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Induction of Nod1 and Nod2 Intracellular Pattern Recognition Receptors in Murine Osteoblasts following Bacterial Challenge

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Osteoblasts produce an array of immune molecules following bacterial challenge that could recruit leukocytes to sites of infection and promote inflammation during bone diseases, such as osteomyelitis. Recent studies from our laboratory have shed light on the mechanisms by which this cell type can perceive and respond to bacteria by demonstrating the functional expression of members of the Toll-like family of cell surface pattern recognition receptors by osteoblasts. However, we have shown that bacterial components fail to elicit immune responses comparable with those seen following challenge with the intracellular pathogens salmonellae and *Staphylococcus aureus*. In the present study, we show that UV-killed bacteria and invasion-defective bacterial strains elicit significantly less inflammatory cytokine production than their viable wild-type counterparts. Importantly, we demonstrate that murine osteoblasts express the novel intracellular pattern recognition receptors Nod1 and Nod2. Levels of mRNA encoding Nod molecules and protein expression are significantly and differentially increased from low basal levels following exposure to these disparate bacterial pathogens. In addition, we have shown that osteoblasts express Rip2 kinase, a critical downstream effector molecule for Nod signaling. Furthermore, to begin to establish the functional nature of Nod expression, we show that a specific ligand for Nod proteins can significantly augment immune molecule production by osteoblasts exposed to either UV-inactivated bacteria or bacterial lipopolysaccharide. As such, the presence of Nod proteins in osteoblasts could represent an important mechanism by which this cell type responds to intracellular bacterial pathogens of bone.

While the primary roles of osteoblasts are to synthesize the components of bone matrix and to control the bone-resorbing activity of osteoclasts, recent studies have revealed an additional function during bone diseases, the initiation and maintenance of inflammatory immune responses. Previous studies by our laboratory have shown that osteoblasts exposed to salmonellae and *Staphylococcus aureus*, the two most common causative agents in bone and joint diseases (23, 32), are a significant source of an array of soluble inflammatory mediators (2–4, 10, 26). Furthermore, we have recently described the surprising ability of osteoblasts to activate T lymphocytes by antigen presentation in the context of major histocompatibility complex II molecules (33) and to express the key costimulatory molecule CD40 (34) following exposure to bacteria. Such a pattern of immune molecule expression is one that can promote the recruitment of leukocytes to sites of bacterial infection and could serve to initiate and sustain inflammatory responses during inflammatory bone diseases, such as osteomyelitis.

The recent demonstration of members of the Toll-like family of cell surface pattern recognition receptors in osteoblasts may represent a means by which these cells recognize bacterial pathogens. Kichuchi and coworkers (21) reported the presence of message encoding Toll-like receptor 2 (TLR2) and TLR4 in murine osteoblasts, and we have confirmed the functional expression of TLR4 (10) on these cells. In addition, we have recently shown that osteoblasts express TLR5 (25), and the presence of TLR9 has been inferred by the ability of activating oligonucleotides to induce production of proinflammatory molecules by these cells (42). However, the expression of these TLRs does not preclude the involvement of other pattern recognition receptors in the initiation of osteoblast immune responses. More importantly, our previous studies have shown that bacterial products and inactivated bacteria are far less potent than live bacteria at eliciting immune molecule production by these cells (2–4, 10). Taken together, these findings suggest that bacterial invasion may be required to evoke maximal responses in osteoblasts.

A family of novel nucleotide-binding oligomerization domain (Nod) proteins has recently been identified in both immune and nonimmune cell types, whose members include at least two that appear to serve as intracellular pattern recognition receptors (as reviewed in reference 17). Nod1 (also designated CARD4) interacts with motifs found in peptidoglycans from gram-negative bacteria (5, 11, 12). In contrast, Nod2 (also designated CARD15) has been suggested to be a more general sensor of bacterial peptidoglycans as it recognizes a minimal motif present in all peptidoglycans (12, 13, 18). Both Nod1 and Nod2 have been reported to associate with Rip2 kinase (also designated RICK and CARDIAK) (6, 22, 29, 41), the activation of which ultimately results in the activation of NF-κB, a pivotal transcription factor in the production of pro-inflammatory mediators. As such, Nod molecules could play an important role in the detection of intracellular pathogens of osteoblasts, including *Salmonella* spp. and *S. aureus* (2, 7, 8, 14), and the initiation of host immune responses.

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In the present study, we demonstrate that isolated cultures of murine osteoblasts differentially express Nod1 and Nod2 following exposure to *Salmonella* spp. and *S. aureus*. Furthermore, we show that a specific ligand for Nod2 augments inflammatory immune responses by activated cells. Taken together, the demonstration that osteoblasts express functional Nod proteins may represent a potentially important mechanism by which this bone cell type can respond to intracellular bacterial pathogens during infectious bone diseases.

MATERIALS AND METHODS

Reagents and solutions. Lipopolysaccharide (LPS; from *Escherichia coli*) and the muramyl dipeptide MurNAc-L-Ala-D-iso-Gln (MDP) were purchased from Sigma Chemical Company (St. Louis, MO). Doses selected represent those that were empirically determined to elicit optimal responses.

Isolation and characterization of murine osteoblasts. Primary osteoblast cell cultures were prepared from BALB/c mouse neonate calvariae by sequential collagenase-protease digestion as previously described by our laboratory (2–4, 10, 25, 33, 34). Osteoblasts isolated in this manner have previously been characterized as being >99% pure cultures as determined by their distinctive morphology and by the expression of type I collagen, osteocalcin, alkaline phosphatase, and parathyroid hormone-cyclic AMP (4). All experiments were performed with osteoblasts grown to the point of confluency in six-well plates or culture flasks, and comparisons were made between each treatment group in that experimental series to insure equal cell numbers.

Exposure of cultured osteoblasts to *S. aureus* or salmonellae. Osteoblasts were exposed to bacteria as previously described by our laboratory (2–4, 10, 25, 33, 34). Confluent cell layers of cells were exposed to the pathogenic *S. aureus* strain UAMS-1 (ATCC 49230), *Staphylococcus carnosus* (ATCC 51365), *Salmonella enterica* serovar Typhimurium strain 12023 (ATCC 14028), *Salmonella enterica* serovar Typhimurium strain SB300, or *Salmonella enterica* serovar Typhimurium strain SB136 at the indicated ratios of bacteria to osteoblasts in growth medium without antibiotics for 45 min at 37°C. Following the infection period, cell cultures were washed with phosphate-buffered saline and incubated in growth medium with 25 μg/ml gentamicin to kill remaining extracellular bacteria. *S. carnosus* is a nonpathogenic staphylococcal species previously reported to be incapable of invasion of osteoblasts (20). *Salmonella enterica* serovar Typhimurium strain SB136 is an invasion-defective mutant strain of *Salmonella enterica* serovar Typhimurium strain SB300, due to a mutation in the *invA* gene that prevents *Salmonella* pathogenicity island-1 type III secretion mechanisms. Attenuated strains of bacteria were a kind gift from Michael C. Hudson at the University of North Carolina at Charlotte.

In some experiments, *S. aureus* or *S. enterica* serovar Typhimurium was exposed to short-wavelength (250-nm) UV light for 5 min. This time period was empirically determined to reduce the number of viable bacteria to <0.01% as determined by colony counting.

Isolation of polyadenylated RNA and semiquantitative RT-PCR. Polyadenylated RNA was isolated from osteoblasts and reverse transcribed as previously described (2–4, 10, 25, 33, 34). PCR was performed on the cDNA product to determine the expression of mRNA encoding Nod1, Nod2, Rip2, interleukin-6 (IL-6), and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) essentially as described previously (2–4, 10, 25, 33, 34). The positive- and negative-strand PCR primers used were GTCCTCAACGAGCATGGCGAGACT and AGCTCATC CAGGCCGTCAA, respectively, to amplify mRNA encoding murine Nod1 (299-bp fragment), GCTGCCAATCTTCACGTCGTC and TAAGTACTGAGGAA GCGAGACTGA, respectively, to amplify mRNA encoding murine Nod2 (273-bp fragment), CTGCACCCGAAGGAGGAACAATCA and GCGCCCATCC ACTCTGTATTAGA, respectively, to amplify mRNA encoding murine Rip2 (276-bp fragment), GATGCAACCAAACTGGATATAATC and GGTCCTT AGCCACTCCTTCTCTG, respectively, to amplify mRNA encoding murine IL-6 (268-bp fragment), and CCATCACCATCTTCCAGGAGCGAG and CACAGTCTTCTGGGTGGCAGTGAT, respectively, to amplify mRNA encoding G3PDH (340-bp fragment). PCR primers were derived from the published sequences of Nod1 (35), Nod2 (19), Rip2 (15), IL-6 (37), and G3PDH (36). Primers were designed by using Oligo 4.0 primer analysis software (National Biosciences Inc., Plymouth, MA) based on their location in different exons of the genomic sequences for each, in addition to their lack of significant homology to sequences present in GenBank (MacVector Sequence analysis software; IBI, New Haven, CT).

PCR amplification of the housekeeping gene G3PDH was performed on cDNA from each sample to insure equal input of RNA and similar efficiencies of reverse transcription. The identities of the PCR amplified fragments were verified by size comparison with DNA standards and by direct DNA sequencing of representative fragments (Davis Sequencing, Davis, CA).

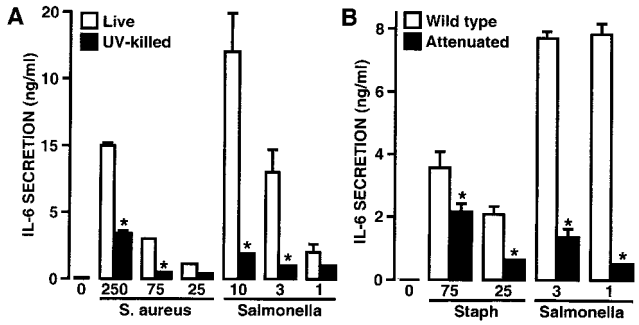

FIG. 1. UV-inactivated bacteria and invasion-defective bacterial strains are weaker stimuli for immune molecule production by osteoblasts than viable wild-type bacteria. Panel A: osteoblasts (2×10^6 per well) were untreated (0) or exposed to either viable or UV-inactivated *S. aureus* (multiplicity of infection [MOI] of 25, 75, and 250 bacteria per cell) or salmonellae (MOI of 1, 3, and 10 bacteria per cell). Panel B: osteoblasts (2×10^6 per well) were untreated (0) or exposed to either wild-type or mutant staphylococci (*S. aureus* and *S. carnosus* at MOIs of 25 and 75 bacteria per cell) or salmonellae (*S. enterica* serovar Typhimurium SB300 and *S. enterica* serovar Typhimurium SB136 at MOIs of 1 and 3 bacteria per cell). At 24 h posttreatment, culture supernatants were taken and assayed for the presence of IL-6 by specific capture ELISA. Data are shown as mean values of triplicate determinations of three separate experiments ± standard error of the mean. Asterisks indicate a significant difference between responses to viable wild-type bacteria and attenuated and inactivated bacteria ($P < 0.05$).

Western blot analysis for Nod1, Nod2, and Rip2 kinase. Western blot analyses for the presence of Nod1, Nod2, or Rip2 kinase in osteoblasts were performed essentially as described previously by our laboratory (25, 33, 34). The primary antibodies used were an affinity-purified rabbit polyclonal antibody directed against human Nod1 (Alpha Diagnostics International Inc., San Antonio, TX), a rabbit polyclonal antiserum against human Nod2 (Cayman Chemical, Ann Harbor, MI), and a mouse reactive affinity-purified rabbit polyclonal antibody directed against Rip2 kinase (Cell Sciences Inc., Canton, MA).

Quantification of IL-6 secretion in osteoblast culture supernatants. Specific capture enzyme-linked immunosorbent assays (ELISA) were performed to quantify IL-6 secretion as described previously (2–4).

Densitometric analyses. Densitometric analyses of Western blots and PCR products were performed using NIH Image (obtained from the NIH web site, http://rsb.info.nih.gov/nih-image). Results are presented as mean values of arbitrary densitometric units corrected for background intensity or as increases over levels in unstimulated cells.

Statistical analysis. The results of the present studies were tested statistically using Student's paired *t* test or one-way analysis of variance and Dunnett's or Bonferroni's multiple comparison tests where appropriate using commercially available software (GraphPad Prism, GraphPad Software, San Diego, CA). Results were determined to be statistically significant when a *P* value of less than 0.05 was obtained.

RESULTS

Active bacterial invasion of murine osteoblasts is required for maximal IL-6 production. To investigate whether active invasion of osteoblasts by viable bacteria is required to elicit optimal immune responses by this cell type, IL-6 production was determined for cells exposed to either viable or UV-inactivated *S. aureus* and salmonellae. As shown in Fig. 1A, UV-killed bacteria elicited significantly less IL-6 production by osteoblasts than cells exposed to viable bacteria. To confirm that this effect was due to an inability of UV-killed bacteria to actively invade osteoblasts rather than an altered ability of

inactivated bacteria to interact with osteoblast cell surface pattern recognition receptors, additional experiments were performed to investigate the relative abilities of invasion-defective mutants of *S. aureus* and salmonellae to elicit IL-6 production.

As shown in Fig. 1B, invasion-defective mutants *Staphylococcus carnosus* and *Salmonella enterica* serovar Typhimurium SB136 were significantly less potent stimuli for IL-6 production by osteoblasts than their wild-type equivalents. Taken together, these results are consistent with our previous findings that bacterial products are far less effective than live bacteria at eliciting immune molecule production by cultured osteoblasts (2–4, 10) and support the notion that viable intracellular bacteria are required to provoke maximal immune responses by these cells.

Induction of mRNA encoding Nod1 and Nod2 in murine osteoblasts following bacterial exposure. To begin to determine how osteoblasts perceive and respond to these disparate intracellular bacterial pathogens, we have investigated the effect of salmonellae and *S. aureus* on the expression of mRNA encoding Nod1 and Nod2 cytosolic pattern recognition receptors in these cells. Murine osteoblasts were untreated or exposed to bacteria at various numbers of bacteria to cells. At 4 and 8 h postinfection, RNA was isolated and semiquantitative reverse transcription (RT)-PCR was performed for the presence of mRNA encoding Nod1 or Nod2.

As shown in Fig. 2, there is modest constitutive expression of mRNA encoding Nod1 in resting cultures of murine osteoblasts with no detectable Nod2 mRNA expression. However, exposure of cells to salmonellae elicited marked increases in the level of expression of mRNA encoding both Nod1 and Nod2 (Fig. 2). Maximal *Salmonella*-induced increases in Nod1 and Nod2 mRNA were 4- and 107-fold, respectively, over constitutive levels, as measured by densitometric analysis. Interestingly, maximal Nod2 mRNA upregulation was observed as rapidly as 4 h postinfection, while Nod1 displayed slower kinetics of induction. *S. aureus* similarly induced the delayed expression of mRNA encoding Nod1 in osteoblasts with a maximal sixfold increase over constitutive levels as measured by densitometric analysis (Fig. 2). In contrast, *S. aureus* failed to induce the expression of Nod2 mRNA (Fig. 2). The identity of the PCR products following bacterial challenge was confirmed by direct sequencing of excised bands (Davis Sequencing, Davis, CA), which demonstrated 99% and 96% homology to published sequences for Nod1 and Nod2, respectively.

In addition, cells were exposed to lipopolysaccharide derived from *E. coli* to test whether extracellular bacterial stimuli can induce expression of mRNA encoding Nod molecules in osteoblasts. LPS is a specific ligand for TLR4 and was used as an external stimulus rather than UV-killed bacteria to circumvent the possible confounding effect of phagocytosed bacteria. As shown in Fig. 2, LPS elicited a modest increase in levels of mRNA encoding Nod1 of 2.6-fold above that seen in resting cells as measured by densitometric analysis. Interestingly, LPS induced a 34-fold increase in levels of mRNA encoding Nod2 as measured by densitometric analysis, approximately a third of the maximal increases observed with viable salmonellae (Fig. 2).

Induction of Nod1 and Nod2 protein expression in osteoblasts following exposure to bacteria. To determine whether the elevations in levels of mRNA encoding Nod1 and Nod2 in murine osteoblasts seen following bacterial challenge translates into the increased expression of protein products, Western blot analyses were performed. Reagents for murine Nod proteins are not currently available, and so polyclonal antibodies directed against human Nod1 and Nod2 proteins were utilized due to their 89% and 78% homology to murine Nod1 and Nod2, respectively (12, 35). Murine osteoblasts were exposed to salmonellae or *S. aureus* at the indicated numbers of bacteria to osteoblasts, and whole-cell protein isolates were obtained at 12 or 24 h prior to Western blot analysis for Nod1 or Nod2.

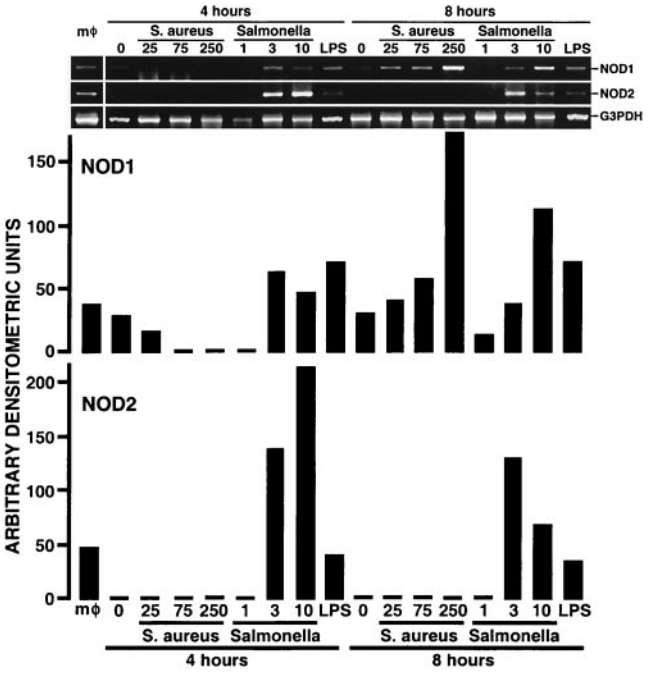

FIG. 2. Expression of mRNA encoding Nod1 and Nod2 in murine osteoblasts following bacterial exposure. Osteoblasts (2×10^6 per well) were untreated (0) or exposed to salmonellae (MOIs of 1, 3, and 10 bacteria per cell), *S. aureus* (MOIs of 25, 75, and 250 bacteria per cell), or LPS (100 ng/ml). RNA was isolated at 4 or 8 h posttreatment, and RT-PCR was performed for the presence of mRNA encoding Nod1 or Nod2. PCR amplification of mRNA encoding G3PDH was performed to ensure that similar amounts of input RNA and similar efficiencies of reverse transcription were being compared. For comparison purposes, RT-PCR was performed on RNA isolated from a similar number of LPS-activated peritoneal macrophages (mϕ). Below, densitometric analysis of this representative experiment is shown as arbitrary densitometric units for Nod1 and Nod2 mRNA expression normalized to G3PDH mRNA levels. These studies were performed three times with similar results.

As shown in Fig. 3A, resting cultures of osteoblasts showed low levels of a protein that migrated to an estimated size of 96 kDa that was close to the predicted size of the Nod1 protein (≈115 kDa). Importantly, the level of expression of this protein was significantly increased at 24 h postinfection with either *S. aureus* or salmonellae (Fig. 3A) ($P < 0.05$). Such induction is in agreement with the inductions in levels of mRNA encoding Nod1 following exposure to either pathogen (Fig. 2). In contrast, osteoblasts demonstrated significant upregulation ($P < 0.05$) of a protein that migrated to a estimated size of 120 kDa that was close to the predicted size of the Nod2 protein (≈130 kDa) at 12 h postinfection with salmonellae but not *S. aureus* (Fig. 3B). Again, such induction is in agreement with

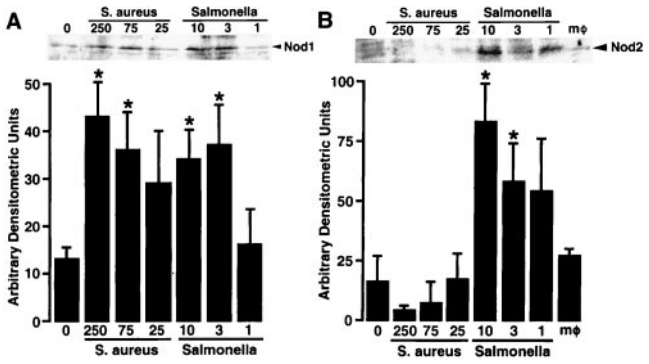

FIG. 3. Elevated Nod1 and Nod2 protein expression in osteoblasts following exposure to *S. aureus* and salmonellae. Osteoblasts were either untreated (0) or exposed to *S. aureus* (MOIs of 25, 75, and 250 bacteria per cell) or salmonellae (MOIs of 1, 3, and 10 bacteria per cell). After 24 h, protein isolates were subjected to Western blot analysis for Nod1 expression (panel A) or after 12 h for Nod2 expression (panel B). Representative immunoblots for each are shown. For comparison purposes, Nod2 protein expression in samples from a similar number of LPS-activated peritoneal macrophages (mϕ) was also analyzed. Below, densitometric analyses of Nod1 ($n = 4$) and Nod2 ($n = 3$) protein bands are shown as arbitrary densitometric units corrected for background intensity in each lane, ± standard error of the mean. Asterisks indicate a significant difference from unstimulated osteoblasts ($P < 0.05$).

the inductions in levels of mRNA encoding Nod2 following exposure to salmonellae but not *S. aureus* (Fig. 2).

Osteoblasts express a pivotal downstream effector molecule for Nod signaling. To begin to determine whether osteoblasts can express functional Nod intracellular pattern recognition receptors, we have investigated whether these cells express Rip2 kinase, a critical effector molecule in both Nod1- and Nod2-mediated cellular activation (1, 16, 29). Cells were untreated or exposed to *S. aureus* (25, 75, and 250 bacteria per cell), salmonellae (1, 3, and 10 bacteria per cell), or LPS (1, 10, and 100 ng/ml). At 4, 8, and 12 h postinfection, RNA was isolated and semiquantitative RT-PCR was performed for the presence of mRNA encoding Rip2. As shown in Fig. 4A, there is strong constitutive expression of mRNA encoding Rip2 in resting cultures of murine osteoblasts with little increase in the level of expression of mRNA following any challenge used.

To further investigate the expression of Rip2 kinase in osteoblasts following bacterial challenge, Western blot analyses were performed. Murine osteoblasts were exposed to salmonellae or *S. aureus* at the indicated numbers of bacteria to osteoblasts, and whole-cell protein isolates were obtained at 24 h prior to Western blot analysis for Rip2 kinase. As shown in Fig. 4B, resting cultures of osteoblasts showed constitutive expression of Rip2 kinase (60 kDa). Importantly, the level of expression of this protein was significantly increased at 24 h postinfection with either *S. aureus* or salmonellae (Fig. 4B), with maximal increases of (1.8 ± 0.1)-fold and (2.5 ± 0.2)-fold, respectively, as determined by densitometric analysis. As such, the constitutive expression of this critical downstream effector molecule and its sensitivity to bacterial challenge provides circumstantial evidence for the functionality of Nod proteins in osteoblasts.

Osteoblast responses are augmented by specific Nod agonists. To test whether activated osteoblasts express functional Nod molecules, we have investigated the effect of MDP, a well-characterized and specific ligand for Nod2 (12, 13, 18), on cytokine production by osteoblasts challenged with LPS- or UV-killed salmonellae. LPS activates osteoblasts via TLR4 ligation independently of Nod2 activation and induces submaximal production of the inflammatory cytokine IL-6 (4). Furthermore, LPS and salmonellae induce the expression of detectable levels of mRNA encoding Nod2 in osteoblasts (Fig. 2).

Osteoblasts were untreated or exposed to LPS (10 and 100 ng/ml) or salmonellae (3 and 10 bacteria per osteoblast) in the presence or absence of MDP (1 μg/ml) for 24 h prior to determination of IL-6 content in culture supernatants by specific capture ELISA. As shown in Fig. 5, MDP alone failed to elicit significant elevations in IL-6 production by osteoblasts. Importantly, MDP significantly ($P < 0.05$) increased the secretion of IL-6 by cells stimulated with either LPS (Fig. 5A) or UV-killed salmonellae (Fig. 5B). In contrast, MDP failed to augment IL-6 production by osteoblasts exposed to UV-killed *S. aureus* (Fig. 5B), consistent with the inability of this bacterium to stimulate Nod2 expression in these cells (Fig. 2 and 3). Taken together, these data suggest that suitably stimulated osteoblasts express functional Nod2 receptors that can respond to MDP and augment inflammatory immune responses by these resident bone cells.

DISCUSSION

There is growing realization that osteoblasts can play an important role in the initiation and maintenance of inflammatory immune responses during bacterial infections of bone.

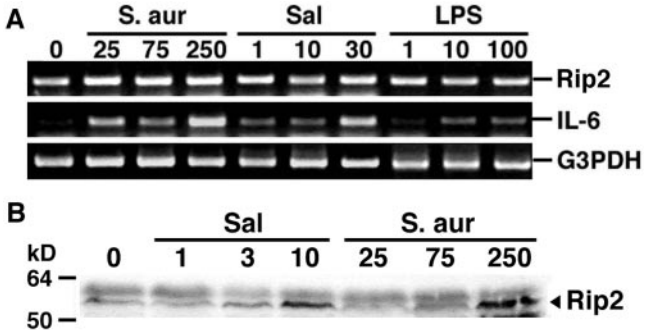

FIG. 4. Expression of Rip2 kinase in murine osteoblasts following exposure to bacterial stimuli. Panel A: Osteoblasts (2×10^6 per well) were untreated (0) or exposed to *S. aureus* (S. aur) (MOIs of 25, 75, and 250 bacteria per cell), salmonellae (Sal) (MOIs of 1, 3, and 10 bacteria per cell), or lipopolysaccharide (LPS) (1, 10, and 100 ng/ml). RNA was isolated at 8 h postinfection and RT-PCR was performed for the presence of mRNA encoding Rip2. PCR amplification of mRNA encoding IL-6 was performed to confirm osteoblast responsiveness to bacterial challenge. PCR amplification of mRNA encoding G3PDH was performed to ensure that similar amounts of input RNA and similar efficiencies of reverse transcription were being compared. These studies were performed three times with similar results. Panel B: Osteoblasts (2×10^6 per well) were untreated (0) or exposed to *S. aureus* (S. aur) (MOIs of 25, 75, and 250 bacteria per cell) or salmonellae (Sal) (MOIs of 1, 3, and 10 bacteria per cell). After 24 h, protein isolates were subjected to Western blot analysis for Rip2 kinase expression. The migration of protein standards of known size is indicated to the left. A representative immunoblot from three separate experiments is shown.

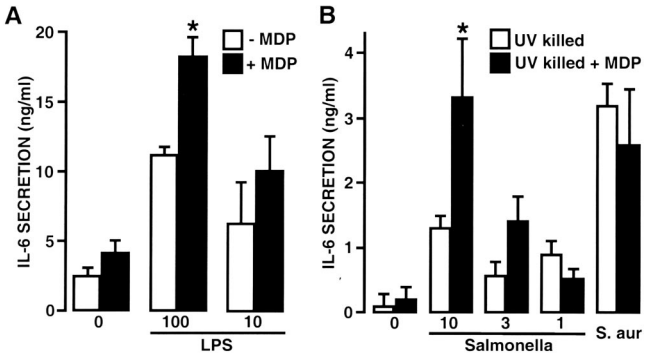

FIG. 5. MDP significantly augments LPS or UV killed bacteria-mediated IL-6 production by osteoblasts. Panel A: Cells (10^7 per well) were untreated (0) or exposed to lipopolysaccharide (LPS) (10 and 100 ng/ml) in the presence or absence of MDP (1 μg/ml). Panel B: Cells (2×10^6 per well) were untreated (0) or exposed to UV-killed salmonellae (MOIs of 1, 3, and 10 bacteria per cell) in the presence or absence of MDP (1 μg/ml). At 24 h posttreatment, culture supernatants were taken and assayed for the presence of IL-6 by specific capture ELISA. For comparison purposes, levels of IL-6 secretion by osteoblasts exposed to UV-killed *S. aureus* (75:1, bacteria to cells) in the absence or presence of MDP are shown. Data are shown as mean values of triplicate determinations of four separate experiments ± standard error of the mean. Asterisks indicate a significant difference in IL-6 production between activated cells in the absence and the presence of MDP ($P < 0.05$).

Bacterial challenge of osteoblasts can initiate the production of an array of immune regulatory molecules (for a review, see reference 32). This pattern of expression is one that could promote the recruitment of leukocytes to sites of bacterial challenge, initiate antigen-specific activation of infiltrating cells, and facilitate the development of cell-mediated immune responses to intracellular pathogens of bone tissue. These findings have identified this cell type as a previously unappreciated component in host responses. However, the mechanisms by which these nonleukocytic cells perceive bacterial pathogens remain unclear.

The recent discovery of the Toll-like family of cell surface receptors for microbial motifs has led to an unprecedented increase in our knowledge of the mechanisms by which the innate immune system recognizes a diverse range of pathogens (28, 39). To date, 11 homologues of these Toll-like receptors (TLRs) have been identified in the human and mouse, and bacterially derived ligands have been defined for TLR2, TLR4, TLR5, and TLR9. Recent studies from our laboratory and others have provided evidence for the functional expression of TLR4 (10), TLR5 (25), and TLR9 (42) on osteoblasts. However, we have shown that neither bacterial components nor UV-killed bacteria are as potent as live bacteria at eliciting gamma interferon-inducible protein 10 (10), MCP-1 (3), or colony-stimulating factor (2) production by osteoblasts. Furthermore, UV-killed bacteria fails to elicit detectable IL-12p70 secretion by these cells (4). These findings suggest that recognition of extracellular bacteria motifs is insufficient to elicit maximal immune responses of osteoblasts and that bacterial invasion may be required for optimal activation.

Nod1 and Nod2 are members of a family of proteins whose members appear to serve as intracellular pattern recognition receptors and/or activators of apoptotic pathways (as reviewed in reference 27). In the present study, we provide evidence for the expression of these novel intracellular pathogen-associated molecular pattern recognition receptors in bacterially challenged murine osteoblasts. We show that, while expression of mRNA encoding the Nod1 and Nod2 is very low or absent in resting cultures of osteoblasts, exposure to salmonellae elicits marked upregulation in levels of mRNA encoding both molecules. In contrast, challenge of osteoblasts with *S. aureus* causes a marked induction in levels of Nod1 mRNA in the absence of detectable Nod2 mRNA expression.

Interestingly, a specific ligand for TLR4 also elicits detectable levels of mRNA encoding Nod2, consistent with the previously documented ability of such ligands to induce Nod2 mRNA expression in a monocytic cell line (19). In addition, the recent availability of polyclonal antibodies to human Nod1 and Nod2 proteins that share 89% and 78% homology to murine Nod proteins, respectively, has enabled us to confirm the presence of these proteins in bacterially challenged osteoblasts by Western blot analysis. Importantly, the upregulations in Nod1 and Nod2 protein levels seen following bacterial challenge mirror the salmonella- and *S. aureus*-mediated increases in mRNA levels encoding these proteins. Furthermore, we have demonstrated the expression of Rip2, a pivotal effector molecule in Nod-mediated cell signaling, in osteoblasts. Such expression is constitutive in resting cell cultures and is significantly upregulated following bacterial challenge. Finally, we have confirmed the ability of a specific ligand for Nod2 to augment inflammatory mediator production by these cells challenged with extracellular bacteria or components. Taken together, these studies provide the first evidence for the functional presence of these novel intracellular bacterial pattern recognition receptor proteins in osteoblasts. The expression of Nod proteins may represent an important mechanism by which these cells perceive intracellular bacteria.

While both Nod1 and Nod2 detect bacterial peptidoglycans, differences have been reported to exist between the specific motifs recognized by each. Nod1 interacts with the naturally occurring peptidoglycan degradation product, GlcNAc-MurNAC-L-Ala-gamma-D-Glu-*meso*-diaminopimelate, which is found in peptidoglycans from gram-negative bacteria (5, 11, 13). In contrast, Nod2 recognizes a minimal muramyl dipeptide motif, MurNAC-L-Ala-D-iso-Gln (MDP), present in all peptidoglycans (12, 13, 17). As such, it has been suggested that Nod1 represents a specific intracellular receptor for gram-negative bacteria, while Nod2 is a more general sensor of bacteria (13).

The gram-positive organism *S. aureus* and gram-negative *Salmonella* species are the two most common causative agents of inflammatory bone and joint disease (23, 32). Salmonellae are well recognized as intracellular pathogens, and we have recently demonstrated the ability of these bacteria to invade and persist within osteoblasts (2). In contrast, staphylococci have historically been regarded as noninvasive extracellular pathogens that damage host bone cells after adhering to the extracellular matrix (9). However, more recent studies have demonstrated that *S. aureus* is internalized by cultured osteoblasts and can persist intracellularly (7, 8, 14).

In the present study we show that salmonella infection rapidly upregulates the expression of both Nod1 and Nod2 in osteoblasts (Fig. 1, 4, and 5). Such a finding is consistent with

the ability of peptidoglycans from this gram-negative species to interact with both Nod homologues. However, our studies utilizing *S. aureus* as the bacterial stimulus yielded more surprising results. This gram-positive bacterium induced the expression of Nod1 despite the previously documented finding that *S. aureus*-derived peptidoglycan fails to activate Nod1 in a transfected cell line (13). Furthermore, this bacterium does not induce the expression of the more general peptidoglycan sensor Nod2 in osteoblasts. Such findings raise the possibility that induction of Nod molecules occurs secondary to signaling via other pattern recognition receptors, rather than as a direct consequence of bacterial ligands interacting with Nod receptors. Support for this notion comes from the finding that LPS can elicit detectable elevations in Nod expression in osteoblasts (Fig. 2) and a monocytic cell line (19) and from the observation that Nod2 is undetectable in unstimulated osteoblasts (Fig. 2 and 3). Furthermore, the Nod2-specific ligand MDP fails to augment *S. aureus*-mediated IL-6 production while significantly elevating secretion elicited by stimuli that induce Nod2 expression (Fig. 5). It is therefore possible that *S. aureus* indirectly induces Nod1 expression following engagement of other pattern recognition receptors.

Both Nod1 and Nod2 have been reported to associate with Rip2 kinase (6, 22, 29, 41), and the present demonstration of the inducible expression of Rip2 in osteoblasts lends credence to the notion that Nod proteins are functional in this cell type. Activation of Rip2 kinase results in the activation of NF-κB, a pivotal transcription factor in the production of cytokines such as IL-1, IL-6, tumor necrosis factor alpha, IL-12, and the induction of costimulatory molecules. Hence, activation of osteoblasts via Nod receptors could underlie, at least in part, the bacterially induced immune molecule production previously reported for this cell type (as reviewed in reference 26). We report that while the Nod2-specific ligand MDP is not a sufficient signal to elicit increases in IL-6 secretion alone, it can significantly augment cytokine production elicited by extracellular stimuli (Fig. 5).

These findings are consistent with previous studies employing MDP that show that this molecule is a relatively weak stimulus in macrophages (30). Furthermore, MDP has been widely shown to synergize with LPS in the production of inflammatory cytokines (24, 31, 38, 40). While it is presently unclear why MDP alone fails to elicit significant cytokine production by osteoblasts, it is possible that initial stimulation is required to induce Nod2 expression (as shown in Fig. 2), thereby rendering osteoblasts sensitive to MDP. If this is correct, then extracellular and intracellular bacterial pattern recognition receptors act in a cooperative manner to initiate maximal osteoblast immune responses. Such a scenario would explain our previous findings that bacterial components and/or UV-killed bacteria are far less effective in initiating immune molecule production by these cells. Taken together, the present demonstration of functional Nod proteins in murine osteoblasts may represent an important mechanism by which this bone cell type can respond to intracellular bacterial pathogens.

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