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Osteoblasts Express NLRP3, a Nucleotide-Binding Domain and Leucine-Rich Repeat Region Containing Receptor Implicated in Bacterially Induced Cell Death

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ABSTRACT: Bacterially induced osteoblast apoptosis may be a major contributor to bone loss during osteomyelitis. We provide evidence for the functional expression in osteoblasts of NLRP3, a member of the NLR family of cytosolic receptors that has been implicated in the initiation of programmed cell death.

Introduction: Osteoblasts undergo apoptosis after exposure to intracellular bacterial pathogens commonly associated with osteomyelitis. Death of this bone-forming cell type, in conjunction with increased numbers and activity of osteoclasts, may underlie the destruction of bone tissue at sites of bacterial infection. To date, the mechanisms responsible for bacterially induced apoptotic osteoblast cell death have not been resolved.
Materials and Methods: We used flow cytometric techniques to determine whether intracellular invasion is needed for maximal apoptotic cell death in primary osteoblasts after challenge with *Salmonella enterica*. In addition, we used real-time PCR and immunoblot analyses to assess osteoblast expression of members of the nucleotide-binding domain leucine-rich repeat region–containing family of intracellular receptors (NLRs) that have been predicted to be involved in the induction of programmed cell death. Furthermore, we have used co-immunoprecipitation and siRNA techniques to confirm the functionality of such sensors in this cell type.
Results: In this study, we showed that invasion of osteoblasts by *Salmonella* is necessary for maximal induction of apoptosis. We showed that murine and human osteoblasts express NLRP3 (previously known as CIAS1, cryopyrin, PYPAF1, or NALP3) but not NLRC4 (IPAF) and showed that the level of expression of this cytosolic receptor is modulated after bacterial challenge. We showed that osteoblasts express ASC, an adaptor molecule for NLRP3, and that these molecules associate after *Salmonella* infection. In addition, we showed that a reduction in the expression of NLRP3 attenuates *Salmonella*-induced reductions in the activity of an anti-apoptotic transcription factor in osteoblasts. Furthermore, we showed that NLRP3 expression is needed for caspase-1 activation and maximal induction of apoptosis in osteoblasts after infection with *Salmonella*.
Conclusions: The functional expression of NLRP3 in osteoblasts provides a potential mechanism underlying apoptotic cell death of this cell type after challenge with intracellular bacterial pathogens and may be a significant contributory factor to bone loss at sites of infection.
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Key words: osteoblasts, apoptosis, inflammation, modeling and remodeling, rodent

INTRODUCTION

Recent studies from our laboratory have shown that osteoblasts exposed to the principle causative agents of osteomyelitis express an array of soluble and cell surface molecules that have the potential to promote immune responses and osteoclast activity at sites of bone infection.[1] Interestingly, our laboratory and others have shown that osteoblasts undergo apoptosis after exposure to intracellular bacterial pathogens commonly associated with osteomyelitis.[2,3] As such, inflammatory bone loss during osteomyelitis may result from increased numbers and activity of bone-resorbing osteoclasts at sites of bacterial infection and the elimination of the cells responsible for new bone matrix deposition. Whereas a proportion of this bacterially induced cell death seems to be attributable to the autocrine and/or paracrine actions of osteoblast-derived TNF-related apoptosis inducing ligand (TRAIL),[3,4] the mechanisms underlying the majority of the observed apoptosis remain unclear.

The demonstration that osteoblasts express members of the Toll-like family of cell surface pattern recognition re-

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ceptors may represent a means by which these cells recognize bacterial pathogens.[5–7] However, our previous studies have shown that bacterial products, inactivated bacteria, and invasion defective strains are far less potent than viable wildtype bacteria at eliciting immune molecule production by osteoblasts.[6,8–10] Furthermore, Thammasitboon et al.[11] have shown that bacterial components such as lipopolysaccharide (LPS) fail to elicit apoptosis in osteoblasts. Taken together, these studies suggest that bacterial invasion is required to evoke maximal responses by these cells. This phenomenon might be explained by our recent demonstration that osteoblasts express NOD1 and NOD2,[12] two members of the nucleotide-binding domain and leucine-rich repeat region containing family of receptor proteins (NLRs) that can serve as intracellular sensors for bacterial peptidoglycans and initiate pro-inflammatory mediator production.[13,14]

Although NOD1 and NOD2 can initiate, augment, or reduce inflammatory mediator production by a variety of cell types,[15] these cytosolic proteins are not widely recognized to participate in the induction of apoptosis. In contrast, two other receptors, NLR family CARD domain containing 4 (NLRC4; previously known as Ipaf, Card12, or CLAN) and NLR family pyrin domain containing 3 (NLRP3; previously known as CIAS1, cryopyrin, PYPAF1, or NALP3) have been implicated in the induction of cell death in response to bacteria and/or their components.[16–19] Both of these molecules can associate with an adaptor protein, apoptosis-associated speck-like protein (ASC) to elicit caspase-1 and caspase-8 activation,[20,21] enzymes that show elevated activity in osteoblasts after bacterial challenge.[3,22] In this study, we determined the relative importance of bacterial invasion in the induction of osteoblast cell death by the intracellular pathogen, *Salmonella*. Furthermore, we studied the functional expression of NLRP3 and NLRC4 in resting and *Salmonella*-exposed cultures of primary murine and human osteoblasts in an attempt to identify the mechanisms linking intracellular invasion to bacterially induced cell death.

MATERIALS AND METHODS

NLRP3-deficient mice

NLRP3-deficient mice, backcrossed for six generations onto a C57BL/6 background, have previously been characterized[17] and were provided to Dr Ting by Millennium.

Isolation and characterization of murine osteoblasts

Primary osteoblast cell cultures were prepared from BALB/c, C57BL/6, and NLRP3-deficient mouse neonate calvariae by sequential collagenase-protease digestion as previously described by our laboratory.[3,4,6–10,12,22–24] 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 PTH-cAMP.[4] All experiments were performed on osteoblasts grown to the point of confluency in 6-well plates or culture flasks, and comparisons were made between the treatment groups in each experimental series to insure equal cell numbers.

Normal human osteoblast cultures

Normal human osteoblast cultures (Clonetics, San Diego, CA, USA) were purchased and propagated as previously described by our laboratory.[8–10,23,24] These commercially available cells have previously been characterized as being authentic osteoblasts.[25]

Exposure of cultured osteoblasts to Salmonella

Osteoblasts were exposed to bacteria as previously described by our laboratory.[6–9,12,22] Confluent cell layers of cells were exposed to the pathogenic *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. After the infection period, cell cultures were washed with PBS and incubated in growth medium with 25 μg/ml gentamicin to kill remaining extracellular bacteria. *Salmonella enterica serovar typhimurium* strain SB136 is an invasion defective mutant strain of *Salmonella enterica serovar typhimurium* strain SB300, because of a mutation in the *invA* gene that prevents *Salmonella* pathogenicity island-1 type III secretion mechanisms. This attenuated bacterial strain was a kind gift from Dr Michael C. Hudson at The University of North Carolina at Charlotte.

Measurement of apoptosis by flow cytometry

Proportions of cells undergoing apoptosis were determined by monitoring apoptotic volume decreases as changes in forward and side scatter characteristics during flow cytometry or by annexin V staining as previously described by our laboratory.[3] Briefly, infected and uninfected cells were incubated with 0.025% trypsin-0.01% EDTA for 10 min at 37°C and collected by centrifugation. Cells were washed with PBS and stained with FITC-conjugated annexin V (BD Biosciences, San Diego, CA, USA) before analysis using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). In some experiments, cells were dual stained with propidium iodide (0.3 μg/ml) to distinguish necrotic (dual positive) from apoptotic (annexin V positive) populations. In each experiment, a minimum of 20,000 cells were counted.

DNA laddering

DNA laddering was performed using a Quick Apoptotic DNA Ladder Detection kit according to the directions provided by the manufacturer (BioVision, Mountain View, CA, USA).

Isolation of RNA and PCR

Poly(A)+ RNA was isolated from osteoblasts and reverse transcribed as previously described.[3,4,6–10,12,22] Real-time PCR was performed on the reverse transcribed cDNA product to determine the expression of mRNA encoding NLRP3, NLRC4, ASC, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) using a SYBR Green approach essentially as described previously.[26,27] Positive and negative strand PCR primers used, respectively, were TG-CAGAAGACTGACGTCTCC and CGTACAGGCAG-

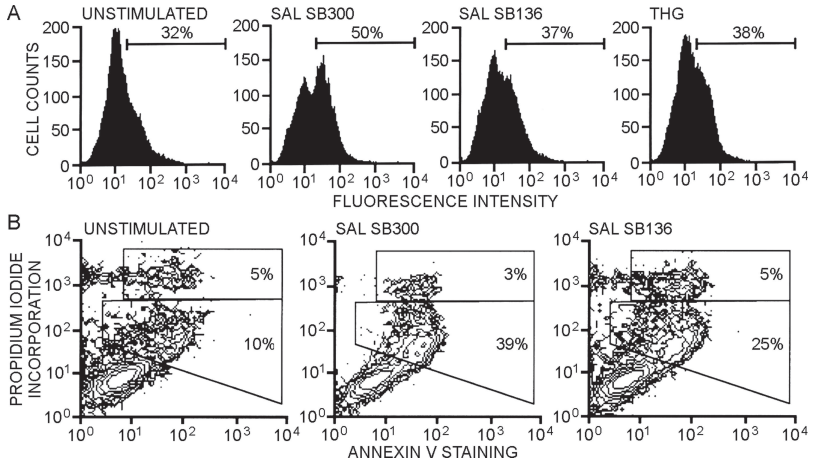

FIG. 1. Invasion-defective *Salmonella* strains are less effective at inducing apoptosis in osteoblasts. (A) Cells (2×10^6 per well) were unstimulated or exposed to either *Salmonella* SB300 or SB136 (m.o.i. of 10 bacteria per cell) for 24 h. Cells were stained with annexin V-FITC and analyzed by flow cytometry. For comparison purposes, cells were treated with the thapsigargin (THG; 1 μM). (B) Osteoblasts (2×10^6 per well) were unstimulated or exposed to either *Salmonella* SB300 or SB136 (m.o.i. of 10 bacteria per cell) for 24 h. Cells were dual stained with annexin V-FITC and propidium iodide and analyzed by flow cytometry.

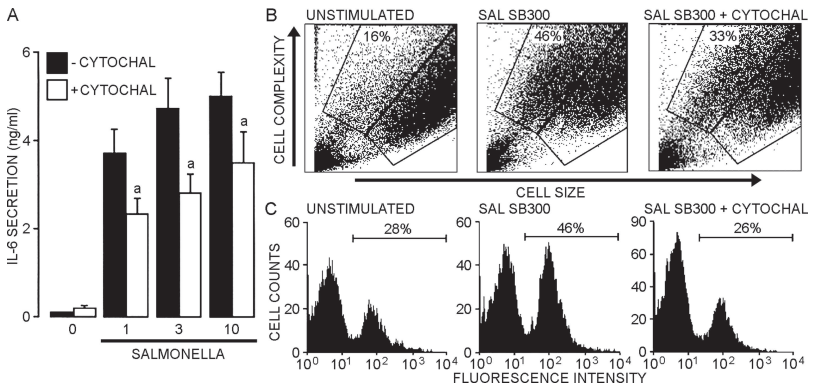

FIG. 2. Bacterial uptake is required for maximal levels of *Salmonella*-induced apoptosis in osteoblasts. Cells (2×10^6 per well) were unstimulated or exposed to either *Salmonella* SB300 (m.o.i. of 1, 3, and 10 bacteria per cell) in the absence or presence of cytochalasin (5 μg/ml) for 24 h. (A) Culture supernatants were assayed for the presence of IL-6 by capture ELISA. (B) The percentage of cells showing an apoptotic volume decrease after challenge with *Salmonella* SB300 at an m.o.i. of three bacteria per cell was assessed by flow cytometry. (C) Cells after challenge with *Salmonella* SB300 at an m.o.i. of three bacteria per cell were stained with annexin V-FITC and analyzed by flow cytometry. [a]Significant difference between IL-6 production in the presence and absence of cytochalasin ($p < 0.05$).

TAGAACAGTTC to amplify mRNA encoding murine NLRP3, AGCATGAACGAGGACGTCCT and GAAGTACAATGCGCTTGCACA to amplify mRNA encoding murine NLRC4, GCAGCTGACTTCCTGGTCT and ACGACTCCAGATAGTAGCTGACA to amplify mRNA encoding murine ASC, and TCCTCCTGGTCGGCGCCT and CCATGTCGCGCAGCACGTT to amplify mRNA encoding human ASC. PCR primers were derived from the published sequences of murine NLRP3,[28] NLRC4,[29] and ASC (accession number: AB032249) and human ASC.[30] All primers were designed by using Oligo 4.0 primer analysis software (National Biosciences, Plymouth, MA, USA) based on their location in different exons of the genomic sequences for NLRP3, NLRC4, and ASC in addition to their lack of significant homology to other published sequences as determined by NCBI Mega BLAST search engine (www.ncbi.nlm.gov/blast).

A Roche Light Cycler 2.0 was used to perform all PCR reactions, and the expression of NLRP3, NLRC4, and ASC products was normalized to the level of expression of mRNA encoding G3PDH determined in parallel PCR reactions. The identities of the PCR amplified fragments were verified by size comparison with DNA standards (Promega, Madison, WI, USA) and by direct DNA sequencing of representative fragments (Davis Sequencing, Davis, CA, USA).

Quantification of IL-6 secretion in osteoblast culture supernatants

Specific capture ELISAs were performed to quantify IL-6 secretion by osteoblasts as described previously by our laboratory.[7,10,12]

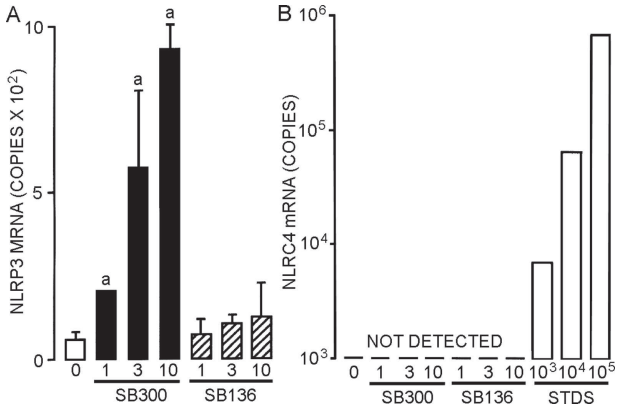

FIG. 3. Expression of mRNA encoding NLRP3 and NLRC4 in resting and *Salmonella*-infected murine osteoblasts. Osteoblasts (2×10^6 per well) were untreated (0) or exposed to *Salmonella* SB300 or SB136 (m.o.i. of 1, 3, and 10 bacteria per cell). RNA was isolated at 4 h after treatment, and real-time PCR was performed for the presence of mRNA encoding (A) NLRP3 or (B) NLRC4. Expression shown is normalized to the expression of mRNA encoding G3PDH quantified in parallel real-time PCR reactions. For comparison purposes, amplification of known quantities of PCR products is shown (STDS). [a]Significant difference in mRNA expression between uninfected and bacterially challenged osteoblasts ($p < 0.05$).

Immunoblot analysis of NLRP3, NLRC4, and ASC

Immunoblot analyses for the presence of NLRP3, NLRC4, and ASC in osteoblasts were performed as described previously by our laboratory.[3,4,6,7,23,24] The primary antibodies used were a commercially available affinity purified rabbit polyclonal antibody directed against human and murine NLR4C (Alpha Diagnostic International), a rabbit polyclonal antibody directed against human and murine ASC (Chemicon International, Temecula, CA, USA), and a rabbit polyclonal anti-serum directed against the amino terminal of human NLRP3 that is cross-reactive with murine NLRP3. This anti-serum was developed in Dr Ting's laboratory, and its specificity has previously been documented.[31]

Immunoblot analysis for NF-kB p65 (RelA) translocation and caspase-1 activation

Activation of the transcriptional activator NF-kB was assessed by nuclear translocation of the p65 subunit (RelA) of NF-kB by immunoblot analysis of nuclear protein isolates as described previously by our laboratory.[32] Immunoblot analysis of caspase-1 activation was performed on whole cell protein isolates using a commercially available goat polyclonal antibody directed against mouse cleaved caspase-1 p10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Co-immunoprecipitation of NLRP3 with ASC

Co-immunoprecipitation was performed essentially as described previously by Clark et al.[33] Briefly, cells (2×10^6) were washed with ice-cold PBS and lysed at 4°C for 2 h in Tris buffered saline with EDTA (150 mM NaCl, 5 mM EDTA, 20 mM Tris, pH 7.5) plus 1% Brij-97 (Sigma-Aldrich) and 10 units/ml aprotinin (Calbiochem, San Diego, CA, USA), 1 mM PMSF, and 1 μg/ml pepstatin A. The lysates were preincubated with protein A agarose beads (Pierce Endogen) for 1 h before incubation with protein A agarose beads-conjugated with antibodies directed against NLRP3 or ASC for 18 h at 4°C. The immunoprecipitated material was subsequently subjected to immunoblot analysis for the presence of ASC or NLRP3 as described above.

Small interfering RNA knockdown of NLRP3 expression

Knockdown of NLRP3 expression was achieved by transfecting osteoblasts with a combination of two commercially available small interfering RNAs (siRNAs) against this molecule (Cias1 Stealth Select RNAi MSS211224 and MSS211223; Invitrogen, Carlsbad, CA, USA) or a siRNA directed against an irrelevant RNA species to ensure the specificity of this knockdown. Osteoblasts were incubated with these siRNA in the presence of lipofectamine (200 pmol/ml; Invitrogen) for 30 min before removal and washing. At 24 h after transfection, knockdown of NLRP3 protein expression was confirmed by immunoblot, and these cells were used in the experiments described.

Densitometric analyses

Densitometric analyses of immunoblots 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 and normalized to the expression of β-actin or as fold increases over levels in unstimulated cells.

Statistical analysis

Results of these studies were tested statistically using Student's paired *t*-test or one-way ANOVA as appropriate using commercially available software (GraphPad Prism; GraphPad Software, San Diego, CA, USA). Fluorescence histograms were directly compared using Kolmogorov-Smirnov statistics using commercially available software (CellQuest; Becton Dickinson). Results were determined to be statistically significant when $p < 0.05$ was obtained.

RESULTS

Intracellular invasion is required for maximal Salmonella-induced apoptosis of primary osteoblasts

Our laboratory has previously shown that UV killed *Salmonella* and invasion defective strains of this bacterium elicit significantly lower inflammatory cytokine production by primary osteoblasts than viable wildtype *Salmonella*.[12] To begin to determine whether bacterial invasion is required from maximal induction of osteoblast apoptosis, primary murine cells were exposed to wildtype *Salmonella* enterica strain SB300 or the invasion defective strain SB136 and levels of apoptosis were assessed by annexin V staining. As shown in Fig. 1A, the annexin V-positive population was significantly elevated ($p < 0.05$) as early as 24 h after *Salmonella* SB300 exposure. Indeed, this induction was mark-

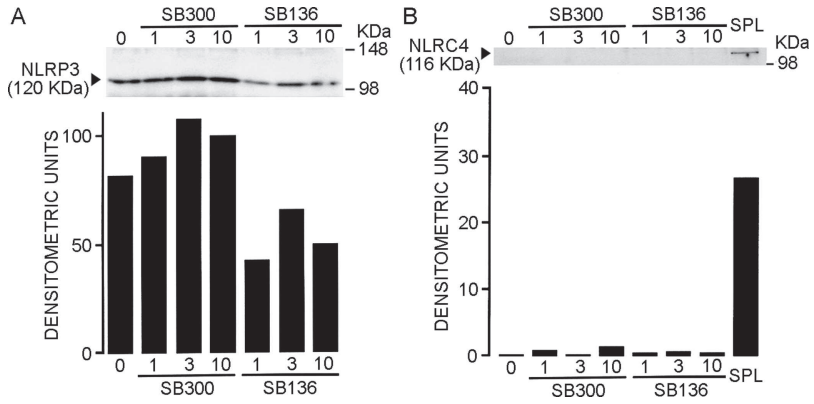

FIG. 4. Expression of NLRP3 protein but not NLRC4 in resting and *Salmonella*-infected murine osteoblasts. Cells (2×10^6 per well) were untreated (0) or exposed to *Salmonella* SB300 or SB136 (m.o.i. of 1, 3, and 10 bacteria per cell). After 24 h, protein isolates were subjected to immunoblot analysis for (A) NLRP3 or (B) NLRC4 expression. Representative immunoblots from four separate experiments are shown. Migration of protein standards of known size is indicated to the right. As a positive control for NLRC4 expression, a spleen total protein isolate was also analyzed (SPL). Below, densitometric analyses of the immunoblot protein bands is shown as arbitrary densitometric units corrected for background intensity and normalized to β-actin expression.

edly higher than that induced by an endosomal Ca^{2+}-ATPase inhibitor, thapsigargin (1 μM; Fig. 1A), or serum starving (data not shown) for the same time period. More importantly, exposure to SB136 resulted in a significantly lower percentage of annexin V-positive cells than the invasive strain ($p < 0.05$). This effect could not be explained on the basis of differences in numbers of necrotic cells because no significant differences were observed in the population of double positive cells stained with annexin V and propidium iodide to assess cell membrane integrity (3% and 5% in SB300 and SB136 treated, respectively) whereas significant differences were observed in annexin V-positive–propidium iodide-negative cells (39%, and 25% in SB300 and SB136 treated, respectively; Fig. 1B). Similarly, osteoblasts exposed to SB300 showed markedly higher levels of apoptotic cell death than SB136-treated cells when qualitatively assessed by DNA laddering (data not shown).

To further assess the relative importance of bacterial invasion in inducing osteoblast apoptosis, cells were exposed to wildtype bacteria in the presence or absence of the endocytosis inhibitor cytochalasin (5 μg/ml). As shown in Fig. 2A, cytochalasin significantly inhibited IL-6 production by osteoblasts exposed to *Salmonella*. Importantly, this agent also significantly reduced the proportion of cell showing an apoptotic volume decrease as shown in the representative experiment shown in Fig. 2B, with a decrease in this population after challenge with *Salmonella* SB300 at an m.o.i. of three bacteria per cell from 34.0 ± 6.0% to 23.5 ± 2.6% ($p < 0.05$; $n = 3$) in the absence or presence of cytochalasin, respectively. Furthermore, cytochalasin diminished the annexin V-positive population after bacterial challenge (46%, and 26% in SB300 and SB136 treated, respectively; $p < 0.05$; Fig. 2C). Taken together, these data indicate that invasion is needed for maximal induction of apoptosis in osteoblasts after bacterial challenge.

Primary osteoblasts express NLRP3 but not NLRC4

To begin to determine the mechanisms underlying osteoblast apoptosis after *Salmonella* invasion, we studied whether these cells express mRNA encoding either NLRP3 or NLRC4. Murine osteoblasts were untreated or exposed to *Salmonella* SB300 or SB136 at varying numbers of bacteria to cells before isolation of RNA at 4 h after infection. As shown in Fig. 3A, resting osteoblasts expressed low but detectable levels of mRNA encoding NLRP3 as determined by quantitative real-time PCR. Interestingly, invasive but not invasion-defective strains of *Salmonella* elicited rapid and marked increases in NLRP3 mRNA expression in a dose-dependent manner (Fig. 3A). The identity of the PCR products was confirmed by comparison of the migration of products with those of known size by agarose gel electrophoresis and by direct sequencing (Davis Sequencing). In contrast, mRNA encoding NLRC4 was not detectable in either resting or bacterially challenged osteoblasts (Fig. 3B).

To confirm that the expression of mRNA encoding NLRP3 results in protein expression, we performed immunoblot analyses using antibodies previously developed to show specificity for NLRP3.[31] As shown in Fig. 4A, unstimulated murine osteoblasts express robust levels of a protein that migrated to the predicted molecular size of NLRP3 (120 kDa). Exposure of murine cells to invasive *Salmonella* elicited only modest elevations in total cellular NLRP3 content at 24 h after infection (Fig. 4A) despite the marked elevations in mRNA (Fig. 3A). Interestingly, and in contrast to SB300, challenge of osteoblasts with noninvasive SB136 *Salmonella* elicited significant ($p < 0.05$) reductions in NLRP3 expression by almost 50% (Fig. 4A). This effect was similarly observed in primary human osteoblasts, in which robust NLRP3 expression was reduced after exposure to invasion defective *Salmonella* (data not shown).

To confirm our real-time PCR data indicating that osteoblasts do not express NLRC4 either constitutively or after bacterial challenge, protein isolates were probed for the presence of this NLR by immunoblot analysis. As shown in Fig. 4B, no significant expression of NLRC4 was detectable

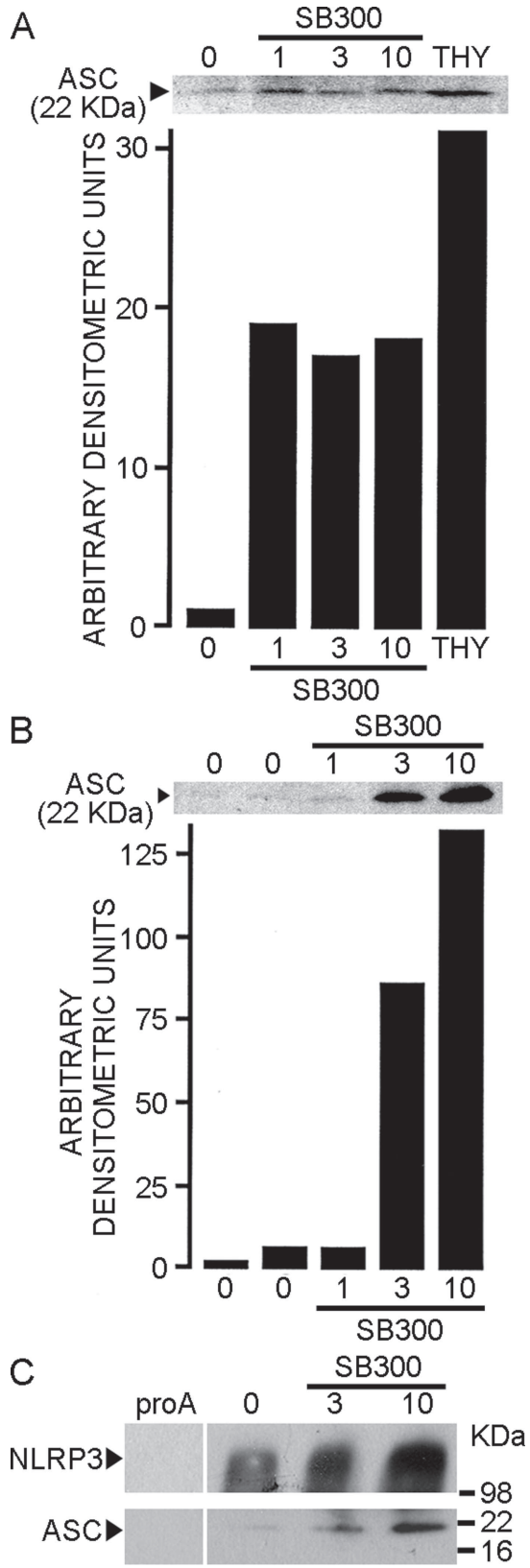

FIG. 5. Osteoblasts functionally express the NLRP3 adaptor molecule, ASC. (A) Murine or (B) human osteoblasts (2×10^6 per well) were untreated (0) or exposed to *Salmonella* SB300 (m.o.i. of 1, 3, and 10 bacteria per cell). After 24 h, protein isolates were subjected to immunoblot analysis for ASC expression. Representative immunoblots from three separate experiments are shown. As a positive control for ASC expression, a murine thymus total protein isolate was also analyzed (THY). Below, densitometric analyses of the immunoblot protein bands is shown as arbitrary densitometric units corrected for background intensity and normalized to β-actin expression. (C) Murine osteoblasts (2×10^6 per well) were untreated (0) or exposed to *Salmonella* SB300 (m.o.i. of 3 and 10 bacteria per cell). After 2 h, protein isolates were immunoprecipitated with antibodies directed against ASC or NLRP3 and subsequently analyzed by denaturing immunoblot for the presence of NLRP3 (top panel) or ASC (bottom panel), respectively. Migration of protein standards of known size is indicated to the right. As a negative control, samples exposed to immunoprecipitation procedure in the absence of antibodies against either NLRP3 or ASC and run on the same immunoblots are shown (proA). These experiments were performed twice with similar results.

in either resting or bacterially challenged osteoblasts, whereas this protein was detectable in murine splenocyte protein isolates.

Osteoblasts functionally express an important adaptor molecule for NLRP3 signaling

To begin to determine whether osteoblasts can express functional NLRP3 cytosolic receptors, we studied whether these cells express ASC, an important adaptor molecule in NLRP3-mediated cellular responses.[15,17] Real-time PCR was performed and revealed that resting murine osteoblasts (2×10^6 cells) express robust levels of mRNA encoding ASC (1.1 ± 0.4 copies $\times 10^5$). Such mRNA expression results in the presence of low but detectable levels of ASC protein as determined by immunoblot analysis (Fig. 5A). Furthermore, cells exposed to *Salmonella* (1, 3, and 10 bacteria per cell) showed significant elevations ($p < 0.05$) in NLRP3 protein expression at 24 h after infection (Fig. 5A). Similarly, low level ASC protein expression in primary human osteoblasts was markedly elevated after infection with *Salmonella* (Fig. 5B).

The constitutive expression of the critical adaptor molecule ASC and the sensitivity of its expression to bacterial challenge provide circumstantial evidence for the functionality of NLRP3 in osteoblasts. To further study whether these molecules form a functional component in the responses of osteoblasts to bacterial infection, we used co-immunoprecipitation techniques to determine if NLRP3 interacts with ASC in cells after challenge with *Salmonella*. Murine osteoblasts were unstimulated or exposed to *Salmonella* (3 and 10 bacteria to each osteoblast), and 2 h after infection, whole cell protein isolates were subjected to immunoprecipitation directed against ASC or NLRP3 and subsequently analyzed by denaturing immunoblot for the presence of NLRP3 or ASC, respectively. As shown in Fig. 5C, infection with *Salmonella* elicited marked elevations in the amount of NLRP3 associated with ASC as determined by both co-immunoprecipitation approaches.

NLRP3 mediates cellular responses in primary osteoblasts

NLRP3 has previously been shown to mediate reductions in the activity of NF-kB,[31] a transcription factor with anti-

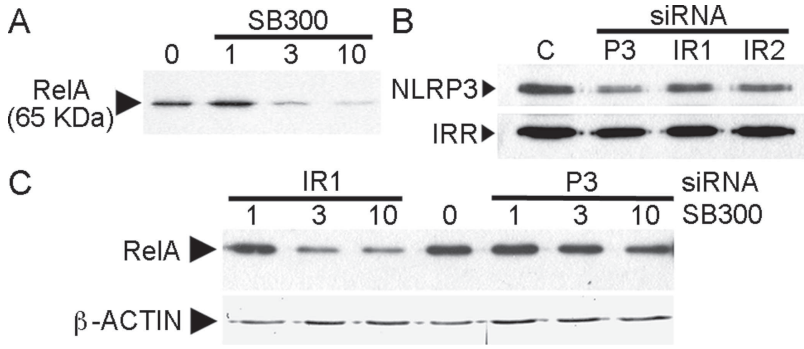

FIG. 6. NLRP3 mediates the ability of *Salmonella* to decrease NF-kB activity in primary murine osteoblasts. (A) Osteoblasts (2×10^6 per well) were untreated (0) or exposed to *Salmonella* SB300 (m.o.i. of 1, 3, and 10 bacteria per cell). After 1 h, nuclear protein isolates were subjected to immunoblot analysis for the p65 subunit of NF-kB (RelA). A representative immunoblot from four separate experiments is shown. (B) Expression of NLRP3 or an irrelevant protein (IRR) in osteoblasts that were untreated (labeled C) or 24 h after transfection with siRNA against NLRP3 (P3) or two siRNA against irrelevant RNA species (IR1 and IR2). The immunoblot shown is representative of four experiments. (C) Osteoblasts transfected with siRNA against NLRP3 (P3) or an irrelevant RNA species (IR1) were untreated (0) or exposed to *Salmonella* SB300 (m.o.i. of 1, 3, and 10 bacteria per cell) for 1 h, and nuclear protein isolates were analyzed for NF-kB p65 subunit (RelA) content. This experiment was performed twice with similar results.

apoptotic activity.[34–36] As shown in Fig. 6A, uninfected cultured osteoblasts exhibit robust NF-kB activity as assessed by nuclear levels of the RelA p65 subunit. Interestingly, exposure to *Salmonella* elicits dose-dependent reductions in NF-kB activity (Fig. 6A). To confirm the functional status of NLRP3 in osteoblasts, we used siRNA techniques to knockdown the expression of this cytosolic protein. As shown in the representative immunoblot in Fig. 6B, transfection of primary murine osteoblasts with siRNA for NLRP3 elicits an ~54% reduction in cellular NLRP3 levels. Importantly, transfection of osteoblasts with siRNA against NLRP3 markedly attenuates bacterially induced reductions in NF-kB activity (Fig. 6C), indicating that the ability of *Salmonella* to diminish the activity of this anti-apoptotic factor is caused, in large part, by the presence of NLRP3.

NLRP3 is required for caspase-1 activation and maximal Salmonella-induced apoptosis of primary osteoblasts

We have previously shown that caspase-1 activity is elevated in osteoblasts after bacterial challenge.[22] To definitively establish a role for NLRP3 in bacterially induced osteoblast cell death, we studied caspase-1 activation and apoptosis in cells derived from animals genetically deficient in the expression of this molecule. Caspase-1 processing is induced in osteoblasts as shown by the appearance of the active caspase-1 p10 subunit and the caspase-1 activation intermediate, p45, as rapidly as 4 h after infection (data not shown) with a maximal induction at 8 h after challenge (Fig. 7A). In contrast, caspase-1 processing is absent in osteoblasts derived from NLRP3-deficient mice ($NLRP3^{-/-}$; Fig. 7A). We have previously shown that infected osteoblasts do not release the caspase-1–dependent cytokines IL-1β and IL-18,[22] but we found that the production of IL-6 by $NLRP3^{-/-}$ cells is significantly lower than that produced by equal numbers of strain matched $NLRP3^{+/+}$ osteoblasts (5.5 ± 0.2 versus 7.6 ± 0.5 ng/ml, respectively, after 24-h *Salmonella* infection at an m.o.i. of 3:1 bacteria to osteoblasts; $p < 0.05$). Currently, the mechanism underlying this effect is not apparent.

Finally, we assessed the relative importance of NLRP3 to the induction of apoptotic cell death in *Salmonella*-infected osteoblasts. As shown in the representative experiment in Fig. 7B, significantly fewer $NLRP3^{-/-}$ osteoblasts undergo apoptotic cell death after *Salmonella* challenge (3:1 bacteria to each osteoblast) than strain-matched $NLRP3^{+/+}$ cells as assessed by apoptotic volume decrease. The population of apoptotic cells after infection was 19.3 ± 1.2% versus 30.7 ± 1.9 to at an m.o.i. of 3:1 ($p < 0.05$; $n = 4$), and 25.9 ± 1.8 versus 43.1 ± 2.3 at an m.o.i. of 10:1 ($p < 0.05$; $n = 4$) in $NLRP3^{-/-}$ cells and $NLRP3^{+/+}$ cells, respectively. Taken together, these data indicate that NLRP3 plays a significant role in the initiation of cell death in bacterially challenged osteoblasts.

DISCUSSION

Osteomyelitis is an intractable and persistent inflammatory disease resulting from bacterial infection of bone tissue. Whereas the increasing incidence and severity of osteomyelitis may be caused by the emergence of antibiotic-resistant strains of the bacteria commonly associated with this condition, the persistence and recurrent nature of such infections may stem from the ability of causative agents to invade and reside within bone-forming osteoblasts. Internalization may provide a means of protection against neutrophil and antibody-mediated immune responses, and may mitigate therapeutic interventions by limiting exposure to antibiotics. For example, *Salmonella* species are intracellular bacterial pathogens that represent one of the most serious pathogens of bone in sickle cell patients and immunosuppressed patients.[37–39] This organism is well known for its ability to invade epithelial cells,[40–42] and we showed that this bacterium can invade primary osteoblasts.[8]

Bacteria and their products can be potent stimulators of

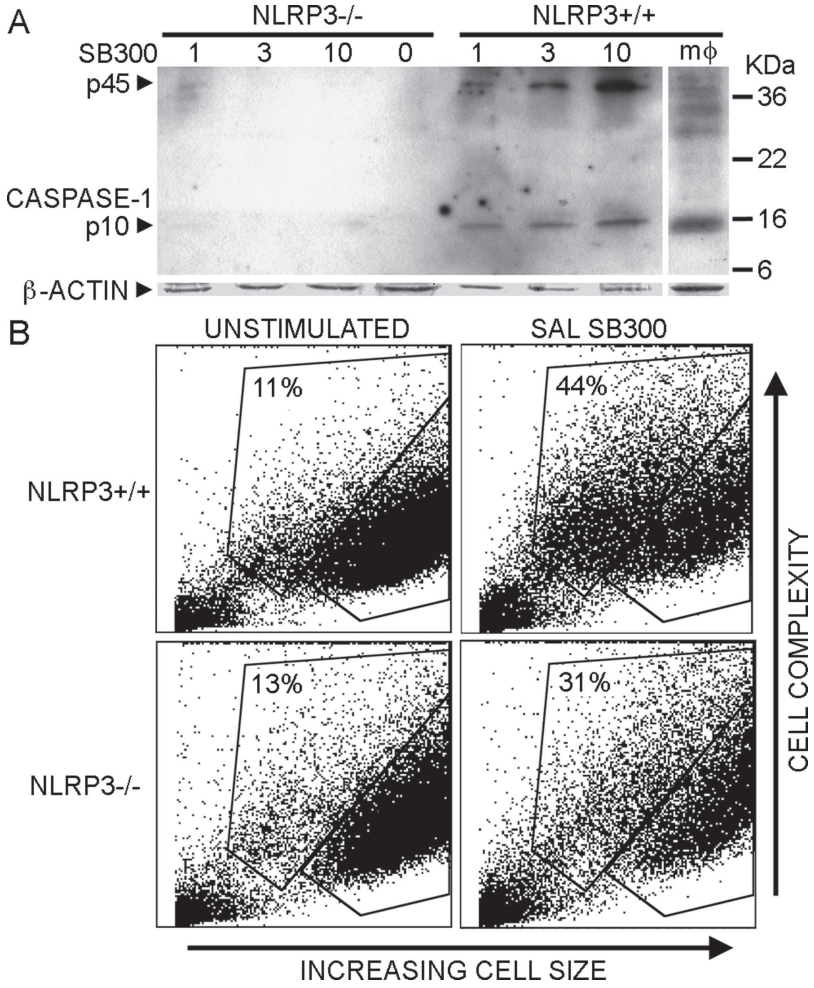

FIG. 7. NLRP3 is required for caspase-1 activation and maximal induction of apoptosis in osteoblasts after challenge with *Salmonella*. (A) Osteoblasts (2×10^6 per well) derived from wildtype ($NLRP3^{+/+}$) or animals genetically deficient in the expression of NLRP3 ($NLRP3^{-/-}$) were untreated (0) or exposed to *Salmonella* SB300 (m.o.i. of 1, 3, and 10 bacteria per cell). After 8 h, protein isolates were subjected to immunoblot analysis for caspase-1. A representative immunoblot from three separate experiments is shown. Bands corresponding to the active caspase-1 p10 subunit and the caspase-1 activation intermediate, p45, are indicated. As a positive control for caspase-1 activation, a total protein isolate from stimulated macrophages was also analyzed (mϕ). (B) Osteoblasts (2×10^6 per well) derived from wildtype ($NLRP3^{+/+}$) or animals genetically deficient in the expression of NLRP3 ($NLRP3^{-/-}$) were unstimulated or exposed to *Salmonella* SB300 (m.o.i. of 10 bacteria per cell). After 24 h, the percentage of cells showing an apoptotic volume decrease was assessed by flow cytometry. The data shown are representative of four experiments.

bone destruction at sites of infection.[43] Bone loss at such sites may result from increased formation and function of bone-resorbing osteoclasts, and we documented the ability of bacterially challenged osteoblasts to produce an array of soluble inflammatory mediators that can promote osteoclastogenesis and osteoclast activity.[1] Importantly, the destruction of bone-forming osteoblasts would be expected to exacerbate bone loss, and laboratories including our own have shown that these cells undergo apoptosis after bacterial challenge.[2,3] In this study, we confirmed that *Salmonella enterica* can elicit significant apoptotic cell death as rapidly at 24 h after infection. Importantly, we showed that intracellular invasion is needed for maximal induction of apoptosis in this cell type as shown by the sensitivity of this response to a pharmacologic inhibitor of endocytosis and the significantly smaller apoptotic response to invasion-deficient *Salmonella* strains (Figs. 1 and 2).

We have previously shown that bacterial components, UV-inactivated bacteria, and invasion-defective strains of bacteria are less effective in eliciting the immune functions of osteoblasts than viable wildtype bacteria.[6,8–10,12] Consistent with these findings, this study showed that cytochalasin significantly attenuates inflammatory cytokine production by invasive *Salmonella*. These observations indicate that invasion is required for optimal osteoblast responses and suggest that the cell surface TLRs expressed by these cells[5–7] are not the only means by which these cells perceive bacterial pathogens. We recently studied the expression by osteoblasts of NOD1 and NOD2, members of the NLR family of cytosolic receptors that have been shown to mediate inflammatory immune responses in other cell types, and showed that these resident bone cells expressed both of these receptors after bacterial challenge.[12] However, neither of these NLR family members is widely rec-

ognized to mediate the induction of apoptosis. In contrast, NLRP3 and NLRC4 have been implicated in caspase activation[17–19,44,45] and the initiation of apoptosis.[16,17,19] In this study, we showed that osteoblasts constitutively express NLRP3 but not NLRC4. Interestingly, infection of these cells with *Salmonella* elicits significantly elevations in mRNA encoding NLRP3 that is associated with the maintenance and perhaps a modest elevation of NLRP3 protein expression in contrast with the significant decrease seen after exposure of osteoblasts to an invasion-defective strain.

In addition to showing that osteoblasts possess robust constitutive levels of NLRP3, we showed that these cells also express ASC, an adaptor molecule that has been shown to mediate the actions of NLRP3.[16,19,44–46] Whereas constitutive levels of ASC seem to be low in resting osteoblasts, robust elevations in expression were observed in cells after *Salmonella* infection. Taken together, the conserved expression of NLRP3 and ASC in both mouse and human osteoblasts, and the sensitivity of such expression to bacterial challenge provide circumstantial evidence of a role for these cytosolic proteins in osteoblast responses to intracellular pathogens. However, we more directly verified NLRP3 functionality in bacterially challenged osteoblasts. First, we showed that NLRP3 associates with ASC after exposure to *Salmonella* as determined by co-immunoprecipitation. Second, we showed that NLRP3 expression knockdown by siRNA attenuates *Salmonella*-induced changes in transcription factor activity. Third, we found that osteoblasts derived from NLRP3-deficient animals produce lower amounts of an inflammatory cytokine than cells derived from wildtype mice. As such, these findings confirm that NLRP3 mediates osteoblast responses to this intracellular bacterial pathogen.

Our previous studies using blocking antibodies have shown that some osteoblast cell death is attributable to the autocrine and/or paracrine actions of TRAIL that is secreted by these cells after bacterial exposure.[3,4] However, a large proportion of this apoptotic cell death is unaffected by anti-TRAIL antibodies.[3] Given that osteoblasts constitutively express NLRP3 and ASC, and the finding that NLRP3 can associate with ASC to elicit caspase-1[16,19,44,45] and perhaps caspase-8 activation,[21] enzymes that show elevated activity in osteoblasts after bacterial challenge,[3,22] this cytosolic NLR may represent an important mechanism underlying apoptosis of osteoblasts after exposure to intracellular bacterial pathogens. This hypothesis is supported by the observation that *Salmonella*-induced decreases in NF-kB activity are markedly attenuated in osteoblasts after siRNA-induced NLRP3 knockdown, because this transcription factor has been reported to be anti-apoptotic in a variety of cell types.[34–36] Furthermore, we showed that *Salmonella*-induced caspase-1 activation is absent in osteoblasts derived from NLRP3-deficient animals. More importantly, we showed that such cells exhibit significantly less apoptotic cell death after *Salmonella* infection than osteoblasts derived from wildtype animals. These data differ from findings in macrophages[18,19] where NLRP3 is not needed for *Salmonella*-induced caspase-1 activation, IL-1β production, or cell death. Instead, these functions are prominently mediated by NLRC4. In our work, NLRP3 is required for caspase-1 activation and for a portion of *Salmonella*-induced cell death in osteoblasts, whereas NLRP4 is not detectable in these cells and is thus unlikely to play a role. Furthermore, earlier work from our laboratory indicates that neither the precursor nor the mature form of IL-1β is produced by infected osteoblasts.[22] Together these studies underscore the differences between osteoblasts and macrophages with a differential reliance on NLRP3 versus NLRC4 after *Salmonella* infection. This illustrates an additional level of complexity in the control of cellular functions by NLR family members.

Although further experimentation will be required to definitively conclude that ASC and alterations in NF-kB activity are essential mechanistic elements in NLRP3-mediated apoptosis in bacterially challenged osteoblasts, this study indicates that this NLR represents an important component underlying the initiation of apoptosis in this bone-forming cell type after challenge with intracellular bacterial pathogens and could, therefore, be a major contributory factor to bone loss at sites of infection.

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