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Stimulation by Toll-Like Receptors Inhibits Osteoclast Differentiation[1]

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Osteoclasts, the cells capable of resorbing bone, are derived from hemopoietic precursor cells of monocyte-macrophage lineage. The same precursor cells can also give rise to macrophages and dendritic cells, which are essential for proper immune responses to various pathogens. Immune responses to microbial pathogens are often triggered because various microbial components induce the maturation and activation of immunoregulatory cells such as macrophages or dendritic cells by stimulating Toll-like receptors (TLRs). Since osteoclasts arise from the same precursors as macrophages, we tested whether TLRs play any role during osteoclast differentiation. We showed here that osteoclast precursors prepared from mouse bone marrow cells expressed all known murine TLRs (TLR1-TLR9). Moreover, various TLR ligands (e.g., peptidoglycan, poly(I:C) dsRNA, LPS, and CpG motif of unmethylated DNA, which act as ligands for TLR2, 3, 4, and 9, respectively) induced NF-κB activation and up-regulated TNF-α production in osteoclast precursor cells. Unexpectedly, however, TLR stimulation of osteoclast precursors by these microbial products strongly inhibited their differentiation into multinucleated, mature osteoclasts induced by TNF-related activation-induced cytokine. Rather, TLR stimulation maintained the phagocytic activity of osteoclast precursors in the presence of osteoclastogenic stimuli M-CSF and TNF-related activation-induced cytokine. Taken together, these results suggest that TLR stimulation of osteoclast precursors inhibits their differentiation into noninflammatory mature osteoclasts during microbial infection. This process favors immune responses and may be critical to prevent pathogenic effects of microbial invasion on bone. *The Journal of Immunology,* **2002, 169: 1516–1523.**

Osteoclasts are multinucleated giant cells residing in bone tissue that resorb calcified matrix (1, 2). Osteoclasts originate from hemopoietic precursor cells that also give rise to macrophages or dendritic cells which mediate immune responses (3–6). TNF-related activation-induced cytokine (TRANCE,[4] also called as receptor activator of NF-κB ligand, osteoprotegerin ligand, or osteoclast differentiation factor) and M-CSF are essential to develop osteoclasts in vivo and in vitro (2).

Many microbial constituents are recognized by Toll-like receptors (TLRs) expressed on macrophages or dendritic cells. TLR engagement can trigger immune responses. Peptidoglycan (PGN), and lipoteichoic acid act as ligands of TLR2 (7, 8). Double-stranded RNA (poly(I:C) RNA), LPS, flagellin, and the CpG motif of unmethylated DNA (CpG DNA) act as ligands of TLR3, TLR4, TLR5, and TLR9, respectively (9–13). In response to TLR ligands, macrophages and dendritic cells produce several inflammatory cytokines such as TNF-α, IL-6, IFN-γ, and IL-12 to activate immune responses (14–17). In addition, TLR stimulation by diverse microbial products directly induces the maturation of dendritic cells, which is an essential step for subsequent adaptive immune responses (15).

LPS, a major constituent of Gram-negative bacteria, has been suggested to be a potent stimulator of bone loss (18–21). LPS has been shown to increase the number of osteoclast precursors via TNF-α action in vivo (19). PGs and the EP4 subtype receptor are implicated in osteoclast differentiation induced by LPS, suggesting an indirect role of LPS in osteoclast differentiation by promoting inflammatory responses accompanied by the production of TNF-α and PGs (19, 20). However, it has recently been shown that LPS can enhance the survival, fusion, and activation of osteoclasts independent of IL-1, TNF-α, and TRANCE (22). In osteoclasts, LPS stimulation induces NF-κB activation, leading to increased survival (22). These observations suggest that bacterial constituents such as LPS may also modulate osteoclasts directly. However, the precise expression profile and function of TLRs in osteoclasts and osteoclast precursors has not been determined.

Since osteoclasts are derived from common precursors for macrophages and dendritic cells, we hypothesized that some of the microbial products interacting with TLRs may modulate osteoclast differentiation, thus contributing to the pathology of bone diseases such as periodontitis, osteomyelitis, and bacterial arthritis caused by microbial infection (18). To explore potential roles of various TLRs during osteoclast differentiation, we have analyzed gene expression patterns of TLRs and their potential function. We show here that mouse osteoclast precursors express all known TLRs, and their stimulation induces the activation of NF-κB and production of the proinflammatory cytokine TNF-α. Unexpectedly, TLR stimulation on osteoclast precursors inhibited osteoclast differentiation induced by M-CSF and TRANCE. This is in contrast to the positive role of TLRs on the immune system. Thus, it appears that TLR stimulation skews the differentiation of common precursors toward immune responses.

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[4] Abbreviations used in this paper: TRANCE, TNF-related activation-induced cytokine; TLR, Toll-like receptor; TRAP, tartrate-resistant acid phosphatase; PGN, peptidoglycan; HPRT, hypoxanthine phosphoribosyltransferase; TRAF, TNFR-associated factor.

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Materials and Methods

Mice

Three- to 4-wk-old C57BL/6 and C3H/HeJ male mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in a specific pathogen-free facility at the University of Pennsylvania (Philadelphia, PA). All procedures were performed according to the University of Pennsylvania Institutional Animal Care and Use Committee Guidelines.

Cytokines and chemicals

A soluble form of recombinant TRANCE was purified from insect cells as described previously (23). Recombinant human M-CSF was obtained from Genetics Institute (Cambridge, MA). LPS from *Escherichia coli* (O55:B5), PGN from *Staphylococcus aureus*, and poly(I:C) RNA were purchased from Sigma-Aldrich (St. Louis, MO), Fluka (Sigma-Aldrich Group), and Amersham Biotech (Piscataway, NJ), respectively. Phosphothioate-stabilized CpG DNA (TCCATGACGTTCCTGATGCT) was synthesized in the core facility of the University of Pennsylvania.

Cells and culture systems

Osteoclast precursors were prepared essentially as described elsewhere (24, 25). In brief, mouse bone marrow cells were cultured in α-MEM (Life Technologies, Grand Island, NY) containing 10% FBS with M-CSF (5 ng/ml) for 12 h in 100-mm diameter dishes (Corning, Glass, Corning, NY; 1×10^7 cells/10 ml/dish) to separate adherent cells and nonadherent cells. Then, nonadherent cells were harvested and cultured with M-CSF (30 ng/ml) in 100-mm diameter dishes (1×10^7 cells/10 ml/dish). After 2 days of culture, floating cells were removed and attached cells were used as osteoclast precursors. To generate osteoclasts, osteoclast precursors were cultured with TRANCE (300 ng/ml) and M-CSF (30 ng/ml) for 3 days in 96-well culture plates (Corning; 2×10^4 cells/0.2 ml/well) or in 60-mm diameter dishes (Corning; 2.5×10^6 cells/5 ml/dish). To purify mature osteoclasts, cells were treated with cell dissociation solution (Sigma-Aldrich) for 5 min, and the sides of the plates were tapped. Most mononuclear cells were detached after tapping, but multinucleated osteoclasts remained attached to the culture plates. More than 98% of cells were tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts after purification (data not shown). To generate osteoclasts from the murine myeloid RAW264.7 cell line (American Type Culture Collection, Manassas, VA), cells were cultured in 96-well culture plates (1×10^3 cells/0.2 ml/well) with TRANCE (300 ng/ml) for 4 days. Old media were replaced with fresh media containing TRANCE (300 ng/ml) on day 3. To generate human osteoclasts, freshly isolated human peripheral blood monocytes were cultured in 96-well culture plates (5×10^4 cells/0.2 ml/well) with TRANCE (300 ng/ml) and M-CSF (30 ng/ml) for 5 days. Old media were replaced with fresh media containing TRANCE (300 ng/ml) and M-CSF (30 ng/ml) on day 3. In some experiments, indicated concentration of PGN, poly(I:C) RNA, LPS, or CpG DNA was added to the cultures with or without TRANCE and M-CSF. All cells were cultured at 37°C and 5% CO_2.

Phagocytosis assay

Fluorescein-conjugated zymosan A (*Saccharomyces cerevisiae*) Bio Particle (Molecular Probes, Eugene, OR) was added to osteoclast precursor and osteoclast cultures in 96-well culture plates (20 μg/0.2 ml/well). After 1 h of culture, cells were washed with PBS to remove the particles that were not incorporated by the cells. Cells were fixed and stained for TRAP, which is strongly expressed in osteoclasts (26). Zymosan particles incorporated by the cells were visualized by UV light illumination under microscopic examination.

Pit formation assay

Mouse osteoclast precursors (5×10^4 cells/0.2 ml/well) or human peripheral blood monocytes (1×10^5 cells/0.2 ml/well) were placed on dentine slices (4 mm in diameter) in 96-well culture plates and cultured for 1 h with M-CSF (30 ng/ml). Dentine slices were then transferred into 48-well culture plates (Corning Glass) by the use of forceps. Cells on dentine slices were cultured in the presence of M-CSF (30 ng/ml) with or without TRANCE (300 ng/ml) for 4 days (mouse) or 6 days (human). In some experiments, mouse IL-1α (10 ng/ml) was added to the cultures. Old media were replaced with fresh media on day 3. After wiping the cells off dentine slices with cotton, the slices were immersed in Mayer's hematoxylin (Sigma-Aldrich) to stain the resorption pits formed by osteoclasts.

PCR amplification of reversed-transcribed mRNA (RT-PCR) and Southern blot analysis

Total RNA from osteoclast precursors and purified mature osteoclasts in culture dishes (60-mm diameter) was prepared using TRIzol solution (Life Technologies) according to the manufacturer's protocols. First-strand cDNA was synthesized from total RNA using Superscript II (Life Technologies) and was subjected to PCR amplification with *Taq* polymerase (Sigma-Aldrich). For Southern blot hybridization, PCR products were separated by electrophoresis on 1.5% agarose gels and transferred to the nylon membranes (GeneScreen; NEN Life Science, Boston, MA). cDNA probes encoding each gene of TLRs and hypoxanthine phosphoribosyltransferase (HPRT) were labeled with ^{32}P using a cDNA labeling kit (Amersham, Arlington Heights, IL). Primers for mouse TLRs and mouse HPRT used in these studies are as follows: TLR1, 5′-CTTCAGACTTCTGACATC CTCTCA-3′ (forward, nt 355–378) and 5′-TTCAAGCACACACTTGAT GTTAGA-3′ (reverse, nt 855–878); TLR2, 5′-TGGAGACGCCAGCTCT GGCTCA-3′ (forward, nt 1953–1974) and 5′-CTGACCCGCCCTTTAAG CTG-3′ (reverse, nt 2313–2332); TLR3, 5′-ATTTAGAGTCCAACGGC TTAGATG-3′ (forward, nt 1749–1772) and 5′-TTCCAGTAAAAAGAG ATCCTCCAG-3′ (reverse, nt 2250–2273); TLR4, 5′-AGTGGGTCAAG GAACAGAAGCA-3′ (forward, nt 1766–1787) and 5′-CTTTACCAGCT CATTTCTCACC-3′ (reverse, nt 2055–2076); TLR5, 5′-GAAAGTAAGA GGTCCAGAAAGCTG-3′ (forward, nt 3748–3771) and 5′-TATAGTTG TGGGGAAGAAAGAAGG-3′ (reverse, nt 4249–4272); TLR6, 5′-GCCTG ACTCTTACAGGTGTGACTA-3′ (forward, nt 1698–1721) and 5′-TTATG ATGGGCAAAATAGAGTTCA-3′ (reverse, nt 2198–2221); TLR7, 5′-GCAATTGTGTACCTGTTCTACTGG-3′ (forward, nt 341–364) and 5′-ACATTATAACATCGAGGGCAATTT-3′ (reverse, nt 845–868); TLR8, 5′-ACATTATAACATCGAGGGCAATTT-3′ (forward, nt 914–938) and 5′-ACTCATCATCGTCTGTTGAGAGAG-3′ (reverse, nt 1412–1435); TLR9, 5′-AGTGTCACTTCCTCAATTCTCTGA-3′ (forward, nt 5–28) and 5′-ACCATTATAGCTCAGGTTCAGCTC-3′ (reverse, nt 508–531); HPRT, 5′-GTAATGATCAGTCAACGGGGGAC-3′ (forward, nt 404–426); and 3′-HPRT, 5′-CCAGCAAGCTTGCAACCTTAACCA-3′ (reverse, nt 557–580). The PCR program was as follows: 35 cycles at 94°C for 30 s, 55°C for 40 s, and 72°C for 1 min for all mouse TLRs and HPRT.

Western and Northern blot analysis

For Western blot analysis, osteoclast precursors were cultured for indicated periods in the presence of PGN (10 μg/ml), poly(I:C) RNA (100 μg/ml), LPS (1000 ng/ml), or CpG DNA (1 μM). Cells were then washed with ice-cold PBS and lysed in sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromphenol blue). Cell lysates were resolved by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA) and probed with anti-phospho-IκBα Abs (1 μg/ml) (New England Biolabs, Beverly, MA) or anti-α-actin (1 μg/ml; Oncogene, San Diego, CA) Abs. For Northern blot analysis, osteoclast precursors in 60-mm diameter dishes were cultured with PGN (10 μg/ml), poly(I:C) RNA (100 μg/ml), LPS (1000 ng/ml), and CpG DNA (1 μM) for the indicated periods, and then were subjected to total RNA isolation using TRIzol (Life Technologies). cDNA probes encoding mouse TNF-α and G3PDH were labeled with ^{32}P using a cDNA labeling kit (Amersham).

Determination of TNF-α production in culture medium

Osteoclast precursors (1×10^5 cells/0.2 ml/well) in 96-well culture plates were cultured with or without PGN (10 μg/ml), poly(I:C) RNA (100 μg/ml), LPS (1000 ng/ml), or CpG DNA (1 μM) for 24 h. Then the quantity of TNF-α protein in the medium was determined by ELISA kit (Quantikine M for mouse TNF-α; R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. The results are expressed as the means ± SD of four cultures. For statistical analysis of the results, groups were compared with Student's *t* test.

Results

Expression pattern of TLRs in osteoclast precursors

Osteoclast precursors were generated from mouse bone marrow cells by M-CSF treatment (see *Materials and Methods*). There were no TRAP-positive or multinucleated cells in osteoclast precursor preparations (Fig. 1*A*, *upper left panel*). After 3 days of culture with TRANCE and M-CSF, both mono- and multinuclear TRAP-positive cells appeared (Fig. 1*A*, *upper right panel*). When osteoclast precursors were cultured on dentine slices with TRANCE and M-CSF, resorption pits were formed on the dentine slices, indicating

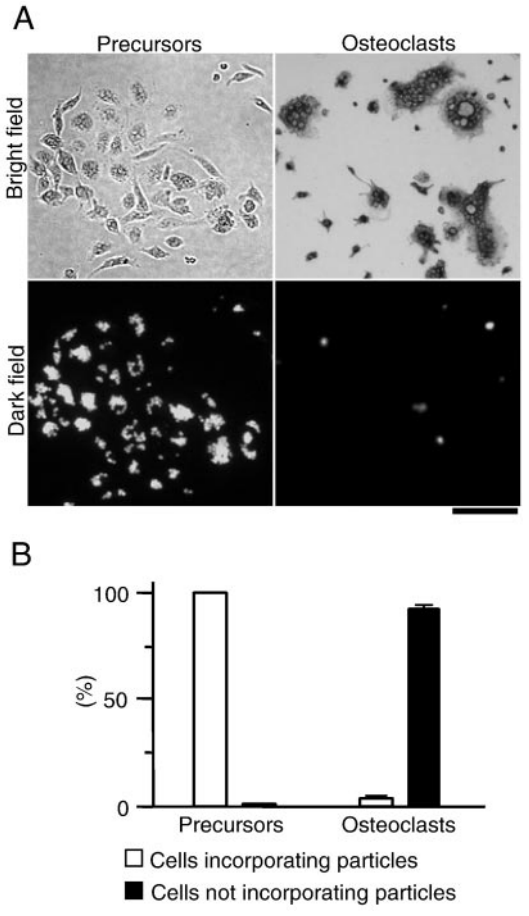

FIGURE 1. Phagocytic capacity of osteoclast precursors and osteoclasts. Osteoclast precursors were generated from mouse bone marrow cells by treatment with M-CSF (30 ng/ml) for 2 days (*A*, *left panels*). Osteoclasts were generated from osteoclast precursors by the treatment with TRANCE (300 ng/ml) and M-CSF (30 ng/ml) for 3 days (*A*, *right panels*). Cells were cultured with FITC-conjugated zymosan particles for 1 h and washed with PBS. Then cells were stained for TRAP and observed by microscopic examination (*A*). TRAP-positive cells appeared as dark-stained cells in the bright field (*A*, *upper panels*). Zymosan particles in cells appeared as bright dots in the dark field with UV light illumination (*A*, *lower panels*). Percentage of osteoclast precursors and osteoclasts that incorporated the zymosan particles in each culture was calculated (*B*). Data are expressed as the mean ± SD of four cultures. Bar, 100 μm.

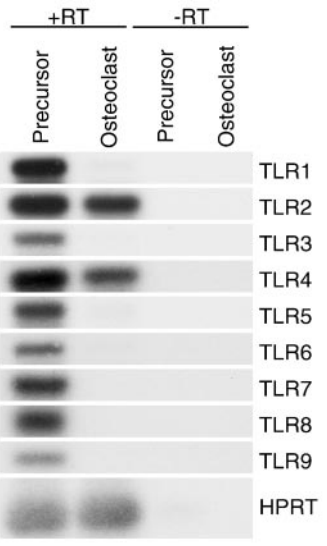

FIGURE 2. Gene expression of TLRs in osteoclast precursors and osteoclasts. Total RNA was prepared from osteoclast precursors and purified mature osteoclasts. Templates for PCR were synthesized with (+RT) or without (−RT) reverse transcriptase. PCR products for mouse TLR1-TLR9 were separated by electrophoresis and then subjected to Southern blot analysis.

that TRAP-positive multinucleated cells are mature osteoclasts (data not shown). Moreover, these precursors differentiated into macrophages in the presence of M-CSF only or dendritic cells in the presence of GM-CSF only (data not shown). When FITC-conjugated zymosan particles were added to the osteoclast precursor culture, >99% of the cells in osteoclast precursor culture incorporated zymosan particles within 1 h by phagocytosis (Fig. 1*A*, *lower left panel*, and *B*). After differentiation into osteoclasts, however, no TRAP-positive cells incorporated zymosan particles (Fig. 1*A*, *lower right panel*, and *B*). These results suggest that osteoclast precursors, which can differentiate into osteoclasts, macrophages, or dendritic cells, possess phagocytic function, which they lose upon differentiation into osteoclasts.

To determine which of the known murine TLRs (TLR1-TLR9) are expressed on osteoclast precursors, RNA from these cells was analyzed by RT-PCR followed by southern blot hybridization. Osteoclast precursors expressed all known TLRs, TLR1-TLR9 (Fig. 2). In contrast, when these cells differentiated into osteoclasts, TLR2 and TLR4 were prominently expressed, suggesting that the gene expression pattern of TLRs is modulated during osteoclast differentiation (Fig. 2).

To test whether TLRs expressed on osteoclast precursors respond to various microbial products, they were stimulated with PGN, poly(I:C) RNA, LPS, or CpG DNA, the ligands for TLR2, TLR3, TLR4, or TLR9, respectively. Despite different kinetics, all of the TLR ligands efficiently induced NF-κB activation, which is a hallmark of TLR stimulation (Fig. 3*A*). Moreover, TLR stimulation in osteoclast precursors by these ligands up-regulated expression of TNF-α (Fig. 3, *B* and *C*). Therefore, TLR stimulation of osteoclast precursors leads to cellular responses similar to those observed in macrophages (14–17).

Effect of TLR stimulation on osteoclast precursors during osteoclast differentiation

To examine the effects of TLR stimulation on osteoclast differentiation, osteoclast precursors were stimulated with PGN, poly(I:C) RNA, LPS, or CpG DNA in the presence of TRANCE and M-CSF. As previously described, combined TRANCE and M-CSF stimulation efficiently differentiated osteoclast precursors into mature, multinucleated TRAP-positive osteoclasts (Fig. 4*A*). When LPS was added to the culture in the presence of TRANCE and M-CSF, it strongly inhibited osteoclast differentiation (Fig. 4*A*). However, osteoclast precursors prepared from C3H/HeJ mice that have a mutation in the gene encoding TLR4 differentiated to osteoclasts even in the presence of LPS. In addition to LPS, all the other TLR ligands tested (PGN, poly(I:C) RNA, and CpG DNA) strongly inhibited osteoclast differentiation (Fig. 4*A*). Cells treated with TLR ligands in the presence of TRANCE and M-CSF for 3 days still incorporated zymosan particles within 1 h (Fig. 4*B*), showing that they maintained their phagocytic capacities rather than differentiating into nonphagocytic mature osteoclasts (Fig. 1). When human peripheral blood monocytes were used as osteoclast precursors, TLR ligands also strongly inhibited osteoclast differentiation induced by M-CSF and TRANCE (Fig. 4*C*). Therefore, these results indicate that TLR stimulation by microbial products elicits

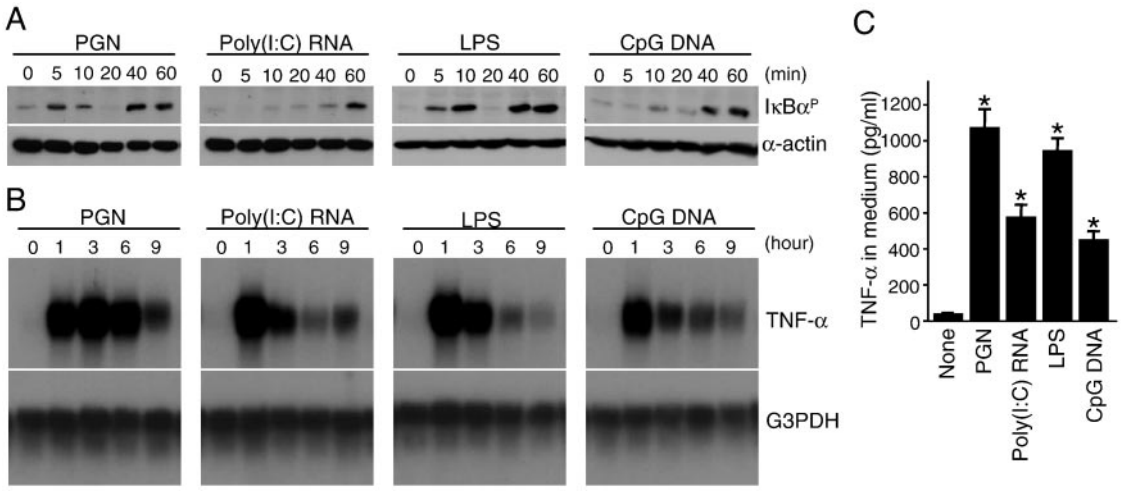

FIGURE 3. Induction of signal transduction and TNF-α production by TLR ligands in osteoclast precursors. *A,* Induction of IκBα phosphorylation by TLR ligands in osteoclast precursors. Osteoclast precursors were cultured in 0.5% FCS-containing medium for 2 h. Cells were then treated with PGN (10 μg/ml), poly(I:C) RNA (100 μg/ml), LPS (1000 ng/ml), or CpG DNA (1 μM). After culturing for indicated times, cell lysates were harvested. Phospho-IκBα and α-actin was detected by Western blotting. *B*, Induction of TNF-α mRNA expression in osteoclast precursors. Osteoclast precursors were treated with PGN (10 μg/ml), poly(I:C) RNA (100 μg/ml), LPS (1000 ng/ml), or CpG DNA (1 μM). After culturing for the indicated times, total RNA was isolated from the cells and the expression of TNF-α mRNA was analyzed by Northern blotting. *C*, Induction of TNF-α production by osteoclast precursors. Osteoclast precursors were cultured in the absence (None) or presence of PGN (10 μg/ml), poly(I:C) RNA (100 μg/ml), LPS (1000 ng/ml), or CpG DNA (1 μM). After culturing for 24 h, the quantity of TNF-α in culture medium was determined by ELISA. Data are expressed as the means $\pm$ SD of four cultures. $*$, Significantly different from the control cultures (None) which were not treated with any TLR ligands; $p < 0.01$.

strong negative signals for osteoclast differentiation from monocytic osteoclast precursors. Furthermore, the combination of TLR ligands in suboptimal concentrations showed strong inhibitory effects on osteoclast differentiation, suggesting that TLRs may act in a cooperative manner (data not shown).

Since the signaling pathway induced by TLRs shares many biochemical components with that of the IL-1R (e.g., myeloid differentiation factor 88, IRAK, and TNFR-associated factor 6 (TRAF6), we tested whether IL-1α can inhibit osteoclast differentiation. Although IL-1α is a potent stimulator of NF-κB and also up-regulates production of TNF-α in bone marrow-derived osteoclast precursors (data not shown), IL-1R stimulation did not antagonize osteoclast differentiation and its function (Fig. 4*D*). These results suggest that signaling pathways specific to TLR, not shared with IL-1R, regulate osteoclast differentiation.

TLR stimulation inhibits osteoclast differentiation induced by TRANCE

To further explore this unexpected, negative effect of TLR stimulation on osteoclast differentiation, the murine myeloid RAW264.7 cell line was treated with TRANCE in the presence or absence of various TLR ligands (Fig. 5). As reported, the RAW264.7 cell line differentiates into mature osteoclasts in response to TRANCE stimulation alone (27). When TLR ligands PGN, LPS, or CpG DNA are added to the culture, TRANCE-induced differentiation of osteoclasts from the RAW264.7 cell line was strongly inhibited. The inhibitory effect of poly(I:C) RNA on TRANCE-induced osteoclastogenesis of the RAW264.7 cell line was not as potent as on bone-marrow derived precursors. This is most likely due to lower expression of TLR3 in the RAW264.7 cell line than in bone marrow-derived osteoclast precursors (data not shown). Taken together, these results suggest that TLR stimulation of specific signaling pathways mediate the inhibition of osteoclast differentiation induced by TRANCE.

Effects of TLR ligands on the survival of mature osteoclasts

Since expression of TLR2 and TLR4 was detected in mature osteoclasts (Fig. 2), we also examined the effects of TLR ligands on the survival of mature osteoclasts. To test the direct effects of TLR ligands on osteoclasts, mature osteoclasts were purified and cultured in the absence or presence of TLR ligands (Fig. 6). In the absence of any stimuli, most multinucleated, mature osteoclasts died within 12 h. However, PGN and LPS induced the survival of mature osteoclasts in a dose-dependent manner (Fig. 6). LPS did not induce the survival of mature osteoclasts prepared from TLR4-deficient C3H/HeJ mice (Fig. 6*A*). On the other hand, neither poly(I:C) RNA nor CpG DNA promoted the survival of mature osteoclasts at all, consistent with the lack of TLR3 and TLR9 expression in these cells (Fig. 6). These results suggest that stimulation by TLRs can directly enhance the survival of mature osteoclasts.

Discussion

Several lines of evidence have established the concept that hemopoietic stem cells differentiate into osteoclasts via the monocyte-macrophage lineage (3–5, 25). Consistent with this, we showed here that osteoclast precursors prepared from bone marrow cells possessed phagocytic function. These cells lost phagocytic function but developed bone-resorbing function upon stimulation with TRANCE and M-CSF. These results suggest that combined signals by M-CSF and TRANCE lead to the down-regulation of gene expression or protein activities involved in phagocytic function and the up-regulation of those involved in bone-resorbing function.

As with various cells in the immune system, osteoclast precursors also expressed TLRs. Stimulation via TLRs induced the activation of NF-κB and up-regulated the expression levels of TNF-α. However, we showed here that TLR ligands are potent inhibitors of osteoclast differentiation induced by M-CSF and TRANCE from mouse bone marrow osteoclast precursors or from human peripheral blood monocytes. Since TRANCE-induced differentiation of the RAW264.7 cell

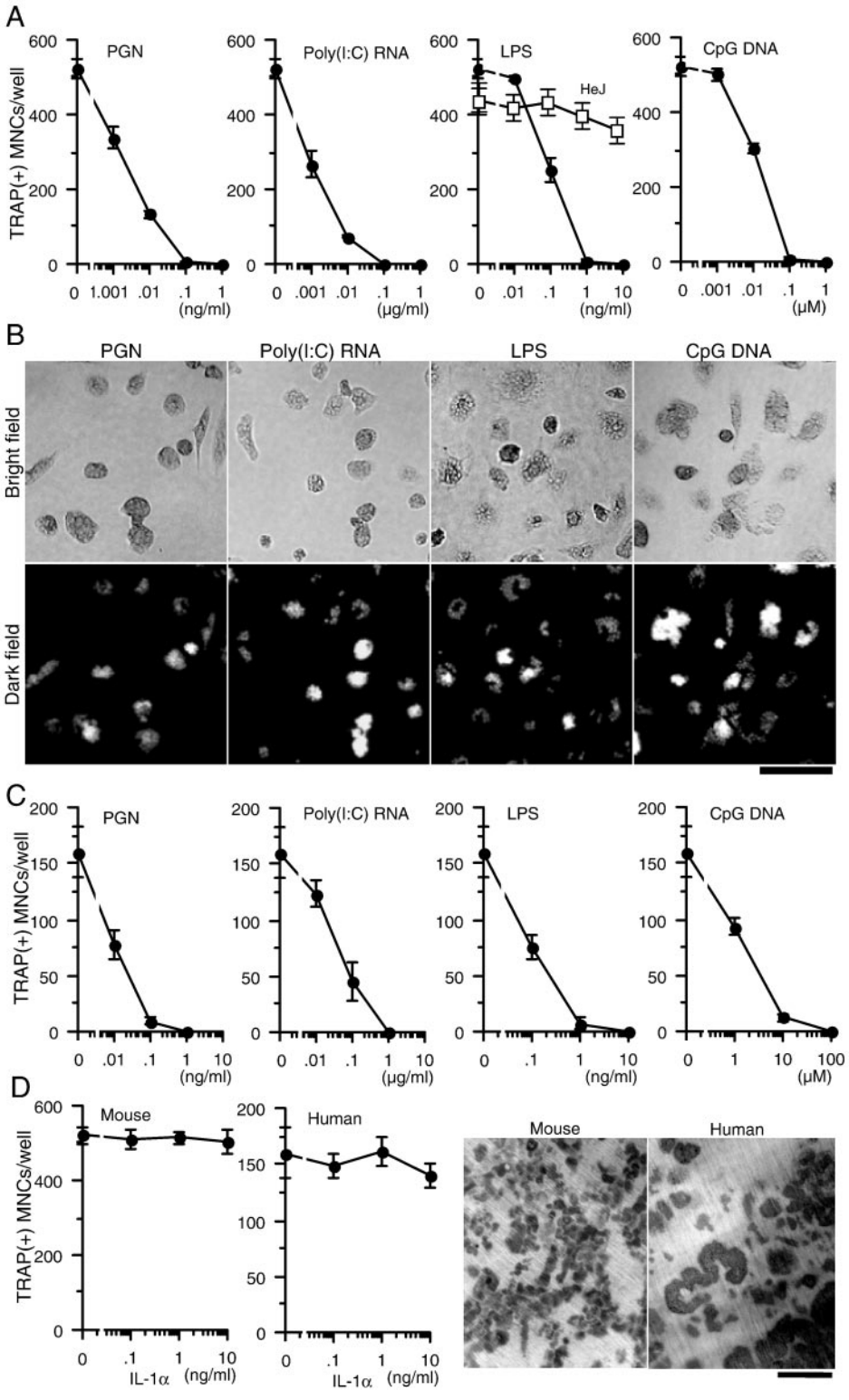

FIGURE 4. Effects of TLR ligands on osteoclast differentiation and phagocytic function. *A* and *B,* Osteoclast precursors derived from bone marrow cells of C57BL/6 (●) or C3H/HeJ (□) mice were cultured with medium containing increasing concentrations of PGN, poly(I:C) RNA, LPS, and CpG DNA in the presence of TRANCE (300 ng/ml) and M-CSF (30 ng/ml) for 3 days. Then cells were cultured with FITC-conjugated zymosan particles for 1 h. After culturing, cells were washed with PBS, fixed, and stained for TRAP. TRAP-positive multinucleated cells ($TRAP^+$ MNCs) having more than three nuclei were counted as osteoclasts (*A*). *B*, Photographs of the cells treated with 1 μg/ml PGN, 1 μg/ml poly(I: C) RNA, 10 ng/ml LPS, and 1 μM CpG DNA in *A*. Note that there are no TRAP-positive cells in the bright field (*B*, *upper panels*). *Upper right panel* in Fig. 1*A* is the control photograph without TLR ligand treatment. FITC-conjugated zymosan particles incorporated by the cells appear as bright dots in the dark field (*B, lower panels*). *C,* Human peripheral blood monocytes were cultured with medium containing increasing concentrations of PGN, poly(I:C) RNA, LPS, or CpG DNA in the presence of TRANCE (300 ng/ml) and M-CSF (30 ng/ml) for 5 days. Then cells were fixed and stained for TRAP. TRAP-positive multinucleated cells ($TRAP^+$ MNCs) having more than three nuclei were counted as osteoclasts. *D,* Mouse osteoclast precursors and human blood monocytes were cultured with medium containing increasing concentrations of IL-1α with TRANCE and M-CSF for 3 and 5 days, respectively. Then cells were fixed and stained for TRAP. TRAP-positive multinucleated cells ($TRAP^+$ MNCs) having more than three nuclei were counted as osteoclasts. The photographs show the dentine slices on which mouse osteoclast precursors and human blood monocytes were cultured with M-CSF (30 ng/ml), TRANCE (300 ng/ml), and IL-1α (10 ng/ml) for 4 days (mouse) and 6 days (human). Dark spots are resorption pits stained with Mayer's hematoxylin. All of the data are expressed as the mean ± SD of four cultures. Bar, 100 μm.

line to osteoclasts can also be inhibited by TLR ligands, it is most likely that TLR ligands inhibit the osteoclastogenic pathway induced by TRANCE. This was rather unexpected since TLR ligands are potent inducers of dendritic cell maturation and macrophage activation (15, 28, 29). In addition, NF-κB activation in osteoclast precursors, which can be induced by TLR stimulation as shown here, has been implicated in the successful differentiation of precursors to mature osteoclasts (30, 31).

IFN-γ, IL-4, and GM-CSF are produced by macrophages and known to inhibit osteoclast differentiation (32–34). To examine the effects of these factors on osteoclast differentiation, we added neutralizing Abs to IFN-γ, IL-4, and GM-CSF with TLR ligands to the cultures. However, none of the neutralizing Abs affected the negative effect of TLR ligands on osteoclast differentiation, suggesting that TLR ligands inhibited osteoclast differentiation independent of IFN-γ, IL-4, and GM-CSF (data not shown).

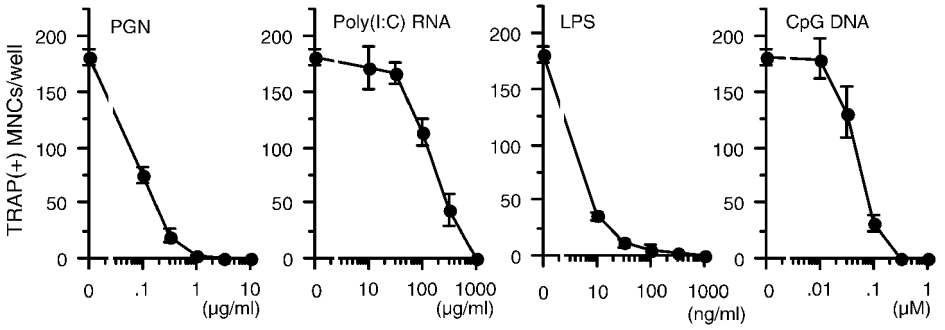

FIGURE 5. Effects of TLR ligands on osteoclast differentiation from the RAW264.7 cell line. RAW264.7 cells were cultured with medium containing increasing concentrations of PGN, poly(I:C) RNA, LPS, or CpG DNA in the presence of TRANCE (300 ng/ml) for 4 days. Then cells were fixed and stained for TRAP. TRAP-positive multinucleated cells (TRAP$^+$ MNCs) having more than three nuclei were counted as osteoclasts. Data are expressed as the means ± SD of four cultures.

It is not clear how TLR stimulation inhibits osteoclast differentiation mediated by TRANCE. The cytoplasmic tails of TLRs are extremely similar to the cytoplasmic domain of the IL-1R called the Toll/IL-1R homology domain (15, 35). The Toll/IL-1R homology domain makes a complex with myeloid differentiation factor 88, IRAK, and TRAF6. TRAF6 signaling induces phosphorylation and degradation of IκBα to activate NF-κB (15, 35–37). However, different from TLR ligands, we showed IL-1α did not inhibit osteoclast differentiation induced by TRANCE. Therefore, signaling molecules unique to TLR stimulation pathway are likely to play roles in the inhibition of osteoclast differentiation, the mechanism of which may be determined by future studies.

Our observation that microbial products inhibit osteoclast differentiation via TLRs is counterintuitive and unexpected, since bacterial infection can cause inflammatory bone diseases such as periodontitis, osteomyelitis, and bacterial arthritis (18). Bone mineral density is reduced in such diseases because of excessive bone resorption by osteoclasts. In addition, LPS has been suggested to be a potent stimulator of bone loss by causing the increase in the number of osteoclasts in mice (18–21). Moreover, it has been recently reported that LPS can also directly enhance the survival of mature osteoclasts (22), suggesting that TLR stimulation provides antiapoptotic signals in mature osteoclasts. Indeed, we showed in this study that mature osteoclasts express TLR4 for LPS. We also showed that mature osteoclasts express TLR2, and PGN (the ligand for TLR2) enhanced the survival of mature osteoclasts (Figs. 2 and 6). However, it appeared that osteoclast precursors were much more sensitive to TLR stimulation. For example, a complete inhibition of osteoclast differentiation can be achieved by 1 ng/ml LPS, at which concentration LPS has no effect on the survival of mature osteoclasts (Figs. 4*A* and 6*A*).

So how can we explain this apparent discrepancy between our observation that TLR stimulation is a potent negative regulator of osteoclastogenesis and that excessive bone resorption by osteoclasts is associated with bacterial infection? It has been shown recently that alveolar bone destruction in periodontitis caused by infection of Gram-negative bacteria is mediated by enhanced

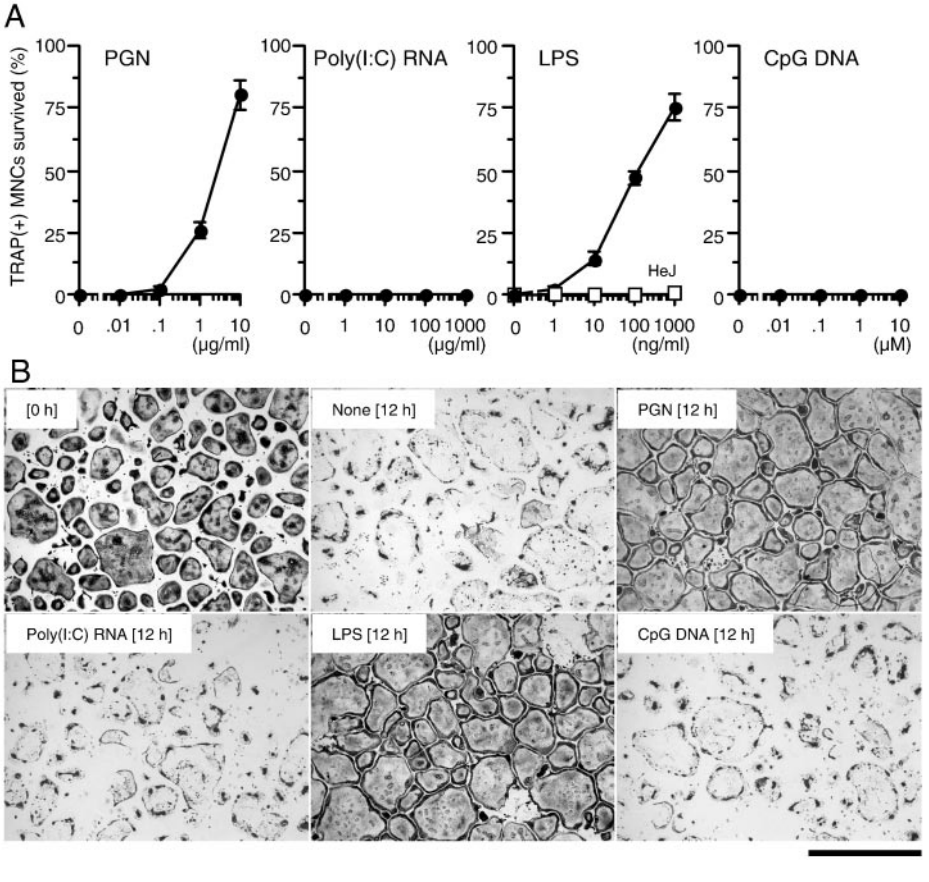

FIGURE 6. Effects of TLR ligands on the survival of mature osteoclasts. *A,* Osteoclasts were generated from osteoclast precursors derived from C57BL/6 (●) or C3H/HeJ (□) mice by the treatment with M-CSF (30 ng/ml) and TRANCE (300 ng/ml). After culturing for 3 days, mature osteoclasts were purified (*B, upper left panel*). Purified mature osteoclasts were cultured in medium containing increasing concentrations of PGN, poly(I:C) RNA, LPS, or CpG DNA. After culturing for 12 h, cells were fixed and stained for TRAP. TRAP-positive multinucleated cells (TRAP$^+$ MNCs) having more than three nuclei were counted as osteoclasts (*A*). LPS treatment did not enhance the viability of mature osteoclasts from TLR4-deficient C3H/HeJ mice (*A*). Data are expressed as the means ± SD of four cultures. *B,* Representative photographs show the cells at 0 and 12 h after culturing with or without 10 μg/ml PGN, 1000 μg/ml poly(I:C) RNA, 1000 ng/ml LPS, or 10 μM CpG DNA. Bar, 500 μm.

osteoclastogenesis due to T cell immunity (38). $CD4^+$ T cell responses to bacterial infection and TRANCE expressed on activated $CD4^+$ T cells are required for increased numbers of osteoclasts and their enhanced activities leading to bone destruction. Moreover, bacterial infection of immunodeficient mice (SCID) did not lead to significant levels of alveolar bone loss, suggesting that bacterial products may not have a direct role in osteoclastogenesis since SCID mice do not have a defect in osteoclast precursors or osteoblasts (38). Therefore, it is likely that bone loss associated with bacterial infection may be an indirect outcome of exacerbated T cell immune responses.

It is becoming clear that TLR stimulation by microbial products such as LPS plays an essential role in inducing inflammatory $CD4^+$ T cell immune responses by regulating many aspects of the innate immune system (36). Most notably, LPS is a potent inducer of dendritic cell maturation, which is required for successful priming of Ag-specific T cells. In addition, TLR ligands can induce the production of various proinflammatory cytokines such as TNF-α, IL-1, or IL-12 by activating macrophages or dendritic cells (15, 16, 39). We showed in this study that, similar to macrophages or dendritic cells, osteoclast precursors also produce proinflammatory cytokines such as TNF-α in response to various TLR ligands. Moreover, although TLR stimulation inhibited osteoclast differentiation, osteoclast precursors treated with TLR ligands still retained high levels of phagocytic activity, which is a major host defense mechanism for the clearance of bacterial infection. Therefore, the net outcome of TLR stimulation in osteoclast precursors is likely to enhance immune responses for bacterial clearance. This enhancement of immune responses can be achieved by promoting cytokine production from precursor cells and by inhibiting their differentiation into nonphagocytic, nonimmune cells such as mature osteoclasts. Because these cells can differentiate into mature osteoclasts if TLR ligands are removed (data not shown), it appears that, after microbial infection is cleared, the presence of residual activated T cells can lead to the differentiation of phagocytic precursors into mature, bone-resorbing osteoclasts.

In addition to promoting inflammatory responses, TNF-α can also enhance the differentiation and survival of osteoclasts and up-regulate M-CSF gene expression in osteoblast/stromal cells (24, 40–43). Therefore, TNF-α produced by osteoclast precursors upon TLR stimulation can enhance osteoclastic bone resorption.

In summary, osteoclast precursors are extremely responsive to microbial constituents via TLRs. Interaction of these microbial products with TLRs on osteoclast precursors appears to favor the role of osteoclast precursors as part of the proinflammatory system by inhibiting their differentiation into mature osteoclasts and by promoting the production of inflammatory cytokines. TLRs are thus likely to regulate the balance of immune responses and bone metabolism during acute attacks of vertebrate hosts by various microbes.

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