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B Lymphocytes Inhibit Human Osteoclastogenesis by Secretion of TGFβ

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Abstract The role of B lymphocytes in osteoclast (OC) formation is controversial, because both stimulatory and inhibitory effects of B-lineage cells on osteoclastogenesis and life span have been reported. In this study, we have investigated the effects of mature B cells on human osteoclastogenesis using cultures of peripheral blood stem cells (PBSC), a system that generates functional OCs in the absence of stromal cells. We report that B cells inhibit the formation of OCs and shorten the life span of mature OCs by secreting transforming growth factor β (TGFβ), a factor that induces apoptosis in these cells. The antiosteoclastogenic effects of B cells are abolished by addition of anti-TGFβ antibody to osteoclast cultures and mimicked by treatment of B cell-deprived PBSC cultures with recombinant TGFβ, thus confirming TGFβ as the B cell produced antiosteoclastogenic activity. Thus, the ability of B cells to downregulate osteoclastogenesis by secretion of the apoptotic cytokine TGFβ provides new insights into the ability of immune cells to regulate OC formation under basal and inflammatory conditions. J. Cell. Biochem. 78:318–324, 2000.

Key words: cytokines; TGFβ; lymphocytes; human osteoclastogenesis

The role of immune cells such as B lymphocytes and T lymphocytes in osteoclastogenesis is poorly understood. T cells and B cells are prominent at sites of inflammation and infection, and the bone marrow is a major reservoir for young or immature lymphocytes as well as mature cells derived from the general circulation. Previous investigations on the effects of B cells on osteoclastogenesis have generated conflicting data. For example, the B cell-deficient mouse μMT/μMT has been found to display decreased trabecular area and increased bone resorption, as compared to B-replete mice of the same strain [Dissanayake et al., 1997], suggesting that B cells inhibit bone resorption and osteoclastogenesis. In contrast, other studies have shown that estrogen deficiency upregulates B lymphopoiesis in the bone marrow [Masuzawa et al., 1994; Erben et al., 1998], suggesting that cells of the B lineage may contribute to the increased osteoclast (OC) production characteristic of estrogen-deficient animals. Thus, the effects of B cells or B cell factors on osteoclastogenesis remain controversial.

Because most of the studies published to date have been conducted in animal models, little information is available on the role of B cells in human osteoclastogenesis. We have recently reported a technique to generate human OCs in culture using peripheral blood stem cells (PBSC), a peripheral blood product enriched (~10–20-fold) in $CD34^+$ stem cells [Matayoshi et al., 1996]. We have now used cultures of PBSC to evaluate the effects of pe-

Abbreviations used: OC, osteoclast; PBSC, peripheral blood stem cells; TRAP, tartrate-resistant acid phosphatase; 7-AD, 7-actinomycin D.

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ripheral blood B cells on the generation of OCs from early monocytic precursors.

Our data demonstrate that B cells are important modulators of osteoclastogenesis and an important source of transforming growth factor β (TGFβ), a factor that inhibits OC formation by inducing apoptosis of early and late OC precursors and mature OCs.

MATERIALS AND METHODS

All reagents were purchased from the Sigma Chemical Corp. (St. Louis, MO), unless otherwise indicated. Recombinant human interleukin (rhIL) 1β was provided by Dompe Pharmaceutical (L'Aquila, Italy) and rhIL-3, recombinant human macrophage colony-stimulating factor (CSF) and recombinant human granulocyte-macrophage (rhGM)-CSF by Genetics Institute (Boston, MA). The specific antiosteoclast antibody 121F was a gift from Dr. P. Osdoby (Washington University St. Louis, MO).

Cell Culture

The RAJI human B-lymphoma and THP-1 human monocytic cell lines were obtained from the ATCC (Manassas, VA), and cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Gaithersburg, MD), penicillin (50 U/ml), and streptomycin (50 μg/ml) in a humidified incubator with 5% CO_2.

Generation of Human Osteoclasts

Human OCs were generated as previously described [Matayoshi et al., 1996]. At the end of the culture period, tartrate-resistant acid phosphatase (TRAP) positive cells exhibiting ≥3 nuclei were counted as mature OCs and TRAP positive cells with ≤2 nuclei were considered OC precursors.

Immunomagnetic Selection or Depletion of Cell Populations

B cell and monocyte populations were purified or depleted from OC cultures using anti-CD19 (B cells) and anti-CD14 (monocytes/macrophages) -coated immunomagnetic Dynabeads (Dynal, Lake Success, NY), according to the manufacturer's instructions. For some experiments, monocytes were semipurified in the absence of CD14 antibodies from B cell-depleted cultures, by adhesion onto plastic dishes for 3 h, and nonadherent cells were removed by three washes of phosphate-buffered saline (PBS). These populations were found to respond in the same manner as monocytes positively selected by magnetic bead and are >99% T and B cell depleted, as assessed by flow cytometry. For immunoselection of B lymphocytes, recovered cells were washed in PBS and attached beads were removed using DETACHaBEAD (Dynal) according to the manufacturer's instructions. B cells positively selected using Dynabeads and DETACHaBEAD are >99% pure and >98% viable after isolation. More than 90% of these cells are in G_0 phase and are not activated by the isolation procedure.

MTT Cell Proliferation Assays

Cell proliferation was determined by MTT assay as described by Mosmann [Mosmann, 1983].

Apoptosis Assays

Annexin-V flow cytometry was used to measure apoptosis, using the Apoptest kit (Nexins Research, Hoeven, The Netherlands), according to the manufacturer's instructions. Briefly, monocytes or mature OCs cultured in P150 dishes (~2×10^5/20 ml) were treated with 2 ng/ml TGFβ (R&D Systems, Minneapolis, MN) or with RAJI or B cell conditioned medium for 4 h. Monocytes were recovered from the dishes by trypsinization, and OCs were recovered as previously described [Matayoshi et al., 1996]. OCs were labeled with 121F anti-OC antibody [Khalkhali-Ellis et al., 1997] and with anti-mouse IgG phycoerythrin secondary antibody. OCs and monocytes were labeled with annexin-V–fluorescein isothiocyanate and 7-actinomycin D before analysis on a three-color Becton-Dickinson, (Franklin Lakes, NJ) flow cytometer (FacScan).

Antibody Depletion Assays

Antibody depletion assays were conducted by preclearing specific cytokines, from B cell and RAJI conditioned medium, before addition to the target cells. Briefly, 1 ml conditioned medium was incubated with either 10 μg of anti-interleukin-8 (IL-8), anti-IL-10, anti-IL-18, anti-interferonγ (IFNγ), or anti-TGFβ panspecific (R&D Systems, Minneapolis, MN). Antibody concentrations were calculated to be capable of neutralizing at least 1 μg/ml of

cytokine, based on the manufacturer's data sheets. After incubation with protein-G agarose, immunocomplexes were pelleted by centrifugation, and the cleared supernatant was added to the cells. Control supernatants were processed in the same manner but received no antibody.

Assay of Human Transforming Growth Factor β_1 by Enzyme-Linked Immunosorbent Assay

Human TGFβ_1 was measured in B cell conditioned medium after acid activation using a Biotrak hTGFβ_1 enzyme-linked immunosorbent assay (ELISA) kit (Amersham International, Arlington Heights, IL), according to the manufacturer's instructions. Background levels of TGFβ_1 present in the serum were also determined.

Statistical Analysis

Group mean values were compared by two-tailed Student's *t* test or one-way analysis of variance, as appropriate.

RESULTS

When cultured in vitro with IL-1, IL-3, and GM-CSF, human PBSC, a mixed population of cells containing T and B lymphocytes, generate large numbers of authentic bone-resorbing OCs [Matayoshi et al., 1996]. Our PBSC culture also contains OC precursors (TRAP-positive cells with ≤2 nuclei) and early OC precursors (TRAP negative, esterase-positive cells of the monocytic lineage). To evaluate the contribution of B cell populations to the process of osteoclastogenesis, PBSC cultures were immunomagnetically depleted of B cells, and the number of OCs formed was compared to those generated in the presence of B cells. To control for fluctuations in the number of early OC precursors plated in B cell-replete and B cell-depleted wells, a control was included in which some B cell depleted cultures were reseeded with B cells at the same original concentration. B cell-depleted PBSC cultures (Fig. 1) produced 3.8-fold more OCs relative to untreated PBSC cultures and 2.3-fold more OC than B cell-replaced PBSC cultures. These data suggest that B cells inhibit OC formation. To determine whether B cells secrete soluble factor(s) capable of inhibiting OC formation, B cells were immunomagnetically removed from PBSC and cultured separately. The addition of

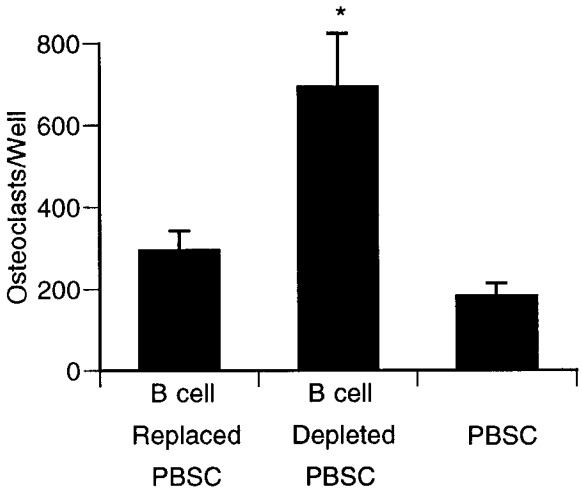

Fig. 1. B cells repress in vitro osteoclast formation, in a human model system. B cells were depleted from PBSC cultures using specific antibodies (CD19) coupled to magnetic beads (B cell-depleted PBSC). To control for fluctuations in the number of early OC precursors plated in B cell-replete and B cell-depleted wells, a control was included in which some B cell-depleted cultures were reseeded with B cells at the same original concentration (B cell-replaced PBSC). After 21 days, the number of mature OCs formed (TRAP positive cells with three or more nuclei) was determined. Data points show the average ± SD of three replicates. The data are representative of five independent experiments. $*P < 0.02$ with respect to the B cell-replaced sample.

increasing concentrations of B cell conditioned medium to PBSC cultures resulted in a dose-dependent inhibition of OC formation (Fig. 2). In additional control experiments, cells that were fed with 50% control medium (incubated in the absence of B cells) showed no change in OC number (data not shown). Although B cells were purified using a positive selection technique, it could be argued that trace contaminating cells present in the B cell culture may be the source of the antiosteoclastogenic activity. To exclude this possibility, we investigated whether inhibitory activity is present in the supernatant of RAJI cells, a B-lymphoma cell line. Figure 3 shows that like B cell conditioned medium, RAJI cell conditioned medium inhibits, in a dose-dependent manner, the number of OCs produced in cultures of B cell-depleted PBSC. This finding confirms that the source of the osteoclastogenic activity is not a contaminating cell, but rather a cell of the B lineage.

The inhibitory factor produced by RAJI cells was found to decrease the number of both OC precursors and mature OCs (Fig. 3). These data suggest that the B cell-produced inhibi-

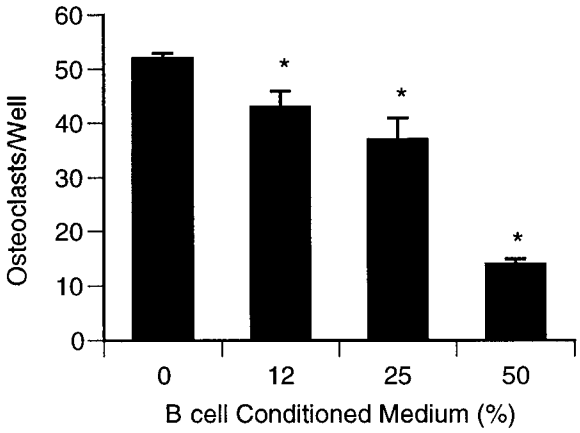

Fig. 2. B cell conditioned medium inhibits in vitro human osteoclast formation in a dose-dependent manner. Human OCs were cultured in the presence of increasing concentrations (up to 50% final volume) of B cell conditioned medium. After 21 days, the number of mature OCs formed (TRAP positive cells with three or more nuclei) was determined. Data points are shown as average ± SD of three replicate samples. The data are representative of three independent experiments. $*P < 0.02$ with respect to 0% conditioned medium control.

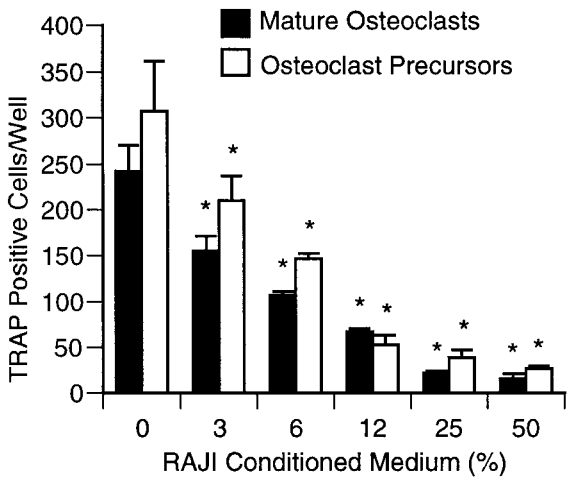

Fig. 3. RAJI conditioned medium inhibits human osteoclast formation in a dose-dependent manner. Human OCs were cultured in the presence of increasing concentrations of RAJI conditioned medium (up to 50% final volume). After 21 days, the number of mature OCs [TRAP-positive (three or more nuclei)] and OC precursors [(TRAP-positive (two or fewer nuclei)] was determined. Data points show the average ± SD of three replicates. The data are representative of three independent experiments. $*P < 0.02$ with respect to 0% conditioned medium.

tory activity "blocks" osteoclastogenesis by decreasing the number of proliferating OC precursors.

We next investigated whether RAJI conditioned medium decreases OC formation by in-

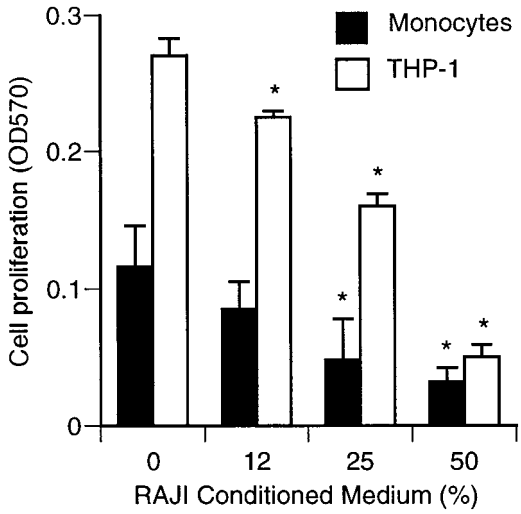

Fig. 4. RAJI conditioned medium induced a dose-dependent inhibition of early OC precursor proliferation. RAJI conditioned medium was tested on the proliferation of human peripheral blood monocytes (early OC precursors) and the monocytic cell line THP-1, which is capable of differentiating into OC-like cells. Cells were cultured in the presence of increasing concentrations of RAJI conditioned medium (up to 50% final volume). After 48 h, cell proliferation was determined by MTT assay. Data points show the average ± SD of three replicates. The data are representative of three independent experiments. $*P < 0.05$ with respect to 0% conditioned medium, for each series.

hibiting the replication of early OC precursors using an MTT proliferation assay. This assay provides a measure of cell number by estimating the number of viable growing cells [Denizot and Lang, 1986]. We evaluated the effects of RAJI conditioned medium on the growth of both peripheral blood monocytes (early OC precursors) and THP-1 cells, a human monocytic line capable of differentiating into multinucleated cells expressing markers of mature OCs [Shozu et al., 1997]. Both human peripheral blood monocytes and THP-1 cells were strongly inhibited by the B cell factor (Fig. 4). Control concentrated medium that had been incubated in the absence of B cells elicited no inhibitory effects at 50% final concentration (data not shown). To further exclude dilution of culture medium with spent medium as being the cause of the antiosteoclastogenic effect, RAJI conditioned medium was concentrated 83-fold using a 10-kDa cutoff centrifugal filter. One and one half microliter of this concentrated extract (equivalent to a 50% final concentration of normal conditioned medium) was then tested on THP-1 target cells in an MTT proliferation assay. This small volume of concentrated

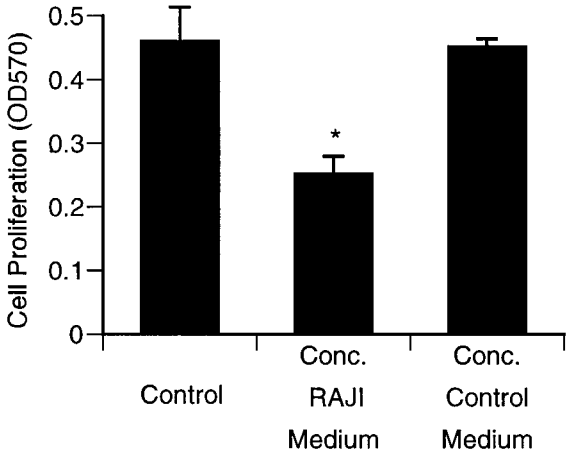

Fig. 5. Small volumes of concentrated RAJI conditioned medium inhibit early OC precursor proliferation. RAJ conditioned medium was concentrated down 83-fold using a 10-kDa cutoff centrifugal filter and 1.5 μl (equivalent to a 50% final dose of dilute medium added to THP-1 monocytic cells) (concentrated RAJI medium). Controls consisted of a group receiving no additional medium (Control) and a control receiving medium that had been incubated in the absence of B cells and concentrated 83-fold (concentrated control medium). After 48 h, cell number was determined by MTT assay. Data points show the average ± SD of three replicates. The data are representative of three independent experiments. $*P < 0.05$ with respect to control.

RAJI medium was found to retain strong inhibitory activity as compared to control concentrated medium that had been preincubated in the absence of B cells (Fig. 5). These findings demonstrate that a specific B cell secreted factor(s) is responsible for the inhibitory activity.

One mechanism by which the inhibitory factor secreted by RAJI and B cells could inhibit osteoclastogenesis is by inducing apoptosis of early OC precursors and/or mature OCs. Thus, the ability of B cell conditioned medium to induce apoptosis of mature OCs and peripheral blood monocytes (early OC precursors) was tested using Annexin-V flow cytometry, a system that identifies the early stages of apoptosis. B cell conditioned medium was found to elicit a 3-fold increase in mature OC apoptosis and a 4.5-fold increase in monocyte apoptosis at 4 h of stimulation (data not shown). Baseline apoptosis was ~10%. Together, the data demonstrate that B cells produce soluble factor(s) that decrease the life span of both mature OCs and OC precursors, as well as limiting the number of early OC precursors, which would also directly contribute to the reduction of mature OCs.

To identify the B cell factor responsible for the inhibitory activity, neutralization experiments were conducted using antibodies directed against cytokines known to inhibit OC formation, including IL-8, IL-10, IL-18, IFNγ, and TGFβ. Although addition of anti-TGFβ antibody to the B cell conditioned medium blocked the antiosteoclastogenic activity, antibodies against IL-8, IL-10, IL-18, and IFNγ had no effect (Fig. 6). This data suggests that B cells secrete the anti-osteoclastogenic factor TGFβ.

To directly assay the levels of TGFβ in B cell conditioned medium, $TGF\beta_1$ was measured using an ELISA specific for human $TGF\beta_1$. Because serum present in tissue culture medium is also known to contain TGFβ, the levels of TGFβ in normal tissue culture medium were also measured. Concentrations of $TGF\beta_1$ as high as 4 ng/ml were identified in B cell conditioned mediums after subtraction of basal serum levels. This corresponds to the presence of 2 ng/ml $TGF\beta_1$ in the OC culture medium, after addition of a 50% final addition of B cell medium

To confirm that TGFβ is capable of inhibiting OC formation at concentrations found to be present in B cell conditioned mediums, rhTGFβ at 2 ng/ml, representative of a 50% final addition of conditioned medium, was added to PBSC cultures, stimulated with IL-1, IL-3, and GM-CSF. These experiments revealed that rhTGFβ decreases OC formation by twofold (Fig. 7). Furthermore rhTGFβ (2 ng/ml) was found to increase OC apoptosis approximately threefold (data not shown), confirming the capacity of TGFβ to inhibit OC formation by an apoptotic mechanism in this culture system.

DISCUSSION

In this study, we have investigated the effects of peripheral blood B cells on OC formation. Our data show that in vitro B cells inhibit osteoclastogenesis and shorten OC life span by secretion of the apoptotic cytokine TGFβ, a factor that has previously been shown to decrease the life span of human [Chenu et al., 1988] and murine [Hattersley and Chambers, 1991; Hughes et al., 1996] OCs. TGFβ is also recognized for its ability to inhibit OC formation, an effect resulting in part from its ability to induce the potent antiosteoclastogenic cytokine osteoprotegerin (OPG) in B cells after CD40 activa-

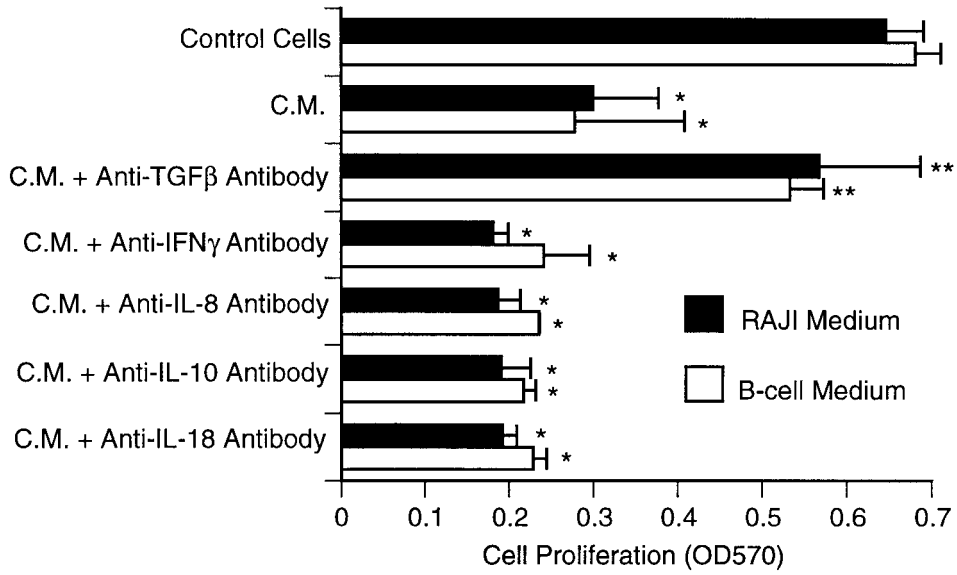

Fig. 6. Antibody neutralization of TGFβ but not of IL-8, IL-10, IL-18, or IFNγ suppresses the inhibitory effect of B cell and RAJI conditioned media. THP-1 early OC precursors were incubated in the presence of B cell or RAJI conditioned medium (50% final volume). Antibodies to IL-8, IL-10, IL-18, IFNγ, and TGFβ were used to neutralize activity of specific cytokines as described in the Materials and Methods section. The ability of specific cytokine-depleted supernatants to block proliferation of THP-1 cells was assessed after 48 h by MTT assay. Data points show the average ± SD of four replicates. The data are representative of three independent experiments. $^{*}P < 0.02$ with respect to control and $^{**}P < 0.5$ with respect to CM.

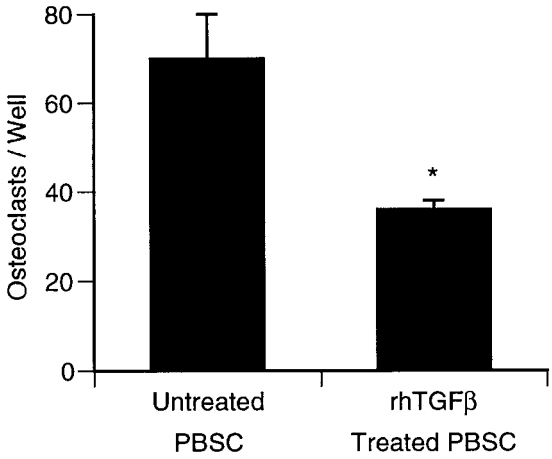

Fig. 7. rhTGFβ represses in vitro human osteoclast formation. Human OCs were generated in the presence or absence of 2 ng/ml of rhTGFβ. The number of mature OCs (TRAP-positive cells with three or more nuclei) were counted after 21 days. Data points show the average ± SD of three replicate samples. The data are representative of three independent experiments. $^{*}P < 0.02$ with respect to untreated PBSC.

tion [Yun et al., 1998], and to elicit the stromal cell production of OPG while simultaneously suppressing the proosteoclastogenic factor OPGL [Takai et al., 1998].

Our data confirm that TGFβ is a potent antiosteoclastogenic cytokine [Chenu et al., 1988; Hattersley and Chambers, 1991; Hughes et al., 1996] and provide the first demonstration that B cells may play an important role in regulating the formation of human OCs in culture by a mechanism involving TGFβ.

The relevance of the anti osteoclastogenic effects of TGFβ in the context of the regulation of bone turnover in vivo remains to be established because TGFβ has complex effects not only on bone resorption, but also on bone formation. Moreover, this cytokine plays a key role in the coupling of bone formation to bone resorption [Erlebacher et al., 1998]. Thus, it is likely that conditions that alter production of TGFβ in the bone marrow may result in appreciable changes in bone turnover and bone mass. In support of this notion are studies in humans showing that polymorphisms in the $TGF\beta_1$ gene associated with the highest serum concentrations of $TGF\beta_{11}$ are found in postmenopausal women with the highest values of bone mineral density [Yamada et al., 1998].

Our observation in vitro attesting to the ability of B cells to repress OC formation and life span are consistent with findings in vivo demonstrating that B cell-deficient mice have lower bone volume and increased bone resorption than B cell-replete mice of the same strain

[Dissanayake et al., 1997]. However, in apparent contrast with our findings, ovariectomy, a condition that stimulates bone resorption, has been found to increase B lymphopoiesis in mice [Masuzawa et al., 1994], and rats [Erben et al., 1998], whereas estrogen, a hormone that represses osteoclastogenesis, inhibits the clonal expansion of B cell precursors [Smithson et al., 1995]. It has also recently been reported that estrogen deficiency is associated with an increase in B220 cells, a B cell precursor population that is now thought to be capable of differentiating into cells of the OC lineage when cocultured with stromal cells [Sato et al., 1999]. Thus, it is likely that unlike mature B cells, which are inhibitory to osteoclastogenesis, the population of pre-B cells formed under conditions of estrogen deficiency not only fail to secrete TGFβ but may also act to increase the pool of OC precursors, thus accounting for this apparent contradiction.

In summary, our findings suggest that B cells may be an important source of TGFβ, a factor inhibitory to OC formation, and that B lymphocytes may thus play an important role in the regulation of osteoclastogenesis.

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