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Regulation of Osteoclastogenesis by Integrated Signals From Toll-Like Receptors

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ABSTRACT

A variety of pathogen-derived molecules have been shown to cause bone loss by enhancing osteoclast differentiation through activation of toll-like receptors (TLRs). The pathogen-derived molecules (TLR-ligands) modulate osteoclastogenesis in a complex manner: inhibition of the osteoclast differentiation factor RANKL in early precursors and osteoclastogenesis stimulation in RANKL-primed cells. Since organisms may be challenged by several TLR ligands at a time, we investigated osteoclastogenesis modulation by simultaneous challenge with different TLR ligands. As an example we used ligands for TLR3 (Synthetic double stranded RNA [dsRNA], polyinosinic-polycytidylic acid [poly(I:C)] mimicking viral dsRNA), TLR4 (lipopolysaccharide [LPS], found in the outer membrane of Gram-negative bacteria) and TLR9 (Synthetic oligodeoxynucleotide mimicking bacterial DNA [CpG-ODN]). In osteoclastogenesis-inhibition, synergy between LPS and CpG-ODN or LPS and poly(I:C) while in stimulation, synergy between LPS and CpG-ODN or CpG-ODN and poly(I:C) were observed. Modulation of molecules involved in osteoclastogenesis (c-Fos, M-CSF receptors [M-CSFR], TNF-α, IL-6, and IL-12 and the three TLRs tested) was examined. The results indicate that M-CSFR plays a role only in the inhibitory effect while c-Fos plays a role in the two effects. TLR3 and TLR9 levels were increased by the TLRs ligands, suggesting that this may be part of the mechanism leading to the synergy. While TLRs activation in RANKL-primed cells, increasing osteoclastogenesis, explains pathogen-induced bone loss, activation of TLRs in early cells inhibiting osteoclastogenesis could attenuate excessive resorption, and promote differentiation of common precursor cells into inflammatory cells. The synergism between TLR ligands enables the individual to initiate response at a lower level of pathogen. J. Cell. Biochem. 115: 2146–2154, 2014.

KEY WORDS: OSTEOCLAST; TOLL-LIKE RECEPTORS; c-Fos; M-CSFR

The discovery of the fundamental role of receptor activator of NF-κB ligand (RANKL), which was previously described for its roles in T-cell activation and dendritic cell survival [Anderson et al., 1997; Wong et al., 1997; Bachmann et al., 1999], as the critical osteoclastogenic factor enabled to induce osteoclasts differentiation in vitro from their precursors in bone marrow, spleen, and peripheral blood monocytes [Lacey et al., 1998]. Thus, it was possible to analyze relatively pure populations of osteoclasts in culture. Consequently, major advances were achieved in our understanding of the factors and the signaling pathways participating in osteoclast differentiation, resorptive activity, and viability. Revealing the involvement of toll-like receptors (TLRs) family in osteoclast biology is one of the topics that benefited from our ability to study relatively pure populations of osteoclasts. TLRs have been mostly studied for their role in mediating the activation of the innate immune system by bacterial and viral factors [Akira et al., 2006]. The TLRs sense and are activated by specific pathogen-associated molecular patterns (PAMPs) [Akira et al., 2006; West et al., 2006].

The skeleton contains numerous blood vessels, tissue surfaces, and bone cells for bacterial colonization and is a major site of bacterial infections. Among bacterial diseases known to severely affect the skeleton, we find caries, periodontitis, periapical infection, osteomyelitis, septic arthritis, and others [Henderson and Nair, 2003].

The mechanism of pathogen-induced bone disease includes activation of TLRs in immune cells by pathogen-derived molecules [Henderson and Nair, 2003]. This activation results in synthesis and release of inflammatory cytokines that are capable of stimulating osteoclastic bone resorption and thus causing bone loss. Using RANKL-induced osteoclast precursors it became apparent that osteoclasts express functional TLRs [Bar-Shavit, 2008]. This was not surprising since osteoclasts are of hematopoietic origin. The most

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studied bacterial factor in this regard is lipopolysaccharide (LPS), found in the outer membrane of Gram-negative bacteria and is recognized by innate immunity cells via TLR4 [Kikuchi et al., 2001]. Bacterial DNA has been shown to be a pathogen-derived structure activating the innate immune system via TLR9 [Hemmi et al., 2000]. This activity depends on unmethylated CpG dinucleotides in particular base contexts ("CpG motif") [Krieg, 2002]. Synthetic oligodeoxynucleotides containing CpG motifs (CpG oligodeoxynucleotides [CpG-ODN]) mimic the bacterial DNA immunostimulatory activity [Yi et al., 1999]. Viral double stranded RNA (dsRNA) is recognized by TLR3 (West et al., 2006). The synthetic dsRNA polyinosinic-polycytidylic acid (poly(I:C)) mimics dsRNA activity [Alexopoulou et al., 2001]. Since infections cause pathological bone loss, it was expected that TLRs ligands would stimulate osteoclastogenesis and resorption. We were the first to show that TLR ligands (CpG-ODN and LPS) exert dual effect on osteoclast precursors. They inhibit the activity of the physiological osteoclast differentiation factor, RANKL, in early precursors, but strongly increase osteoclastogenesis in RANKL-pretreated osteoclast precursors [Zou and Bar-Shavit, 2002; Zou et al., 2002]. The anti-osteoclastogenic effect of TLR ligands was confirmed by others [Takami et al., 2002].

The organism is often challenged by more than one TLR ligand at a time. Therefore, we studied in this communication how osteoclastogenesis is modulated by simultaneous challenges with different TLR ligands. As an example we used for this study the ligands for TLR3, TLR4, and TLR9.

MATERIALS AND METHODS

MICE

BALB/c male mice (7–9-weeks old; Harlan Laboratories) were housed in the animal facility at the Hebrew University Faculty of Medicine under specific pathogen-free conditions. The Institutional Animal Care and Use Committee at the Hebrew University approved all protocols.

CELLS

Primary bone marrow macrophages (BMMs) were isolated from the mice. Femora and tibia were aseptically removed. The bone ends were cut, and marrow cells were obtained by flushing the bones with α-MEM medium supplemented with 10% fetal bovine serum (FBS) in the presence of antibiotics (penicillin and streptomycin) and glutamine (Biological Industries Ltd., Bet Haemek, Israel). Cells were centrifuged (1,000 rpm, 10 min) and cultured in α-MEM containing 10% CMG14-12 culture supernatant, which served as a source of M-CSF [Takeshita et al., 2000] for overnight in 100-mm diameter tissue culture plates (1×10^7 cells/10 ml/plate) to separate adherent and non-adherent cells. Then, non-adherent cells were harvested and cultured in 10% M-CSF in tissue culture plates. After 2 days of culture, floating cells were removed and attached cells were used as osteoclast precursors (OCPs).

IN VITRO OSTEOCLASTOGENESIS

Inhibition protocol. OCPs were cultured in α-MEM containing 10% FBS, 5% M-CSF, and RANKL (5 ng/ml) (R&D Systems Inc., MN) without or with poly(I:C), CpG-ODN (5′-TCCATGACGTTCCTGACGTT-3′) (InvivoGen, Toulouse, France) or LPS (Sigma–Aldrich) in 96-well culture plates (2×10^4 cells/0.2 ml/well) for 4 days (medium was changed on day 3).

Stimulation protocol. OCPs were cultured with α-MEM containing 10% FBS, 5% M-CSF, and RANKL (2.5 ng/ml) for 3 days. Then, the medium was changed and the cells were cultured without RANKL in α-MEM containing 10% FBS and 5% M-CSF without or with poly(I:C), CpG-ODN, or LPS for additional 24 h.

TARTRATE-RESISTANT ACID PHOSPHATASE (TRAP) STAINING

A commercial TRAP staining kit (Sigma–Aldrich) was used according to the manufacturer's instructions. TRAP-positive cells containing more than 20 nuclei were scored.

WESTERN BLOTTING

OCPs were seeded (2×10^6 cells/4 ml/60 mm plate) and treated as specified in the experiment. The whole cell lysates were prepared by lysis in 100 ml of RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 5 mM PMSF, 17 mM β-glycerophpsphate, 2.5 mM sodium pyrophosphate, 5 mM EDTA, and 1:100 protease inhibitors cocktail (Sigma–Aldrich)) at 4 °C for 30 min. The cell lysates were subjected to centrifugation at 12,000 g at 4 °C for 15 min. The supernatants were saved and protein content was determined using Coomassie Plus Protein Assay Reagent (Pierce Biotechnology, Inc., IL). Sixty microgram of lysates were loaded onto 7.5 or 10% SDS-polyacrylamide gels. Proteins were transferred onto a nitrocellulose membrane (Millipore Corporation, MA). Membranes were blocked for 1 h with 5% nonfat milk in TBST (10 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20). Rabbit anti-human M-CSFR (1:2000) and rabbit anti-human c-Fos (1:1000) were purchased from Santa Cruz Biotechnology, CA. Mouse anti-human TLR3 (1:1000), rabbit anti-human TLR4 (1:2000), and mouse anti-human TLR9 (1:1000) were purchased from Abcam, Cambridge, UK. (All anti-human antibodies mentioned are also suitable for mouse.) Mouse anti-mouse β-actin (1:10,000) was purchased from Sigma–Aldrich, goat anti-mouse antibody conjugated to horseradish peroxidase (1:10,000) and goat anti-rabbit antibody conjugated to horseradish peroxidase (1:10,000) were purchased from Jackson ImmunoResearch Laboratories, PA. Immunoreactivity was assayed using an ECL chemiluminescence kit (Pierce Biotechnology) according to the manufacturer's instructions.

ISOLATION OF RNA AND REAL-TIME PCR

OCPs were plated (2×10^6 cells/4 ml/60 mm plates) and treated as specified in the experiment. Total RNA was extracted with TRI reagent (Sigma–Aldrich) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA using the High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, CA) according to the manufacturer's instructions. Twenty microliter reaction mixtures were incubated for 10 min at 25 °C and then 2 h at 37 °C. The primers used were: M-CSFR: 5′-TCTCGCTTGGTCTGAACCCCT-3′, 5′-TCGGTGCAAATACAGCTGGGC-3′; TLR3: 5′-TTTGCAAAGAAGATAAAGCAGGTCT-3′, 5′-CCTTTCATGATTVAGCCCAGAT-3′; TLR4: 5′-CTTGAATCCCTGCATAGAGGTAGTT-3′, 5′-TGTTCTTGGTTGAAGAAGGAATGTC-3′. TLR9: 5′-CTGAGAGACCCTGGTGTGGAAC-3′, 5′-TCCTTCGACGGAG-

AACCATG-3′. IL-12: 5′-GGAGGTCAGCTGGGAGTACC-3′, 5′-AGGAACGCACCTTTCTGGTT-3′. IL-6: 5′-TTCACAAGTCGGAGGCTT-3′, 5′-CAGTTTGGTAGCATCCAT-3′. TNF-α: 5′-TCTCAGCCTCTTCTCATTCCTGCT-3′, 5′-AGAACTGATGAGAGGGAGGCCATT-3′. GAPDH: 5′-CCTGGAGAAACCTGCCAAG-3′, 5′-CAACCTGGTCC-TCAGTGTAGC-3′. Real-time PCR was performed in triplicates in 10 μl reactions using an Applied Biosystems 7300 sequence detection system. The relative level of each mRNA was determined using the comparative C_T method for relative quantification with GAPDH as endogenous references.

CALCULATIONS

Synergy. A combination of TLRs was considered synergetic, if the value obtained by the combination was significantly higher ($P < 0.05$) than the sum of the values obtained with each TLR alone.

Inhibition level. This was calculated by dividing the value (osteoclast number, band density in Western analyses, real-time-PCR data) of the control group by the value of TLR ligand treatment group.

STATISTICAL ANALYSIS

Results are expressed as mean ± SD. Mann-Whitney *U* test was used for the analyses.

RESULTS

MODULATION OF OSTEOCLASTOGENESIS BY SIMULTANEOUS SIGNALS FROM TLRs

Poly(I:C), LPS, and CpG-ODN, the ligands of TLR3, TLR4, and TLR9, respectively, inhibit osteoclastogenesis when added to early osteoclast precursors. To examine if simultaneous treatment of the cells with the TLRs ligands results in additive or synergistic effect we first determined the dose response of each TLR ligand inhibitory effect. OCPs were incubated with RANKL (5 ng/ml) for 4 days in the absence or presence of LPS (bars 2–5), CpG-ODN (bars 6 and 7), or poly(I:C) (bars 8–10) at different doses. As shown in Figure 1A, a dose-dependent reduction in osteoclastogenesis was observed for each ligand. The reduction in osteoclastogenesis at the lowest TLR ligand concentrations were 11% with 0.25 ng/ml LPS (bar 5); 15% with 10 nM CpG-ODN (bar 7) and no reduction was seen with 10 ng/ml poly(I:C) (bar 10).

For the combination experiment (Fig. 1B) we used the lowest concentration of each of the TLR ligands. OCPs were incubated with RANKL (5 ng/ml) for 4 days in the absence (bar 1) or presence of LPS, CpG-ODN, or poly(I:C), separately or in combination (bars 2–8). TRAP staining showed that LPS, CpG-ODN, or poly(I:C), in low concentrations, did not significantly block RANKL-induced osteoclast differentiation. The inhibition was calculated as detailed in methods section. The inhibition by LPS was 1.03-fold (bar 2), by CpG-ODN 1.25-fold (bar 3), and by poly(I:C) 1.24-fold (bar 4) as compared to cells without TLRs ligands (bar 1). Treatments with LPS together with CpG-ODN (bar 5) and LPS together with poly(I:C) (bar 6) inhibited RANKL-induced osteoclastogenesis 10.80- and 2.10-fold, respectively. On the other hand, CpG-ODN together with poly(I:C) (bar 7) caused only 1.53-fold inhibition. Incubation with the three ligands together (bar 8) caused 15-fold inhibition. Thus, LPS and CpG-ODN exhibited a strong synergistic effect; a lower synergy was observed with LPS and poly(I:C) and no synergy was seen with CpG-ODN and poly(I:C) or with the three ligands together.

Next we examined how simultaneous treatment of RANKL-primed OCPs affects osteoclastogenesis. In these conditions, as we have shown before [Zou and Bar-Shavit, 2002; Zou et al., 2002], TLRs activation increases osteoclastogenesis. As in the inhibition protocol, we first determined the ligands concentrations with

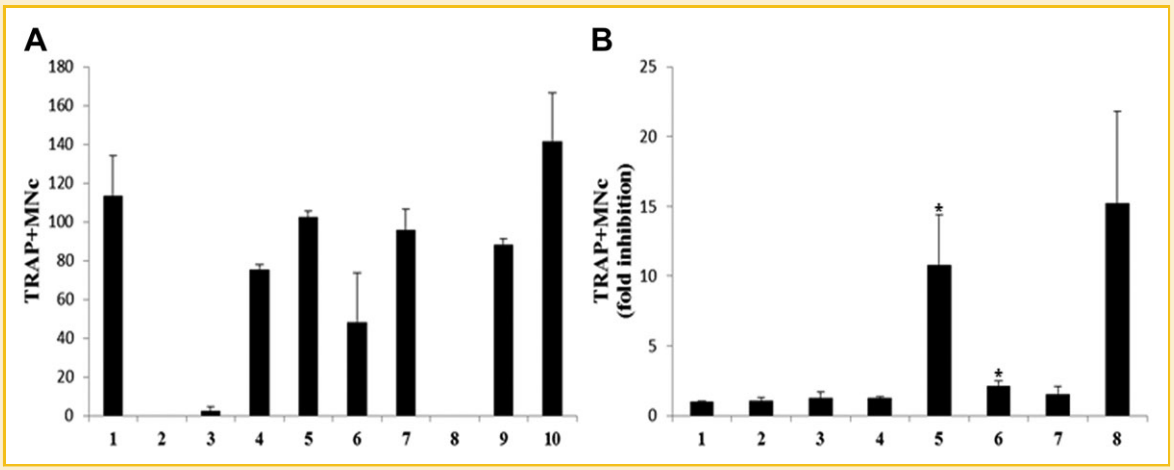

Fig. 1. Inhibition of RANKL-induced osteoclastogenesis by TLR ligands. (A) OCPs were incubated with RANKL (5 ng/ml) for 4 days. LPS (25, 5, 1, and 0.25 ng/ml, bars 2, 3, 4, and 5, respectively); CpG-ODN (100 and 10 nM, bars 6 and 7, respectively) or poly(I:C) (1, 0.1, and 0.01 μg/ml, bars 8, 9, and 10, respectively) were included for the duration of the experiment. Number TRAP positive multinucleated cells per 96 well is shown. (B) OCPs were incubated with RANKL (5 ng/ml) for 4 days without (1) or with LPS (0.25 ng/ml) (bars 2, 5, 6, and 8), CpG-ODN (10 nM) (bars 3, 5, 7, and 8), or poly(I:C) (0.1 μg/ml) (bars 4, 6, 7, and 8). Fold inhibition is shown. Results are expressed as mean ± SD of three different experiments. *Synergistic effect.

minimal effects. OCPs were incubated with RANKL (2.5 ng/ml) for 3 days. Then, cells were incubated without or with LPS, CpG-ODN, or poly(I:C) at different doses in the absence of RANKL. As shown in Figure 2A, dose-dependent osteoclastogenesis was observed. Concentrations of 0.5 ng/ml LPS, 10 nM CpG-ODN, and 0.1 μg/ml poly(I:C) increased osteoclastogenesis by 1.66-, 7.00- and 1.33-fold, respectively.

For the combinations experiment OCPs were treated for 3 days as for the experiment shown in Figure 2A, and then, in the absence of RANKL, were incubated with or without LPS, CpG-ODN, or poly(I:C), separately or in combinations for 24 h. Treatment with LPS, CpG-ODN, or poly(I:C) at low concentrations (Fig. 2B, bars 2–4), exhibited minimal effects on osteoclastogenesis (increases of 1.5-, 1.5-, and 3.0-fold, respectively), as compared to control cells (bar 1). Treatment with LPS together with CpG-ODN (bar 5), LPS together with poly(I:C) (bar 6), and CpG-ODN together with poly(I:C) (bar 7) increased osteoclastogenesis by 49-, 7-, and 32-fold, respectively. The combination of the three ligands together (bar 8) increased osteoclastogenesis by 55-fold. Thus, synergy was observed only with the combinations of LPS with CpG-ODN and CpG-ODN with poly(I:C).

MODULATION OF M-CSFR EXPRESSION BY SIMULTANEOUS SIGNALS FROM TLRs

Since M-CSF/M-CSFR interaction is required for osteoclastogenesis [Yoshida et al., 1990], we examined the effects of TLR ligands on M-CSFR expression. Each of the TLR ligands reduced M-CSFR expression in a concentration-dependent manner (Fig. 3A). The inhibition was observed after treatment with LPS, CpG-ODN, or poly (I:C) for 7 h (left section) and 3 days (right section). Low concentrations of ligands were used for the combination experiments. The inhibitory effect was synergistic with every combination of two TLR ligands at both 7 h (Fig. 3B, left) and 3 days (Fig. 3B, right). M-CSFR protein expression was reduced at 7 h by LPS (bar 2), CpG-ODN (bar 3), and poly(I:C) (bar 4) by 1.3-, 1.9-, and 1.5-fold, respectively. The combinations of LPS together with CpG-ODN (bar 5), LPS together with poly(I:C) (bar 6), and CpG-ODN together with poly(I:C) (bar 7) reduced M-CSFR expression by 3.2-, 3.4-, and 4.5-fold, respectively. The combination of the three TLR ligands (bar 8) did not exert additional inhibitory effect. M-CSFR protein expression was reduced at 3 days by LPS (bar 2), CpG-ODN (bar 3), and poly(I:C) (bar 4) by 1.2-, 1.3-, and 1.3-fold, respectively. The combinations of LPS together with CpG-ODN (bar 5), LPS together with poly(I:C) (bar 6), and CpG-ODN together with poly(I:C) (bar 7) reduced M-CSFR expression by 2.4-, 3.9-, and 2.7-fold, respectively. The combination of the three TLR ligands (bar 8) did not exert any additional inhibitory effect. On the other hand, no significant reduction of M-CSFR mRNA levels was observed with the TLR ligands (Fig. 3C).

As shown in Figure 3D the TLRs ligands did not affect significantly M-CSFR expression either separately or in combinations in RANKL-pretreated OCPs.

MODULATION OF RANKL-INDUCED c-Fos BY SIMULTANEOUS SIGNALS FROM TLRs

The transcription factor c-Fos plays a key role in osteoclast differentiation [Wang et al., 1992]. OCPs were incubated with RANKL (5 ng/ml) (Fig. 4A upper part the Western, lower part-densitometry) without (lane 1) or with LPS (0.5 ng/ml) (lanes 2,5,6,8), CpG-ODN (10 nM) (lanes 3,5,7,8), or poly(I:C) (10 ng/ml) (lanes 4,6,7,8) for 3 days. Western analysis of c-Fos protein expression was performed. The densitometry reveals that LPS (bar 2), CpG-ODN (bar 3), and poly(I:C) (bar 4) reduced c-Fos expression by 1.4-, 1.3-, and 1.1-fold, respectively, as compared to control (bar 1). Treatment

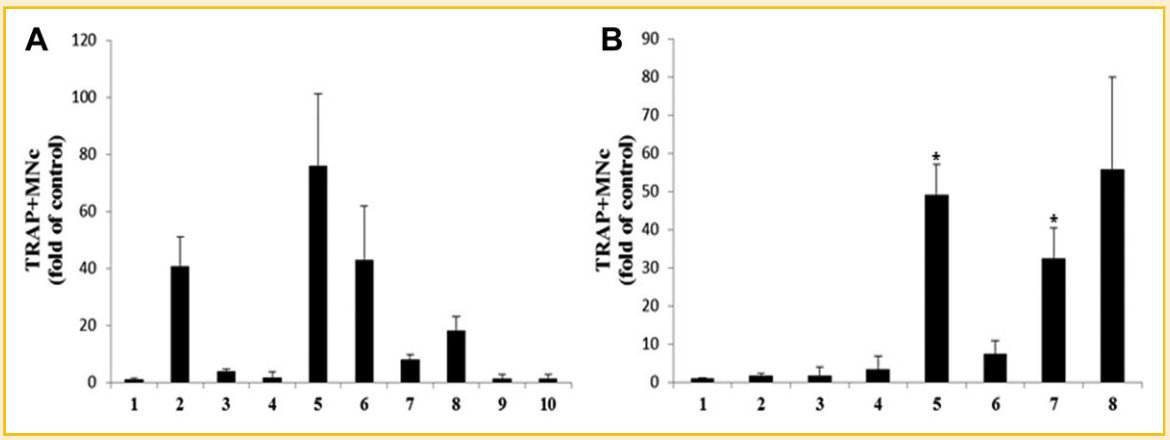

Fig. 2. Stimulation of osteoclastogenesis by TLR ligands in RANKL-primed OCPs. (A) OCPs were incubated with RANKL (2.5 ng/ml) for 3 days. Then medium was replaced and cells were incubated in the absence of RANKL without (bar 1) or with LPS (5, 1, and 0.5 ng/ml, bars 2, 3, and 4, respectively), CpG-ODN (100, 50, and 10 nM, bars 5, 6, and 6, respectively), or poly(I:C) (1, 0.1, and 0.01 μg/ml, bars 8, 9, and 10, respectively) for additional 24 h. (B) OCPs were incubated for 3 days with RANKL (2.5 ng/ml). Then the medium was replaced and cells were incubated without (bar 1) or with LPS (0.5 ng/ml) (bars 2, 5, 6, and 8), CpG-ODN (10 nM) (bars 3, 5, 7, and 8), or poly(I:C) (0.1 μg/ml) (4, 6, 7, and 8) for additional 24 h. *Synergistic effect. In both panels number of TRAP positive multinucleated cells is presented as fold of control. Results are expressed as mean ± SD of three different experiments.

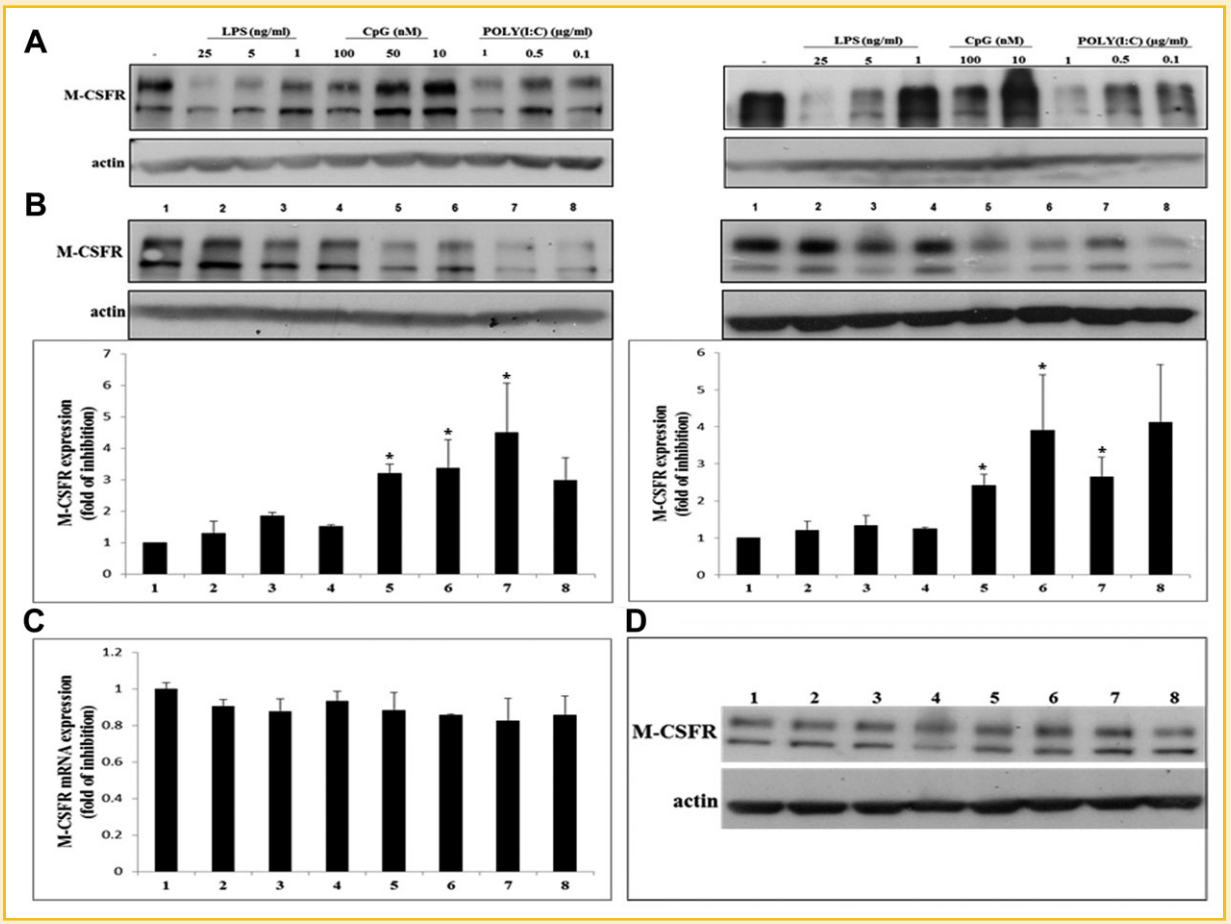

Fig. 3. Modulation of M-CSFR expression by TLR ligands. (A) OCPs were incubated with RANKL (5 ng/ml) for 7 h (left) or 3 days (right). LPS, CpG-ODN, or poly(I:C) at the indicated doses were included for the whole experiment. Western was performed as described in Materials and Methods. (B) OCPs were incubated with RANKL (5 ng/ml) for 7 h (left) or 3 days (right). LPS (0.5 ng/ml) (lanes 2, 5, 6, and 8), CpG-ODN (10 nM) (lanes 3, 5, 7, and 8), or poly (I:C) (0.1 μg/ml) (lanes 4, 6, 7, and 8) were present throughout the experiment. Western (upper part) and densitometric analyses (average from three experiments) (lower part) are presented. (C) M-CSFR mRNA levels were determined by real-time PCR. GAPDH mRNA was used for normalization. (D) OCPs were incubated for 3 days with RANKL (2.5 ng/ml). Then medium was changed and cells were incubated in the absence of RANKL and without (1) or with LPS (0.5 ng/ml) (lanes 2, 5, 6, and 8), CpG-ODN (10 nM) (lanes 3, 5, 7, and 8), or poly(I:C) (0.1 μg/ml) (lanes 4, 6, 7, and 8) for an additional 8 h. *Synergistic effect.

of LPS with CpG-ODN (bar 5), LPS with poly(I:C) (bar 6), and CpG-ODN with poly (I:C) (bar 7) reduced c-Fos expression by 3.1-, 2.1-, and 1.5-fold, respectively. Thus, only the first two combinations exhibited synergy, in correlation with the ligands' effects on the osteoclastogenesis (Fig. 1B).

We next examined modulation of c-Fos expression by TLRs ligands in RANKL-primed OCPs (Fig. 4B upper part the Western, lower part-densitometry). Densitometry shows that LPS (bar 2), CpG-ODN (bar 3), and poly(I:C) (bar 4) at low concentrations increased 1.42-, 1.30-, and 1.37-fold, respectively c-Fos expression. The combination of LPS and CpG-ODN (bar 5), the combination of LPS and poly(I:C) (bar 6), and the combination of CpG-ODN with poly(I:C) (bar 7) increased c-Fos expression by 7.5-, 1.7-, and 6.5-fold, respectively, as compared to control (bar 1). The combinations of the three ligands together (lane 8) increased c-Fos expression by of 3.1-fold. Thus, the combinations of LPS and CpG-ODN and CpG-ODN with poly(I:C) exhibited synergy, in correlation with the ligands' effects on the osteoclastogenesis (Fig. 2B).

MODULATION OF IL-12 EXPRESSION BY SIMULTANEOUS SIGNALS FROM TLRs

The cytokine IL-12 is known for its anti-osteoclastogenic activity [Nagata et al., 2003; Amcheslavsky and Bar-Shavit, 2006]. Figure 5 shows that 4 h incubation of OCPs with LPS (bar 2), CpG-ODN (bar 3), or poly(I:C) (bar 4) increased IL-12 mRNA levels by 300-, 60-, and 200-fold, respectively. Simultaneous incubation of LPS together with CpG-ODN (bar 5) and LPS together with poly(I:C) (bar 6) increased the mRNA levels of IL-12 by 600- and 2500-fold, respectively. Thus, these combinations were synergistic. On the other hand, no synergy was observed when CpG-ODN and poly(I:C) were incubated together (bar 7) (250-fold increase) or when all three TLR ligands were incubated together (bar 8) (1480-fold increase).

MODULATION OF TNF-α AND IL-6 BY SIMULTANEOUS SIGNALS FROM TLRs IN RANKL-PRETREATED OCPs

TNF-α and IL-6 are potent activators of osteoclastogenesis and their expression is increased during osteoclastogenesis [Zou et al., 2001;

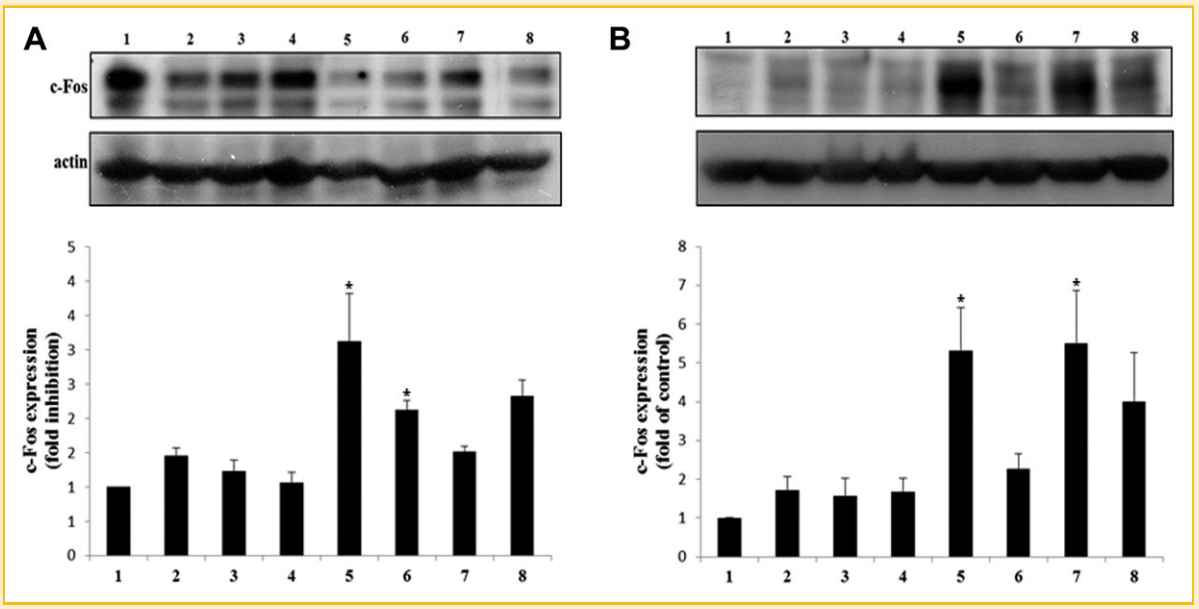

Fig. 4. Modulation of c-Fos expression by TLR ligands. (A) OCPs were incubated with RANKL (5 ng/ml) without (lane 1) or with LPS (0.5 ng/ml) (lanes 2, 5, 6, and 8), CpG-ODN (10 nM) (lanes 3, 5, 7, and 8), or poly(I:C) (0.1 μg/ml (lanes 4, 6, 7, and 8) for 3 days. Western (upper part) and densitometric analysis (average from three experiments) (lower part) are presented. (B) OCPs were incubated for 3 days with RANKL (2.5 ng/ml). Then medium was changed and cells were incubated in the absence of RANKL and without (1) or with LPS (0.5 ng/ml) (lanes 2, 5, 6, and 8), CpG-ODN (10 nM) (lanes 3, 5, 7, and 8) or poly(I:C) (0.1 μg/ml) (lanes 4, 6, 7, and 8) for an additional 8 h. Western (upper part) and densitometric analysis (lower part) are presented. *Synergistic effect.

Kudo et al., 2003]. Figure 6 shows that LPS, CpG-ODN, and poly(I:C) increased TNF-α (left panel) and IL-6 (right panel) mRNA abundance in RANKL-pretreated precursors (5.0-, 4.5-, 5.5-fold for TNF-α and 10.0-, 58.0-, 35.0-fold for IL-6, respectively). Combined treatment with LPS and CpG-ODN or with LPS and poly(I:C) resulted in 21.0- and 19.0-fold for TNF-α and in 427- and 113-fold increases for IL-6, respectively. The combination of CpG-ODN with poly(I:C) increased TNF-α and IL-6 expression by 10- and 99-fold, respectively. The combination of the three ligands together increased TNF-α and IL-6 expression by 27- and 250-fold, respectively. Thus, for both of these cytokines, the combinations of LPS with CpG-ODN and LPS with poly(I:C) exhibited synergy.

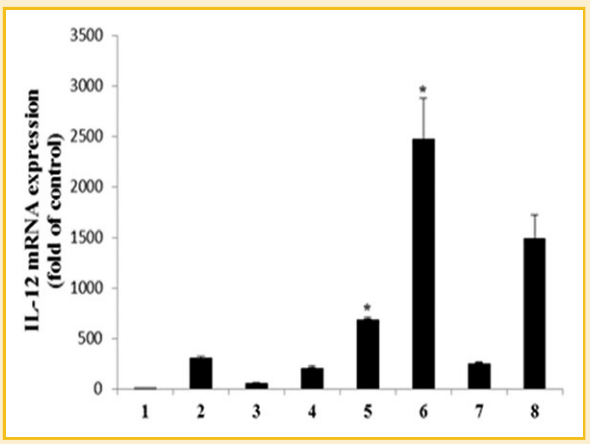

Fig. 5. Modulation of IL-12 expression by combination of TLR ligands. OCPs were incubated for 4 hours without (bar 1) or with LPS (0.1 ng/ml) (bars 2, 5, 6, and 8), CpG-ODN (10 nM) (bars 3, 5, 7, and 8), or poly(I:C) (5 ng/ml) (bars 4, 6, 7, and 8). IL-12 mRNA was determined by real-time PCR. GAPDH mRNA was used for normalization. The experiment was carried out three times. *Synergistic effect.

MODULATION OF TLRs EXPRESSION BY TLR LIGANDS

Up-regulation of TLRs in response to a variety of TLR ligands has been reported previously (Paul-Clark et al., 2006; Ilievski and Hirsch, 2010). Increased expression of a TLR induced by ligands of other TLRs is a potential mechanism to mediate synergistic effects of these ligands. As shown in Figure 7A, TLR3 mRNA level was increased by LPS, CpG-ODN, and poly(I:C) 17-, 8-, and 13-fold, respectively, and TLR9 mRNA level was increased by LPS, CpG-ODN, and poly(I:C) 6-, 2-, and 4-fold, respectively, l s were increase. In contrast, no significant increase was observed in TLR4 mRNA level with any of the ligands. Similar patterns of regulation were obtained when TLRs protein expression was determined by Western analysis (Fig. 7B).

DISCUSSION

TLRs, the focus of this study, sense pathogen-derived molecules and initiate the inflammatory reactions of innate immune cells. TLRs are also expressed in osteoclasts, and their activation affects these cells differentiation and activity.

Several potential mechanisms have been proposed to explain how bacteria might cause bone destruction. In addition to release of inflammatory cytokines from the immune system, these include the release of substances acting directly on the bone matrix, the release of factors capable of directly or indirectly stimulating bone-resorbing cells and the release of factors capable of inhibiting

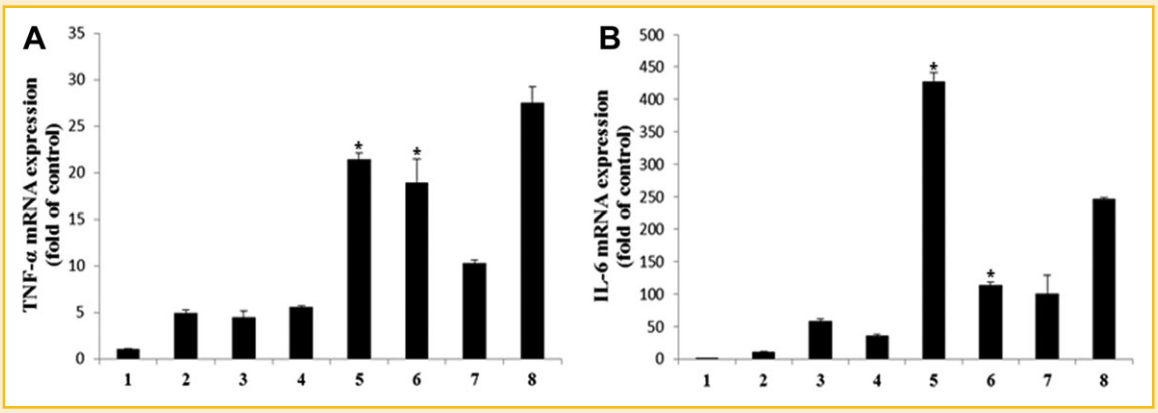

Fig. 6. Modulation of TNF-α and IL-6 expression by TLR ligands in RANKL-pretreated cells. OCPs were incubated for 3 days with RANKL (2.5 ng/ml). Then medium was changed and cells were incubated for 1 hour (A for TNF-α measurement) or 4 hours (B for IL-6 measurement). The cytokines mRNA levels were determined by real-time PCR. GAPDH mRNA expression was used for normalization. *Synergistic effect.

bone-forming cells and invading bone cells, causing apoptosis or other effects [Henderson and Nair, 2003]. The expression of functional TLRs by osteoclasts raises the possibility of direct modulation of these cells' differentiation and resorptive activity by pathogen-derived TLR ligands. While many studies indeed show stimulation of osteoclasts by TLR ligands it is recognized now that at least some of the TLR ligands modulate osteoclasts in a dual manner: they stimulate osteoclastogenesis in precursors committed to the lineage (pretreated with RANKL), but inhibit osteoclastogenesis in more primitive precursors (that were not pre-exposed to RANKL) [Zou and Bar-Shavit, 2002; Zou et al., 2002].

Studies in which resorption is examined following bacterial infection generally show an increase in bone loss, consistent with increased osteoclastogenesis and in correlation with bone loss observed in patients suffering infections [Henderson and Nair, 2003]. These studies, however, do not directly address the impact of the activation of TLRs on osteoclast differentiation in vivo. The injection of LPS into mice induces osteoclast differentiation in a p55TNF receptor-dependent manner (Abu-Amer et al., 1999). Hayashi et al. (2003) examined the effects of LPS and CpG-ODN injection on osteoclasts and found that bone marrow cells harvested from LPS- and CpG-ODN- injected mice exhibited increased osteoclastogenic potential.

We [Amcheslavsky and Bar-Shavit, 2007] found that the osteoclastogenic potential of bone marrow is increased following CpG-ODN injection. If the in vivo situation is similar to that observed in vitro, one would expect to find conditions in which in vivo activation of TLRs reduces osteoclastogenesis. Alternatively, TLR-mediated stimulation of osteoclastogenesis may be more pronounced than any TLR-mediated inhibitory effect, in which case the inhibitory effect would not be observed in vivo. In all the examined studies, only stimulation of osteoclast differentiation was observed.

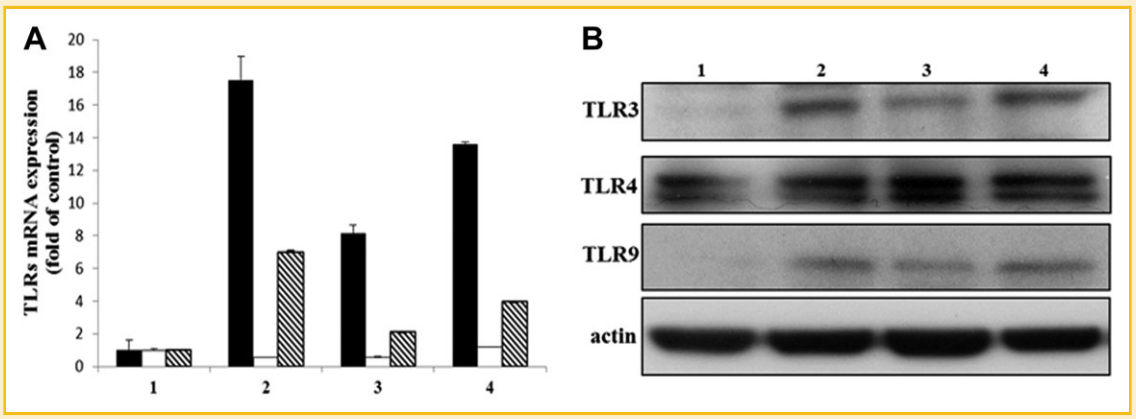

Fig. 7. Modulation of TLRs expression by TLR ligands. (A) OCPs were incubated without (group 1) or with LPS (25 ng/ml, group 2), CpG-ODN (100 nM, group 3), or poly(I:C) (1 μg/ml, group 4) for 5 h. TLR3 (solid bars), TLR4 (empty bars), and TLR9 (striped bars) mRNA levels were determined by real time PCR. GAPDH mRNA was used for normalization. The basal level for each TLR was given the value 1. (B) Cells were incubated without (lane 1) or with LPS (25 ng/ml, lane 2), CpG-ODN (100 nM, lane 3), or poly(I:C) (1 μg/ml, lane 4) for 18 h. Western blot analysis of each TLR was performed.

We injected mice with 5-fluorouracil (5FU) in order to observe inhibition of osteoclast differentiation in vivo. This chemical is known to be toxic to cycling cells in the bone marrow, and therefore its application causes an increase in the proportion of early hematopoietic precursor population [Van Zant, 1984]. Consistent with the in vitro findings, injection of CpG-ODN into 5FU-treated mice decreased the osteoclastogenic potential of the bone marrow. Therefore, in both the in vitro and in vivo situations, the activation of TLRs (at least TLR9) results in both stimulation and inhibition of osteoclast differentiation. While the activation of TLRs in committed osteoclasts results in increased osteoclastogenesis and is probably the mechanism by which pathogen-induced bone loss occurs, the inhibition of osteoclastogenesis by TLR activation in early precursor cells may play a role in reducing the excessive bone loss caused by pathogenic infection and shifting the balance between the bone and immune systems during infection to recruit immune cells.

TLRs are evolutionary conserved, from the worm Caenorhabditis elegans to mammals [Akira et al., 2006]. To date, 11 human TLRs and 13 mouse TLRs have been identified [West et al., 2006]. Probably the infected organism is challenged with more than one PAMP at the same time. We therefore examined how osteoclast lineage cells will be affected by simultaneous challenges of more than one PAMP.

We examined modulation of both early and committed (RANKL-primed) osteoclast precursors. As an example we analyzed the combinations of the ligands of TLR3, TLR4, and TLR9. In both cases, committed precursors (Table I) and early precursors (Table II) some combinations resulted in a synergistic effects on osteoclastogenesis. In committed precursors (Table I) simultaneous activation of TLR3 and TLR9, and of TLR4 and TLR9 resulted in synergistic effects. No synergy was observed in simultaneous activation of TLR3 and TLR4. In early precursors (Table II) simultaneous activation of TLR3 and TLR4, and of TLR4 and TLR9 resulted in synergistic effects. No synergy was observed in simultaneous activation of TLR3 and TLR9. We also initiated studies to reveal the mechanisms involved in the synergy. In light of the pivotal role of c-Fos in mediating osteoclastogenesis we examined the effects of the various treatments on this protein expression. We see in Table I overlapping in the combinations that exhibit synergy on osteoclastogenesis and on c-Fos stimulation. Similarly, Table II shows the overlapping in the combinations that inhibit osteoclastogenesis and reduce c-Fos expression. Activation of M-CSFR by MCSF is also essential for osteoclastogenesis. Indeed the combinations that exhibit synergy in inhibition of osteoclastogenesis of the non-committed precursors also exhibit synergy in reducing M-CSFR expression (Table II). However, at the mRNA level, no inhibition was observed with the combinations of TLR ligands. Therefore, it is possible that the inhibitory effect of the TLR ligands on M-CSFR expression is due to modulation of protein translation and/or stability. In order to assign a role for M-CSFR it is important to identify the membrane form of the receptor. Flow-cytometry failed, since the presence of M-CSF was required in order to observe the inhibitory effect. Unfortunately, the presence of M-CSF inhibited the binding of anti-M-CSFR antibodies to its antigen. However, it was previously shown that the heavier ("slower") band shown in Figure 3 is the membrane form [Ji et al., 2009] and the quantification shown was of the membrane form. In contrast (Fig. 3D), the TLRs ligands did not affect significantly M-CSFR expression either separately or in combinations in RANKL-pretreated OCPs. The inflammatory cytokines IL-6 and TNF-α play a role in osteoclastogenesis [Zou et al., 2001; Kudo et al., 2003]. There was an overlap in the stimulation of osteoclastogenesis and of these cytokines' expression in the combination of TLR4 and TLR9, but not in the combinations of TLR3 and TLR4 and of TLR3 and TLR9 (Table I), suggesting that these cytokines participate only in the synergy of TLR4 and TLR9. IL-12 was identified as an anti-osteoclastogenic cytokine and indeed the combinations that synergize in inhibition of osteoclastogenesis also synergize in stimulating IL-12 expression (Table II). The simultaneous stimulation of the three TLRs never resulted in an additional synergy (Tables I and II).

Modulation of TLR expression by activation of other TLR is a potential mechanism to mediate synergistic effects. To this end, we incubated OCPs with either TLR3, TLR4, or TLR9 ligands. All treatments up-regulated TLR3 and TLR9 mRNA and protein expression. In contrast, TLR4 expression was not affected by any of the TLR ligands. These observations are consistent with published

TABLE I. Modulation of RANKL-Primed OCPs by Combinations of TLR-Ligands

TLR ligand	Osteoclastogenesis	c-Fos	IL-6	TNF-α
LPS + CpG	Synergy	Synergy	Synergy	Synergy
LPS + Poly (IC)	No synergy	No synergy	Synergy	Synergy
CpG + Poly (IC)	Synergy	Synergy	No synergy	No synergy
LPS + CpG + Poly (IC)	No synergy	No synergy	No synergy	No synergy

TABLE II. Modulation of Non-committed OCPs by Combinations of TLR-Ligands

TLR ligand	Osteoclastogenesis	c-Fos	M-CSFR	IL-12
LPS + CpG	Synergy	Synergy	Synergy	Synergy
LPS + Poly (IC)	Synergy	Synergy	Synergy	Synergy
CpG + Poly (IC)	No synergy	No synergy	Synergy	No synergy
LPS + CpG + Poly (IC)	No synergy	No synergy	No synergy	No synergy

studies (Paul-Clark et al., 2006; Ilievski and Hirsch, 2010) and suggest that the synergistic effect seen with two TLR ligands may be caused, at least in part, through upregulation of a given TLR by a ligand of another TLR.

It is planned to further analyze the mechanisms of the synergies described above by analyses of earlier events in the cascades. The significance of the synergism between the TLR ligands is that it enables the individual to initiate response at low level of the pathogen. While on one hand the pathological bone loss may be initiated in low levels of infection, on the other hand the PAMPs will also promote the differentiation of the precursor toward the innate immune system to eradicate the pathogen at the low concentration.

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REFERENCES

Abu-Amer Y, Ross FP, Edwards J, Teitelbaum SL. 1997. Lipopolysaccharide-stimulated osteoclastogenesis is mediated by tumor necrosis factor via its P55 receptor. J Clin Invest 100:1557–1565.

Akira S, Uematsu S, Takeuchi O. 2006. Pathogen recognition and innate immunity. Cell 124:783–801.

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413:732–738.

Amcheslavsky A, Bar-Shavit Z. 2006. Interleukin (IL)-12 mediates the anti-osteoclastogenic activity of CpG-oligodeoxynucleotides. J Cell Physiol 207:244–250.

Amcheslavsky A, Bar-Shavit Z. 2007. *In vivo* activation of toll-like receptor 9 modulates osteoclastogenesis. J Bone Miner Res 22(S1):s151.

Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe MC, DuBose RF, Cosman D, Galibert L. 1997. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature 390:175–179.

Bachmann MF, Wong BR, Josien R, Steinman RM, Oxenius A, Choi Y. 1999. TRANCE, a tumor necrosis factor family member critical for CD40 ligand-independent T helper cell activation. J Exp Med 189:1025–1031.

Bar-Shavit Z. 2008. Taking a Toll on the bones: regulation of bone metabolism by innate immune regulators. Autoimmunity 41:195–203.

Hayashi S, Yamada T, Tsuneto M, Yamane T, Takahashi M, Shultz LD, Yamazaki H. 2003. Distinct osteoclast precursors in the bone marrow and extramedullary organs characterized by responsiveness to Toll-like receptor ligands and TNF-alpha. J Immunol 171:5130–5139.

Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S. 2000. A Toll-like receptor recognizes bacterial DNA. Nature 408:740–745.

Henderson B, Nair SP. 2003. Hard labour: bacterial infection of the skeleton. Trends Microbiol 11:570–577.

Ilievski V, Hirsch E. 2010. Synergy between viral and bacterial toll-like receptors leads to amplification of inflammatory responses and preterm labor in the mouse. Biol Reprod 83:767–773.

Ji JD, Park-Min KH, Shen Z, Fajardo RJ, Goldring SR, McHugh KP, Ivashkiv LB. 2009. Inhibition of RANK expression and osteoclastogenesis by TLRs and IFN-gamma in human osteoclast precursors. J Immunol 183:7223–7233.

Kikuchi T, Matsuguchi T, Tsuboi N, Mitani A, Tanaka S, Matsuoka M, Yamamoto G, Hishikawa T, Noguchi T, Yoshikai Y. 2001. Gene expression of osteoclast differentiation factor is induced by lipopolysaccharide in mouse osteoblasts via Toll- like receptors. J Immunol 166:3574–3579.

Krieg AM. 2002. CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol 20:709–760.

Kudo O, Sabokbar A, Pocock A, Itonaga I, Fujikawa Y, Athanasou NA. 2003. Interleukin-6 and interleukin-11 support human osteoclast formation by a RANKL-independent mechanism. Bone 32:1–7.

Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165–176.

Nagata N, Kitaura H, Yoshida N, Nakayama K. 2003. Inhibition of RANKL-induced osteoclast formation in mouse bone marrow cells by IL-12: involvement of IFN-gamma possibly induced from non-T cell population. Bone 33:721–732.

Paul-Clark MJ, McMaster SK, Belcher E, Sorrentino R, Anandarajah J, Fleet M, Sriskandan S, Mitchell JA. 2006. Differential effects of Gram-positive versus Gram-negative bacteria on NOSII and TNFalpha in macrophages: role of TLRs in synergy between the two. Br J Pharmacol 148:1067–1075.

Takami M, Kim N, Rho J, Choi Y. 2002. Stimulation by Toll-like receptors inhibits osteoclast differentiation. J Immunol 169:1516–1523.

Takeshita S, Kaji K, Kudo A. 2000. Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. J Bone Miner Res 15:1477–1488.

Van Zant G. 1984. Studies of hematopoietic stem cells spared by 5-fluorouracil. J Exp Med 159:679–690.

Wang ZQ, Ovitt C, Grigoriadis AE, Möhle-Steinlein U, Rüther U, Wagner EF. 1992. Bone and haematopoietic defects in mice lacking c-fos. Nature 360:741–745.

West AP, Koblansky AA, Ghosh S. 2006. Recognition and signaling by Toll-like receptors. Annu Rev Cell Dev Biol 22:409–437.

Wong BR, Rho J, Arron J, Robinson E, Orlinick J, Chao M, Kalachikov S, Cayani E, Bartlett FS, III, Frankel WN, Lee SY, Choi Y. 1997. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c- Jun N-terminal kinase in T cells. J Biol Chem 272:25190–25194.

Yi AK, Peckham DW, Ashman RF, Krieg AM. 1999. CpG DNA rescues B cells from apoptosis by activating NF-kB and preventing mitochondrial membrane potential disruption via a chloroquine-sensitive pathway. Int Immunol 11:2015–2024.

Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD, Nishikawa S. 1990. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature 345:442–444.

Zou W, Bar-Shavit Z. 2002. Dual modulation of osteoclast differentiation by lipopolysaccharide. J Bone Miner Res 17:1211–1218.

Zou W, Hakim I, Tschoep K, Endres S, Bar-Shavit Z. 2001. Tumor necrosis factor-alpha mediates RANK ligand stimulation of osteoclast differentiation by an autocrine mechanism. J Cell Biochem 83:70–83.

Zou W, Schwartz H, Endres S, Hartmann G, Bar-Shavit Z. 2002. CpG oligonucleotides: novel regulators of osteoclast differentiation. FASEB J 16:274–282.