Differential roles for NOD2 in osteoblast inflammatory immune responses to bacterial pathogens of bone tissue

Vinita S. Chauhan and Ian Marriott

Correspondence

Ian Marriott

imarriot@uncc.edu

Department of Biology, University of North Carolina at Charlotte, Charlotte, NC 28223, USA

Osteoblasts produce an array of immune molecules following bacterial challenge that can contribute to inflammation and the recruitment of leukocytes to sites of infection during bone diseases such as osteomyelitis. However, the mechanisms by which osteoblasts perceive and respond to facultative intracellular pathogens such as *Salmonella* species and *Staphylococcus aureus* have not been determined. Recently, our laboratory has described the expression in osteoblasts of members of the nucleotide-binding domain leucine-rich repeat region containing family of proteins that include nucleotide-binding oligomerization domain-2 (NOD2), a molecule that functions as an intracellular receptor for bacterial peptidoglycans. In the present study, we demonstrate that NOD2 expression is required for select inflammatory mediator production by osteoblasts following infection with the invasive pathogen *Salmonella*. In contrast, we have found that the inflammatory immune responses of osteoblasts to the passively internalized bacterial species *Staphylococcus aureus*, heat-killed pathogenic *Salmonella*, a non-invasive *Salmonella* strain and specific Toll-like receptor ligands are not reduced in the absence of NOD2 expression but are, in fact, elevated. Based upon these findings, we suggest that NOD2 serves differential roles in osteoblasts, promoting inflammatory responses to invasive bacteria while tempering cell responses to extracellular and/or passively internalized bacterial species.

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INTRODUCTION

Studies from our laboratory have demonstrated that osteoblasts exposed to *Salmonella enterica* and *Staphylococcus aureus*, the principle causative agents of osteomyelitis, express an array of soluble and cell surface molecules that could promote immune responses and osteoclast activity at sites of bone infection (as summarized by Marriott, 2004 and Marriott *et al.*, 2007). The demonstration that osteoblasts express members of the Toll-like family of cell surface pattern recognition receptors (TLR) may represent a means by which these cells respond to such pathogens (Kikuchi *et al.*, 2001; Gasper *et al.*, 2002; Madrazo *et al.*, 2003). However, our previous studies have shown that bacterial products, inactivated bacteria and invasion-defective strains are far less potent than viable pathogenic bacteria at eliciting these responses (Bost *et al.*, 1999, 2000, 2001; Gasper *et al.*, 2002). These results suggest that bacterial invasion is required for maximal osteoblast responses and point to the involvement of additional intracellular sensors for bacterial pathogens or their products.

Consistent with this hypothesis, we have recently demonstrated that osteoblasts express nucleotide-binding oligomerization domain-2 (NOD2) (Marriott *et al.*, 2005), a member of the novel nucleotide-binding domain leucine-rich repeat region containing family of proteins (NLR) that functions as an intracellular receptor for bacterial peptidoglycans (Girardin *et al.*, 2003; Inohara *et al.*, 2003). The functional nature of such expression was inferred from the ability of TLR ligands or bacterial challenge to upregulate the expression of both NOD2 and Rip2 kinase (also designated RICK and CARDIAK) (Marriott *et al.*, 2005), a critical downstream effector molecule in the NOD2 signalling pathway (Chin *et al.*, 2002; Kobayashi *et al.*, 2002). Furthermore, we demonstrated that muramyl dipeptide (MDP), a ligand for NOD2 (Girardin *et al.*, 2003; Inohara *et al.*, 2003), significantly augments TLR-induced inflammatory cytokine production by osteoblasts (Marriott *et al.*, 2005). It is therefore possible that NOD2 acts as a sensor for facultative intracellular bacterial species such as *Salmonella* and *Staphylococcus aureus* in this cell type.

In the present study, we have assessed the role played by NOD2 in the inflammatory responses of primary osteoblasts to invasive and extracellular and/or passively internalized bacterial species. We report that NOD2

Abbreviations: GFP, green fluorescent protein; MDP, muramyl dipeptide; NLR, nucleotide-binding domain leucine-rich repeat region; NOD2, nucleotide-binding oligomerization domain-2; TLR, Toll-like receptor.

expression contributes to inflammatory cytokine production by *Salmonella*-infected osteoblasts. In contrast, osteoblast responses to *Staphylococcus aureus* are augmented in the absence of NOD2 expression and resemble the enhanced responses observed with inactivated *Salmonella*, a non-invasive *Salmonella* strain, and ligands for cell surface TLRs.

METHODS

Reagents and solutions. LPS isolated from *Escherichia coli* ($>500\,000$ EU mg^{-1} ; $\geq 97\%$ by TLC) and the MDP MurNAc-L-Ala-D-isoGln ($\geq 98\%$ by TLC) were purchased from Sigma Chemical Company. An activating oligonucleotide, GCTCATGACGTTCC-TGATGCTG, previously described as a specific agonist for TLR9 (Sweet *et al.*, 2002), was synthesized by Integrated DNA Technologies. Endotoxin-free flagellin protein preparations were isolated from *Salmonella typhimurium* serotype 12023 as previously employed (Madrazo *et al.*, 2003). Doses selected represent those that were empirically determined to elicit optimal responses.

Wild-type and NOD2-deficient mice. B6.129S1-*Nod2*^{tm1Flv/J} mice that are NOD2-deficient (NOD2^{-/-}) and bred for >10 generations onto a C57BL/6 background, as well as wild-type C57BL/6j mice, were purchased from Jackson Laboratory.

Isolation and characterization of murine osteoblasts. Primary osteoblast cell cultures were prepared from C57BL/6 and NOD2-deficient mouse neonate calvariae by sequential collagenase-protease digestion as previously described by our laboratory (Bost *et al.*, 1999; Marriott *et al.*, 2005; McCall *et al.*, 2008). 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-cAMP (Bost *et al.*, 1999).

Exposure of cultured osteoblasts to *Salmonella enterica* or *Staphylococcus aureus*. Osteoblasts were exposed to bacteria as previously described by our laboratory (Bost *et al.*, 1999; Marriott *et al.*, 2005; McCall *et al.*, 2008). Confluent cell layers of cells were exposed to the pathogenic *Salmonella enterica* serovar Typhimurium strain SB300, *Salmonella enterica* serovar Typhimurium strain SB136 or *Staphylococcus aureus* UAMS-1 (ATCC 49230) 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 PBS and incubated in growth medium with 25 μg gentamicin ml^{-1} to kill remaining extracellular bacteria. *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. *Staphylococcus aureus* UAMS-1 is a clinical isolate and capable of inducing inflammatory responses in osteoblasts. In some experiments, osteoblasts were exposed to green fluorescent protein (GFP)-expressing *Staphylococcus aureus* (*Staphylococcus aureus* RN4220 p^{SB2030}) (Reott *et al.*, 2008). SB136 and GFP-expressing *Staphylococcus aureus* were kind gifts from Dr Michael C. Hudson at The University of North Carolina at Charlotte.

Quantification of inflammatory cytokine and chemokine secretion in bone protein extracts and osteoblast culture supernatants. Specific capture ELISAs were performed to quantify IL-6, IL-12p70, MCP-1, IP-10 and RANKL levels in culture supernatants as

described previously by our laboratory (Bost *et al.*, 1999; Marriott *et al.*, 2005; McCall *et al.*, 2008).

Immunoblot analysis of NOD2 and GRIM-19. Immunoblot analyses for the presence of NOD2 and GRIM-19 in osteoblasts were performed as described previously by our laboratory (Gasper *et al.*, 2002; Marriott *et al.*, 2005; McCall *et al.*, 2008). The primary antibodies used were a rabbit serum antibody directed against NOD2 (IMGEX) and a mouse antibody that is reactive against murine GRIM-19 (BD Transduction Laboratories). To assess total protein loading in each well, immunoblots were reprobed with a goat anti-mouse β -actin antibody (Santa Cruz Biotechnology).

Co-immunoprecipitation of Rip2 kinase with NOD2. Co-immunoprecipitation was performed as described by our laboratory (McCall *et al.*, 2008). Protein A agarose beads (Pierce Endogen) were pre-incubated with antibodies directed against NOD2 for 1 h prior to incubation with cell lysates for 18 h at 4 °C. The immunoprecipitated material was subsequently subjected to immunoblot analysis for the presence of Rip2 kinase (Assay Designs). Since most standard housekeeping proteins are lost during this two-step process, we have determined the level of expression of an unidentified non-specific protein (irrelevant protein) that elutes in the immunoprecipitated material to confirm equal loading in each lane.

Measurement of bacterial burden and apoptotic volume decreases by flow cytometry. Bacterial burden in osteoblasts challenged with GFP-expressing *Staphylococcus aureus* was assessed by flow cytometry (FACSCalibur; Becton Dickinson) as previously described by our laboratory (McCall *et al.*, 2008). In some experiments, proportions of cells undergoing apoptosis were determined by measuring apoptotic volume decreases as changes in forward and side scatter characteristics by flow cytometry as previously described by our laboratory (McCall *et al.*, 2008).

Densitometric analyses. Densitometric analyses of immunoblots were performed using NIH Image (obtained from the NIH website: <http://rsb.info.nih.gov/nihi-image>). Results are presented as arbitrary densitometric units corrected for background intensity and normalized to the expression of β -actin, or as fold increases over levels in unstimulated cells.

Statistical analysis. All results are presented as the mean $\pm$ SEM and were tested statistically by Student's *t*-test and one- or two-way analysis of variance (ANOVA) with Tukey's post-hoc test, as appropriate, using commercially available SAS v9.1.3 software. Results were considered to be statistically significant at a probability of <0.05 .

RESULTS AND DISCUSSION

NOD2 expression is required for maximal expression of select inflammatory mediators by primary osteoblasts following *in vitro* *Salmonella* infection

Osteomyelitis is a refractory bone disease and can be recurrent with attacks occurring following treatment even when causative organisms can no longer be isolated from sites of infection (as discussed by Marriott *et al.*, 2007). An explanation for these phenomena may lie in the ability of the causative agents of osteomyelitis to invade and persist within resident bone cells. Internalization may provide a

means of protection against neutrophil and antibody-mediated immune responses, and may complicate treatment of bone infections by limiting exposure to antibiotics. *Salmonella* is a widely studied invasive bacterial pathogen, and we have previously demonstrated the ability of this bacterium to invade and persist within osteoblasts (Bost *et al.*, 2001). Such a finding is not surprising since *Salmonella* is well known for its ability to invade non-leukocytic cells (Ohl & Miller, 2001). The recent description of the NLR family of cytosolic proteins that can function as pattern recognition receptors for bacterial motifs has provided a potential mechanism by which both immune and non-immune cell types can perceive intracellular bacterial pathogens (as reviewed by Ting & Davis, 2005; Strober *et al.*, 2006). We have previously described the expression of several NLR family members, including CIITA and NLRP3, in osteoblasts (Schrum *et al.*, 2003; McCall *et al.*, 2008). Importantly, we have also demonstrated that these cells can express NOD2 (Marriott *et al.*, 2005), a NLR that can recognize a minimal bacterial peptidoglycan motif that is found in both Gram-positive and Gram-negative organisms (Girardin *et al.*, 2003; Inohara *et al.*, 2003).

While distinct NOD2-mediated responses to intracellular bacteria have been described in immune cells including macrophages (Kobayashi *et al.*, 2005; Leber *et al.*, 2008), in the present study we have performed experiments using isolated primary cultures of osteoblasts derived from NOD2^{+/+} and NOD2^{-/-} animals to determine whether this intracellular pattern recognition receptor similarly mediates inflammatory immune responses of these resident bone cells to *Salmonella*. As shown in Fig. 1(a), NOD2^{-/-} derived osteoblasts produce significantly lower levels of IL-6 at 12 h following *Salmonella* infection than cells from NOD2^{+/+} animals. Interestingly, our data suggest the presence of redundant mechanisms underlying perception of *Salmonella* by osteoblasts as the differences in the maximal production of IL-6 in the absence or the presence of NOD2 could be overcome when cells were exposed to high bacterial numbers (Fig. 1a). The importance of NOD2 expression in *Salmonella*-induced osteoblast immune responses was not restricted to IL-6 as the production of a second key inflammatory cytokine, IL-12p70, was similarly lower in the absence of NOD2 at 24 h post-infection (Fig. 1b). However, NOD2 does not appear to be important for all osteoblast immune responses to this invasive bacterial pathogen as evidenced by the absence of an effect of NOD2 deficiency on the production of the inflammatory chemokine MCP-1 at 12 h (data not shown) or 24 h (Fig. 1c) post-infection.

Yang *et al.* (2005) have previously suggested that NOD2-mediated signalling can exacerbate TLR-induced increases in the level of mRNA encoding the osteoclastogenic factor RANKL in osteoblasts. We have therefore determined whether NOD2 deficiency affects RANKL production by osteoblasts following *Salmonella* challenge. In the present

Fig. 1. NOD2 expression is required for maximal expression of select inflammatory mediators by primary osteoblasts following *in vitro* *Salmonella* infection. Cells (2×10^6) derived from wild-type (NOD2^{+/+}; black bars) or NOD2-deficient (NOD2^{-/-}; white bars) mice were untreated (0) or exposed to *Salmonella* (1:1, 3:1, 10:1 and 30:1 bacteria to each osteoblast). At 12 (a; $n=7$) or 24 (b, c; m.o.i. of 10:1; $n=3$) h post-infection, culture supernatants were isolated and levels of IL-6, IL-12p70 and MCP-1 were assessed by specific capture ELISA. Asterisks indicate significant differences between similarly treated NOD2^{+/+} and NOD2^{-/-} derived cells ($P < 0.05$).

study, we have found no significant difference in bacterially induced RANKL secretion in the presence or absence of NOD2 expression (31.5 ± 3.3 pg ml⁻¹ in NOD2^{+/+} derived cells versus 32.2 ± 0.1 pg ml⁻¹ in NOD2^{-/-} derived cells following *Salmonella* challenge at an m.o.i. of 10:1; $n=4$). Taken together, these data indicate that NOD2 plays a significant role in the detection of an invasive pathogen by osteoblasts and the subsequent production of select cytokines that could promote protective immune responses or progressive inflammatory damage.

Production of select inflammatory mediators by osteoblasts following exposure to *Staphylococcus aureus* is elevated in the absence of NOD2

We have compared our findings obtained with this invasive bacterial species with those obtained with a second pathogen of bone tissue, *Staphylococcus aureus*, an organism that is passively internalized by osteoblasts (Ellington *et al.*, 1999). As shown in Fig. 2(a), *in vitro* exposure to *Staphylococcus aureus* is a potent stimulus for

Fig. 2. Expression of select inflammatory mediators by osteoblasts following *in vitro* exposure to *Staphylococcus aureus* is elevated in the absence of NOD2. Cells (2×10^6) derived from wild-type (NOD2^{+/+}; black bars) or NOD2-deficient (NOD2^{-/-}; white bars) mice were untreated (0) or exposed to *Staphylococcus aureus* (75:1, 100:1 and 250:1 bacteria to each osteoblast). At 12 (a) or 24 (b–d) h post-challenge, culture supernatants were isolated and levels of IL-6, IL-12p70, IP-10 and MCP-1 were assessed by specific capture ELISA. Asterisks indicate significant differences between similarly treated NOD2^{+/+} and NOD2^{-/-} derived cells ($n=3$; $P<0.05$).

IL-6 production by osteoblasts at 12 h following challenge. Interestingly, and in contrast to the responses to *Salmonella*, osteoblast production of IL-6 is elevated in cells isolated from NOD2^{-/-} mice relative to wild-type-derived cells. Again, this effect was not restricted to IL-6 and *Staphylococcus aureus*-induced production of IL-12p70 (Fig. 2b) and IP-10 (Fig. 2c) was similarly higher in NOD2^{-/-} cell cultures at 24 h following bacterial challenge. Consistent with our findings with *Salmonella*, the influence of this NLR appears to be selective as there were no significant differences in the production of MCP-1 by these cells in the absence or presence of NOD2 expression at 12 h (data not shown) or 24 h (Fig. 2d) following bacterial challenge, or in the level of production of RANKL (34.3 ± 1.7 pg ml⁻¹ in NOD2^{+/+} derived cells versus 32.6 ± 1.1 pg ml⁻¹ in NOD2^{-/-} derived cells following *Staphylococcus aureus* challenge at an m.o.i. of 75:1; $n=4$).

It is conceivable that the absence of NOD2 leads to a higher bacterial burden within *Staphylococcus aureus*-infected osteoblasts thereby providing a more potent stimulus for inflammatory mediator production. To investigate this possibility, we have assessed the bacterial burden of

NOD2^{+/+} and NOD2^{-/-} derived cell cultures following challenge with GFP-expressing *Staphylococcus aureus*. As shown in Fig. 3, NOD2^{-/-} derived cells demonstrated a modest but statistically significant ($P<0.05$; $n=5$) increase in the proportion of osteoblasts harbouring bacteria at 24 h following *in vitro* challenge than similarly challenged wild-type cells. Another possibility is that the presence or absence of NOD2 could influence osteoblast survival following infection. However, no differences in the proportion of cells undergoing apoptotic volume decreases were observed between bacterially challenged NOD2^{+/+} and NOD2^{-/-} cells as determined by flow cytometric analysis (data not shown). Perhaps more importantly, differences in bacterial burden or changes in lethality fail to explain why the production of certain inflammatory mediators such as IL-6, and IL-12p70 and IP-10 is either promoted or decreased in the presence or absence of NOD2 expression, while the production of other soluble proteins including MCP-1 and RANKL is unaltered.

Inflammatory cytokine production by osteoblasts exposed to non-invasive *Salmonella* is elevated in the absence of NOD2

Alternatively, the apparent differences in NOD2-mediated cell responses to *Salmonella* and *Staphylococcus aureus* could stem from the dissimilar mechanisms by which these organisms are internalized by osteoblasts. While both of these organisms are facultative intracellular pathogens of osteoblasts, *Salmonella* utilizes a type III secretion system to actively invade host cells (Ohl & Miller, 2001), while the

Fig. 3. Osteoblast cultures derived from NOD2-deficient animals demonstrate a somewhat higher bacterial burden following exposure to *Staphylococcus aureus* than similarly challenged wild-type derived cells. Cells (2×10^6) derived from wild-type (NOD2^{+/+}; black bars) or NOD2-deficient (NOD2^{-/-}; white bars) mice were untreated (0) or exposed to GFP-expressing *Staphylococcus aureus* (25:1, 75:1 and 250:1 bacteria to each osteoblast). At 24 h post-treatment, cells were analysed for the proportion of cells demonstrating GFP-associated fluorescence as determined by flow cytometry. Asterisks indicate significant differences between similarly treated NOD2^{+/+} and NOD2^{-/-} derived cells ($n=5$; $P<0.05$).

entry of *Staphylococcus aureus* into osteoblasts is passive (Ellington *et al.*, 1999). To test this hypothesis, we examined cell responses to heat-killed pathogenic *Salmonella* or a non-invasive *Salmonella* strain in the absence or presence of NOD2 expression. As shown in Fig. 4, and consistent with our previous studies (Bost *et al.*, 1999; Marriott *et al.*, 2005), IL-6 production by osteoblasts

exposed to either killed invasive *Salmonella* (Fig. 4a) or SB136, an invasion-deficient strain of *Salmonella* (Fig. 4b), is markedly lower than that seen following infection with an invasive strain (Fig. 2a). Interestingly, and in contrast to IL-6 levels following infection with viable invasive *Salmonella*, osteoblast production of IL-6 is elevated in cells derived from NOD2^{-/-} mice following exposure to killed *Salmonella* (Fig. 4a) or SB136 (Fig. 4b) relative to wild-type derived cells in a similar manner to that seen following *Staphylococcus aureus* challenge. Taken together, these data suggest that NOD2 differentially influences osteoblast immune responses to actively invasive versus passively internalized bacteria.

Inflammatory cytokine production by osteoblasts exposed to select TLR ligands is elevated in the absence of NOD2

These findings suggest that NOD2 mediates osteoblast immune responses to actively invasive bacterial pathogens, but attenuates responses to external/passively internalized organisms. To further test this hypothesis, we have investigated cell responses to specific ligands for various TLRs in the absence or presence of NOD2 expression. As shown in Fig. 5, exposure of osteoblasts to LPS, flagellin or an activating oligonucleotide (CpG), which are ligands for TLR4, TLR5 and TLR9, respectively, induces the expression of IL-6, while MDP alone fails to induce inflammatory cytokine production by osteoblasts (data not shown) as previously reported by our laboratory (Marriott *et al.*, 2005). Similar to our results with *Staphylococcus aureus* and non-invasive *Salmonella*, IL-6 production by LPS- or flagellin-challenged osteoblasts was higher in cells derived from NOD2^{-/-} mice than wild-type-derived osteoblasts (Fig. 5a, b). Interestingly, this phenomenon appears to be restricted to cell surface TLR types as IL-6 responses to a specific ligand for TLR9, an endosomal pattern recognition receptor, were not significantly different between cells derived from NOD2^{+/+} and NOD2^{-/-} animals (Fig. 5c).

Consistent with this finding, we have confirmed that the NOD2 ligand, MDP, can augment the expression of IL-6 elicited by either LPS or flagellin but not CpG, and it is important to note that MDP-mediated augmentation of LPS and flagellin-induced IL-6 production is not apparent in similarly treated cells derived from NOD2^{-/-} mice (Fig. 5). These findings are consistent with the studies of Netea *et al.* (2005) demonstrating that MDP can modulate TLR2- and TLR4-mediated cytokine responses in human mononuclear cells, but not those initiated by TLR9. Taken together, these data suggest that NOD2 can limit cell surface TLR-mediated inflammatory cytokine responses in osteoblasts, although it remains to be determined whether such an effect represents a constitutive activity of NOD2 in its unbound state or reflects the actions of an as yet undetermined endogenous ligand.

Fig. 4. Expression of IL-6 by osteoblasts following *in vitro* exposure to heat-killed *Salmonella* or a non-invasive *Salmonella* strain (SB136) is elevated in the absence of NOD2. (a) Cells (2×10^6) derived from wild-type (NOD2^{+/+}; black bars) or NOD2-deficient (NOD2^{-/-}; white bars) mice were untreated (0) or exposed to heat-killed *Salmonella* (30:1 bacteria to each osteoblast) in the presence or absence of MDP ($5 \mu\text{g ml}^{-1}$) for 24 h. (b) Cells (2×10^6) derived from NOD2^{+/+} (black bars) or NOD2^{-/-} mice (white bars) were untreated (0) or exposed to SB136 (3:1, 10:1 and 30:1 bacteria to each osteoblast) for 12 h. Levels of IL-6 in culture supernatants were assessed by specific capture ELISA. Asterisks indicate significant differences between similarly treated NOD2^{+/+} and NOD2^{-/-} derived cells while the dagger symbol indicates a significant difference between groups in the absence or presence of MDP ($n=3$; $P<0.05$).

Fig. 5. Expression of IL-6 by osteoblasts following *in vitro* exposure to select TLR ligands is elevated in the absence of NOD2. Cells (2×10^6) derived from wild-type (NOD2^{+/+}; black bars) or NOD2-deficient (NOD2^{-/-}; white bars) mice were untreated (0) or exposed to LPS (100 ng ml^{-1}), flagellin (FLG; 100 ng ml^{-1}) or an activating oligonucleotide (CpG; $1 \text{ } \mu\text{g ml}^{-1}$) in the absence or presence of MDP ($5 \text{ } \mu\text{g ml}^{-1}$). At 12 (a) or 24 (b, c) h post-challenge, culture supernatants were isolated and levels of IL-6 were assessed by specific capture ELISA. Asterisks indicate significant differences between similarly treated NOD2^{+/+} and NOD2^{-/-} derived cells while the dagger symbol indicates a significant difference between groups in the absence or presence of MDP ($n=3$; $P<0.05$).

***Salmonella*, but not *Staphylococcus aureus*, elicits activation of a NOD2-associated downstream effector molecule**

Finally, we have investigated the relative ability of *Salmonella* and *Staphylococcus aureus* to elicit activation of NOD2-associated downstream effector molecules. Rip2 kinase is a critical downstream effector molecule in the NOD2 signalling pathway in other cell types (Chin *et al.*, 2002; Kobayashi *et al.*, 2002). As shown in the representative immunoblot of three separate experiments in Fig. 6(a), we have used co-immunoprecipitation techniques to demonstrate that infection of osteoblasts with *Salmonella* elicits association of NOD2 with Rip2 kinase. *Salmonella*-infected osteoblasts show an almost eightfold induction at an m.o.i. of 10:1 bacteria to each osteoblast over the low level of association observed in uninfected cells. In contrast, exposure of osteoblasts to *Staphylococcus aureus* fails to induce appreciable NOD2/Rip2 association (Fig. 6a) despite being able to elicit a significant increase in NOD2 protein expression (Fig. 6b).

While Rip2 kinase activation has been widely shown to be a critical step in the initiation of cellular responses following NOD2 ligation, at least one report has suggested that NOD2 can associate with a second adaptor/effector molecule, GRIM-19 (gene associated with retinoid-interferon-induced mortality-19) (Barnich *et al.*, 2005). To determine whether this molecule has the potential to play a role in NOD2-mediated osteoblast responses to bacterial challenge, we have investigated the expression of GRIM-19 in this cell type at rest and following *Salmonella* or *Staphylococcus aureus* infection. As shown in the representative immunoblot of three separate experiments in Fig. 6(c), osteoblasts fail to express detectable levels of this adaptor molecule constitutively. Importantly, while GRIM-19 protein expression was induced in cells at 24 h following *Staphylococcus aureus* or *Salmonella* exposure, osteoblasts failed to express detectable levels of this molecule up to 2 h following bacterial challenge (Fig. 6c). As such, it appears unlikely that GRIM-19 plays a significant role in rapid NOD2-mediated osteoblast cell responses to bacterial pathogens.

Together, these findings suggest that the inflammatory immune responses of osteoblasts to invasive bacterial pathogens such as *Salmonella*, but not passively internalized bacterial species including *Staphylococcus aureus*, are mediated at least in part via NOD2 activation of Rip2 kinase.

Conclusions

The present study supports and extends our earlier description of constitutive NOD2 expression in osteoblasts and the ability of bacterial pathogens and/or their components to upregulate such expression (Marriott *et al.*, 2005). Furthermore, the present findings are consistent with the recent work of other investigators describing the

Fig. 6. *Salmonella* elicits more robust activation of NOD2-associated downstream effector molecules in osteoblasts than *Staphylococcus aureus*. (a) Osteoblasts (2×10^6 per well) were untreated (0) or exposed to *Staphylococcus aureus* (75:1 and 250:1 bacteria to each osteoblast) or *Salmonella* (3:1, 10:1 and 30:1 bacteria to each osteoblast). After 2 h, protein isolates were immunoprecipitated with antibodies directed against NOD2 and subsequently analysed by denaturing immunoblot for the presence of Rip2 kinase. As a negative control, samples exposed to the immunoprecipitation procedure in the absence of antibodies against Rip2 kinase and run on the same immunoblots are shown (ProA). As a positive control, samples from thymus tissue were subjected to the same procedure and run on the same immunoblot (+). To confirm equal loading in each lane, the expression of an irrelevant protein present in the immunoprecipitated material is shown (IRR). (b) Cells (2×10^6 per well) were untreated (0) or exposed to *Staphylococcus aureus* (25:1, 75:1 and 250:1 bacteria to each osteoblast) for 12 h prior to whole-cell protein isolation and immunoblot analysis for NOD2 expression. (c) Cells (2×10^6 per well) were untreated (0) or exposed to *Staphylococcus aureus* (25:1, 75:1 and 250:1 bacteria to each osteoblast) or *Salmonella* (3:1, 10:1 and 30:1 bacteria to each osteoblast) for 2 or 24 h prior to protein isolation and immunoblot analysis for GRIM-19 expression. Bar graphs showing densitometric analysis of each representative immunoblot from three separate experiments normalized to the expression of an irrelevant protein (IRR) or β -actin are shown.

expression of NOD2 in primary murine osteoblasts (Yang *et al.*, 2005), and osteoblast and cementoblast-like cell lines (Nemoto *et al.*, 2008). We have previously demonstrated that a NOD2 ligand can augment the production of IL-6 by stimulated osteoblasts (Marriott *et al.*, 2005) and similar results have been obtained in cementoblasts (Nemoto *et al.*, 2008). The data presented in this study support the functional expression of this NLR in primary osteoblasts and suggest that NOD2 plays differential roles in the regulation of immune responses by this cell type. As might be predicted, this intracellular pattern recognition receptor

appears to play an important role in the generation of osteoblast inflammatory responses to an invasive bacterial species. However, NOD2 also appears to play an alternative function in osteoblasts, namely the suppression of inflammatory mediator production by extracellular and/or passively internalized bacterial pathogens. At the present time, the reasons for this unexpected second function are unclear but it is tempting to speculate that such suppression might represent a protective host mechanism to limit infection-associated inflammatory damage in bone tissue. The idea that NLRs can also suppress innate immune responses is

supported by the recent demonstration that NOD1 deficiency elevates acute inflammation in a murine model of arthritis (Joosten *et al.*, 2008). While further study will be required to resolve this issue, it is apparent that NOD2 plays a significant role in the control of the innate immune functions of osteoblasts.

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