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B cells activated in the presence of Th1 cytokines inhibit osteoclastogenesis

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Abbreviations: $B_{IFN-\gamma}$, B cells activated with anti-IgD mAb, IL-4, anti-CD40 mAb, and IFN-γ; B_{IL-2}, B cells activated with anti-IgD mAb, IL-4, anti-CD40 mAb, and IL-2; BTh2, B cells activated with anti-IgD mAb, IL-4, and anti-CD40 mAb; MNC, multinuclear cell; OPG, osteoprotegerin; RT, reverse transcription; sRANKL, soluble receptor activator of NFκB ligand; TRAP, tartrate resistant alkaline phosphatase

Abstract

Host immune response has been considered as an important disease-modifying factor of periodontitis, however, which immune cell(s) or factor(s) are involved in the destruction of periodontium remains unclear. Previously, we reported that osteoclastogenesis is enhanced by activated B cells but suppressed by activated $CD8^+$ T cells. We present new data that B cells activated in the presence of Th1 cytokines inhibit osteoclastogenesis. Purified murine B cells were activated with anti-IgD mAb, IL-4, and anti-CD40 mAb, in the absence (B_{Th2}) or presence of Th1 cytokines, either IL-2 (B_{IL-2}) or IFN-γ ($B_{IFN-\gamma}$). Each activated B cell population was co-cultured with RAW264.7 cells in the presence of soluble receptor activator of NF-κB ligand (sRANKL), and the effect on osteoclastic differentiation was evaluated. While B_{Th2} increased osteoclastogenesis, B_{IL-2} and $B_{IFN-\gamma}$ suppressed it profoundly. To verify the mediating molecule(s), we analyzed cytokine profiles of the activated B cells. Compared to B_{Th2}, B_{IL-2} expressed increased amount of IFN-γ and $B_{IFN-\gamma}$ expressed decreased amounts of IL-4, IL-5, and IL-10. IFN-γ was a key negative regulator of osteoclastic differentiation, and mediated the inhibition by B_{IL-2}. These results suggest that Th1 cytokines may have new important roles in resistance to periodontitis, acting directly on osteoclasts or indirectly through B cells.

Keywords: B lymphocyte subsets; cytokines; interferon type II; interleukin-2; osteoclasts; Th1 cells

Introduction

Although microbial plaque is a primary etiologic agent responsible for periodontitis, host immune response has long been considered as an important disease-modifying factor. Considering the protective function of immune response, to determine which immune cell(s) or factor(s) are involved in the destruction of periodontium rather than protection is important to predict disease course and plan treatment. People with defects in neutrophil function demonstrated that neutrophil has a pivotal role in the protection from periodontitis (Cianciola *et al*., 1977; Clark *et al*., 1977; Anderson *et al*., 1985). Chronically inflamed periodontal tissues are infiltrated densely with lymphocytes as well as phagocytic leukocytes. To understand the role of the infiltrated lymphocytes in the pathogenesis of periodontitis, many investigators studied the number and proportion of lymphocyte populations and established a concept that the change from a stable to a progressive lesion was associated with the increased infiltration of B cells and plasma cells (Mackler *et al*., 1977; Seymour *et al*., 1979; Malberg *et al*., 1992). However, the role of B cells in the pathogenesis of periodontitis has been controversial: while some insisted that plasma cell predominance *per se* causes periodontal bone loss (Seymour, 1991; Kinane, 2000), some insisted that periodontal destruction does not result from the conversion of a predominantly T cell to a predominantly B cell lesion, but rather from episodes of acute inflammation (Page, 1986).

To clarify this issue, we studied the effect of each activated lymphocyte subset on osteoclast differentiation and activation *in vitro* and reported previously that osteoclastogenesis is enhanced by activated B cells but suppressed by activated $CD8^+$ T cells (Choi *et al*., 2001). However, we subsequently observed that B cells could also suppress osteoclastogenesis depending on the activation protocol used. In this study, we demonstrated that B cells activated in the presence of Th1 cytokines suppressed osteoclastogene-

sis. In addition, we studied cytokine profiles of B cells activated in the absence or presence of Th1 cytokines, and have shown that IFN-γ was a key negative regulator of osteoclastic differentiation and mediated the inhibition of osteoclastogenesis by B cells activated in the presence of IL-2.

Materials and Methods

Mice and reagents

Four to six week-old C57BL/6 female mice (Han-Lim Laboratory Animal Co. Kyung-gi, Korea), were used for all experiments. Recombinant murine (rm) sRANKL, rmIFN-γ, rmIL-2, and rmIL-10 were purchased from Peprotech (London, UK). Other cytokines including recombinant human (rh) TGF-β, rmIL-4, rmIL-13, and rmTNF-α were purchased from R&D systems (Minneapolis, MN). All monoclonal antibodies were purchased from BD PharMingen (San Diego, CA).

Activation of B cells

Mononuclear leukocytes were isolated from splenocytes by Ficoll separation, and then subjected to T-cell depletion using anti-CD3 Ab-conjugated Dynabeads (Dynal, Lake Success, NY). The purified B cells were re-suspended in α-MEM complete medium (10% fetal bovine serum, 2 mM glutamine, penicillin, streptomycin) at 2×10^6 cells/ml and activated for 2 days with 1 mg/ml anti-IgD Ab clone 217-170, 0.2 mg/ml anti-CD 40 Ab clone HM40-3, and 20 IU/ml rmIL-4, in the absence or presence of Th1 cytokines, either 100 IU/ml rhIL-2 (Chiron Therapeutics, Emeryville, CA) or 50 ng/ml rmIFN-γ. We designate the B cells activated by three different protocols B_{Th2}, B_{IL-2}, and $B_{IFN-\gamma}$, respectively. Following B cell-specific activation, few granulocytes survived and most monocytes remained attached to the culture plate. The purities of the activated B cells were confirmed by flow cytometry before co-culture and they were between 95-98%.

In vitro osteoclastogenesis and co-culture with activated B cells

Murine monocyte cell line RAW264.7 was purchased from ATCC (Bethesda, MD). After expansion, cells at passage 4 or 5 were kept as frozen vials and freshly thawed cells were used for all experiments. For tartrate resistant alkaline phosphatase (TRAP) stain and TRAP enzyme activity assay, RAW264.7 cells were plated into 96-well plates at 2,500 cells/0.15 ml/well in α-MEM complete medium with murine soluble RANKL (20 ng/ml). On day 2, the medium with sRANKL was replaced with fresh one and co-cultured with activated B cells (5×10^5 cells/well) for another 3 days. For resorption assay, RAW264.7 cells were plated in 48-well Osteoclast Activity Assay System plates (OAAS; Oscotec, Korea) at 4,500 cells/0.3 ml/well in α-MEM complete medium with murine soluble RANKL (20 ng/ml) for 2 days, and then co-cultured with activated B cells (10^6 cells/well) for another 6 days, replacing them once with new activated B cells on day 5. After co-culture, the supernatants were saved and kept in -70°C for ELISA assay. For neutralization of IFN-γ, various amounts of anti-INF-γ mAb, clone H22 were added with B_{IL-2} cells from day 2 to 5.

TRAP activity assay

After wash, the entire adherent cells in each well were lysed with 50 μl RIPA buffer. TRAP activity was measured by the conversion of α-naphthyl phosphate (2.5 mM; Sigma) to α-naphthol in the presence of 25 mM L-tartrate solution (Sigma). Absorbance was measured at 405 nm using a microplate reader.

TRAP stain

After wash, cells were fixed with 3% formaldehyde in acetone for 30 s. Cells were stained for TRAP using a leukocyte acid phosphatase staining kit (No. 387, Sigma). $TRAP^+$ cells with three or more nuclei were regarded as osteoclasts and counted under an inverted-phase contrast microscope.

Resorption assay

After termination of culture, all remaining cells on the OAAS plates were lyzed using 1 M NaOH. The image (×40) of an entire well was captured, and resorbed area was measured using a computer-assisted image analyzer (ImagePro v4.0; Media Cybernetics, Bethesda, MD).

Reverse transcription (RT)-PCR

Total RNA was extracted from B cells that were activated for 2 days with three different protocols. Total RNA (1 μg) was subjected to RT with $(dT)_{16}$ and 1 μl of Superscript II enzyme (GibcoBRL, NY) in 20 ml reaction mix. After 1 h at 42°C, 1 μl and serially diluted samples (1/5 and 1/25) were subjected to PCR in cycling conditions: 94°C, 20 s; 60°C, 30 s; 72°C, 1 min for 35 cycles. Actin gene amplified for 25 cycles served a control for RNA input. Primer sequences used are listed in Table 1.

ELISA

The amounts of IL-2, IL-4, IL-10, and IFN-γ secreted into media during co-culture with activated B cells,

Table 1. Primer sequences used in RT-PCR.

Primer names	Sequences
Actin-F	ATGTGCAAGGCCGGCTTCGCGGGCGAC
Actin-R	GATGTCCACGTCACACTTCATGATGGA
RANKL-430F	TGAAAACGCAGATTTGCAGGA
RANKL-739R	CATGTTAGAGATCTTGGCCCA
OPG-160F	GAAGAACCCATCTGGACATTT
OPG-520R	TTTTGCAAACTGTGTTTCGCT
RANK-181F	AACCAGGAAAGTACCTGTCCT
RANK-735R	TGTAGTAAACGCCGAAGATGA
TNFa-163F	CATGAGCACAGAAAGCATGAT
TNFa-440R	CTACAGGCTTGTCACTCGAAT
TGFb-239F	ACCTACCTTTCCTTGGGAGA
TGFb-849R	TGCTCCACACTTGATTTTAA
IL2-20F	CTCACAGTGACCTCAAGTCCT
IL2-577R	TTGTAAGCAGGAGGTACATAGTTA
IFNg-502F	TCAATGAGCTCATCCGAGTG
IFNg-929R	TGGTCAGTGAAGTAAAGGTA
TNFb-150F	TCTGGTTCTCCACATGACACT
TNFb-630R	AAGTCCCGGATACACAGACTT
IL4-180F	AGAGATCATCGGCATTTTGAA
IL4-420R	CGATGAATCCAGGCATCGAAA
IL5-170F	TTGACAAGCAATGAGACGATG
IL5-428R	ACTCTGTACTCATCACACCAA
IL10-53F	CTTGCAGAAAAGAGAGCTCCA
IL10-392R	TTGATTTCTGGGCCATGCTTC
IL13-178F	ATTGAGGAGCTGAGCAACATC
IL13-435R	TGTGTAGCTGAGCAGTTTTGT

were measured using ELISA kits from R&D systems. Cytokines in the media from the culture of osteoclasts alone were also measured.

Statistics

Unless stated otherwise, Student *t*-test was performed to compare experimental wells with control wells that were cultured with sRANKL alone.

Results

B cells activated in the presence of Th1 cytokines suppress osteoclastogenesis

Previously, we reported that activated B cells enhance osteoclastogenesis (Choi *et al.*, 2001). However, we subsequently observed that the activated B cells could have different effects on osteoclastic differentiation depending on the activation protocol: while B cells activated with PWM following T cell depletion enhanced osteoclastogenesis, B cells activated in the presence of T cells followed by T cell depletion suppressed it (data not shown). These observations suggested that some factor(s) from T cells changed the phenotype of the activated B cells, thereby effects on osteoclastogenesis. Since the suppressive effect was further aggravated by addition of IL-2, we hypothesized that Th1 cells as well as Th2 cells might have a role in B cell activation. The exact mechanism of signaling or activation by PWM is not known, therefore, we modified our activation protocol to test our hypothesis. B cells activated with anti-IgD Ab, anti-CD 40 Ab, and IL-4, simulated Th2 cell-dependent B cell activation (B_{Th2}). To simulate B cell activation under the influence of Th1 cells as well as Th2 cells, purified B cells were incubated with anti-IgD Ab, anti-CD 40 Ab, and IL-4 plus either IL-2 ($B_{IL\text{-}2}$) or IFN-γ ($B_{IFN\text{-}\gamma}$). For *in vitro* osteoclastogenesis, we adopted a well-established model system using RAW-264.7 cells that comprise a pure population of monocytic lineage and easily differentiate into osteoclasts after 5 to 6 days in culture in the presence of sRANKL (Hsu *et al.,* 1999; Shevde *et al.,* 2000). On day 2 when we started co-culture with activated B cells, all cells still were mononuclear and less than 20% were $TRAP^+$. Following co-culture, the influence of activated B cells on the osteoclast differentiation was evaluated by the formation of $TRAP^+$ multinuclear cell (MNC), TRAP activity, and resorption of coated minerals. All three assays showed that B_{Th2} increased osteoclastogenesis but either $B_{IL\text{-}2}$ or $B_{IFN\text{-}\gamma}$ suppressed it substantially (Figure 1).

The phenotypes of activated B cells

These results suggested that the presence of Th1 cytokines during activation altered the phenotypes of activated B cells. B cells secrete various cytokines that affect osteoclastogenesis positively or negatively. We studied the expression profile of pro- and anti-inflammatory, Th1, and Th2 cytokines as well as RANKL, osteoprotegerin (OPG) and RANK using semi-quantitative RT-PCR (Figure 2A). First, there was no difference in the expression of the key regulators of osteoclastogenesis, RANKL and OPG: all expressed high levels of RANKL but no OPG. The expression of RANK was not observed, either. All three types of B cells expressed similar levels of a pro-inflammatory cytokine, TNF-α, but an anti-inflammatory cytokine, TGF-β in different levels, being $B_{IFN\text{-}\gamma} > B_{IL\text{-}2} > B_{Th2}$. We had expected that the presence of Th1 cytokines during activation might skew the cytokine profile toward Th1, but such a pattern was

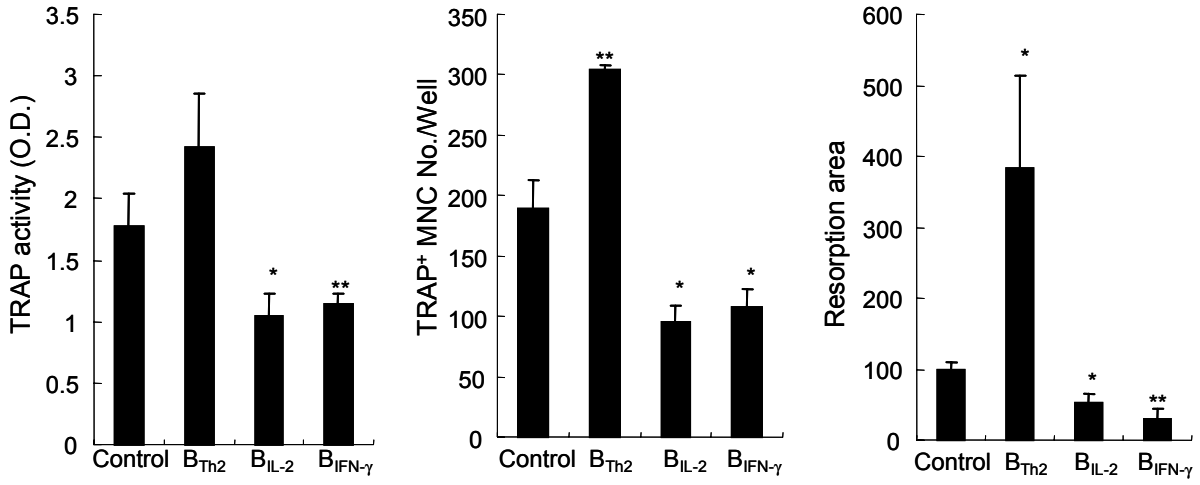

Figure 1. B cells activated in the presence of Th1 cytokines inhibit osteoclastogenesis. RAW264.7 cells plated in 96-well or 48-well OAAS plates were cultured in the presence of 20 ng/ml sRANKL. Co-culture with activated B cells was performed from day 2 to 5 at 5×10^5 cells/well in 96-well plate (TRAP stain and TRAP activity assay) or from day 2 to 8 at 10^6 cells/well in 48-well OAAS plates. Resorption area was expressed as % to control wells in which RAW264.7 cells were cultured without co-culture with B cells. Each column represents the mean and SEM of triplicate wells; $*P < 0.05$, $**P < 0.01$.

not observed clearly. In general, all three types of B cells expressed both Th1 and Th2 cytokines. However, increased expression of IFN-γ by B_{IL-2} and decreased expression of Th2 cytokines including IL-4, IL-5, and IL-10 by $B_{IFN-\gamma}$ were noted. Since IL-4, IL-2, and IFN-γ were added in saturating levels during activation, B cells might have turned off their own synthesis, producing artificially low mRNA results by RT-PCR. Therefore, we also measured the amounts of the cytokines secreted into supernatants upon co-culture by ELISA. As shown in Figure 2B, the results by ELISA quite well reflected the results by RT-PCR: B_{IL-2} expressed a high level of IFN-γ and $B_{IFN-\gamma}$ expressed the lowest levels of IL-4 and IL-10.

IFN-γ is a key negative regulator of osteoclast differentiation and mediates the suppressive effect of B_{IL-2} cells

Interestingly, $TRAP^-$ MNCs, rather than $TRAP^+$ MNCs, were often observed upon co-culture with B_{IL-2} cells (Figure 3A). These $TRAP^-$ MNCs did not have actin rings at the periphery (data not shown), and they phagocytosed beads (Figure 3A), a characteristic of giant cells. IFN-γ is known to induce the formation of giant cells and indeed as small as 1 ng/ml of IFN-γ could convert the developing osteoclasts into giant cells (Figure 3B).

These results indicated that IFN-γ must be a major factor mediating the suppressive effect by B_{IL-2}, and it was clearly confirmed by the use of anti-IFN-γ neutralizing mAb (Figure 4). In the control experiment without B_{IL-2}, the anti-IFN-γ mAb did not have much effect on either TRAP activity or formation of $TRAP^+$ MNC. In contrast, upon co-culture with B_{IL-2}, the anti-IFN-γ mAb reversed the suppressive effect of B_{IL-2} completely ($TRAP^+$ MNC) and increased the level of TRAP activity even higher than that of control. It suggested that IFN-γ is a key suppressor secreted from B_{IL-2} and with the function of IFN-γ blocked, B_{IL-2} may also enhance osteoclastogenesis.

B_{IL-2} express cytokines that are known to antagonize IFN-γ mediated activation of macrophage: IL-4, IL-13, IL-10, and TGF-β (Gazzinelli *et al.*, 1992; Hausmann *et al.*, 1994; Paludan *et al.*, 1997; Paludan *et al.*, 1998). We tested if these cytokines can antagonize the effect of IFN-γ on osteoclasts, too. In addition, we tested TNF-α that induces high expression of tumor necrosis factor receptor-associated factor 6 (TRAF6) since IFN-γ inhibits osteoclastogenesis by degrading TRAF6 (Takayanagi *et al.,* 2000; Zhang *et al.,* 2001). 2 ng/ml IFN-γ decreased the TRAP activity and $TRAP^+$ MNC about 50%, a similar level obtained by co-culture with B_{IL-2}. Therefore, instead of co-culture with B_{IL-2}, 2 ng/ml IFN-γ plus various cytokines were added from day 2 to day 5 during osteoclastogenesis using RAW264.7 cells. Among tested cytokines (TNF-α, IL-4, IL-13, IL-10, and TGF-β), only TNF-α showed a slight antagonistic effect but it never antagonized the effect of IFN-γ completely even at high concentration (Figure 5).

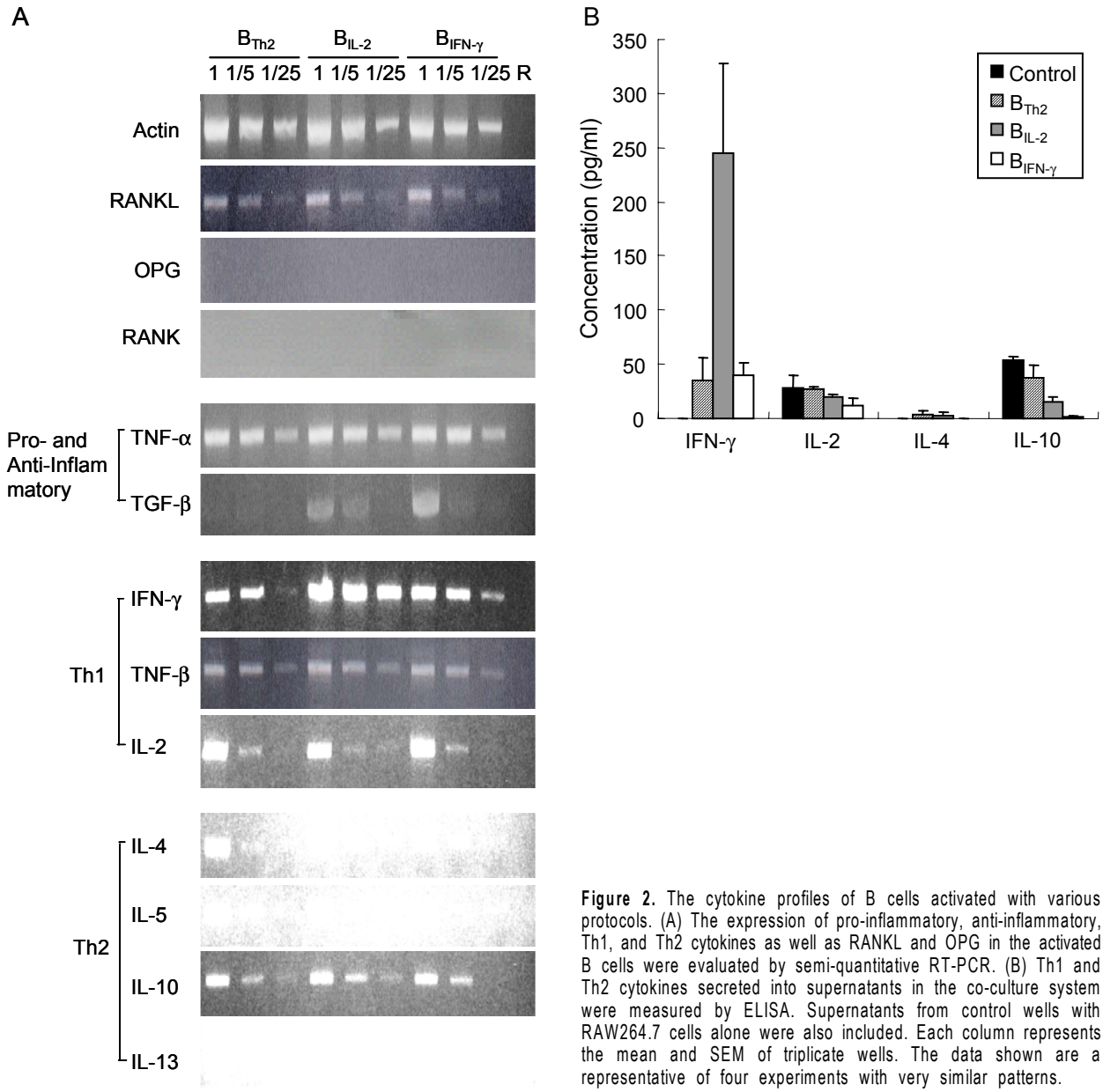

Figure 2. The cytokine profiles of B cells activated with various protocols. (A) The expression of pro-inflammatory, anti-inflammatory, Th1, and Th2 cytokines as well as RANKL and OPG in the activated B cells were evaluated by semi-quantitative RT-PCR. (B) Th1 and Th2 cytokines secreted into supernatants in the co-culture system were measured by ELISA. Supernatants from control wells with RAW264.7 cells alone were also included. Each column represents the mean and SEM of triplicate wells. The data shown are a representative of four experiments with very similar patterns.

Discussion

In this study, we have demonstrated that cytokine environment during B cell activation can change the phenotype of the activated cells. Recently, it was shown that B cells co-cultured with Th1 cells or Th2 cells differentiated into two populations of effector B cells that secrete Th1 cytokines and Th2 cytokines, respectively (Harris *et al.*, 2000). Although such a clear polarized pattern was not observed, B_{IL-2} expressed an increased level of IFN-γ and $B_{IFN-\gamma}$ expressed decreased levels of Th2 cytokines including IL-4, IL-5, and IL-10 compared with B_{Th2} (Figure 2A and 2B). Several cytokines and surface molecules, rather than a single cytokine, may be required to induce completely polarized effector cells. In addition, activated B cells may have heterogeneous phenotypes between two completely polarized types depending on their micro-environment during activation. B cells first encounter Ag and undergo initial activation in the T cell zone of peripheral lymphoid organs, where the Th1/Th2 cytokine balance in the vicinity of B cells during activation is known to affect the phenotypes of activated B cells.

B_{Th2} expressed pro-osteolytic factors including RANKL, TNF-α, and TNF-β but low levels of strong suppres

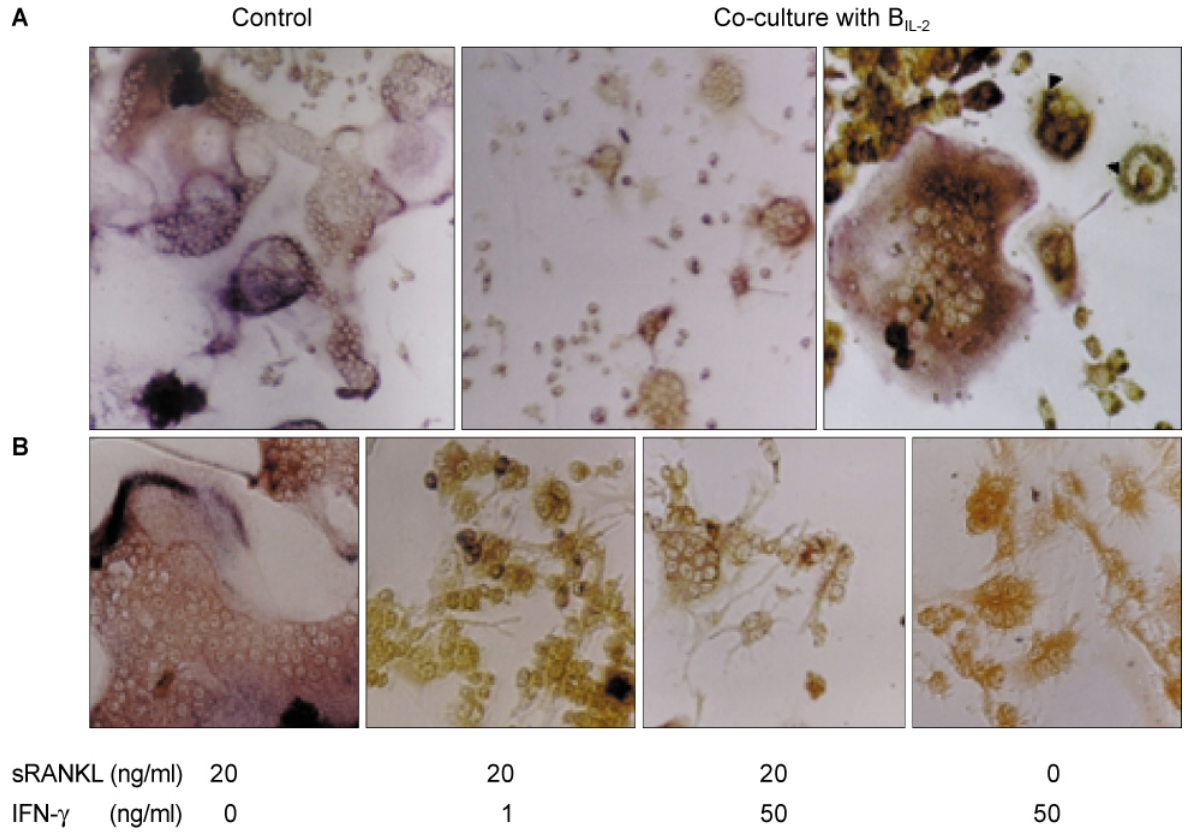

Figure 3. Co-culture with B_{IL-2} or IFN-γ induced formation of giant cells. A. RAW264.7 cells were cultured with sRANKL (20 ng/ml) alone for 5 days (left panel) or co-cultured with B_{IL-2} from day 2 to 5 (right panel) and stained for TRAP. Arrowheads indicate phagocytosed beads. On day 5, cells were stained for TRAP and photographed under an inverted-phase-contrast microscope (×100). B. RAW264.7 cells were cultured with various amounts of sRANKL and IFN-γ. On day 5, cells were stained for TRAP and photographed under an inverted-phase-contrast microscope (×100).

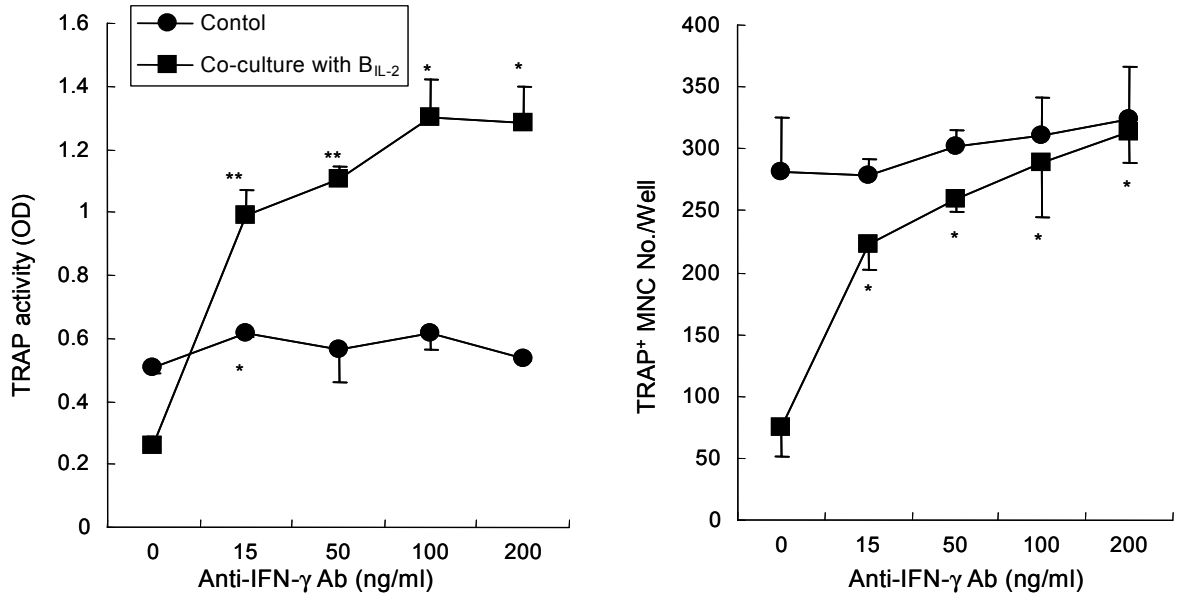

Figure 4. Neutralization of IFN-γ reversed the suppressive effect of B_{IL-2}. Various amounts of anti-IFN-γ neutralizing mAb were added with B_{IL-2} cells from day 2 to 5 during 5 day-sRANKL-induced osteoclastogenesis. Data shown are a representative of two similar results, mean and SEM of triplicate well; $^*P < 0.01$, $^{**}P < 0.001$.

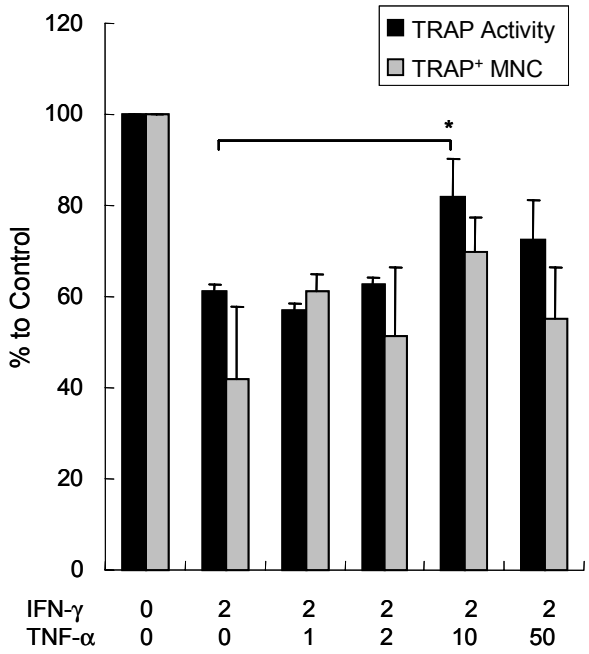

Figure 5. Antagonization of IFN-γ by TNF-α. Various amounts of TNF-a along with 2 ng/ml IFN-γ were added from day 2 to 5 during 5 day-sRANKL-induced osteoclastogenesis. Data shown are a representative of three similar results, mean and SEM of triplicate wells. A bracket indicates compared two groups by student *t*-test; $*P < 0.01$.

sive factors such as IFN-γ (Figure 2A and 2B), and enhanced osteoclastogenesis (Figure 1). Abundant expression of RANKL and support of osteoclast differentiation by B cells were recently reported by other investigators, too (Manabe *et al*., 2001). When we stained B cells with succinimidyl ester of carboxyfluorescein diacetate (CFSE) and co-cultured with RAW264.7 cells, none of the differentiated osteoclasts had fluorescence (data not shown), excluding the possibility that B cell themselves differentiate into osteoclasts as suggested from the shared lineage in these two cells (Sato *et al*., 2001). Compared with $B_{Th2,}$ B_{IL-2} expressed high levels of IFN-γ as well as RANKL, TNF-α, and TNF-β (Figure 2A). Induction of many giant cells by co-culture with B_{IL-2} suggested that IFN-γ had an important role in the inhibitory action, which was confirmed by the use of anti-IFN-γ neutralizing Ab (Figure 4). B_{IL-2} also expressed cytokines that are known to antagonize IFN-γ mediated activation of macrophage: IL-4, IL-13, 1L-10, and TGF-β (Gazzinelli *et al*., 1992; Hausmann *et al*., 1994; Paludan *et al*., 1997; Paludan *et al*., 1998). However, none of these could reverse IFN-γ mediated inhibition of osteoclastogenesis. TNF-α that induces the expression of TRAF6 (Kaji *et al*., 2001; Zhang *et al*., 2001) showed a slight antagonistic effect, but never reversed the inhibitory effect completely (Figure 5). These results suggested that: 1) IFN-γ uses different signal pathways in macrophages and osteoclasts; 2) IFN-γ is a key negative regulator of osteoclastogenesis even in the presence of RANKL, TNF-α and TNF-β.

How $B_{IFN-\gamma}$ inhibited osteoclastogenesis is not clear at present. TGF-β might have a role as shown by Weitzmann *et al.,* although the role of TGF-β in osteoclastogenesis is controversial (Weitzmann *et al.,* 2000). Otherwise, cytokines that we did not analyze or unknown factors produced by $B_{IFN-\gamma}$ may have played a role.

In summary, we have demonstrated that Th2-dependent B cells and B cells activated in the presence of Th1 cytokines, IL-2 or IFN-γ, have opposite effects on osteoclastogenesis *in vitro*, acting positively and negatively, respectively. In addition, we have shown that IFN-γ is a key negative regulator of osteoclastogenesis that mediated the inhibitory effect of B_{IL-2} in the presence of two major osteoclast inducing factors, RANKL and TNF-α. Our findings suggested that activated B cells infiltrated into inflammation sites should not be regarded as cells of a uniform phenotype and Th1 cytokines might protect against bone destruction in an inflammatory bone disease such as periodontitis, acting directly on osteoclasts or indirectly through B cells.

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