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Activated Invariant NKT Cells Regulate Osteoclast Development and Function

Ming Hu,* J. H. Duncan Bassett,†,‡ Lynett Danks,‡ I. Peter G. T. Howell,¶ Ke Xu,* Emmanuel Spanoudakis,∥ Ioannis Kotsianidis,‡ Alan Boyde,‖ Graham R. Williams,* Nikki Horwood,∥ Irene A. G. Roberts,* and Anastasios Karadimitris*

Invariant NKT (iNKT) cells modulate innate and adaptive immune responses through activation of myeloid dendritic cells and macrophages and via enhanced clonogenicity, differentiation, and egress of their shared myeloid progenitors. Because these same progenitors give rise to osteoclasts (OCs), which also mediate the egress of hematopoietic progenitors and orchestrate bone remodeling, we hypothesized that iNKT cells would extend their myeloid cell regulatory role to the development and function of OCs. In this study, we report that selective activation of iNKT cells by α-galactosylceramide causes myeloid cell egress, enhances OC progenitor and precursor development, modifies the intramedullary kinetics of mature OCs, and enhances their resorptive activity. OC progenitor activity is positively regulated by TNF-α and negatively regulated by IFN-γ, but is IL-4 and IL-17 independent. These data demonstrate a novel role of iNKT cells that couples osteoclastogenesis with myeloid cell egress in conditions of immune activation. The Journal of Immunology, 2011, 186: 2910–2917.

Osteoclasts (OCs), along with osteoblasts, play an essential role in bone remodeling (10). In addition, OCs—when activated in response to various stimuli, including receptor activator of NFκB ligand (RANKL)—facilitate egress of hematopoietic progenitor and mature cells from the BM to the periphery (11, 12). RANKL, together with M-CSF, is also essential for OC development from CFU macrophages, the same myeloid progenitors that give rise to monocytes-macrophages and mDCs (13–15). However, whether iNKT cells promote the activation of OCs as well as macrophages and mDCs, perhaps via effects on their common progenitor, is not known. We therefore investigated the hypothesis that iNKT cells, under conditions of immune activation, are able to regulate the development and function of OCs and their progenitors.

Materials and Methods

Animals

iNKT cell-deficient TCRα18−/− (3), Cdl1d1−/− (4), and TNFαR55−/− (16) mice have been described and were bred with wild type (WT) mice in the Medical Research Council Imperial College Animal Facility, Hammersmith Hospital, London. BALB/c IFN-γ−/− and IL-4−/− mice were provided by A. O’Garra (National Institute of Medical Research, London, U.K.). Approval for the use of mice was obtained under the authority of a U.K. Home Office Project License.

Reagents

αGC (provided by Stephan Gadola, University of Southampton, Southampton, U.K.) was dissolved in PBS at 200 μg/ml and stored at 4°C. Mouse macrophage colony stimulating factor (m-CSF) and mRANKL were obtained from Peprotech, and anti-mouse IL-17 blocking Ab and IgG control were obtained from R&D Systems (Abingdon, U.K.).

In vivo assays

For in vivo activation of iNKT cells, 2 μg αGC diluted in 200 μl PBS was injected i.p. into each mouse. Anti–IL-17 blocking Ab or IgG isotype control (50 μg per mouse) were administered by tail vein injection 3 h prior to αGC injection.

Flow cytometry

BM cells were stained with anti–CD115-PE, anti–CD117-allophycocyanin, anti–CD3-FITC, anti–B220-FITC, and anti–Mac1-FITC (from BD Pharmingen) using standard protocols. Data acquisition was performed on a
two-laser FACS Calibur (Becton Dickinson), and analysis was performed with FlowJo software.

**Histology and histomorphometry**

Mouse lower limbs were fixed for 24 h in 10% neutral buffered formalin and decalcified in 10% EDTA, pH 7.4, for 14 d. Decalcified bones were embedded in paraffin, and 3-μm longitudinal midline sections were cut onto Super Frost plus slides (VWR), deparaffinized in xylene, and rehydrated. At each 80-μm level, serial sections were stained with H&E and Masson trichrome, and OC numbers were determined after staining sections for tartrate-resistant acid phosphatase (TRAP) activity. Staining using a Sigma TRAP kit (386A-1KT) was performed according to manufacturer’s instructions, except that the incubation time was 4 h. Sections were photographed using an Olympus BX51 microscope and DP71 digital camera. OC numbers and total bone surface were determined using Osteomeasure XP v1.2 software of a 1-mm² area of the secondary spongiosa of the proximal tibia, 500-μm below the growth plate.

**Analysis of growth plate dimensions**

Mouse lower limbs were fixed for 24 h in 10% neutral buffered formalin and decalcified in 10% EDTA (pH 7.4) for 28 d. Decalcified bones were embedded in paraffin and 3-μm longitudinal midline sections. Sections were stained with van Gieson and alcin blue. Reserve zone, prehypertrophic zone, hypertrophic zone, and total growth plate heights were determined at four separate positions across the growth plates using digital images of proximal tibia sections (17). Results from two different levels of sectioning were compared to ensure consistency of the data.

**Faxitron point projection microradiographic analysis**

Upper limbs and tail vertebrae were stored in 70% ethanol at 4˚C. Soft tissue was removed and 10-μm–resolution scale images were obtained using a Faxitron MX20 point projection x-ray source and digital imaging system operating at 26 kV and 5 x 5 magnification (Qados, Cross Technologies, Sandhurst, Berkshire, U.K.). Magnifications were calibrated by imaging a digital micrometer, and long bone lengths were determined using ImageJ 1.41 software (http://rsb.info.nih.gov/ij/). Cortical bone thickness and diameter were determined in at least 10 locations in the middistal humerus diaphysis (Fig. 6). The relative mineral content of calcified tissues was determined using ImageJ software by comparison with three standards included in each image frame (18). Increasing gradations of mineralization density were represented in 16 equal intervals by a pseudocolor scheme for presentation of digital images. The distributions of mineralization densities were analyzed by the Kolmogorov-Smirnov test.

**Backscattered electron scanning electron microscopy**

Analysis of bone microarchitecture was determined by backscattered electron scanning electron microscopy (BSE-SEM). Freshly dissected lower limbs were fixed and stored in 70% ethanol. Bones were opened longitudinally and cleaned by maceration with an alkaline bacterial pronase. Samples were coated with carbon and imaged using backscattered electrons, generally at a 20-kV beam potential. The images provide the best and most detailed view of bone surfaces, from which surface activity states (forming, resting, resorbing, resorbed) can be investigated. Cortical and trabecular bone surfaces were analyzed, and the fraction of bone surface with evidence of osteoclastic resorption pits was determined (17).

The distribution of skeletal micromineralization densities was examined by BSE-SEM and quantified by digital image analysis at the subcubic micron-resolution scale (quantitative BSE [qBSE]). Fixed femurs were embedded in poly-methyl-methacrylate. Block faces were cut through the midline of specimens, which were then polished, coated with carbon, and analyzed by BSE-SEM (17). The mineralization densities of trabecular and cortical bone were determined by comparison with halogenated dimethacrylate standards. Increasing gradations of micromineralization density were represented in eight equal intervals by a pseudocolor scheme for presentation of digital images. Femur and caudal vertebra bone volume (BV) as a proportion of tissue volume (BV/TV) was quantified in qBSE images using ImageJ (17).

**ELISAs**

ELISA kits for mRANKL, IFN-γ, and IL-4 were obtained from R&D Systems Europe (Abingdon, U.K.). The Ratlaps C-terminal telopeptides of collagen type II (CTX) ELISA kit was obtained from Nordic Bioscience Diagnostics (Herlev, Denmark). ELISA assays were performed according to the manufacturer’s instructions.

**Quantitative RT-PCR**

BM cells from αGCF-treated mice were flushed from femurs and tibias at different time points after αGCF injection and lysed in Trizol solution. Total RNA was extracted according to the manufacturer’s instructions (Invitrogen, Paisley, U.K.). One microgram of total RNA was reverse-transcribed into cDNAs using the Superscript III cDNA synthesis kit (Invitrogen). TaqMan real-time quantitative PCR was performed using TNFα-specific primer-probe (Assay ID: Mm04435258_m1; Applied Biosystems, Warrington, U.K.).

**Statistics**

Normally distributed data were analyzed by Student t test; p values <0.05 were considered significant. Relative and cumulative frequency histograms of micromineralization densities from WT and Cdl1d-/- mice were analyzed using the Kolmogorov-Smirnov test.

**Results**

**In vitro activated iNKT cells promote OC progenitor expansion**

Activation of iNKT cells with αGCF in vivo leads to rapid egress of myeloid progenitors and mature forms into peripheral blood (9), representing an important aspect of the ability of activated iNKT cells to recruit effectors of the cellular innate immune system. In agreement with previous data (9), we found that within 3 h of αGCF administration, the frequency of Mac1+ myeloid cells decreased in BM and concomitantly increased in peripheral blood (Fig. 1A, 1B).

OC progenitors (OCPs) are myeloid cells that have the capacity to differentiate to granulocytes, monocytes, and monocyte-derived mDCs (13). The same progenitors develop into OCs under the drive of RANKL (13, 19). It was shown previously that BM CD3⁻B220⁻CD11b⁻c-fms⁻c-Ki6h cells are highly enriched in OCPs as assessed by in vitro, M-CSF- and RANKL-dependent assays (13, 19). We confirmed that, in the presence of M-CSF and RANKL, flow-sorted, highly purified CD3⁻B220⁻CD11b⁻c-fms⁻c-Ki6hi cells form a large number of TRAP⁺, mature OCs displaying polymerized F-actin upon staining with phalloidin. By contrast, CD3⁻B220⁻CD11b⁻c-fms⁻c-Ki6hi cells formed no, or very few, mature OCs (Fig. 1C, 1D).

Because the CD3⁻B220⁻CD11b⁻c-fms⁻c-Ki6hi cells were shown to be bona fide OCPs, we tested whether αGCF-activated iNKT cells would promote the development of BM OCPs and mature OCs. We found that 3 d post-treatment of WT mice with αGCF, OCP cells were increased by 5.8-fold (p < 0.01) compared with PBS-treated controls (Fig. 2A, 2B). This finding was an iNKT cell- and CD1d-dependent effect, because the increase in OCP frequency in response to αGCF was not observed in TCRJα⁻/⁻ or Cdl1d⁻/⁻ mice (Fig. 2A, 2B).

**In vivo activated iNKT cells lead to accelerated maturation and activation of OCs**

To test whether increased OCP frequency induced by αGCF led to enhanced OC maturation and activation, BM cells from WT mice were collected and plated in vitro in the presence of M-CSF and RANKL 72 h after αGCF injection. Consistent with the in vivo results, day 3 TRAP⁺ multinucleated and day 4 mature OCs derived from αGCF-treated mice developed in vitro in higher numbers, suggesting increased OC clonogenicity and accelerated differentiation. This effect was also iNKT cell-dependent because it was not observed in TCRJα⁻/⁻ and Cdl1d⁻/⁻ mice. (Fig. 2C, 2D). During the same period, serum CTX levels, a specific marker of in vivo OC activation and bone resorption, were increased by 30% and remained increased in αGCF-treated WT mice over a
after a rapid egress of myeloid cells in response to GC. While a 2.5-fold increase in the frequency of myeloid cells in PB is observed at 3 h, their frequency in BM decreases by 2-fold, suggesting a rapid egress of myeloid cells in response to GC. B, Cumulative data of the frequency of myeloid cells (data shown as mean ± SD; n = 3 mice at each time-point). C, Flow cytometric identification of BM OCPs as c-fmshi c-Kithi (right dot blot, G1) gated on CD3+ B220+ CD11b+ cells (left dot blot). D, In vitro clonogenicity and generation of mature OCs from highly purified CD3+ B220+ CD11b+ c-fmsic-Kithi cells in response to M-CSF and RANKL. The left panel shows mature OCs identified either by TRAP staining or phallloidin staining to demonstrate actin polymerization. The right panel shows the number of mature OCs 4 d after plating 6500 CD3+ B220+ CD11b+ c-fmsic-Kithi or 6,500 and 65,000 cells as shown in G2 in Fig. 2C. Data are shown as mean ± SD of triplicate wells and representative of two independent experiments.

Intramedullary kinetics of in vivo activated mature OCs

To visualize mature OC development in the BM after αGC administration, TRAP staining in histologic sections of BM was performed. This staining showed that although the overall number of OCs did not change during a 72-h period after injection of αGC (data not shown), within 16 h and maximally at 24 h (Fig. 3A, 3B) the numbers of OCs attached to the trabecular and periosteal bone surfaces (OCS/BSA in Fig. 3B, left) were decreased. Interestingly, their numbers increased by ∼5-fold in the intramedullary space at 24 h and returned to baseline levels at 72 h (Fig. 3B, right). Given the increase in OCPs and their accelerated maturation and increase in CTX levels (Fig. 2E), these observations suggest that αGC provokes accelerated turnover of overactive OCs as well as their redistribution from the periosteal and trabecular surfaces into the BM cavity. Therefore, αGC-dependent immune activation coordinately couples differentiation and egress of myeloid cells with enhanced OC development, altered intramedullary localization, and increased resorptive activity of mature OCs, indicating a central role of iNKT cells in linking myelopoiesis with OC function. IFN-γ is a negative regulator of iNKT cell-induced OC development

RANKL is the most potent pro-osteoclastogenic cytokine. High levels of RANKL as result of T cell activation lead to OC activation and bone destruction in experimental and clinical arthritis (20). We measured serum levels of RANKL in response to αGC over a period of 72 h (p < 0.01 at 72 h; Fig. 2E) while remaining unchanged between baseline and 72 h in TCRJα18−/− and Cd1d−/− mice (Fig. 2F). Thus, αGC promotes in vivo osteoclastogenesis under conditions of immune activation in an iNKT cell-dependent manner.

FIGURE 1. In vivo activated iNKT cells promote mature myeloid cell egress and OCP expansion. A, Myeloid cell egress in response to αGC. Mac−/−Gr−/− mature myeloid cells in BM and peripheral blood (PB) as assessed by flow cytometry at baseline and 3 h after αGC injection in WT mice. While a 2.5-fold increase in the frequency of myeloid cells in PB is observed at 3 h, their frequency in BM decreases by 2-fold, suggesting a rapid egress of myeloid cells in response to αGC. B, Cumulative data of the frequency of myeloid cells (data shown as mean ± SD; n = 3 mice at each time-point). C, Flow cytometric identification of BM OCPs as c-fmshi c-Kitih (right dot blot, G1) gated on CD3+ B220+ CD11b− cells (left dot blot). D, In vitro clonogenicity and generation of mature OCs from highly purified CD3+ B220+ CD11b+ c-fmsic-Kithi cells in response to M-CSF and RANKL. The left panel shows mature OCs identified either by TRAP staining or phallloidin staining to demonstrate actin polymerization. The right panel shows the number of mature OCs 4 d after plating 6500 CD3+ B220+ CD11b+ c-fmsic-Kithi or 6,500 and 65,000 cells as shown in G2 in Fig. 2C. Data are shown as mean ± SD of triplicate wells and representative of two independent experiments.

FIGURE 2. αGC-induced maturation and activation of OCs. A, Flow cytometric analysis of BM OCPs 72 h after WT C57BL/6 mice received a single αGC (2 μg) or PBS injection i.p. OCPs are identified as c-fmsic-Kithi gated on CD3+ B220+ CD11b− cells (left dot blot). B, Frequency of OCPs in C57BL/6 WT, TCRJα18−/−, and Cd1d−/− mice 72 h after treatment with PBS or αGC (n = 10 for WT; n = 4 for TCRJα18−/− and Cd1d−/− mice). Data are shown as mean ± SD. **p < 0.01 by Student t test. C, In vitro assay of OCs derived from BM of WT mice injected with either PBS or αGC. BM cells were obtained from mice 72 h after injection and plated in the presence of M-CSF and RANKL. On days 3 and 4 of the culture, cells were stained with TRAP to determine multinucleated and mature OCs. More and larger multinucleated and mature OCs were observed in the αGC-treated group. D, Total numbers of day 3 and day 4 mature and multinucleated OCs generated from in vitro differentiation of BM cells obtained from WT, TCRJα18−/−, and Cd1d−/− mice treated with PBS or αGC. One representative of three experiments is shown; data are shown as mean ± SD of triplicates assays. E, Serum CTX levels at baseline and different time points after αGC injection into WT mice (n = 6 for 0 and 72 h; n = 2 for 16 and 24 h). Data are shown as mean ± SD. **p < 0.01 by Student t test. F, Serum CTX levels at baseline and at 72 h after αGC injection into TCRJα18−/− and Cd1d−/− mice (n = 3 for each time point).
72-h period. Serum RANKL levels were the same in αGC-treated WT and PBS-treated animals (Fig. 4A), suggesting that soluble RANKL is not a major mediator of the pro-osteoclastogenic effect of αGC-activated iNKT cells.

We therefore tested the role of IFN-γ and IL-4, the two archetypal Th1 and Th2 cytokines, in this process. First, we measured serum levels, and in agreement with previous reports (7, 21) we found that a single injection of αGC leads to a rapid secretion of IL-4 and IFN-γ in the first 3–6 h in WT but not TCRJα182/2 mice (Fig 4B, 4C). To investigate the relative contribution of IFN-γ and IL-4, we injected αGC into WT, IL-42/2, and IFN-γ2/2 BALB/c mice. We found that in each strain, OCP frequency was significantly higher in mice treated with αGC compared with PBS controls (p < 0.01 for αGC versus PBS in all three strains). However, although the fold increment of OCP frequency in response to αGC was similar between WT and IL-42/2 mice (IL-42/2, 2.1-fold versus WT, 2.3-fold; p > 0.05; Fig. 4D, 4E), it was significantly higher in IFN-γ2/2 mice (IFN-γ2/2, 6.2-fold versus WT, 2.3-fold; p < 0.01; Fig. 4D, 4E), suggesting that IFN-γ is an important negative regulator of the pro-osteoclastogenic effect of activated iNKT cells.

TNF-α is a positive regulator of iNKT cell-induced OC development

In search of positive regulators of iNKT cell-mediated enhancement of OC development, we tested the roles of IL-17 and TNF-α. IL-17, the prototypic Th17 cytokine, was shown recently to be secreted by activated iNKT cells (22) and to promote OC development (23, 24). Similarly, TNF-α is a strongly pro-osteoclastogenic cytokine (25, 26).

We found that OCP frequency in C57BL/6 WT mice treated with αGC plus anti-IL-17 Ab [previously shown to effectively neutralize IL-17 in vivo (27)] was not different to αGC-plus-IgG control-treated mice (Fig. 5A, 5B) indicating that IL-17 is not a major regulator of OC development in this context. To test the role of TNF-α, mice lacking the TNF-αR p55 subunit were injected with αGC. The p55 but not the p75 subunit of the TNF-αR was previously shown to mediate the pro-osteoclastogenic effect of TNF-α (25, 26). We found that, although in response to αGC, the frequency of OCPs in TNF-αR2/2 mice rose by 2.3-fold. Compared with PBS controls, the increment was significantly lower compared with C57BL/6 WT controls (TNF-αR2/2, 2.3-fold versus WT, 6-fold; p < 0.01; Fig. 5C, 5D), suggesting that TNF-α is indeed a major mediator of the pro-osteoclastogenic effect of αGC-activated iNKT cells. Consistent with these results, αGC injection triggered an up to 10-fold increase of TNF-α mRNA production in total BM cells (Fig. 5E).

These findings suggest that αGC-induced osteoclastogenesis is independent of IL-4 and IL-17, whereas IFN-γ and TNF-α are negative and positive regulators of this process, respectively.
**FIGURE 4.** Role of IFN-γ and IL-4 in αGC-enhanced OC development. A, Serum RANKL in response to αGC. Serum RANKL was measured at different time points by ELISA following injection of αGC or PBS into WT C57BL/6 mice (n = 3 mice per time point). B and C, Serum levels of IFN-γ and IL-4 as determined by ELISA at different time points after αGC injection in C57BL/6 WT and TCR Jα18−/− mice. Values are mean ± SD from three mice at each time point. D and E, Frequency of OCP in IFN-γ−/− and IL-4−/− mice at 72 h after treatment with PBS or αGC. Because both IFN-γ−/− and IL-4−/− mice were on the BALB/c background, BALB/c WT mice were used as controls (n = 7–9 for each strain). **p < 0.01, ns (not significant) refers to comparisons of the fold increment between groups by Student’s t test.

**FIGURE 5.** Role of IL-17 and TNF-α in αGC-enhanced OC development. A and B, Frequency of OCPs in C57BL/6 WT mice treated with αGC and anti–mIL-17 blocking Ab. C57BL/6 WT mice were injected i.v. with isotype IgG or anti–IL-17 blocking Ab 3 h prior to αGC injection. Flow cytometric analysis of OCPs was performed 72 h later (n = 4). C and D, Frequency of OCPs in TNF-αR55−/− and C57BL/6 WT mice 72 h after treatment with PBS or αGC (n = 4 for each strain). **p < 0.01, ns (not significant) refers to comparisons of the fold increment between groups by Student’s t test. E, TNF-α mRNA levels relative to GAPDH in total BM cells at the indicated time points after injection of αGC as assessed by Taqman real-time quantitative PCR (n = 3 mice per time point).

Skeletal phenotype of Cd1d−/− mice

iNKT cells are known to be autoreactive—that is, they are activated by endogenous ligands in steady state in the absence of exogenous glycolipid Ags (28). It is possible that this background iNKT activation might be important also for steady state OC development and activation; if so, then chronic lack of iNKT cells might result in perturbed skeletal phenotype and bone development. To address this prediction, we assessed skeletal developmental and adult bone structure in Cd1d−/− mice using several independent specific and sensitive methods (Fig. 6).

First, as shown by histologic analysis of the growth plates, endochondral ossification is not affected in 11-wk-old female Cd1d−/− mice as compared with age- and gender-matched WT controls (Fig. 6A).

Furthermore, Faxitron radiographic analysis showed that linear growth, diaphyseal diameter, cortical bone thickness and adult bone morphology in Cd1d−/− mice are similar to WT controls (Fig. 6B). These data are consistent with the in vitro OC development assays and serum CTX levels in Cd1d−/− and TCR Jα18−/− mice, which were different in WT mice (Fig. 2). However, further analysis indicated that bone mineral content was increased in adult Cd1d−/− mice (Fig. 6B). Because cortical bone thickness was similar in WT and Cd1d−/− mice, the increased bone mineral content determined by Faxitron analysis suggested a phenotype of increased cortical bone mineralization.

To investigate bone structure in detail, we first analyzed bone microarchitecture by BSE-SEM. As shown in Fig. 6C, Cd1d−/− mice had increased trabecular BV in long bones (23% increase in BV fraction [BV/TV]) compared with WT and vertebrae (19% increase in BV/TV).

qBSE-SEM analysis demonstrated increased cortical bone mineralization density in femurs from Cd1d−/− mice (Fig. 6E, 6F), which was accompanied by reduced osteoclastic resorption surfaces on endostal (35% lower in Cd1d−/− mice) and trabecular (26% reduced) bone, although these reductions did not reach statistical significance.

These detailed studies indicate that, although Cd1d−/− mice have normal endochondral ossification, the increased bone mineralization and BV result from low bone turnover with impaired osteoclastic bone resorption, which is consistent with a role for iNKT cells in OC development and activation even under basal conditions.

**Discussion**

iNKT cells have the unique property of modulating immune responses by recruiting and activating innate cellular components, including NK cells and mature myeloid cells such as monocytes-macrophages and granulocytes (2, 9, 29). Our work adds OCs, a specialized form of macrophage, to the cellular circuit that is mobilized and activated by iNKT cells to promote immediate and effective innate immune responses.

Our findings suggest that activated iNKT cells act on OC development, maturation, and activation. Indeed, the frequency of...
OCPs increases in the BM in response to αGC in an iNKT cell- and Cd1d-dependent manner. In addition, the in vivo clonogenic assays reflect enhanced commitment and accelerated differentiation toward precursors and mature OCs. The modest but sustained increase in serum CTX levels provides an in vivo measurement of increased OC activity and bone resorption. Kollet et al. (11, 30) have shown that activated OCs, through their proteolytic activity, contribute significantly to myeloid cell egress and mobilization in response to growth factors such as G-CSF, stress such as bleeding, and inflammatory stimuli such as LPS. Previous work has demonstrated the ability of αGC-activated iNKT cells, through secretion of GM-CSF and IL-3, to mobilize myeloid cells to the periphery (8, 9)—a finding also observed in our studies. These observations strongly support the hypothesis that the promotion of OC development and activation by iNKT cells contributes significantly to the ability of these cells to mobilize myeloid innate effectors during the course of an immune response.

Direct visualization of the mature OC in the BM in response to αGC revealed a complex, dynamic picture. First, in contrast to the increased frequency of OCP, OC clonogenic and resorptive activity, overall numbers of TRAP+ cells (i.e., mature osteoclasts) on histological sections were not increased. This apparent discrepancy would suggest that αGC induces higher turnover and hyperactivity of OCs that would be supported by their accelerated development and maturation in vitro (Fig. 2). It was also interesting to observe the redistribution of a large proportion of mature OCs from the bone surfaces to the intramedullary area. It remains to be determined whether this phenomenon is of functional significance and whether it is linked to the role of OCs in the mobilization of progenitor and mature myeloid forms.
Activated iNKT Cells Regulate Osteoclasts

As discussed above, and similar to LPS stimulation, activation of iNKT cells, these two cytokines are major determinants of OC development. Previous work showed that the net effect of IFN-γ on OC development and function is determined by the balance of its direct inhibitory effect on hematopoiesis and OCPs (32, 33) and its ability to promote osteoclastogenesis through the induction of RANKL expression on activated T cells (20, 34, 35). Our finding that IFN-γ is a negative regulator of aGC-promoted osteoclastogenesis is consistent with previous observations showing that IFN-γ inhibits osteoclastogenesis when nonspecific T cell activation occurs in the course of an innate immune response, such as in response to LPS (35). In that context, IFN-γ inhibits OC development through ubiquitination and proteasome degradation of TRAF6, an adapter protein required for RANK-dependent NF-κB and Jnk signaling (35).

As discussed above, and similar to LPS stimulation, activation of iNKT cells by aGC results in rapid secretion of IFN-γ by iNKT cells and in nonspecific activation and IFN-γ secretion by T and NK cells (2, 29, 36), hence the enhanced frequency of OCP in response to aGC in IFN-γ−/− mice.

Activation of T cells and macrophages by iNKT cell-derived IFN-γ also leads to secretion of TNF-α (37), a strongly pro-osteoclastogenic cytokine. In an inflammatory context, TNF-α enhances osteoclastogenesis in a RANKL-dependent manner either directly, by promoting commitment of progenitors to OC lineage and differentiation, or indirectly by stimulating secretion of RANKL and M-CSF by osteoblasts (25, 26). We addressed the role of TNF-α using mice that lack expression of p55R—that is, the TNF-αR that in contrast to p75R was shown previously to mediate the pro-osteoclastogenic effect of TNF-α (38). Indeed, the enhancing effect of aGC on OCP was significantly dampened in TNFp55R−/− mice, suggesting that TNF-α is a major mediator of the effects of aGC and iNKT cells in osteoclastogenesis.

Given that overall in response to aGC osteoclastogenesis is enhanced, we presume that the positive effect of TNF-α in this process prevails over the suppressive effect of IFN-γ, such that the pro-osteoclastogenic effect of iNKT cell activation is coupled with myeloid cell egress.

We found that aGC-mediated promotion of OC development is not regulated by IL-4, the prototypical Th2 cytokine that negatively regulates osteoclastogenesis by inhibiting maturation of OC precursors (39–41), or by IL-17, a recently described strongly pro-osteoclastogenic cytokine (23). Similarly, serum RANKL levels were not increased in response to aGC, suggesting that soluble RANKL is not a major mediator of OC development in this process. However, it is possible that membrane-bound RANKL (e.g., on activated T cells, osteoblasts, or other stroma cells) could contribute along with TNF-α in the enhanced development and activation of OCs in response to iNKT cell activation.

The ability of activated iNKT cells to modulate OC function raised the prospect of these cells being required for OC development and activation under basal conditions. Indeed, iNKT cells are known to be autoreactive and in an activated state in vivo, even in the absence of exogenous antigenic challenge [reviewed in Ref. 28]. Cord blood iNKT cells, which bear markers of Ag experience and activation, are a characteristic example of this endogenous activation of iNKT cells (42, 43). It is possible that the baseline activation of iNKT cells might have an effect on OC function and turnover and might ultimately result in impaired bone resorption and increased bone mass over time. Accordingly, detailed analysis of bone structure and mineralization in 11-wk-old Cd1d−/− mice revealed a mild phenotype characterized by increased BV/TV and increased bone mineralization density, accompanied by evidence of reduced osteoclastic bone resorption.

We propose that the effects of iNKT cells on OC function cover a spectrum that ranges from baseline, homeostatic, chronic, low-level OC activation to the immediate and robust OC activation in response to exogenous stimuli, such as the one provided by aGC.

Exogenous activation of iNKT cells results in secondary activation of T cells, which are likely to be a major source of the cytokines modulating OC function. Indeed, T cells are a major source of pro-osteoclastogenic cytokines and indispensable for OC activation and bone loss in the context of immune activation in inflammatory and autoimmune diseases (13, 44–46). To our knowledge, our data provide evidence for the first time of the involvement of an immunoregulatory T cell subset (i.e., iNKT cells) in this process.

Based on this and previous studies (8, 9), we propose a model integrating the role of iNKT cells in myeloid cell recruitment during an immune response (Fig. 7). iNKT cells promote activation of mature myeloid cells (i.e., macrophages, mDCs, OCs) and enhance the clonogenic activity and differentiation of their common progenitors and egress of their mature forms. Activation of OCs by iNKT cells further potentiates this process. Our findings also suggest that therapeutic manipulation of iNKT cells to control chronic inflammatory conditions, such as experimental (47) and rheumatoid arthritis (40, 48–50), might be useful for controlling the associated cytokine-induced OC activation and bone destruction (20).

Disclosures
The authors have no financial conflicts of interest.

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