5-azacytidine-induced protein 2 (AZI2) regulates bone mass by fine-tuning osteoclast survival

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Running title: Role of AZI2 in osteoclastogenesis

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Key words: Osteoclast, Osteoporosis, AZI2

Background: 5-azacytidine-induced protein 2 (AZI2) is critical in GM-CSF induced dendritic cell differentiation.

Results: AZI2 deficiency enhances osteoclast survival, leading to decreased bone mass in vivo.

Conclusion: AZI2 suppresses osteoclast survival by inhibiting c-Src activation.

Significance: This is the first report showing the osteoprotective function of AZI2.

Summary

5-azacytidine-induced protein 2 (AZI2) is a TNFR-associated family member-associated NF-κB activator binding kinase 1 binding protein that regulates the production of IFNs. A previous in vitro study showed that AZI2 is involved in dendritic cell differentiation. However, the roles of AZI2 in immunity and its pleiotropic functions are unknown in vivo. Here we report that AZI2 knockout mice exhibit normal dendritic cell differentiation in vivo. However, we found that adult AZI2 knockout mice have severe osteoporosis, due to increased osteoclast longevity. We revealed that the higher longevity of AZI2-deficient osteoclasts is due to an augmented activation of proto-oncogene tyrosine-protein kinase (c-Src), which is a critical player in osteoclast survival. We found that AZI2 promotes c-Src activity, by inhibiting the heat shock protein 90 (Hsp90)—a chaperone involved in c-Src dephosphorylation. Furthermore, we demonstrated that AZI2 indirectly inhibits c-Src, by interacting with the Hsp90 co-chaperone Cdc37. Strikingly, administration of a c-Src inhibitor markedly prevented bone loss in AZI2 knockout mice. Together, these findings indicate that AZI2 regulates bone mass by fine-tuning osteoclast survival.
Role of AZI2 in osteoclastogenesis

Introduction

Bone degrading osteoclasts are large multinucleated cells of myeloid lineage origin (1). It has been established that osteoclast differentiation and activation is induced by RANK ligand (RANKL) expressed on bone forming osteoblasts. After binding of RANKL to its receptor RANK, transcription factors such as nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) (2) (3), Jun dimerization protein 2 (Jdp2) (4) (5), c-Fos (6), and NF-κB (7) are activated and osteoclast specific genes are induced. Once the differentiation process is completed, osteoclasts undergo apoptosis. Recently, it has been suggested that the longevity of osteoclasts controls bone destruction. For example, osteoblast derived cytokines such as macrophage-colony stimulating factor (M-CSF) (8) and osteopontin (OPN) (9) promote osteoclast survival. After the stimulation of osteoclast precursors by such survival factors, proto-oncogene tyrosine-protein kinase (c-Src), a non-receptor tyrosine kinase is activated and induces resistance to apoptosis (10, 11). c-Src activation is orchestrated by interplay between several other proteins. A recent report suggests that heat shock protein 90 (Hsp90), a molecular chaperone required for the stability of target proteins, suppresses c-Src activation (12). Moreover, previous studies revealed that Cdc37, a cochaperone of Hsp90, directly binds to Hsp90 to strengthen interactions between Hsp90 and its binding partners, leading to enhanced chaperone activity of Hsp90 (13). Importantly, Hsp90 inhibitors such as 17-AAG disrupts the Hsp90-c-Src association, leading to the bone degrading activity of osteoclasts via activation of c-Src. Therefore, the Hsp90 mediated regulation of the c-Src pathway is thought to be a novel therapeutic target for bone destructive diseases (12).

Regulation of osteoclast formation is also modulated by innate immune cytokines, such as IFNs (14). Type-I IFNs such as IFN-β are induced by RANKL and inhibit osteoclast differentiation (15). Virus induced type-I IFN production is mediated by TNFR-associated factor family member-associated NF-κB activator binding kinase 1 (TBK1) and IFN regulatory factor 3 (IRF3). Among the TBK1 binding proteins, TRAF family member-associated NF-κB activator (TANK), 5-azacytidine-induced protein 2 (AZI2), and TBK1 binding protein 1 (TBKBP1) have been implicated in the production of type-I IFNs (14). However, our previous knockout study revealed that TANK is dispensable for the production of IFNs (16), but indispensable for the suppression of excessive osteoclast formation via the inhibition of TNF receptor associated factor 6 (TRAF6) activation (17). We also generated AZI2 and TBKBP1-deficient mice, and revealed that both genes were dispensable for IFN production (18). However, to our surprise, AZI2-deficient bone marrow cells exhibited impaired granulocyte-macrophage colony-stimulating factor (GM-CSF) mediated dendritic cell differentiation in vitro (18). GM-CSF is involved in the survival, maturation, proliferation and differentiation of myeloid cells including dendritic cells (19). GM-CSF is also important in alveolar protein clearance, because GM-CSF neutralizing autoantibodies are involved in the pathogenesis of pulmonary alveolar proteinosis (PAP) (20). Bronchoalveolar lavage (BAL) from PAP patients and GM-CSF-deficient mice contains lipoproteinaceous material consisting of surfactant proteins and alveolar macrophages in BALs exhibit a large foamy appearance (20, 21). Importantly, such macrophages are also severely deficient in ATP-binding cassette sub-family G member 1 (ABCG1), a group of transmembrane proteins that play pivotal roles in mediating the cellular efflux of lipids, and peroxisome proliferator-activated receptor (PPAR)γ, a critical regulator of lipid homeostasis (22). Furthermore, lung tissues from PAP patients and GM-CSF-deficient mice exhibited many PAS positive alveolar fillings leading to respiratory distress (20, 21, 23).

Despite the importance of AZI2 in GM-CSF signaling in vitro, the roles of AZI2 in immunity, lung homeostasis, and their functions in bone metabolism remain to be studied in vivo. Here we explored the role of AZI2 in host defense and bone homeostasis. We report that AZI2 is dispensable for GM-CSF signaling in vivo, but we found that AZI2-deficient mice have severe osteoporosis due to enhanced osteoclast...
survival.

**Experimental procedures**

**Mice-AZI2 and TBKBP1-deficient mice were generated as previously described (18) and maintained in specific-pathogen-free conditions. All animal experiment was performed in accordance with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases (Osaka University, Osaka, Japan).**

**Cells and reagents**-For BAL harvest, the thoracic cavity was opened. After cannulating the trachea, BAL fluid was collected by injecting PBS. T cells, B cells and dendritic cells were isolated from total splenocytes by anti-Thy-1.2, anti-B220 and anti-CD11c magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively. Splenic CD11b+ macrophages were sorted by a FACSria (BD Biosciences, Bedford, MA). To generate Th cells, CD4+ cells were isolated by anti-CD4 magnetic beads (Miltenyi Biotec) and used to generate Th1, Th2, or Th17 cells, as previously described (24). Synovial fibroblasts were prepared as previously described.(25) For CD4+ T proliferation assays, splenic CD11c positive cells were stimulated with 1 μg/ml LPS for 24 h and irradiated (3000 rad). Balb/c derived splenic CD4+ T cells were mixed with irradiated cells, and after 72 h, CD4+ T proliferation was measured as previously described (18). LPS from *Salmonella minnesota* strain Re-595 was purchased from Sigma-Aldrich. Other reagents purchased included recombinant murine RANKL (462-TR, R&D Systems), murine M-CSF (315-02, PeproTech), murine OPN (441-OP-200, R&D Systems), c-Src inhibitor AZ0530 (1010-80, AdooQ Bioscience), Mouse Cross Linked C-Telopeptide of Type I Collagen ELISA Kit (USCN Life Science Inc.), RANKL ELISA Kit (MTR00, R&D Systems), OPG ELISA Kit (MOP00, R&D Systems), Cell Death Detection ELISA (Roche), ELISA kits for TNF, IL-6 and IL-12 (R&D Systems) and ALP quantification kit (LabAssay, Wako). Nuclear extracts were prepared as described (16) and the DNA-binding of NF-κB p65 and NFATc1 were quantified using a TransAM Transcription Factor Assay (Active Motive, Carlsbad, CA). Antibodies for FACS analysis were purchased from BD Biosciences. Data were collected by FACSCalibur (BD Biosciences) and analyzed by FlowJo (Ashland, OR).

**In vivo immunization**-Mice were intraperitoneally immunized with 10 μg OVA (Sigma) plus alum (LG6000 LSL, Cosmo Bio). Serum OVA-specific IgG1 and OVA-specific IgM were measured by mouse anti-OVA IgG1 ELISA kit (500830, Cayman Chemical) and mouse anti-OVA IgM ELISA kit (600-170-OGM, Alpha diagnostic), respectively.

**In vivo infection**- *S. aureus* was cultured in tryptic soy broth for 15 h at 37°C. Mice were infected intravenously with a PBS solution containing 5×10^7 S. aureus.

**Analysis of in vivo bone phenotype**-To check the bone formation rate, double calcein labeling was performed as described (26). For bone histomorphometric analysis, tibias were stained with Villanueva Bone Stain, and embedded in MMA. Serial longitudinal sections (6-mm thick) were generated and each section was observed by a Histometry RT Camera (System Supply Co., Ltd., Nagano, Japan). Distal portions of femurs were analyzed by three-dimensional μCT using Scan-Xmate RB080SS110 (Comscan Techno Co., Ltd., Sagamihara, Japan) and TRI/3D-Bon software (Ratoc System Engineering Co., Ltd., Tokyo, Japan). Bone microarchitectural parameters were quantified in the trabecular regions at 0.1 to 1.5 mm from the chondro-osseous junction.

**Immunoblotting and immunoprecipitation**-Western blotting was performed as described previously (16). Proteins were detected using anti-phospho-Src (#2101, Cell Signaling) and anti-actin (C-11, Santa Cruz Biotechnology) antibodies. For immunoprecipitation, cell lysates were incubated with protein A-Sepharose (GE Healthcare) containing 3 μg anti-Cdc37 antibodies (E-4; Santa Cruz Biotechnology), anti-AZI2 antibodies (ab65242, Abcam), anti-Hsp90 antibodies (3389-100, Bio Vision) or...
control IgG for 1 h at 4°C. The immunoprecipitants were washed, eluted and then analyzed by western blotting as described previously (16). Immunoprecipitated proteins were detected using ImmunoCurz IP/WB Optima System (Santa Cruz Biotechnology, Inc.) antibodies. To detect activation of Caspase3, anti-cleaved Caspase3 antibody (Cell signaling) was purchased.

**Osteoclast and osteoblast culture-M-CSF-derived macrophages (MDMs) were prepared as described (27) and used as osteoclast precursors. Osteoclast differentiation was induced in the presence of 25 ng/ml M-CSF for various times and with various concentrations of RANKL. TRAP staining was performed as described (17). For the osteoclast survival assay, MDMs were cultured with RANKL for 84 h. Then, RANKL and M-CSF were removed and osteoclasts were cultured for an additional 36 h. The survival rate was quantified by counting morphologically intact osteoclasts. For the osteoclast/osteoblast coculture assay, calvarial cells (5×10^5) and MDMs (3×10^5) were cultured in the presence of PGE2 and 1α,25(OH)2D3. For analysis of osteoclast resorptive activity, MDMs were plated on bone-resorption assay plates (Iwai Chemical Company, Tokyo, Japan). After 5 d of RANKL stimulation, plates were immersed in 1 M NH4OH, and pit numbers were counted.**

**Calvariae from newborn mice were digested in α-MEM containing 0.1% collagenase and 0.2% dispase at 37°C for 20 min. Mesenchymal stem cells from compact bone were prepared as described (28). To generate osteoblasts in vitro, calvarial cells or mesenchymal stem cells were cultured in an osteoblast-inducer reagent (Takara). ALP and calcified nodules in osteoblast cultures were detected using a TRAP/ALP staining kit (Wako, Tokyo, Japan) and Calcified Nodule Staining Kit (AK-21; Primary Cell Co., Ltd., Hokkaido, Japan), respectively. Calcium concentrations were measured using a kit (Metalloassay LS-MPR, AKJ Global Technology).**

**Calcium imaging-Cells were plated on poly-L-lysine-coated glass-bottom dishes and cultured with Fura2/AM. Fluorescent activities were analyzed as previously described (29).**

**PCR-RNA was extracted using TRIzol Reagent (Invitrogen Life Science Technologies, Carlsbad, CA) and reverse transcribed by ReverTra Ace (Toyobo Co., Ltd., Japan). Quantitative real-time PCR (qPCR) was performed using an ABI PRISM 7500 Real Time PCR System. TaqMan Assays-on-Demand primers (Applied Biosystems, Foster City, CA) were purchased.**

**Viral gene transfer/knockdown-AZI2 was cloned into a retroviral vector and transfected into the packaging cell line PlatE. Retroviral gene transduction of MDMs was performed as previously described (30). After transduction, cells were stimulated with RANKL. Cdc37 shRNA lentiviral particles (sc-35043-V, Santa Cruz Biotechnology Inc.), and control particles (sc-108080, Santa Cruz Biotechnology Inc.) were purchased. For lentiviral gene transfer, virus was added to wells with polybrene. After 20 h, cells were stimulated with RANKL to induce osteoclasts.**

**Statistical analysis-Student’s t-test was used to evaluate statistical significance, with significance set at p < 0.05.**

**Results**

**AZI2 is dispensable for in vivo myeloid cell differentiation and function-Because AZI2 is critical for in vitro GM-CSF signaling, we hypothesized that AZI2-deficient mice would exhibit PAP. We first evaluated the population of myeloid and lymphoid cell lineages in AZI2-deficient mice, but hematopoiesis was normal (Fig. 1A). Unexpectedly, the number of alveolar macrophages in AZI2-deficient mice was normal. Moreover, alveolar macrophages in AZI2-deficient mice was normal. Moreover, alveolar macrophages presented normal morphology, cytokine production, and lipid metabolism associated gene expression (Fig. 1B-E). Furthermore, alveolar structures of AZI2-deficient mice were normal and PAS positive alveolar fillings were not detected in these mice (Fig. 1F). Because GM-CSF-induced AZI2-deficient dendritic cells have shown impaired TLR signaling and antigen-presenting activity(18), we evaluated the production of cytokine and acquired immune...**
functions in AZI2-deficient mice. However, AZI2 deficiency had no impact on the production of pro-inflammatory cytokines or survival in response to LPS (Fig. 1G), *Staphylococcus aureus* (Fig. 1H), and *Candida albicans* (data not shown). Additionally, the production of ovalbumin (OVA)-specific IgM and IgG1 in response to intra-peritoneal injection of OVA plus alum was normal in AZI2-deficient mice (Fig. 1I). Furthermore, AZI2 deficiency had no impact on T cell activation by CD11c positive splenocytes (Fig. 1J). Thus, these findings indicate that TBK1 binding protein AZI2 is dispensable for myeloid cell differentiation and functions *in vivo*.

**AZI2-deficient mice are osteoporotic**—During the flushing of bone marrow from 11-wk-old mice, we noticed that AZI2-deficient femurs were more fragile compared to wild-type control mice. Thus, this observation prompted us to explore the role of AZI2 in bone homeostasis. Body weight and femur length were normal in AZI2-deficient mice, but microcomputed tomographic (µCT) analysis of the distal portion of femurs revealed dramatically impaired trabecular bone volume and number compared with wild-type mice (Fig. 2A–C). Bone histomorphometric analysis of proximal tibias from AZI2-deficient mice showed a significant reduction of the trabecular bone area accompanied by a dramatic increase of osteoclast number and eroded area (Fig. 2D-G). In contrast, osteoblast parameters and bone forming rate were normal (Fig. 2F, G). Consistent with the high number of osteoclasts, the bone mineral density in cortical areas of femurs was significantly decreased and the serum bone resorption marker CTX1 was increased, in AZI2-deficient mice (Fig. 2H, I). Thus, these data clearly indicate that AZI2-deficient mice are osteoporotic. In contrast, femurs and osteoclasts from TBKBP1-deficient mice were normal (data not shown).

**Osteoblast functions are normal in AZI2 deficiency**—Because the osteoporotic phenotype of AZI2-deficient mice could be due to impaired osteoblast functions, we explored the role of AZI2 in osteoblasts. First, we found that the expression level of AZI2 in osteoblasts considerably decreases during differentiation (Fig. 3A). Next, we cultured calvarial cells with osteoblast inducing medium and analyzed their calcification potential. However, AZI2 deficiency did not affect bone nodule formation (Fig. 3B, C). Furthermore, the expression levels of osteoblast differentiation markers such as ALP, RANKL, OPG and Runx2 were normal (Fig. 3D, E). Recently, it has been reported that osteoblast derived factors such as EphB4 and Sema3a are involved in osteoclast suppression. Because these osteoblast-osteoclast communicating factors are critical for bone remodeling, we further checked the expression of these genes, but AZI2 deficiency had no impact on gene expression (Fig. 3E). Collectively, these findings suggest that AZI2 does not affect osteoblast differentiation and function both *in vitro* and *in vivo*.

**Increased survival of AZI2-deficient osteoclasts**—AZI2 is broadly expressed in various organs, especially bone marrow, kidney, spleen and brain (Fig. 4A). This suggests that AZI2 is predominantly expressed in hematopoietic and neural systems. We also analyzed the AZI2 expression levels in various cells, and found that AZI2 expression is lower in osteoclasts compared to lymphocytes, myeloid cells, osteoblasts, and synovial cells (Fig. 4B). Importantly, osteoclasts from old mice exhibited significantly lower expression of AZI2 compared with young mice, and the expression level of AZI2 in MDMs gradually decreased during RANKL-induced osteoclastogenesis (Fig. 4C, D). To explore the role of AZI2 during osteoclastogenesis, we analyzed the differentiation of AZI2 knockout mouse-derived MDMs into osteoclasts. Three days after RANKL-induced osteoclastogenesis, the number of osteoclasts generated was comparable between AZI2-deficient MDMs and wild-type MDMs (Fig. 4E, F). However, 5 days after RANKL stimulation, wild-type cells began to die and the number of TRAP positive multinucleated cell was lower, compared to day 4 (Fig. 4E, F). In contrast, AZI2-deficient cells exhibited a high number of TRAP positive multinucleated cells, even at day 5 (Fig. 4E, F). Confirming the improved osteoclast activity of
AZI2-deficient osteoclasts, the number of bone resorption pits generated by AZI2-deficient osteoclasts was significantly higher compared to wild-type osteoclasts. However, this improvement of osteoclast activity was not due to an enhanced osteoblast-supporting function (Fig. 4G–I). Importantly, osteoclast survival assay showed that AZI2-deficient osteoclasts have a significantly slower apoptosis (Fig. 4J, K). Confirming this slower apoptosis rate, AZI2-deficient osteoclasts have a lower concentration of cleaved caspase 3, which is a critical executioner of apoptosis (Fig. 4L). Furthermore, slower apoptosis level in AZI2-deficient osteoclasts was partially rescued by AZI2 expression (Fig. 4M). Nevertheless, the expression levels of osteoclastic genes (Fig. 5A), calcium oscillation, and activation of osteoclasts inducing transcription factors such as NFATc1 and NF-κB (Fig. 5B, C) were similar in both AZI2-deficient osteoclasts and wild-type osteoclasts. Together, these observations clearly show that AZI2-deficient osteoclasts are defective for apoptosis pathways, but exhibit normal differentiation.

AZI2 regulates c-Src activation—To gain insight into the mechanism underlying the aberrant osteoclastogenesis in AZI2-deficient mice, we analyzed RANKL and M-CSF signaling. First, because AZI2 is implicated in the production of IFN-β, and because RANKL can also induce IFN-β, we quantified the expression of IFN-β induced by RANKL in AZI2-deficient MDMs (Fig. 6A). However, AZI2-deficient MDMs exhibited normal IFN-β expression, in response to RANKL (Fig. 6A). Next, because M-CSF and OPN promote osteoclast survival via activation of c-Src, we stimulated AZI2-deficient osteoclasts with M-CSF or OPN and we measured the level of phosphorylated c-Src level (pY416 src) by western blotting (Fig. 6B). Intriguingly, the activation of c-Src was enhanced in AZI2-deficient cells, in response to M-CSF or OPN stimulation (Fig. 6B), and a c-Src inhibitor AZD0530 suppressed excessive osteoclast generation in AZI2-deficient cells (Fig. 6C-E). To clarify the molecular mechanisms leading to an enhanced c-Src activation in AZI2 cells, we measured the expression levels of Hsp90, which is a potent inhibitor of c-Src activation (Fig. 6F). Unexpectedly, Hsp90 expression in AZI2-deficient osteoclasts was normal (Fig. 6F). Notably, a Hsp90 inhibitor, 17-AAG, significantly increased osteoclast numbers in wild-type cells, but AZI2-deficient cells did not respond to this inhibitor (Fig. 6G, H). Moreover, the expression levels of Cdc37, which is critical binding partner of Hsp90 for the inhibition of c-Src, were comparable between wild-type and AZI2 knockout cells (Fig. 6I). Because the Hsp90 inhibitor had no effect on osteoclast formation in AZI2-deficient cells, we predicted that AZI2 may be a component of the functional Hsp90-Cdc37 complex. To confirm this hypothesis, we examined the association of AZI2 with Cdc37. As expected, an association between Hsp90 and Cdc37 was observed by immunoprecipitation of mouse primary MDMs (Fig. 6J). Intriguingly, an association between AZI2 and Cdc37 was also identified (Fig. 6J). Furthermore, retroviral expression of AZI2 significantly rescued excessive osteoclast formation in AZI2-deficient cells, but not in Cdc37 knock-downed AZI2-deficient cells (Fig. 6K–M). Thus, these findings clearly indicate that the Hsp90-Cdc37-AZI2 complex is critical for the suppression of c-Src activation in osteoclasts. Finally, oral administration of a c-Src inhibitor, AZD0530, significantly rescued the osteoporotic phenotype of AZI2-deficient mice (Fig. 6N–P). Based on these data, we concluded that AZI2 regulates bone mass by fine-tuning c-Src activation in osteoclasts.

Discussion
Previous reports demonstrated that among the TBK1 binding proteins, TANK, AZI2, and TBKBP1 regulated the production of type-I IFNs in vitro (14). In contrast, our knockout mouse studies revealed that TANK, AZI2, and TBKBP1 are dispensable for IFN production in vivo. In addition, we have demonstrated that AZI2 is indispensable for in vitro GM-CSF-induced dendritic cell differentiation (18) and it has been reported that GM-CSF signaling is critical for alveolar protein clearance by macrophages (20, 21). Here, to our surprise, we could not observe any immunological or alveolar abnormalities in AZI2-deficient mice. Although GM-CSF is
predominantly used as an inducer of dendritic cells in vitro, GM-CSF-deficient mice exhibit normal dendritic cell populations (20, 21). Thus, we speculate that in vivo GM-CSF signaling is dispensable for dendritic cell differentiation, but indispensable for proper functions of lung macrophages. Most likely, additional studies are needed to clarify the molecular functions of AZI2 in dendritic cells, but our data clearly indicate that the in vitro signaling defects of GM-CSF in AZI2-deficient cells are compensated by unknown mechanisms in vivo. While we did not detect any immunological or alveolar abnormalities in AZI2-deficient mice, we discovered that they are osteoporotic. We found that c-Src activation is enhanced in AZI2-deficient osteoclasts, when they are stimulated by osteoclast survival factors such as M-CSF or OPN. Recently, it was reported that disruption of Hsp90-c-Src binding by 17-AAG stimulates c-Src activation (12) and previous reports also suggested that Cdc37 is critical for the Hsp90 inhibitory function on c-Src (13). Here, we demonstrate that AZI2 inhibits the c-Src repressive activity of Hsp90, by directly binding to Cdc37. Moreover, because the expression of AZI2 gradually decreases during osteoclast differentiation, while the expression of Hsp90 and Cdc37 increases during this process, AZI2 expression levels might regulate the c-Src repressive activity of the Hsp90-Cdc37 complex.

In our previous knockout study we have found that TANK negatively regulates osteoclast formation by suppressing the activation of TRAF6 (16). In this report, we show that AZI2, but not TBKBP1, also functions as negative regulator of osteoclast formation by suppressing the activity c-Src. Therefore, we suggest that these TBK1-binding partner proteins are indispensable for proper bone homeostasis. Although, further experiments are required to understand the precise molecular mechanisms of AZI2 in osteoclastogenesis, our data clearly indicate that AZI2 plays a pivotal role by fine-tuning osteoclast survival. Moreover, our findings provide a basis for AZI2-stimulating therapeutic strategies to treat osteoporosis and rheumatoid arthritis.

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Acknowledgments
We would like to thank E. Kamada for secretarial assistance, and Y. Fujiwara and M. Kumagai for technical assistance.

Footnotes
This research was supported by a research fellowship from the Japan Society for the Promotion of Science (JSPS) for the Promotion of Science for Young Scientists, JSPS KAKENHI for Grant-in-Aid for Challenging Exploratory Research, a grant from the Osaka University MEET project, a grant from the Astellas Foundation for Research on Metabolic Disorders, a grant from Naito Foundation, a grant from SENSIN Medical Research Foundation, a grant from Japan Intractable Disease Research Foundation and a vision research grant from Takeda Science Foundation to K. Maruyama. The founders have no role in experimental design, data analysis, or preparation of the manuscript.

Author contributions
K.M. initiated, designed and performed most of the experiments. The manuscript was written by K.M. S.U. and O.T. provided the AZI2-deficient mice. M.F., T.S., T.K. and M.M. assisted to the experiments (Figures 1-6). S.A. supervised overall research. All authors reviewed the manuscript.

Competing financial interests
The authors declare no competing financial interests.

The abbreviations used are: RANKL, receptor activator of NF-κB ligand; OPG, osteoprotegerin; MDMs, M-CSF derived macrophages; μCT, micro computed tomography; TRAP, tartrate resistant acid phosphatase; BMD, bone mineral density

Figure legends

**FIGURE 1.** AZI2 is dispensable for *in vivo* myeloid cell differentiation and function. (A) Splenic cells were stained with the indicated antibodies and analyzed by FACS. (B) CD11c+ cell numbers from BAL of AZI2-deficient mice. Representative pictures of CD11c+ cells. (C) Expression of ABCG1 and PPARγ in CD11c+ cell from BAL. (D) CD11c+ cells in (B) were sorted and stimulated with LPS for 24 h. Cytokine productions were measured by ELISA. (E) Mice were intravenously infected with *S. aureus*. After 24 h, BAL cells were harvested and IFN-β mRNA levels were measured by qPCR. (F) Representative histological images of lung (upper: HE staining; lower: PAS staining). (G) Serum cytokine concentrations after intra-peritoneal injection of 1 mg/ml LPS. (H) *S. aureus* was intravenously injected into mice and survival monitored for the indicated times. (I) *S. aureus* was intravenously injected into mice and survival monitored for the indicated times (n=5). (I) Mice were immunized with OVA plus Alum on days 0 and 14. Serum levels of OVA-specific IgM and OVA-specific IgG1 were measured by ELISA. (J) Splenic CD11c+ cells were stimulated with LPS for
24 h and irradiated. CD4+ T cells from Balb/c mice were added to the culture, and proliferation of T cells was quantified by [3H] thymidine incorporation. Error bars, S.E. n = 3, unless indicated.

**FIGURE 2.** AZI2-deficient mice (10-wk-old female mice) are osteoporotic. (A) Representative μCT images of distal femurs from *(upper: longitudinal view; lower: axial view)*. (B) Representative 3D μCT view of metaphyseal portion of femur. (C) Bone morphometric analysis of distal femurs by μCT. Error bars, S.E. *p < 0.05, (n = 5). (D) Representative images of proximal portion of tibias. (E) Representative images of osteoclasts in proximal portion of tibias (TRAP staining). (F) Representative images of double calcein staining. (G) Bone histomorphometric analysis of metaphyseal portion of tibias. Error bars, S.E. *p < 0.05, (n = 4). (I) Serum levels of bone metabolism markers. S.E. *p < 0.05, (n = 4). BV/TV, bone volume per tissue volume; Tb.Th, trabecular bone thickness; Tb.N, trabecular bone number; Tb.Sp, trabecular bone spacing; Ob.S/BS, osteoblast surface per bone surface; Ob.N/BS, osteoblast number per bone surface; Es/BS, eroded surface per bone surface; Oc.N/BS, osteoclast number per bone surface; Es/BS, eroded surface per bone surface; MAR, mineral apposition rate; MS/BS, mineralized surface per bone surface; BFR, bone formation rate.

**FIGURE 3.** Osteoblastogenesis is normal in AZI2-deficient cells. Calvariae were cultured in osteoblast inducing medium and (A) AZI2 expression levels or (B) calcium concentrations were measured. (C) Representative images of calcified nodules. (D) Calvariae were cultured with osteoblast inducing medium and ALP activities were measured. Representative ALP staining pictures at day 10 are shown in right panels. (E) qPCR analysis of osteoblast differentiation markers from cells in (C). Error bars, S.E. *p < 0.05, (n = 3).

**FIGURE 4.** Increased survival of AZI2-deficient osteoclasts. (A, B) Indicated organs (A) and cells (B) were collected and AZI2 expression levels were analyzed by qPCR. (C) Bone marrow cells from variously aged mice were harvested and osteoclastogenesis was induced by RANKL stimulation. Expression of AZI2 was measured by qPCR. (D) AZI2 expression levels during RANKL-induced osteoclastogenesis were quantified by qPCR. (E) MDMs were cultured in the presence of 75 ng/ml RANKL for the indicated times. Representative TRAP staining is shown. (F) TRAP positive cell numbers in (E) were counted. (G, H) MDMs were cultured on dentine slices resorption pits and analyzed under a microscope (G). Pit numbers were counted (H). (I) Bone marrow cells and calvariae were co-cultured and TRAP positive cell numbers counted. Representative images are shown (right). (J) MDMs were cultured in the presence of 75 ng/ml RANKL. Histone-DNA levels in culture supernatants were quantified by ELISA. (K) Osteoclast survival assay was performed. Giant MNC = >10 nuclei/TRAP positive MNC. (L) Cleaved caspase3 levels in osteoclast survival assay were measured by western blotting. (M) Osteoclast survival assay was performed after infection of retrovirus encoding AZI2 (RV-AZI2). Error bars, S.E. *p < 0.05, (n = 4).

**FIGURE 5.** Differentiation is normal in AZI2-deficient osteoclasts. (A) MDMs were cultured in the presence of 75 ng/ml RANKL. Cells were harvested for the indicated times and osteoclast differentiation markers were measured by qPCR. Error bars, S.E., (n = 3). (B) MDMs were treated with 175 ng/ml RANKL. Nuclear extracts were harvested and DNA binding activity of NFATc1 and NF-κB were measured. Error bars, S.E., (n = 3). (C) MDMs were treated with 100 ng/ml RANKL for 24 h and calcium imaging was performed. Representative traces of fura-2 fluorescence ratios are shown.

**FIGURE 6.** Enhanced e-Src activation in AZI2-deficient osteoclasts. (A) MDMs were stimulated with 300 ng/ml RANKL for the indicated times. IFN-β expression levels were measured by PCR. (B) MDMs were cultured with 75 ng/ml RANKL for 60 h and then starved for 6 h, removed and stimulated with 300 ng/ml M-CSF medium or replated on OPN (20 μg/ml) coated dishes. Cells were
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harvested at the indicated times and p-Src levels quantified by western blotting. (C-E) AZI2-deficient MDMs were treated with 75 ng/ml RANKL plus Src inhibitor AZD0530 (0.25 μM) for 4 d. Representative TRAP staining (C), p-Src protein levels (D) and TRAP positive cell numbers (E). (F) MDMs were stimulated with 75 ng/ml RANKL and Hsp90 levels measured by qPCR. (G, H) MDMs were treated with 75 ng/ml RANKL plus Hsp90 inhibitor 17-AAG (30 nM) for 4.5 d. Representative TRAP staining (G) and TRAP positive cell numbers (H). (I) MDMs were stimulated with 75 ng/ml RANKL and Cdc37 levels measured by qPCR. (J) Immunoprecipitation of wild-type MDM lysates. After anti-Cdc37, anti-AZI2, and anti-Hsp90 immunoprecipitation, precipitates were analyzed by western blotting using the indicated antibodies. (K-M) Representative TRAP staining of osteoclasts after infection of retrovirus encoding AZI2 (RV-AZI2), empty retrovirus (RV-empty), lentivirus encoding shCdc37 (LV-shCdc37) and lentivirus encoding nontargeting shRNA (LV-shNT) (K). TRAP positive cell numbers (L). Expression levels of AZI2 and Cdc37 were measured by western blotting (M). (N-P) Nine-week-old AZI2-deficient mice were orally inoculated with AZD0530 (50 mg/kg, 4 times/wk). After 5 wk, bones were analyzed by μCT. Representative μCT images of distal femurs (N), bone morphometric analysis of distal femurs (O) and bone histomorphometric analysis of metaphyseal portion of tibias (P). ES/BS, eroded surface/bone surface; Ob.S/BS, osteoblast surface/bone surface; MAR, mineral apposition rate; BFR, bone forming rate. Error bars, S.E. *p < 0.05, (n = 3).
Figure 1

A. Flow cytometry analysis of spleen cells from +/+ and −/− mice after S. aureus infection.

B. Serum concentration of CD11c+ cells in BALB/c mice.

C. Relative expression of ABCG1 and PPARγ.

D. Serum concentration of TNF-α, IL-6, and IL-12.

E. Relative expression of IFN-β.

F. Histological analysis of spleen sections from +/+ and −/− mice.

G. Serum concentration of TNF-α, IL-6, and IL-12.

H. Survival rate of mice after S. aureus infection.

I. Serum concentration of OVA-specific IgM and IgG1.

J. Serum concentration of CD4+ T cells in BALB/c mice following LPS injection.
Fig. 2

(A) Bone microarchitecture images of wild-type (+/+) and osteopetrotic (-/-) mice.

(B) BVTV (%) and Tb.Th (μm) bar graphs comparing wild-type (+/+) and osteopetrotic (-/-) mice.

(C) Tb.N (1/mm) and Tb.Sp (μm) bar graphs comparing wild-type (+/+) and osteopetrotic (-/-) mice.

(D) Histological images of wild-type (+/+) and osteopetrotic (-/-) mice showing bone erosion.

(E) Immunohistochemical staining for osteocalcin in wild-type (+/+) and osteopetrotic (-/-) mice.

(F) Fluorescence microscopy images of bone tissue in wild-type (+/+) and osteopetrotic (-/-) mice.

(G) Ob.S/BS (%), Ob.N/BS (μm), Oc.S/BS (%), and Oc.N/BS (μm) bar graphs comparing wild-type (+/+) and osteopetrotic (-/-) mice.

(H) BMD (mg/cm²) graph showing changes in BMD from distal to proximal regions in wild-type (+/+) and osteopetrotic (-/-) mice.

(I) CTXI (ng/ml), RANKL (pg/ml), OPG (pg/ml), and ALP (OD405nm) bar graphs comparing wild-type (+/+) and osteopetrotic (-/-) mice.

Maruyama et al. Fig. 2
Maruyama et al. Fig. 3
5-azacytidine-induced protein 2 (AZI2) regulates bone mass by fine-tuning osteoclast survival
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J. Biol. Chem. published online February 17, 2015

Access the most updated version of this article at doi: 10.1074/jbc.M114.631374

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