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Inhibition of Bone Remodeling in Young Mice by Bisphosphonate Displaces the Plasma Cell Niche into the Spleen

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The bone marrow provides niches for early B cell differentiation and long-lived plasma cells. Therefore, it has been hypothesized that perturbing bone homeostasis might impact B cell function and Ab production. This hypothesis is highly relevant for patients receiving long-term treatment with antiresorptive drugs. We therefore analyzed the humoral immune response of mice chronically treated with ibandronate, a commonly used bisphosphonate. We confirmed the increased bone mass caused by inhibition of osteoclast activity and also the strongly reduced bone formation because of decreased osteoblast numbers in response to ibandronate. Thus, bisphosphonate drastically inhibited bone remodeling. When ibandronate was injected into mice after a primary immunization to mimic common antiresorptive treatments, the generation of the various B cell populations, the response to booster immunization, and the generation of plasma cells were surprisingly normal. Mice also responded normally to immunization when ibandronate was applied to naive mice. However, there, ibandronate shunted the homing of bone marrow plasma cells. Interestingly, ibandronate reduced the numbers of megakaryocytes, a known component of the bone marrow plasma cell niche. In line with normal Ab responses, increased plasma cell populations associated with increased megakaryocyte numbers were then observed in the spleens of the ibandronate-treated mice. Thus, although inhibition of bone remodeling disturbed the bone marrow plasma cell niche, a compensatory niche may have been created by relocating the megakaryocytes into the spleen, thereby allowing normal B cell responses. Therefore, megakaryocytes may act as a key regulator of plasma cell niche plasticity.

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B one is the preferential homing site for hematopoietic stem cells (HSCs) from which all hematopoietic cells can be generated and is therefore the primary hematopoietic organ of the adult (1, 2). Bone is also the preferential homing site for subclasses of mature B and T cells such as CD8-positive and CD4-positive memory T cells and regulatory T cells (3, 4) as well as long-lived plasma cells (5–8).

Cells governing bone turnover (i.e., the bone-resorbing osteoclasts and the bone-forming osteoblasts) were proposed to participate in the building and the maintenance of the diverse niches for hematopoietic cells. Indeed, HSC niches appear stabilized by mature osteoblasts (9) and destabilized by bone resorption by osteoclasts (10). The common denominator of various models for the HSC niche is the participation of mesenchymal stem cells (MSCs) or at least early pluripotent mesenchymal stromal cells (MSCs) as an essential component of the HSC niche architecture (11). These MSCs are the precursors for chondrocytes, adipocytes, and osteoblasts (12).

The various differentiation states of bone marrow mesenchymal stromal cells appear not only to contribute to the HSC niches but also seem to provide a dynamic niche supporting the differentiation of B cells (11, 13, 14). B cells are indeed permanently generated in high numbers in the bone marrow, where they undergo their first steps of differentiation from common lymphoid progenitors (CLPs) to pre–pro-B cells, pro-B cells, and finally pre-B cells (15). This differentiation is facilitated by defined subtypes of mesenchymal stromal cells. For instance, CLPs are localized on the endosteal niches that encompass osteoblasts and CXCL12-abundant reticular cells. These niches support the commitment of CLPs into pro-B cells that further migrate toward IL-7–producing stromal cells when differentiating into pro-B cells. The release of pro-B cells from the IL-7–positive stromal cell niches allows their maturation into pre B cells and finally to immature B cells leaving the bone marrow into the circulation. Thus, HSC differentiation into immature B cells occurs at niches that are associated with different stages of differentiation of MSCs to mineralizing osteoblasts (15).
Importantly, long-lived plasma cells home back to the bone marrow to specific niches partly built by CXCL12-abundant reticular cells, megakaryocytes, and eosinophils. This niche provides all the signals necessary to store plasma cells including APRIL, IL-6, BAFF, CXCL12, IL-5, and TNF-α (16, 17). Thus, the bone environment creates survival niches for HSCs, early B cell populations, as well as Ig-producing plasma cells (7). Therefore, all these observations clearly point to a modular role of the osteoblastic lineage in regulating B cell generation and function (11, 13).

Postdevelopmentally, bone is a very dynamic organ constantly regenerated by a process called bone remodeling. Bone remodeling is the result of the activity of the bone-resorbing osteoclasts balanced by the activity of the bone-forming osteoblasts (18, 19). On the one hand, the highly dynamic nature of the bone is apparently difficult to reconcile with the apparent stability of HSC and long-lived plasma cell niches. On the other hand, these dynamics could be considered to be necessary for the mobilization and differentiation of hematopoietic cells as well as for the proper function or homing of plasma cells.

The homing capacity of the bone marrow certainly depends on the proper maintenance of the cellular components of the niches. Therefore, it is hypothesized that modulation of bone remodeling should affect these niches and the homing of hematopoietic cells in the bone marrow. This idea is supported by several observations: 1) the expansion of the HSC niche following the inductive process of osteoblast differentiation by PTH (9); 2) the switch of hematopoiesis from the bone marrow to the spleen as well as the decreased number of bone marrow HSCs following ganciclovir-mediated killing of type I collagen expressing cells (i.e., of both the lining cells and the mature osteoblasts) (20); and 3) the increase in immature hematopoietic progenitor cells observed after stimulation of osteoclastogenesis by RANKL, and the opposite effect of the antiresorptive drug calcitonin (10).

The fact that bone remodeling is a cycle initiated by activation of bone resorption by osteoclasts resulting in a secondary osteoblast differentiation and therefore bone formation clearly suggests that B cell generation and function should react to any alteration of this cycle. Thus, blocking bone resorption could disturb the process of B cell differentiation in the bone marrow or the homing of plasma cells and thereby Ab production. This hypothesis is clinically relevant for osteoporosis and multiple myeloma, a plasma cell malignancy associated to bone fracture. Indeed, antiresorptive drugs (i.e., bisphosphonates) are commonly given to patients suffering from multiple myeloma to protect them from the severe bone complications because of the disease progression. In that case, unexplained antitumor activities of bisphosphonates were reported that might be related to disturbances of the plasma cell niche. Similarly, anti-resorptive therapies, the standard treatments against postmenopausal osteoporosis are given to elderly patients who already have been exposed to pathogens and vaccinated and thus would primarily affect either the persistence of Ab production or most importantly the restimulation of Ab secretion. In addition, bisphosphonates are also the treatment of choice of young growing patient with osteogenesis imperfecta, where the treatment would rather be affecting the establishment the early immune response.

To test these hypothesis, we compared the effects of ibandronate, a bisphosphonate commonly used in clinic, on the generation of B cell populations and on the response to immunization in young naive as well as in preimmunized adult mice. We observed that, in addition to blocking bone resorption by osteoclasts, ibandronate treatment drastically inhibits bone formation as a consequence of decreased osteoblast numbers. However, surprisingly, this blockade of bone remodeling did neither affect B cell generation nor did it impair responses to immunization. Nevertheless, when given to naive mice, plasma cell homing into the bone marrow was strongly impaired and relocated to the spleen. This phenotype was associated to a bisphosphonate-induced relocation of the megakaryocytes from the bone marrow to the spleen where they associated with plasma cells. Thus, these results confirm the importance of megakaryocytes in building the plasma cell niche and also shed a new light on their potential role in regulating the plasticity of the plasma cell niche.

Materials and Methods

Mice, ibandronate treatment, and immunization

All mice were of C57BL/6N background (Charles River Laboratories) and were maintained in the pathogen-free facility of the University of Erlangen-Nuremberg, according to the local institutional guidelines.

Two different protocols were applied to mice. In the first one, to mimic commonly used antiresorptive treatment of elderly mice, mice received first a T-dependent primary immunization at the age of 9.5 wk and a secondary immunization at the age of 14 wk before daily s.c. injection with 160 μg/kg/d ibandronate (Bondronat; Roche) dissolved in PBS from an age of 15 wk until sacrificed at an age of 24.5 wk. T cell-dependent primary immunization was performed at the age of 9.5 wk by i.p. injection of 50 μl 4-hydroxy-3-nitrophénylacetyl hapten coupled to keyhole limpet hemocyanin (NP-KLH) (loading 15) (Biosearch) dissolved in 100 μl PBS and 100 μl Imject Alum Adjuvant (Thermo Scientific). Secondary immunizations were given without adjuvant at the age of 14, 18.5, and 23 wk (results are presented in Figs. 1–5). In the second protocol, ibandronate was injected to naive mice until sacrificed at the age of 15.5 wk. Immunizations were performed 5 wk after the beginning of ibandronate treatment at the age of 9.5 wk as follow: T cell–independent immunizations was performed at an age of 9.5, 11.5, and 13.5 wk by injection of 50 μg 4-hydroxy-3-nitrophénylacetyl hapten coupled to AminoethylCarboxyMethyl-Ficol (NP-AECM-FICOLL) (loading 77) (Biosearch) dissolved in 200 μl PBS. T cell–dependent primary immunization was performed at the age of 9.5 wk and secondary immunizations at the age of 11.5 and 13.5 wk (results are presented in Figs. 5–8).

ELISA and serum analysis

Blood was drawn by retro-bulbar puncture from mice anesthetized i.p. with 10 μl/g Rompun/Ketavet anesthetic (20 mg/ml xylazine, 100 mg/ml ketavet). For serum analysis, the following ELISA kits were used: Quantikine ELISA Mouse TRANCE/RANKL/ TNF-α, Mouse Osteoprotegerin/ TNFRSF11B Immunoassay (R&D Systems), Mouse Osteocalcin EIA Kit (ICN, Costa Mesa, CA), Mouse Osteoprotegerin/ OPG EIA Kit (Hycultec), RatLaps (CTX-I) EIA (ids), and Mouse IgM, IgG1, and IgG3 Ready-SET-Go ELISA (Bioscience). For the measurement of total and high-affinity Abs, ELISA plates were coated with NP-BSA with a loading of 26 and 4, respectively.

Histology and histomorphometry

Spleens were cryogenically frozen in Tissue-Tek OCT Compound (Sakura) and cut using a CM 3050 S Cryostat microtome (Leica) for 10-μm sections. The sections were fixed and permeabilized by incubation with −20°C acetone for 2 min, rehydrated, and blocked before staining with the Abs: CD41-FITC (BD Pharmingen), x L Chain-PE, and λ L Chain-PE (http://www.antibodies-online.com). Sections were mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories). Immunofluorescence microscopy was performed with a Leica TCS SP5 confocal microscope and the Leica LAS AF software. For bone histomorphometry, all mice received two i.p. injections of 5 mg/kg calcitonin (Sigma-Aldrich) dissolved in 2% NaHCO3 and 2 of 2 before sacrifice. Von Kossa/van Gieson, toluidine blue, and tartrate-resistant acid phosphatase (TRAP) staining on tibiae were performed as described previously (21). Briefly, right tibiae were decalcified in 20% EDTA at 4°C for 4 d before paraffin embedding and cutting of 1-μm sections. TRAP activity staining was performed by incubation with 10 mM sodium tartrate dissolved in 40 mM acetate buffer (pH 5) and then 0.1 mg/ml Naphthol AS-MX Phosphate (number N-5000; Sigma Aldrich) dissolved in 2% NaHCO3, 9 and 2 before sacrifice. On Kossa/van Gieson, tibiae were hydrated in ascending alcohol concentrations and embedded in methyl methacrylate. Five-micrometer sections were cut with a Microtec rotation microtome (Techno-Med). The sections were stained by toluidine blue (circular histomorphometry) or with 1% toluidine blue solution (pH 4.5) for 30 min or by von Kossa/van Gieson (static histomorphometry) by incubation in a multistep procedure with 3% silver nitrate 5 min, 5% sodium thiosulfate for 5 min, and van Gieson solution (0.25% acid fuchsin,
0.5% nitric acid [con], 10% glycerine, and picric acid to saturation) for 20 min. Unstained 12-μm sections were used to measure fluorescent calcein-labeled bone surfaces (BS) at 495-nm wavelength (dynamic histomorphometry). Bone parameter analysis was done using the OsteoMeasure histomorphometry system (OsteoMetrics), according to American Society for Bone and Mineral Research standards (22).

**FACS analysis**

Spleen, thymus and femoral bone marrow were removed, minced, and passed through a 70-μm cell strainer. After centrifugation (1500 × g, 30 s/ml) erythrocyte lysis was performed by administration of 5 ml lysis buffer (10 mM KHCO3, 155 mM NH4Cl, and 0.1 mM EDTA [pH 7.43]) for 5 min and stopped with 5 ml PBS (2 mM EDTA and 0.1% BSA). For each single or multilabeling, 106 cells were stained for 30 min with the following Abs: CD4-PE, CD8a-FITC, CD19-PE, CD21-FITC, CD23-PE, CD45R/B220-PerCP, CD138-APC, IgD-FITC, IgM-PE, and IgM-Cy5 (BD Pharmingen); and κ L Chain-PE and λ L Chain-PE (http://www.antibodies-online.com). A Gallios Flow Cytometer and the Kaluza Flow Analysis Software (Beckman Coulter) were used for analysis.

**Statistical analysis**

All data are presented as means ± SEM. Statistical analysis was performed using unpaired, two-tailed Student t test. A p value < 0.05 was considered statistically significant.

**Results**

**Increased bone mass because of reduced bone remodeling in ibandronate-treated mice**

To test the hypothesis that antiresorptive drugs affect either the induction or persistence of Ab production, we performed experiments in which adult mice were immunized and boosted prior or after administration of ibandronate. First, we established the efficacy of the antiresorptive drugs. An increased bone mass was clearly observed on sections of bone from ibandronate-treated mice (Fig. 1A). The increased bone mass was confirmed by histomorphometric quantification of the bone volume per tissue volume (BV/TV) and associated to increased trabecular thickness (Tb.Th) and numbers (Tb.N) (Fig. 1B). As expected and in agreement with the pharmacological properties of ibandronate, osteoclast numbers (Oc.N/bone perimeter [B.Pm]) and osteoclast-covered (Oc.S/BS were not significantly altered by ibandronate (Fig. 1C, 1D). Accordingly, the ratio of circulating RANKL to OPG was unchanged (Supplemental Fig. 1). However, the bisphosphonate drastically reduced the resorptive activity of osteoclasts as measured by the decreased concentration of C-terminal collagen cross-links (CTX) in the sera (Fig. 1D). In addition, ibandronate treatment strongly suppressed bone formation as shown by the reduced bone formation rate (BFR), which was due to the combined decreases in the mineralizing surface (MS/BS) and in the mineral apposition rate (MAR) (Fig. 1E, 1F). These effects are certainly the consequence of a drastic reduction in the numbers of osteoblasts (N.Ob/B.Pm) and in the BS covered by osteoblasts (Fig. 1G, 1H). This latter fact was confirmed by the strong decrease in the levels of circulating osteocalcin, a marker of osteoblast activity, in the sera of ibandronate-treated mice (Fig. 1H). Thus, our experimental setting in mice recapitulated the known pharmacological effect of ibandronate in humans (23, 24). Hence, this treatment can be used to analyze the effect of bisphosphonate treatment on the B cell mediated immune response.

**FIGURE 1.** Increased bone mass because of inhibition of bone remodeling in ibandronate-treated pre-immunized mice. (A) Von Kossa staining of undecalcified sections of tibias of 24-wk-old preimmunized mice treated for 2 mo with ibandronate compared with untreated control mice. (B) Histomorphometric quantification of trabecular BV/TV, Tb.Th, and Tb.N. (C) TRAP staining to reveal the presence of multinuclear osteoclasts in red. (D) Histomorphometric quantification of the number of osteoclasts per bone perimeter (N.Oc/B.Pm), the osteoclast-covered bone surface (Oc.S/BS), and ELISA quantification of CTX fragment corrected by the BS as serum marker for osteoclast activity. (E) Double calcein labeling of trabecular bone to reveal the bone formation activity. (F) Quantification of the MS per BS or the MAR and of the BFR. (G) Toluidine blue staining of undecalcified sections of tibias; osteoblasts are indicated by the arrows. (H) Histomorphometric quantification of the N. Ob (N.Ob/B.Pm) the osteoblast-covered BS (Ob.S/BS) and the levels of circulating osteocalcin (Ocn). *p < 0.05, **p < 0.001.
Ibandronate treatment of preimmunized mice does not affect B cell differentiation

Having established our animal model, we first performed FACS analysis to characterize the potential consequences of ibandronate treatment on the generation of T and B lymphocytes. Normal numbers and proportion of CD4 and CD8 single- or double-positive T cells were found in the thymus and in the spleen, indicating that treatment with ibandronate had no major effect on the generation of T cells (Supplemental Fig. 2A–D). Numbers and frequencies of CD19-positive cells in the bone marrow were unchanged as was the proportion of B220-positive cells in the spleen despite a small decrease in their total numbers (Fig. 2A, 2B). Similarly, the proportion and numbers of immature and recirculating B cells in the bone marrow (Fig. 2C, 2D), of B1 and B2 B cells in the peritoneum (Supplemental Fig. 2E, 2F), and of the splenic marginal zone B (MZB) cells, transitory 1 (T1) and T2 as well as follicular B (FO) cells, also were unchanged (Fig. 2E–H). Thus, when injected to preimmunized mice, despite a general decreased number of splenic B cells, ibandronate had no remarkable effect on the relative differentiation of T and B cell populations.

Ibandronate does not impair the response to booster immunization

Because Ag-independent B cell generation was unchanged, we next asked whether Ag-dependent B cell differentiation could be affected by bisphosphonate. We therefore monitored the quality of the immune response by measuring the levels of Ag-specific IgM, IgG, and IgG1 circulating in the blood of mice immunized with the T cell–dependent Ag NP-KLH (Fig. 3A). Ibandronate did not affect the production of any of the three Ag-specific Igs: IgM, IgG1 (Fig. 3B, 3C), or IgG (data not shown). Affinity maturation also properly occurred as demonstrated by the generation of high-affinity NP-reactive IgG1 (Fig. 3D) or IgG (data not shown) after booster immunization. In agreement, ibandronate treatment did reduce neither the generation of bone marrow plasma cells (Fig. 3E and F) nor the numbers of bone marrow megakaryocytes (Fig. 3G, 3H) that are known to participate in the plasma cell niches (17). Thus, inhibiting bone resorption by ibandronate had no significant functional consequence on the response of mice to boosting immunization.

**FIGURE 2.** No effect of ibandronate on the bone marrow and splenic B cell population of preimmunized mice. (A) Representative dot blot of FACS analysis of the CD19 B cell population in the bone marrow of ibandronate-treated and untreated control mice. (B) FACS quantification of the proportion as well as total number of B cells defined as CD19-positive cells in the bone marrow and as B220-positive cells in the spleen. (C) Representative dot blot of FACS analysis of the IgD- and IgM-positive cells in the bone marrow. (D) Quantification of the proportion and total number of immature B cells defined as IgM positive and IgD negative and of the recirculating B cells defined as IgM and IgD double-positive cells in the bone marrow. (E) Representative dot blot of FACS analysis of the CD23- and CD21-positive cells in the spleen. (F) Quantification of the proportion and total number of the MZB cells defined as CD21 high and CD23 negative and of the FO cells defined as CD21 low and CD23 positive in the B220 positive population. (G) Representative dot blot of FACS analysis of the IgD- and IgM-positive cells in the spleen. (H) Quantification of the proportion and total number of the T1 and MZB cells (T1/MZB) defined as IgD low and IgM-positive cells, of the T2 B cells defined as IgD high and IgM high double-positive cells, and of the FO B cells defined as IgD-positive and IgM low cells in the spleen (n = 5 mice/group, the data are the mean ± SEM). *p < 0.05, **p < 0.01.
Increased bone mass and decreased bone turnover in young naive mice after chronic ibandronate treatment

Although treating preimmunized mice with ibandronate did not have any deleterious effect on B cell–mediated responses, it was still a possibility that immune responses in naive mice could be affected. We therefore chronically pretreated naive mice with ibandronate and first characterized the bone parameters before initiating T cell–independent or T cell–dependent immunization. As expected, a drastic increased bone mass and trabecular numbers and thickness were observed after daily injection of the bisphosphonate (Fig. 4A, 4B). The increased bone mass was again associated with a drastic inhibition of bone remodeling as shown by the strong decrease in the BFR (Fig. 4C) because of a strong reduction in the number of osteoblasts and of the BS covered by osteoblasts (Fig 4D). Under those conditions, osteocyte numbers per bone area also were decreased (Fig. 4D). Osteoclast numbers and osteoclast-covered BS were not reduced but rather highly increased (Fig. 4E, 4F), most likely as a consequence of the increased concentration of circulating RANKL and the unchanged OPG levels (Fig. 4G). However, as expected, the resorptive activity of osteoclasts was still drastically reduced as shown by the measurement of the concentration of circulating CTX in the blood (Fig. 4F). Thus, ibandronate treatment of young naive mice resulted in an increased bone mass associated with a secondary blockade of bone remodeling.

Immunization does not modify the effects of ibandronate treatment on bone

Having demonstrated the increased bone mass in chronically ibandronate-treated naive mice, we performed T cell–independent or T cell–dependent immunization (Fig.5A) and measured the effect of immunization on bone remodeling in mice treated with ibandronate compared with untreated controls. We did not observe any significant difference in the BV (BV/TV) between controls and mice immunized with the T cell–independent Ag or with the T cell–dependent Ag, respectively (Fig. 5B). Immunizations had no effect on osteoblast parameters (Fig. 5C). Finally, none of the two protocols of immunization affected osteoclast numbers (Fig. 5D). We concluded that the activation of B cell–mediated humoral responses did not have any detectable impact on bone remodeling, even in the presence of bisphosphonates.
Long-term ibandronate treatment decreases B cell generation

Having established the bone phenotype of naive mice chronically treated with ibandronate, we analyzed the potential effects of the drug on lymphocyte populations in this experimental setting. Chronic administration of ibandronate to naive mice resulted in a reduced cellularity of the bone marrow (Supplemental Fig. 3A), most likely as a consequence of the severely reduced bone marrow space. In contrast, the cellularity of the spleen and thymus was unaffected (Supplemental Fig. 3B, 3C). No change in the numbers and proportion of the T cell subpopulations in the thymus or in the spleen was observed in ibandronate-treated naive or immunized mice at the end of the experiment (15.5 wk old) (Supplemental Fig. 3D, 3E). Although ibandronate had no effect on the B cell populations of the bone marrow or the spleen at the time of the first immunization (Fig. 6A), clearly decreased numbers of both bone marrow and splenic B cells were observed at the end of the experiment in both controls and immunized mice (Fig. 6B, Supplemental Fig. 3F–I). However, ibandronate never affected the relative proportion of immature or recirculating B cells in the bone marrow (Fig. 6C, 6D). In contrary, a mild decrease in the proportion of T2 and FO B cells, accompanied by an increased proportion of MZ B cells, was observed in the spleens of ibandronate-treated mice at the end point of the experiment (Fig. 6E–G). Thus, when first given to naive mice, long-term treatment of ibandronate specifically decreased the absolute number of bone marrow and splenic B cells as well as shifting the proportions of T2 and FO B cells toward MZ B cells.

Impaired plasma cell homing in the bone marrow of chronically ibandronate-treated mice

The decreased proportions of T2 and FO B cells suggested that the B cell–mediated immune response could be reduced in the bisphosphonate-treated mice. To test this hypothesis, we measured the levels of Ag-specific Ig circulating in immunized or nonimmunized mice. Unexpectedly, pretreatment with ibandronate did not modify the response to T cell–independent immunization as shown by the unchanged levels of circulating Ag-specific IgM, IgG, and IgG3 (Fig. 7A–C). In agreement, the response to T cell–dependent immunization also was unaffected as shown by the similarly increased levels of Ag-specific IgM and IgG1 measured following immunization (Fig. 7D, 7E) or IgG (data not shown). Again, ibandronate did not disturb affinity maturation as shown by the similarly increased levels of NP4-reactive IgG (data not shown) and IgG1 (Fig. 7F). This conclusion was supported by measuring the titers of total NP-reactive IgG1 and high-affinity NP-reactive IgG1 before and after booster immunization. Indeed, a clear similar increase in the titers of NP4-reactive IgG1 was observed in both ibandronate-treated and control mice (Fig. 7G–I). Because secretion of Ig depends on the generation of Ab-secreting plasma cells that are preferentially localized in the bone marrow, we next assessed numbers of this B cell population in the bone marrow of immunized mice. As expected, in the control group, both protocols of immunization induced increased numbers and proportions of CD138 and kL chain double-positive cells that characterize Ab-producing plasma cells (Fig. 7J, 7K). This increase in bone marrow plasma cells was significantly impaired in ibandronate-pretreated mice (Fig. 7J, 7K).
The recently described bone marrow plasma cell niche is composed of at least two elements: the eosinophils and the megakaryocytes (16, 17). Interestingly, the reduced accumulation of plasma cells in the bone marrow was associated with an ibandronate-dependent strong decrease in the numbers of megakaryocytes in the bone marrow (Fig. 8A, 8B). Thus, chronic treatment with ibandronate, when initiated in young naive mice, reduced megakaryocyte numbers and impaired the homing of plasma cells to the bone marrow.

Relocation of megakaryocytes into the spleen after chronic ibandronate treatment

Despite a near absence of bone marrow plasma cells, the response to immunization of ibandronate-treated mice was normal, suggesting the presence of a compensatory niche for plasma cells in these mice. To address this question, we performed immunostaining for CD41 as marker for megakaryocytes and κ/λ L chain for plasma cells on frozen sections of the spleen. We observed more CD41-positive megakaryocytes in nonimmunized as well as in TI and in TD-immunized ibandronate-treated mice when compared with their respective control mice (Fig. 8C). These observations were confirmed by quantitative histomorphometric analysis of the staining (Fig. 8D). Importantly, in agreement with the proposed role of megakaryocytes in the building of the plasma cells niche, increased proportions of megakaryocytes were found associated with κ/λ L chain–positive cells in immunized mice regardless of the bisphosphonate treatment (Fig. 8C, 8E). These data suggest that the decreased homing of plasma cells in the bone marrow of ibandronate-treated mice is likely compensated by an expansion of the megakaryocyte-dependent plasma cell niches in the spleen. If a relocation of megakaryocytes from the bone marrow to the spleen is responsible for the displacement of the plasma cell niche, we hypothesized that this relocation of megakaryocytes should precede the generation of plasma cells. To test this hypothesis, we quantified the presence of megakaryocyte in the bone marrow and spleen of ibandronate-treated mice at the time of the first immunization (i.e., at the age of 9.5 wk). Indeed, a drastic decrease in the numbers of megakaryocytes was observed already in the bone marrow of the ibandronate-treated mice that was associated to concomitantly increased number of splenic megakaryocytes (Fig. 8F, 8G). Finally, we asked whether the decreased numbers of megakaryocytes was solely due to a reduction of the bone marrow space or an active consequence of blocking bone resorption. To do this, we first quantified megakaryocytes in the bone marrow of Src knockout mice that develop osteosclerosis because of a cell autonomous defect in osteoclast activity (25, 26). Second, we quantified megakaryocytes in the bone marrow of mice with a gain of function mutation of LRP5 (LRP5[G170V]) (27, 28) that leads to the development of osteosclerosis because of a cell autonomous increase in bone formation by osteoblasts. Increased bone mass leading to decreased bone marrow space was observed in both models (Supplemental Fig. 4A, 4B, 4E, 4F). No reduction in the numbers of megakaryocytes was observed in the bone marrow of the LRP5-mutated mice (Supplemental Fig. 4G, 4H); this also was the case in another osteopetrotic mouse model, namely the Frl transgenic mouse (data not shown) (29). In contrast, no megakaryocytes were detected in the bone marrow of the Src-deficient mice (Supplemental Fig. 4C, 4D). These data strongly suggest that bone resorption by osteoclasts is necessary for the localization of megakaryocytes into the bone marrow.

Discussion

This work analyzes the potential consequences of commonly used antiresorptive treatments, such as the bisphosphonate ibandronate, on B cell–mediated immune responses. The experiments originally were designed to come as close as possible to human situations, where bisphosphonates are used either for the treatment of osteoporosis in aged patients having developed a full immune repertoire, or in young patients, as in osteogenesis imperfecta. These young patients are in their growing phase and should have a less developed immune repertoire. Thus, in one experimental setting, we treated young naive growing mice with bisphosphonate to analyze the consequence of the treatment on the establishment of the immune response. In the other experimental setting, we treated adult already immunized mice with ibandronate to analyze the effect of the drug on the maintenance of the immune response.

We demonstrate that, when given to young naive mice, ibandronate strongly reduces plasma cell homing into the bone marrow. This effect appears being the consequence of the reduced bone marrow niche most likely built by megakaryocytes and compensated by increased megakaryocyte and plasma cell numbers in the spleen. We also clearly show that plasma cell as well as megakaryocyte homing in the bone marrow is only affected when suppression of bone resorption occurs in the young growing mice. This conclusion also is supported by the data showing that a developmental reduction of the bone marrow space by blocking bone resorption (Src-deficient mice) but not by increasing bone formation (LRP5 gain of function) abolishes megakaryocyte homing to the bone marrow.

In agreement with the known pharmacological effects of this class of antiresorptive drugs, ibandronate significantly inhibited bone resorption while not decreasing osteoclast numbers (30), thereby resulting in net gain in bone mass (23, 24). However, inhibiting bone resorption by ibandronate also resulted in a secondary drastically decreased bone formation by osteoblasts. Thus ibandronate substantially impairs bone remodeling. This was not the exclusive result of long-term ibandronate treatment because, as also reported by others (24), very similar effects were found when ablating osteoclasts by short-term treatment with the natural...
RANKL antagonist osteoprotegerin (OPG) (data not shown). In addition, similar responses to the other commonly used bisphosphonate zoledronic acid have been reported (31). Interestingly, ibandronate also reduces the number of osteocytes. This decrease in osteocyte numbers may account in part to the increased osteoclast numbers. Supporting this idea, death of osteocytes is a known trigger of osteoclastogenesis (32), and osteocytes recently have been identified as the main source of RANKL stimulating bone resorption during bone remodeling (33, 34). Thus, our data illustrate the actual concept of the coupling between bone resorption and formation that regulates bone remodeling. Antiresorptive drugs disrupt the coupling with bone formation by inhibiting bone destruction by osteoclasts, resulting in fewer osteoblasts and finally nonrenewal or death of osteocytes that stimulates the recruitment of new osteoclasts. This conclusion is based on the specifically decreased proportion of B cells in the bone marrow of the bisphosphonate-treated mice. These data confirm the observation reported using zoledronic acid (31). In particular, the decrease in osteoblast numbers that we observed in ibandronate-treated mice support concepts that osteoblasts support B lymphogenesis (39–42). The proportion of splenic B cells also was specifically decreased by long-term treatment with ibandronate, most likely as a consequence of the reduced generation of B cells in the bone marrow. Thus, our data are in agreement with a specific role of the bone environment in B cell generation (14).

When analyzing lymphogenesis, we observed that T cell generation was not affected in ibandronate-treated mice as judged by unchanged numbers and respective proportions of CD4- and CD8-positive thymic and splenic cell populations. These data are in agreement with reports by others, revealing that the generation of T cells from the thymus was normal in the absence of bone marrow progenitors in adult mice (37, 38). Although focusing on B cell–mediated responses, we did not extensively analyze the functionality or the different T cell populations. However, we could still demonstrate that the function of Th cells were not drastically affected, as judged by the normal response to T cell–dependent immunization that we observed in all the different settings of bisphosphonate treatment.

We rather found that long-term treatment with ibandronate specifically affected B cell generation. This effect was independent of generally decreased bone marrow cellularity caused by the drastic increased bone mass. This conclusion is based on the specifically decreased proportion of B cells in the bone marrow of the bisphosphonate-treated mice. These data confirm the observation reported using zoledronic acid (31). In particular, the decrease in osteoblast numbers that we observed in ibandronate-treated mice support concepts that osteoblasts support B lymphogenesis (39–42). The proportion of splenic B cells also was specifically decreased by long-term treatment with ibandronate, most likely as a consequence of the reduced generation of B cells in the bone marrow. Thus, our data are in agreement with a specific role of the bone environment in B cell generation (14). Among the splenic B cells, we observed that ibandronate treatment could induce a mild but specific decrease in the proportion of transitory T2 and FO cells. However, this did not have any functional consequences on the levels of circulating Ig or on the capacity of the mice to respond to T cell–dependent immunization. In fact, despite a strong inhibition of bone remodeling, chronic treatment of naive or preimmunized mice with ibandronate did not
have any major functional consequence on T cell–independent and T cell–dependent immune responses. These data, which suggest a normal generation and function of plasma cells, are surprising given the proposed preferential homing of plasma cells into the bone marrow (5). This situation was indeed the case when ibandronate was given to already immunized mice, an experimental setting that mimics the antiosteoporotic treatment in aging human. In this case, numbers and proportion of bone marrow plasma cells were normal. In contrast, we observed a drastic inhibition of plasma cell homing into the bone marrow when ibandronate was chronically given to young growing mice before the first immunization. Thus, the generation of a functional bone marrow niche for plasma cells would depend on bone remodeling, but its persistence would not.

Despite the impaired homing of plasma cells to the bone marrow, the treatment with ibandronate never affected the actual Ab response to boosting immunization as shown by the normal affinity maturation observed in the chronically ibandronate-treated mice. Two possibilities could explain this observation: first, a reduced but sufficient volume of functional bone marrow niche is still present for which newly generated plasma cells can compete (43). Second, the plasma cell niche is relocating to another organ. Our data, establishing the presence of more megakaryocytes known to be a component of the plasma cell niche in the spleen (17), clearly favor the second hypothesis. Reinforcing this model, megakaryocytes were indeed found associated with plasma cells in the spleen. Our conclusion also is supported by the strong decrease in the numbers of bone marrow megakaryocytes and their concomitant increase in the spleen of ibandronate-treated naive mice. Importantly, the decreased number of megakaryocytes in the bone marrow and their increased numbers in the spleen were observed even before immunizing the mice. Thus, the reduction of megakaryocytes and their relocation occurred independently of the immune response. This concept is supported by the decreased homing of plasma cells observed in the bone marrow of mice with reduced megakaryocytes and their increased homing following the stimulation of megakaryocytogenesis (17). Whether the other

FIGURE 7. Unchanged response to T cell–independent or T cell–dependent immunization in chronically ibandronate-treated mice. ELISA measurement of the relative amounts of NP26-specific reactive IgM (A), NP26-specific reactive IgG (B), and NP26-specific reactive IgG3 (C) in the sera of the mice following T cell–independent (TI) immunization. ELISA measurement of the relative amounts of NP26-specific reactive IgM (D), NP26-specific reactive IgG1 (E), and NP4-specific reactive IgG1 (F) in the sera of the mice following T cell–dependent (TD) immunization. The arrows indicate the timing of immunization. Quantification of the titers of NP26-specific reactive IgG1 (G) and of NP4-specific reactive IgG1 (H) in TD-immunized mice at the age of 11.5 and 15.5 wk. (I) Calculation of the titer ratio between the NP4- and NP26-reactive IgG1. FACS analysis (J) and quantification (K) of the proportion of k/λ L chain and CD138 double-positive plasma cells in the bone marrow of the mice (n = 5 mice/group, the data are the mean ± SEM). **p < 0.01, ***p < 0.001.
component of the bone marrow plasma cell niche, identified so far, namely the eosinophil (16), is similarly affected by bisphosphonate remains to be addressed. Nevertheless, one important observation of our work is that the depletion of the megakaryocytes from the bone marrow and their increase in the spleen only occurred when ibandronate was given prior to the first immunization. These data imply 1) that the presence of megakaryocytes; the right pictures are representative high magnifications that reveals the interaction between megakaryocytes and plasma cells (white arrows) in the spleen of TD-immunized mice. (D) Histomorphometric quantification of the megakaryocyte density and (E) histomorphometric quantification of the proportion of megakaryocytes associated with plasma cells in the spleen. Histomorphometric quantification of the number of megakaryocytes in the bone marrow (F) and the spleen (G) in the ibandronate-treated and control nonimmunized mice at the time of the first immunization (9.5 wk old) (n = 5 mice/group, the data are the mean ± SEM). *p < 0.05, **p < 0.01, ***p < 0.001.

Whether the decreased number of megakaryocytes in the bone marrow is only a consequence of a long-term impairment of bone remodeling or a direct effect of ibandronate on megakaryocytes is unknown and has to be further investigated. However, our comparative analysis of megakaryocyte numbers of osteopetrotic and osteosclerotic mice clearly suggests that bone resorption by osteoclasts is a prerequisite for the establishment of the megakaryocyte niche in the bone marrow. This property may have important therapeutic implications because it can partly explain the bisphosphonate-mediated antitumor activity observed in multiple myeloma even in the absence of established bone complications (44, 45). In addition, these data suggest that long-term treatment with bone anabolic treatments would have less chance to affect immune responses than antiresorptive drugs.

In summary, we demonstrate the absence of any drastic consequence on B cell function in response to chronic inhibition of bone remodeling by the antiresorptive bisphosphonate ibandronate. However, this widely used bone intervention can decrease plasma cell homing to the bone marrow niche as a potential consequence of decreasing the number of megakaryocytes. Importantly, this work that strengthens the role of bone in the development of the B cells/plasma cell lineage and supports the concept of bone marrow niches uncovers the potential role of megakaryocytes in regulating the plasticity of the plasma cell niche.

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Disclosures
The authors have no financial conflicts of interest.
References


Supplementary Figure 1. Unchanged levels of circulating RANKL and OPG in ibandronate treated pre-immunized mice. ELISA measurement of RANKL and OPG levels in the sera of ibandronate treated mice compared to controls. Calculation of the ratio RANKL/OPG.
Supplementary Figure 2. Unchanged T and B cell populations in ibandronate treated pre-immunized mice. FACS analysis of T cell populations in the thymus (A,B) and spleen (C,D) and of B cells population in the peritoneal cavity (E,F), the data are expressed as proportion of gated cells (%) or as absolute cell numbers (Total cells).
Supplementary Figure 3. Decreased total cell numbers in the bone marrow of chronically ibandronate treated mice (A), unchanged total cell numbers in the spleen (B) and thymus (C). No change in numbers of T cell subtypes in the thymus (D) and spleen (E) of chronically ibandronate treated naïve mice. Decreased absolute numbers of B cells in the bone marrow (F) and spleen (G). Decreased numbers of immature and recirculating B cells in the bone marrow (H) and of T1/MZB, T1 and FO B cells in the spleen (I).
Supplementary Figure 4. (A) Histology of tibias of c-Src deficient mice compared to wild type littermates (B) Histomorphometric quantification of the bone parameters. (C) Histology of the bone marrow, megakaryocytes are indicated by the black arrows in the wild type. (D) Histomorphometric quantification of the density of megakaryocytes in the bone marrow (nd=non-detectable). Histology of the tibia of LRP5 (G170V) mutated mice (E) and of the bone marrow (G). Histomorphometric quantification of the bone parameters (F) and of the density of megakaryocytes (H).