D systems) for 2 days. Ten ng/ml RANKL (R&D systems) was added to the media and the cultures were continued for an additional 4 days and then TRAP stained. For osteoblastic co-cultures, osteoblastic cells were isolated as described. An osteoblast feeder layer was established by culturing 1.4x10⁴ cells/well in 96 well plates in complete α-MEM supplemented with 1x10⁻⁹ M 1,25 dihydroxyvitamin D3 (Sigma, D1530). After 2 days 8.75x10⁴ whole marrow cells
from either normal or leukemic mice were added to each well. Half media changes were performed every 2 days. Cultures were ended and TRAP stained after 7 days.

For cultures utilizing bovine bone wafers, an IsoMet low speed saw with a diamond blade was used to cut 300μm thick wafers of bone from a 4mm square pillar of devitalized bovine cortical bone to produce 300μm x 4mm x 4mm bone wafers.

**Analysis of relative gene expression by real-time RT-PCR**

Total mRNA was extracted using the RNeasy kit (Qiagen) according to manufacturer’s instructions. The Quantitect Reverse Transcription kit (Qiagen) was used to transcribe cDNA which was then diluted 1:50 in water, combined with SYBR Green PCR master mix (BioRad) and amplified using MyiQ Single Color PCR detection systems and software under the following conditions: 95°C for 3 minutes followed by 40 cycles of 95°C for 15 seconds and 58°C (Osteocalcin) or 60°C (CCL-3) for 30 seconds. Data were analyzed using the relative standard curve method, normalized to β-actin. Murine β-actin 5’ primer:

GCCACTGCGCATCCTCTT,

3’ primer: GGAACCGCCTCGTTGCAATAG.

Murine Osteocalcin 5’ primer: CCGCCTACAAACGCATCTACG,

3’ primer :GAGAGAGGACAGGGAGGATCAAG.

Murine **CCL3** 5’ primer :AAGGATACAAGCGACGAGCGAGTACAG,

3’ primer :TGCAGAGTGTCTAGGTACAGAGAA
Human gene expression was determined using Taqman technology (Applied Biosystems, Foster City, CA). Specific Taqman probes were obtained from Applied Biosystems: CCL3 (Hs00234142_m1) and GAPDH (Hs02758991_g1) cDNA amplification was performed according to manufacturers specifications using a LightCycler 480 II (Roche).

**Co-cultures and conditioned media**

CFU-OB cultures were started as previously described. After 4 days in culture osteogenic media was added and 1x10^6 marrow cells from either normal or leukemic animals were added to the cultures. In addition separate cultures containing normal CFU-OB cultures and 1x10^6 bone marrow cells from leukemic mice were used to produce conditioned media that was added to normal CFU-OB cultures starting on the same day as the bone marrow cells from leukemic mice were added to the co-cultures.

**Scanning Electron Microscopy**

Bovine bone wafers were removed from culture media and fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 24 hours at 4°C. The next day the wafers were postfixed in cacodylate buffered 1.0% osmium tetroxide, processed through a graded series of ethanol to 100% (x3), processed into a series of mixtures of 100% ethanol and hexamethyldisilazane (HMDS) before transitioning to 100% HMDS (x3). The last exchange of 100% HMDS covering the wafers was allowed to evaporate at room temperature overnight in an
uncovered 12 well plate in a fume hood. The dried wafers were mounted onto aluminum stubs, sputter coated for 90 seconds with gold, and imaged using a Zeiss Supra 40VP Field Emission scanning electron microscope.

**Light Microscopy**

Histology slides were viewed at room temperature with a CKX41 upright microscope (Olympus, Tokyo, Japan). Cell culture dishes were viewed at room temperature with a BX41 inverted microscope (Olympus). All images were obtained with a DP70 digital microscope camera and DPController software (Olympus).

**Statistical Analysis**

For quantitative assays, treatment groups were reported as mean plus or minus SEM and compared using the Student t test in GraphPad Prism, Version 5.0b (GraphPad Software). Statistical significance was established at $P \leq 0.05$.

**Results**

*An immunocompetent in vivo model where progression of leukemia is identified first in marrow, then spleen and peripheral blood*

We utilized a previously characterized model of acute leukemia (Figure 1a-c)\textsuperscript{16,17}. Leukemic cells can be identified and quantified based on their expression of Green Fluorescent Protein (GFP) and Yellow Fluorescent Protein...
(YFP) (fig.1d). In this model, secondary transplantation of the leukemia does not require irradiation of the recipient mice. Since our goal was to study the intact microenvironment, we chose to use as our experimental mice (heretofore designated as leukemic mice) the secondary transplantation recipients (fig.1c) and as controls sex and age-matched normal mice. Leukemic mice have rapid progression of disease that results in the accumulation of leukemic cells in the marrow identified by morphology, immunohistochemistry (fig.1e) and flow cytometric analysis (fig.1f) though total cellularity of the marrow is unchanged (29.94 ± 2.48 vs 27.18 ± 2.69, mono-nuclear marrow cells/hindlimb, Normal vs Leukemic, n=13 from 3 separate experiments p=0.4595). Leukemic cells also accumulate rapidly in the spleen (fig.1g), followed by the blood (fig.1h). This sequence suggests initial engraftment of leukemic cells in the marrow and spleen followed by migration into the bloodstream.

**Leukemia decreases osteoblastic cells**

Examination by immunohistochemistry of sections from the long bones demonstrated a severe decrease in osteopontin+ endosteal osteoblastic cells in leukemic compared to normal mice (fig.2a,b). Osteoblastic cells actively lay down bone; therefore a decrease in bone formation would be expected with osteoblastic inhibition. Global bone formation correlates well with serum osteocalcin levels, which were strongly suppressed in leukemic mice compared to sex and age-matched controls (fig.2c). Since osteocalcin is specifically expressed in mature osteoblastic cells, we measured osteocalcin expression in cells obtained from the long bones (femur and tibia) by collagenase digestion.
after bulk hematopoietic cells are removed by flushing. As expected, osteocalcin expression was present only in non-hematopoietic, CD45- cells and in leukemic mice there was a nearly 400-fold decrease in osteocalcin expression (fig.2d), demonstrating loss of osteocalcin+ cells from the marrow microenvironment. Osteocalcin expression was already decreased at day 6, when only 10-15% of marrow mononuclear cells are leukemic (fig.1f). To quantify the presence of osteoprogenitors in this cell pool, we performed bone nodule analysis and found that cells from the leukemic mice had reduced capacity to form alkaline phosphatase and von kossa positive colonies (fig.2e). Notably, a small number of bone-attached CD45+ cells were still present, and in the cultures from leukemic mice, leukemic cells were detected by fluorescence, although they represented only a very small proportion of the total cells (data not shown). To identify more primitive osteoprogenitors and exclude leukemic cells from the bone nodule cultures from leukemic mice, marrow from leukemic mice was depleted of leukemic cells by FACS prior to plating. Marrow from leukemic mice had reduced osteoprogenitor numbers compared to normal sorted marrow (fig.2f). These data suggest that, in addition to impaired function, osteoblastic and to a lesser degree osteoprogenitor numbers are decreased in the long bones of leukemic mice.

**Leukemia induces bone loss**

Functional inhibition of osteoblastic cells would be expected to result in decrease in bone structures. Leukemic mice exhibited qualitative loss of
trabecular structures, both in the metaphyseal region and the secondary ossification centers of the long bones, as well as cortical thinning (fig.3a,b). At day 10, quantification of mineralized bone volume (BV/TV) by micro-CT analysis demonstrated severe loss of trabecular (fig.3c,d,e) and cortical (fig.3f) bone in femora and tibiae of leukemic mice compared to age and sex-matched controls. Trabecular number and thickness were decreased (fig.3g,h) and trabecular spacing was increased (fig.3i) in leukemic mice, consistent with alterations in the bone microenvironment and loss of trabecular bone volume. Therefore, in mice, development of AML results in net loss of mineralized bone.

Leukemic environment transiently increases osteoclast numbers in vivo

Bone formation and resorption are closely linked; therefore bone loss could result from increased bone resorption, in addition to decreased bone formation. C-terminal telopeptides (CTX) are released from the bone matrix when it is resorbed and are an established measure of global bone resorption. When serum CTX levels were measured in the sera from leukemic and normal mice, there was no measured change in CTX levels from leukemic mice at any time during disease progression (fig.4a).

To quantify osteoclasts, histological sections from normal and leukemic mice were stained for Tartrate Resistant Acid Phosphatase (TRAP) activity. Multinucleated TRAP⁺ cells were scored as osteoclasts (fig.4b). In leukemic mice, osteoclasts were mildly increased 6 days after induction of disease, but were decreased by 10 days in leukemic mice compared to controls (fig.4b,c).
Measurement of serum TRACP 5b, has been validated as a measure of global osteoclast numbers. In normal mice, the TRACP 5b serum levels were stable, contrasting with leukemic mice in which global TRACP 5b levels were initially mildly increased and fell below normal by day 10 mirroring the histologic findings (fig.4d). These data suggest that, in our model, there is an initial and transient increase in osteoclastic cells, but as the disease progresses to overt leukemia, osteoclastic cell numbers decline.

*Leukemic cells do not differentiate into osteoclasts and do not resorb bone matrix*

As osteoclasts are derived from the myeloid lineage, it is possible that leukemic cells may differentiate into osteoclasts. Therefore, we evaluated the ability of spleen-derived leukemic cells to differentiate into osteoclasts in vitro. Under osteoclastogenic conditions, cells from normal spleens produce abundant osteoclasts (fig.5a,c). In striking contrast, GFP+/YFP+ leukemic cells isolated from spleens produce no TRAP+ osteoclasts (fig.5b,c).

Although leukemic cells do not differentiate into TRAP+ osteoclasts in vitro, they may be able to resorb bone matrix. To determine if leukemic cells resorb bone directly, we sorted GFP+/YFP+ cells from the spleens of leukemic mice and cultured them on bovine bone wafers. Scanning electron microscopic images were obtained to visualize the surface of the bone wafers, where osteoclastic activity is identified by the presence of resorption pits with a rough appearance that were abundant in normal controls (fig.5d). While viable...
leukemic cells were observed to adhere to the surface of the bone wafers, the bone matrix in wells containing leukemic cells remained smooth and lacked resorption pits (fig.5e). The amount of bone resorption was determined by the quantification of bovine CTX in the culture media. Normal osteoclast precursors under osteoclastogenic conditions readily resorb bone, as was demonstrated by the increase in bovine CTX. In contrast, leukemic cells under the same conditions did not increase bovine CTX (fig.5f). These data suggest that GFP+/YFP+ leukemic cells do not directly contribute to bone resorption.

To determine if the initial increase in osteoclasts in vivo was the result of pro-osteoclastogenic signals from the leukemic clone, we cocultured marrow cells from either leukemic or normal mice with a normal osteoblastic feeder layer in the presence of 1,25 dihydroxyvitamin D3. Under these culturing conditions, osteoclast formation was much more abundant in normal marrow cells as compared to leukemic (fig. 5g,h,i). These data confirm the inability of leukemia cells to form mature osteoclasts in vitro and suggest that leukemic cells require a full leukemic microenvironment to stimulate the in vivo transient increase in osteoclasts.

Treatment with zoledronic acid blocks bone resorption but only partially rescues bone loss in leukemic mice

To determine whether loss of bone formation or increased numbers of osteoclasts was responsible for the loss of bone in leukemic animals we treated...
mice with Zoledronic acid (ZA), a highly potent osteoclastic inhibitor \(^{25}\) prior to transplantation with leukemic cells (fig.6a). This treatment was sufficient to inhibit the activity of osteoclasts (fig. 6b). The serum levels of osteocalcin, a well-established bone formation marker, were significantly decreased in normal ZA-treated mice (142 ± 28 vs 49 ± 1 ng/ml, vehicle vs. ZA-treated mice p=0.0296). This homeostatic compensatory decrease in bone formation in response to effective osteoclastic inhibition explains the lack of significant ZA-dependent increases in bone volumes in wildtype mice (fig.6d,e). In leukemic mice treated with ZA simultaneous to control mice treated with PBS (fig.4a), serum CTX dropped to subnormal levels (fig.6c), confirming the inhibitory effect of ZA on osteoclasts in the setting of leukemia. Loss of trabecular bone volume was ameliorated by treatment with ZA (fig.6d). Trabecular number (fig.6f) and spacing (fig.6g) were also rescued, consistent with ZA-dependent protection of the trabecular bone volume loss induced by leukemia. In contrast, cortical bone volume loss remained unchanged by inhibition of resorption (fig.6e). When disease burden was quantified in leukemic mice treated with ZA, there was a mild decrease in disease burden in the marrow (Supplemental fig.1a), but not in spleen (Supplemental fig.1b) or peripheral blood (Supplemental fig.1c). In this model the phenotype of the leukemia stem cell (LSC) has been previously defined \(^{16}\) (Supplemental fig.1d), however treatment with ZA did not result in measurable changes in the frequency of LSC in marrow (Supplemental fig.1e) or spleen (Supplemental fig.1f). Moreover, there were no changes in progression of disease or mortality in ZA-treated leukemic mice (data not shown). These data
suggest that inhibition of bone resorption only partially reverses leukemic-induced bone loss without significantly changing disease progression.

**Expression of the chemokine CCL3 is increased in malignant marrow cells in leukemic mice**

Osteoblastic inhibition by leukemia may be mediated by cell contact and/or by secreted molecules. CFU-OB co-cultures of wildtype pre-osteoblasts with murine leukemic cells or with conditioned media generated from murine leukemic cells both showed a similar lack of alkaline phosphatase positive colonies (fig. 7a) suggesting that a secreted factor(s) is (are) sufficient to suppress colony formation. CCL3 is a pro-inflammatory cytokine in the CC chemokine family that has been implicated in the pro-osteoclastic actions observed in multiple myeloma\(^{26,27}\). In addition, data have recently suggested that CCL3 inhibits osteoblastic cells in mice and in human in vitro studies\(^{28}\). The level of CCL3 in the media was elevated in both co-cultures and conditioned media (fig. 7a). CCL3 protein levels were also increased in the marrow plasma and blood serum from leukemic animals compared to normal controls (fig. 7b). To determine the cellular source of CCL3, mRNA was measured by quantitative real time RT-PCR in total mRNA of marrow mononuclear cells, cells digested from bone fragments of the hindlimbs that were separated based on surface CD45 positivity, and sorted GFP\(^+\)/YFP\(^+\) leukemic cells. Bone-associated CD45\(^+\) cells from leukemic mice expressed higher levels of CCL3 mRNA compared to normal controls (fig. 7c). In sorted cells, GFP\(^+\)/YFP\(^+\) leukemic cells demonstrated the
highest level of \textit{CCL3} expression (fig. 7c). To assess whether \textit{CCL3} is also upregulated in patients with AML, \textit{CCL3} expression was quantified in sorted CD34+CD38-CD123+ human marrow cells, and found to be increased in marrow samples from AML patients compared to normal controls (fig.7d). In addition CCL3 protein levels were elevated in the marrow plasma from AML patients compared to normal controls (fig.7f). This data is in agreement with the analysis of microarray data previously published by Bullinger et al. in which 58 of 75 AML samples had elevated levels of \textit{CCL3} (Supplemental fig.2).29

\textbf{Discussion}

It has long been known that osteoblastic cells support and expand HSCs in vitro\textsuperscript{30}, and co-transplantation of osteoblastic cells with HSCs can increase engraftment rate\textsuperscript{31}. Work in our laboratory and others first identified osteoblastic cells as a regulatory component in the HSC niche through genetic means\textsuperscript{2,3}. It has become evident that osteoblastic cells can both stimulate\textsuperscript{2,3} and limit HSC expansion\textsuperscript{32,33}, promote quiescence\textsuperscript{34-36}, initiate HSC mobilization\textsuperscript{37}, and integrate sympathetic nervous system and HSC regulation\textsuperscript{38}. In addition, bone progenitor dysfunction is sufficient to induce myelodysplasia and secondary leukemia\textsuperscript{39}. Finally, recent data suggest that mesenchymal stem cells, which give rise to cells of the osteogenic lineage, regulate HSCs\textsuperscript{40}. In addition to increasing evidence that osteoblastic lineage cells act as orchestrators of HSC behavior, data strongly suggest that osteoblastic dysfunction results in pancytopenia\textsuperscript{4,41}.

Pancytopenia is the cause of significant morbidity in leukemia, and yet the
mechanisms by which leukemia causes anemias remain poorly understood. We have shown that induction of myeloid leukemia in an in vivo immunocompetent non-irradiated murine microenvironment induces severe functional inhibition of osteoblastic cells even when the burden of disease is relatively low and when leukemic cells are undetectable in blood. Therefore, osteoblastic damage by leukemia may be due to specific leukemic-initiated interactions rather than as a consequence of systemic disease.

Osteoblastic inhibition in leukemia resulted in decreased bone formation and net bone loss, particularly in cortical bone, where inhibition of osteoclast activity could not compensate for the leukemic-dependent bone loss. While there is little data reporting the effects on bone in adult AML, these data are consistent with findings at diagnosis in pediatric acute leukemia, where decreased markers of bone formation prior to corticosteroid treatment have been documented in numerous studies. This disruption in bone formation may be reversible as, in spite of corticosteroid treatment in this disease, bone formation markers improve with reduction of disease burden after chemotherapy.

Bone turnover is a closely regulated event in which bone formation and bone resorption are tightly coupled. In the setting of dramatic loss of bone formation, a compensatory loss of osteoclastic bone resorption would be expected. In contrast, there was a mild and transient increase in osteoclastic cells in mice with leukemia. Moreover, leukemic-induced trabecular bone loss was completely blocked by osteoclast inhibition. Therefore, leukemia initiation results in uncoupling of bone formation and bone resorption. This effect is likely
responsible for the trabecular loss, and may be important in the early stages of
disease. We speculate that the decrease in osteoclastic number seen at later
time points may be due, in part, to the leukemia-induced block in hematopoietic
differentiation that could limit the pool of hematopoietic osteoclast precursors.
This result also indicates a potential role for osteoclastic inhibition, particularly at
time of recurrence where the percent of blasts present in the marrow is relatively
low and hematopoietic differentiation is preserved.

The mechanisms by which AML inhibits osteoblastic cells may include
both direct and indirect actions of leukemia on their microenvironment.
However, review of microarray data comparing gene expression in total RNA
obtained from sorted lin−GFP+YFP+ cells to lin−GFP−YFP− cells from this mouse
model indicated no significant changes in the expression of genes encoding
cytokines with potent inhibitory activity against osteoblastic cells, such as dkk1,
CCL2, SOST and noggin (Supplemental Table 1), suggesting they are not likely
direct mediators of the leukemia-induced osteoblastic defect. In contrast, we
have shown significantly increased expression of the gene encoding the
chemokine CCL3. Receptors for CCL3 (CCR1 and CCR5) are present on
osteoblastic cells 45,46, and recent data have demonstrated the inhibitory effects
of CCL3 on both murine and human osteoblastic cells 28. Our findings reveal that
CCL3 is highly expressed both by our leukemic model as well as in human AML.
Further experiments are needed to determine if CCL3 is indeed the key mediator
of AML-dependent effects on the marrow microenvironment. While initial studies
identified CCL3 as an inhibitor of proliferation that may have differential effects
on benign compared to malignant primitive hematopoietic cells its role in AML remains unclear. This chemokine also has strong pro-osteoclastic effects that may at least in part explain the transient increase in TRAP+ cells found in our model, consistent with the uncoupling of bone formation and bone resorption found in our model. Of note, strategies for CCL3 inhibition have already been developed for the treatment of myeloma, where CCL3 is an important mediator of osteolytic disease. Therefore our findings, if verified in human AML, may suggest a therapeutic approach in which CCL3 inhibition may be used to ameliorate osteoblastic dysfunction and accelerate recovery of normal hematopoiesis in the setting of leukemia treatment.

In summary, findings in our model support the concept that leukemia disrupts the normal marrow microenvironment, and particularly targets cells that have been demonstrated to support and regulate HSCs. Since the loss of normal hematopoietic function during human leukemia is a major determinant of morbidity and mortality, our data support investigation of a human osteoblastic defect as a result of AML. If osteoblastic dysfunction is confirmed in human AML, mitigation of osteoblastic defects in the setting of chemotherapy may provide a novel therapeutic strategy to accelerate hematopoietic recovery.

Acknowledgements

This work was supported by the Wilmot Scholar Cancer Research Award and the Pew Scholar in Biomedical Sciences Award (to L.M.C). The authors would like to thank Drs. Marshall Lichtman and James Palis for helpful discussion, Karen
Bentley of the University of Rochester School of Medicine and Dentistry Electron Microscope Research Core facility for the SEM analysis, Michael Thullen for micro-CT analysis, and Mark LaMere for assistance in all studies involving human samples.

Author Contribution: B.J.F and J.M.A. performed experiments; L.P.X., M.W.B. C.T.J. provided advice; and B.J.F and L.M.C. designed the research, analyzed experiments and wrote the manuscript. The authors declare no conflict of interest.
References cited


28


Figure Legends

Figure 1: Murine model of bcCML.

A) Murine Stem Cell Virus (MSCV) construct containing BCR/ABL and GFP. B) MSCV construct containing Nup98/HoxA9 and YFP. C) Schematic representation of the transplant strategy used to produce the leukemic mice utilized. D) Flow cytometric gating strategy to identify leukemic cells as GFP and YFP positive. E) Representative anti GFP immunohistochemistry of the femur’s marrow space at the metaphysis. GFP is visualized by brown staining, with a hematoxylin counterstain. F) Flow cytometric data represents bcCML cells as a percentage of total marrow mononuclear cells. G) Flow cytometric data representing bcCML cells as a percentage of total spleen, and H) total peripheral blood mononuclear cells over the course of 10 days. * p≤0.05, ** p≤0.01, *** p≤0.001. n=5 mice per time point. Bar indicates SEM in this and subsequent experiments.

Figure 2: Leukemia decreases osteoblastic number and function.

A, B) Osteopontin immunohistochemistry was performed on paraffin embedded sections, representative images are shown of A) a naïve femur, and B) a leukemic femur. Osteopontin positive cells are stained brown and sections were counterstained with hematoxylin (blue). Arrowheads indicate osteopontin+ cells. C) Quantification of serum osteocalcin measured by ELISA. D) Real Time RT-PCR quantifying Osteocalcin RNA expression in osteoblast-like cells collected from the long bones of normal or leukemic mice at day 6 or 11 and magnetically separated based on CD45 expression, statistical significance was determined compared to Naïve mice (day 0) (n=5 samples per experimental group). E, F) CFU-OBs formed per well from E) whole marrow after 28 days in culture and F) cells collected by collagenase digestion of bone fragments after 15 days in culture. * p≤0.05, ** p≤0.01, *** p≤0.001.

Figure 3: Leukemic environment induces bone loss.

A, B) Representative images of H&E stained Paraffin sections of the distal femur from a A) naïve and B) Leukemic mouse 10 days after transplant. C, D) Representative micro-CT images from the metaphysis of the femur from C) naïve and D) leukemic mice. E) Micro-CT analysis of femur trabecular bone volume/total volume and F) femur cortical bone volume/total volume. G) Trabecular number H) Trabecular thickness and I) Trabecular spacing. * p≤0.05, ** p≤0.01. n=4 mice per experiment.

Figure 4: Leukemic environment mildly and transiently increases osteoclastic numbers in vivo.
A) Serum levels of carboxy-terminal collagen cross links (CTX) a marker of bone resorption as measured by ELISA.  B) Representative images of paraffin sections stained for the osteoclastic marker tartrate resistant acid phosphatase (TRAP). TRAP$^+$ cells are pink and highlighted by arrowheads.  C) Quantification of multi-nucleated TRAP positive cells in a 1mm square area just proximal of the distal growth plate in the femur from sections represented by the panels in B.  D) Serum levels of TRACP 5b, the osteoclast specific TRAP enzyme, measured by ELISA. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001. Each point indicates an individual mouse in this and subsequent experiments. n=5 mice per experiment.

**Figure 5: Leukemia cells do not differentiate into osteoclasts and do not resorb bone.**

Representative light micrographs of A) normal and B) leukemic spleen cells under osteoclastogenic conditions in vitro. Pink cells are positive for TRAP activity. C) Quantification of TRAP$^+$ cells in A and B. D) Low and high power scanning electron micrographs of osteoclasts on bovine bone wafers. E) Low and high power scanning electron micrographs of leukemia cells on bovine bone wafers. F) ELISA quantification of CTX released into culture media during culture of cells with bovine bone wafers. G, H) Representative light micrographs of co-cultures containing osteoblasts and G) normal marrow cells and H) leukemic marrow cells. I) Quantification of TRAP$^+$ cells formed per well from osteoblastic co-cultures with normal and leukemic marrows. * p ≤ 0.05, *** p ≤ 0.001 n=3-4 mice per treatment group.

**Figure 6: Zoledronic acid rescues trabecular but not cortical bone loss.**

A) Schematic for the treatment schedule of leukemic and normal mice with zoledronic acid (ZA). Leukemia was initiated on day 0 following 2 weeks of ZA treatment. Injection of ZA denoted by arrows. B) Serum CTX levels in normal mice following 2 weeks of treatment with ZA. C) Serum CTX levels in normal and leukemic mice following the ZA treatment schedule. D) Trabecular bone volume/total volume E) cortical bone volume/total volume F) trabecular number and G) trabecular spacing in normal or leukemic mice as quantified by micro-CT analysis following treatment with zoledronic acid. ** p ≤ 0.01, *** p ≤ 0.001. n=4 mice per treatment group in panels D-G.

**Figure 7: CCL-3 expression is increased in malignant cells from leukemic mice**

A) Top panel: Representative wells from CFU-OB cultures stained for alkaline phosphatase activity (pink). Bottom panel: CCL3 levels in culture media from CFU-OB cultures. B) CCL3 protein levels in murine model of AML compared to normal controls. C) Relative expression of CCL3 in bone marrow mononuclear cells isolated from whole bone marrow, cells sorted for GFP and YFP expression
according to fig.1d, and cells liberated from bone fragments by collagenase
digestion and magnetically separated based on CD45 cell surface expression.
D) Relative expression of human CCL3 in primitive CD34+CD38-CD123+ AML
cells compared to normal controls each bar represents a single AML sample
normalized to 3 normal controls. E) CCL3 protein levels in human AML patient
marrow plasma, each bar represents a single AML marrow sample compared to
7 normal controls. * p ≤ 0.05, ** p ≤ 0.01. Each data point represents an individual
mouse in panels A and B and D, n=3 mice per treatment group in panel C.
Figure 1

A: LTR Bcr/Abi IRES GFP LTR

B: LTR Nup98/HoxA9 pgk YFP LTR

C: Infect LSK

1° Transplant

BCR/ABL + GFP

15 days

2° transplant of leukemic cells

Experimental Animals

D: Time After Injection

Marrow

GFP/YFP Positive Cells (% Total Marrow)

WT Day 5 Day 6 Day 7 Day 8 Day 9 Day 10

G Spleen

GFP/YFP+ cells (% of Total Cells)

WT Day 5 Day 6 Day 7 Day 8 Day 9 Day 10

Blood

GFP/YFP+ cells (% of Total Cells)

WT Day 5 Day 6 Day 7 Day 8 Day 9 Day 10

E: GFP+ cells

F: YFP+ cells

G: YFP+ cells

H: YFP+ cells

Marrow

Spleen

Blood

GFP/YFP+ cells (% of Total Cells)

WT Day 5 Day 6 Day 7 Day 8 Day 9 Day 10

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Figure 2

A. Naïve Femur

B. Leukemic Femur

C. Serum OC

D. OC Expression

E. CFU-OB

F. CFU-OB
Figure 3

A. Naïve Femur

B. Leukemic Femur

C. Naïve

D. Leukemic d10

E. Trabecular BV/TV

F. Cortical BV/TV

G. Trabecular Number

H. Trabecular Thickness

I. Trabecular Spacing
Figure 4

A. Serum CTX

B. Naïve vs. Leukemic d6 vs. Leukemic d10

C. TRAP Positive Cells

D. Serum TRACP 5b
Figure 5

A) Normal

B) Leukemic

C) Spleen Culture

D)

E)

F) Culture Media CTX

G)

H)

I) Marrow Co-Culture
Functional inhibition of osteoblastic cells in an in vivo mouse model of myeloid leukemia

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