Alendronate augments lipid A-induced IL-1β release by ASC-deficient RAW264 cells via AP-1 activation

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Received June 17, 2023; Accepted September 26, 2023

DOI: 10.3892/etm.2023.12276

Abstract. Alendronate (ALN) is an anti-bone-resorptive drug with inflammatory side effects. ALN upregulates lipid A-induced interleukin (IL)-1α and IL-1β release by J774.1 cells via apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) activation. The present study examined whether ALN augmented lipid A-induced proinflammatory cytokine production using ASC-deficient mouse macrophage-like RAW264 cells. Pretreatment of RAW264 cells with ALN significantly augmented lipid A-induced IL-1β release, although ALN did not upregulate the expression of Toll-like receptor 4, myeloid differentiation factor 88 (MyD88) and caspase-11. Moreover, pretreatment of caspase-11-deficient RAW264.7 cells with ALN significantly augmented lipid A-induced IL-1β release. Notably, ALN upregulated the activation of FosB, c-Jun or JunD, but not c-Fos or NF-κB in RAW264 cells. Furthermore, pretreatment with the activator protein 1 (AP-1) inhibitor SR11302, but not the c-Fos inhibitor T-5224, before addition of ALN inhibited ALN-augmented IL-1β release by lipid A-treated RAW264 cells. SR11302 also reduced ALN-augmented lactate dehydrogenase release by the cells. These findings collectively suggested that ALN augmented lipid A-induced IL-1β release and cell membrane damage in ASC-deficient RAW264 cells via activation of AP-1, but not NF-κB.

Introduction

Nitrogen-containing bisphosphonates (NBPs) are used to treat bone diseases, such as osteoporosis, because they induce the apoptosis of osteoclasts and inhibit bone resorption (1,2). However, NBPs also affect other cells, such as macrophages, via inhibition of the mevalonate pathway and activation of caspase-8, which initiates apoptosis (3-5). Moreover, NBPs have inflammatory side effects and cause medication-related osteonecrosis of the jaws (MRONJ) (6). Alendronate (ALN), an NBP, reduces angiotensin II-induced cardiac fibrosis and hypertrophy and reportedly augments cell membrane damage and the lipid A-induced release of proinflammatory cytokines by macrophages through activation of the nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome *in vitro* and *in vivo* (7-10). However, NBPs may reduce the risk of pneumonia in patients with hip fracture, although the reason is unclear (11).

Bacterial infection has been suggested to be a cause of MRONJ (6). Lipid A is a moiety of lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria and a major cause of inflammation (4,10). It is recognized by caspase-11 and Toll-like receptor (TLR) 4, which are LPS receptors, and activates macrophages that affect osteoclast differentiation by producing proinflammatory cytokines (12). Myeloid differentiation factor 88 (MyD88) is an adaptor molecule downstream of TLR4, and caspase-11 activates the NLRP3 inflammasome (13,14). Inflammasomes are large multimolecular complexes that include apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and NLRP3, and activate caspase-1, which promotes mature interleukin (IL)-1β release (15,16). Our previous study reported that ALN augments the lipid A-induced release of IL-1α and IL-1β by mouse macrophage-like J774.1 cells via the activation of ASC (4,10). IL-1α and IL-1β are proinflammatory cytokines produced by microbial components, such as LPS, and directly and indirectly activate osteoclasts (17). IL-1α is also constitutively expressed by various cells, unlike IL-1β. Thus, infectious diseases can disturb the balance of IL-1α and IL-1β production. As ALN increases the number of osteoclasts (18), it is necessary to inhibit ALN-augmented IL-1 release during long-term use of ALN. J774.1 cells, macrophages that are of the same lineage as osteoclasts and are representative of the innate immune system, are often used to examine the action of NBPs (2,19). Mouse macrophage-like RAW264 cells are also frequently used in molecular studies of NBPs.

Activator protein-1 (AP-1) and NF-κB regulate gene expression by binding to the promoter region of target genes (12).

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Key words: nitrogen-containing bisphosphonate, alendronate, apoptosis-associated speck-like protein containing a caspase recruitment domain, activator protein 1, interleukin-1β

Transcription factors play important roles in the production of proinflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNF-α. Homodimers or heterodimers of FosB, c-Fos, c-Jun and JunD constitute AP-1 (20). Heterodimers of p65 and p50 subunits compose a canonical NF-κB, whereas a non-canonical NF-κB consists of p52 and RelB subunits (12). ASC is required for not only mature IL-1β release but also AP-1 and NF-κB activation, although LPS can induce IL-1α and IL-6 production by ASC-deficient cells (21). Thus, there is a need for palliative agents other than anti-ASC antibodies for necrotizing inflammatory diseases caused by ALN (10). MyD88, a TLR4 adaptor protein, is upstream of AP-1 and NF-κB, although TLR4 can also signal via the MyD88-independent pathway (13,22).

NEDD4-binding protein 1 (N4BP1) is a nuclear RNase that degrades viral mRNA species in macrophages and primary T cells (23). N4BP1 also inhibits the production of proinflammatory cytokines and chemokines by inactivating AP-1 and NF-κB (22,24). The cleavage of N4BP1 by caspase-8 upregulates TLR4 ligand-induced cytokine production in mice, suggesting that ALN may degrade N4BP1 through the activation of caspase-8 (8).

In view of these collective findings from the literature, the present study investigated whether ALN induced AP-1 and NF-κB activation and augmented the lipid A-induced release of IL-1α and IL-1β by mouse macrophage-like RAW264 cells, which do not express ASC (25).

Materials and methods

Reagents. Inhibitors were dissolved in dimethyl sulfoxide and diluted in high-glucose Dulbecco's Modified Eagle Medium (DMEM; cat. no. D5796; MilliporeSigma) before use. Lipid A (compound 506), an agonist of caspase-11 and TLR4, was purchased from Peptide Institute, Inc. ALN was purchased from LKT Laboratories, Inc. and dissolved as previously described (4). AP-1 inhibitors SR11302 (cat. no. 16338) and T-5224 (c-Fos inhibitor; cat. no. 22904) were obtained from Cayman Chemical Company. Rat monoclonal anti-caspase-11 antibody (cat. no. NB120-10454) was purchased from Novus Biologicals, LLC; this antibody can detect p43, p38 and p26. Rabbit polyclonal anti-ASC antibody (N-15; cat. no. #sc-22514-R) and anti-actin antibody (I-19; cat. no. #sc-1616-R) were purchased from Santa Cruz Biotechnology, Inc. Rabbit monoclonal anti-N4BP1 antibody (EPNCIR118; cat. no. ab133610) was obtained from Abcam. Rabbit monoclonal anti-MyD88 antibody (D80F5; cat. no. #4283), anti-α-tubulin antibody (11H10; cat. no. #2125), and horseradish peroxidase (HRP)-conjugated anti-rabbit (cat. no. #7074) and anti-rat (cat. no. #7077) IgG antibodies were purchased from Cell Signaling Technology, Inc. Alexa Fluor™ 488-conjugated mouse anti-mouse TLR4 monoclonal antibody (cat. no. UT41) and Alexa Fluor™ 488-conjugated IgG1κ isotype control (clone no. P3.6.2.8.1; cat. no. 53-4714-42) were purchased from Thermo Fisher Scientific, Inc.

Cell culture. J774.1 cells and RAW264 cells were obtained from the RIKEN Bioresource Center. RAW264 cells were cultured in high-glucose DMEM containing 100 units/ml penicillin, 100 μg/ml streptomycin (Thermo Fisher Scientific, Inc.) and 10% heat-inactivated fetal bovine serum (FBS; HyClone; Cytiva), in a 5% CO_2 incubator at 37˚C. RAW264 cells were used as confluent monolayers at passages 4 through 9. J774.1 cells were cultured and used as previously described (4). Caspase-11 deficient RAW264.7 cells (RAW-ASC-KO-CASP11 cells) were purchased from InvivoGen and cultured according to the manufacturer's instructions. RAW-ASC-KO-CASP11 cells were used as confluent monolayers at passages 3 through 7.

Cell viability. RAW264 cells ($1x10^5$ cells/well) were grown in 96-well flat-bottomed plates for 18 h. The concentrations of ALN were determined as administered ALN is sequestered in bones, from where it is released very slowly and, thus, blood cells around bones experience high concentrations of ALN (26). Cells were pretreated with or without ALN (1, 10 or 100 μM) for 24 h, washed twice with serum-free high-glucose DMEM and incubated in the presence or absence of lipid A (1, 10 or 100 ng/ml) in medium containing 10% FBS for 24 h. MTS solution (Cell Titer 96® AQueous One Solution Assay; Promega Corporation) was then added directly to each well and incubated for 1 h at 37˚C. Cell viability was evaluated by measuring the reduction of MTS to formazan by living cells. Absorbance was measured at 490 nm with a reference at 655 nm using an iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Inc.).

Lactate dehydrogenase (LDH) assay. The levels of LDH activity in supernatants, which were assessed to evaluate cell membrane damage, were determined using the Cytotoxicity Detection kit (cat. no. 11644793001; Roche Diagnostics GmbH). Cells treated with 2% Triton X-100 were used as positive controls with a total activity of 100%. The amount of formazan formed was determined by measuring absorbance at 490 nm (reference, 655 nm), as aforementioned.

Cytokine measurements. RAW264 cells ($1x10^5$ cells/well) grown as aforementioned were washed once with serum-free DMEM and incubated for 24 h with or without 100 μM ALN in DMEM containing 10% FBS at 37˚C. The cells were then washed twice with serum-free high-glucose DMEM and incubated for an additional 24 h in high-glucose DMEM with or without lipid A at the indicated concentrations at 37˚C. For inhibition assays, cells were pretreated with 3 or 10 μM inhibitors for 1 h at 37˚C before ALN or lipid A addition. Culture supernatants were collected after centrifugation at 500 x g for 5 min at 4˚C, and levels of secreted mouse proinflammatory cytokines were measured by the following enzyme-linked immunosorbent assay (ELISA) kits: Mouse IL-1β uncoated ELISA kit (cat. no. #88-7013-88; Thermo Fisher Scientific, Inc.), mouse IL-1α DuoSet ELISA (cat. no. DY400-05; R&D Systems, Inc.), mouse IL-6 DuoSet ELISA (cat. no. DY406-05; R&D Systems, Inc.) and mouse TNF-α DuoSet ELISA (cat. no. 410-05; R&D Systems, Inc.).

Protein extraction and transactivation analysis. Cells ($2.5x10^6$ cells) were cultured in 60 mm dishes (Falcon) for 18 h, washed once with serum-free high-glucose DMEM and treated with or without 100 μM ALN for 24 h at 37˚C. Cytosol or nuclear extracts were prepared with the Nuclear Extract Kit (Active Motif, Inc.) according to the manufacturer's protocol as previously described (12). Protein concentration was

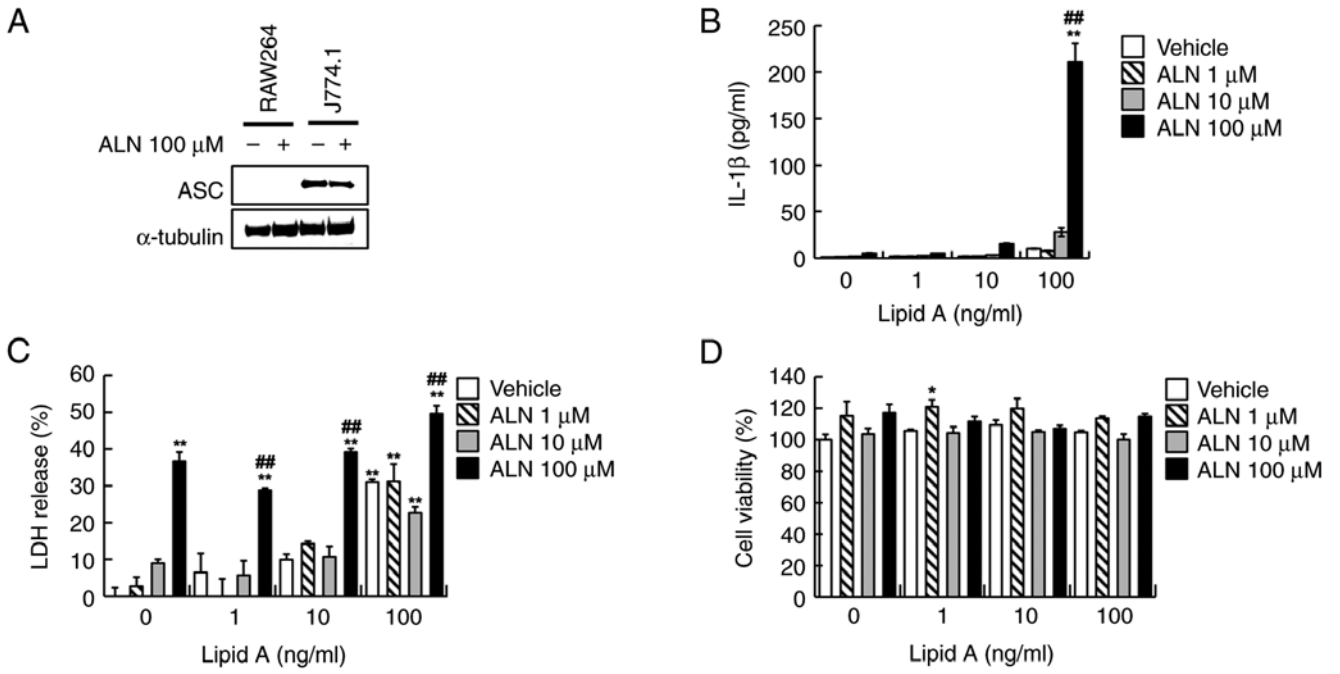

Figure 1. Pretreatment of RAW264 cells with ALN augments lipid A-induced IL-1β release by ASC-deficient RAW264 cells. (A) Western blotting of ASC in the cytosol of RAW264 cells and J774.1 cells. (B) IL-1β release. RAW264 cells were pretreated with vehicle or 100 μM ALN for 24 h. Cells were then washed and treated with or without 100 ng/ml lipid A for 24 h. (C) LDH release and (D) cell viability. The optical density (absorbance at 490 nm-absorbance at 655 nm) of cells incubated in medium alone without ALN pretreatment was set at 100%. compared with vehicle. *P<0.05 and **P<0.01 compared with 0 ng/ml lipid A + vehicle group. $^{\#\#}$P<0.01 compared with the same concentration of lipid A alone. ALN, alendronate; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; IL, interleukin; LDH, lactate dehydrogenase.

determined using a BCA™ protein assay kit (Pierce; Thermo Fisher Scientific, Inc.).

AP-1 and NF-κB activation were measured using TransAM ELISA kits (cat. nos. 44296 and 43296; Active Motif, Inc.). Oligonucleotides containing AP-1 or NF-κB consensus binding sites were immobilized in each well. Nuclear extracts (10 μg for c-Fos, c-Jun, JunD, p65 and p50; 5 μg for p52 and RelB) were added to each well, and the plate was incubated according to the manufacturer's protocol (12). Absorbance was read on a spectrophotometer at 450 nm (reference, 655 nm).

Flow cytometry. RAW264 cells ($2.5x10^6$ cells/60 mm dish) were treated as aforementioned. Cells were harvested, washed with PBS containing 2% FBS three times and incubated for 5 min at room temperature with a human FcR blocking reagent (Clear Back; MBL International Co.). Cells were then incubated for 30 min at room temperature with Alexa Fluor™ 488-conjugated mouse anti-mouse TLR4 monoclonal antibody (1:5). To prepare controls, cells were incubated with Alexa Fluor™ 488-conjugated mouse IgG1 κ isotype control (1:5). Cells were washed with PBS containing 2% FBS and analyzed using a flow cytometer (CytoFLEX S; Beckman Coulter, Inc.). Data were analyzed using Kaluza Analysis 2.1 software (Beckman Coulter, Inc.).

Western blotting. Cytosol or nuclear extracts (30-40 μg/lane) of cells were fractionated by a 5-20% gradient SDS-PAGE gel (ATTO Corporation) and transferred to a Hybond-P PVDF membrane (Cytiva) by electroblotting. The molecular mass of a given protein was estimated by comparison with the positions of standard proteins (Bio-Rad Laboratories, Inc.). The blot was blocked for 1 h with 5% (wt/vol) skim milk in TBS containing 0.1% Tween 20 (TBS-T) at 25˚C and then incubated overnight at 4˚C with primary antibodies specific for mouse ASC (1:3,000), caspase-11 (1:4,000), MyD88 (1:4,000), N4BP1 (1:4,000), α-tubulin (1:4,000) or actin (1:2,000) in Can Get Signal 1 solution (Toyobo Life Science). The blot was then washed three times with TBS-T, followed by incubation for 1 h with a suitable HRP-conjugated, affinity-purified anti-rabbit or anti-rat IgG antibody (1:4,000) in Can Get Signal 2 solution at 25˚C. After the wash, the blot was analyzed using EzWestLumi plus and LuminoGraph I (ATTO Corporation). Images were obtained with an Image Saver 6 (ATTO Corporation) and analyzed with CS Analyzer 4.0 (ATTO Corporation).

Statistical analysis. Statcel4 (The publisher OMS) was used for all statistical analyses. After Bartlett's test, data were analyzed using one-way analysis of variance (ANOVA) with Tukey's post hoc test or the unpaired Student's t-test. All experiments were repeated at least three times. Results are presented as mean ± standard error (SE) of triplicate wells. P<0.05 was considered to indicate a statistically significant difference.

Results

ALN augments lipid A-induced IL-1β release by RAW264 cells. Unlike J774.1 cells, RAW264 cells do not express ASC (Fig. 1A). The present study first examined whether pretreatment with ALN augmented 100 ng/ml lipid A-induced IL-1β release by RAW264 cells, with the assumption that exposure to oral bacteria during administration of ALN triggers MRONJ. Pretreatment with 100 μM ALN for 24 h significantly

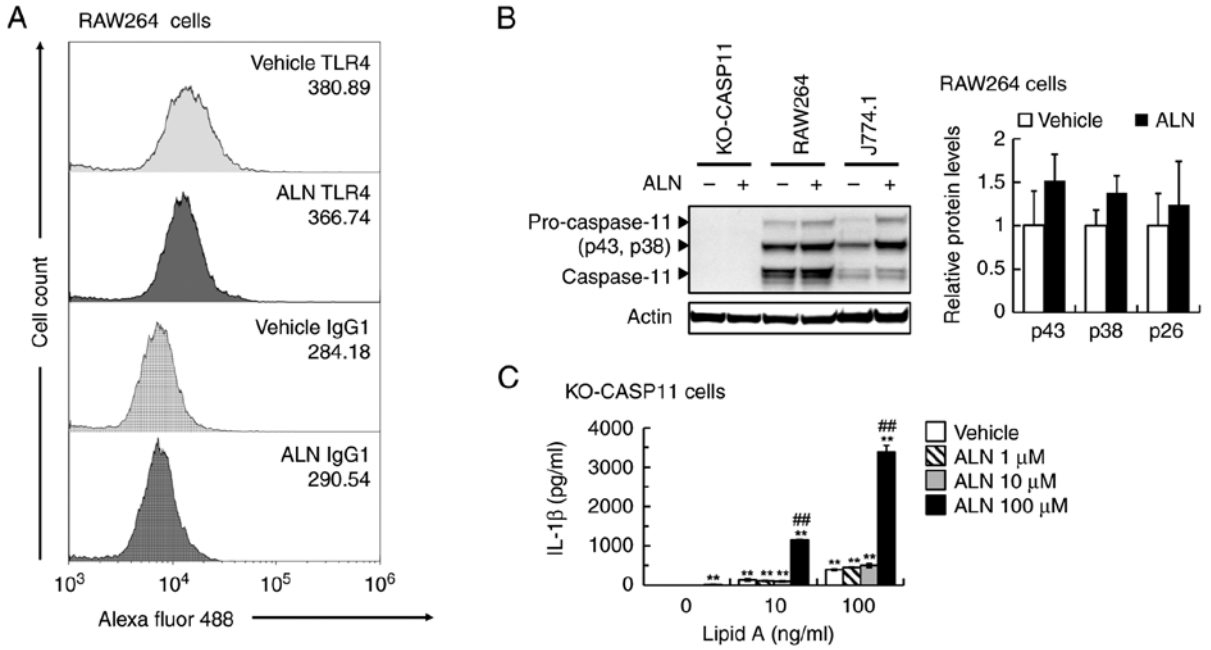

Figure 2. Effects of ALN on expression of TLR4 and caspase-11. (A) Flow histograms of TLR4 expression on RAW264 cells are shown. RAW264 cells were treated with vehicle (gray area) or 100 μM ALN (black area) for 24 h. Treated cells were stained with a specific antibody for AlexaFlour 488-conjugated TLR4 or IgG1 as negative controls. Values indicated in the histograms represent the mean fluorescence intensity of cells based on three independent experiments. (B) Western blotting of caspase-11 in the cytosol of RAW264 cells. (C) Effect of ALN on lipid A-induced IL-1β release by KO-CASP11 cells. **P<0.01 compared with 0 ng/ml lipid A + vehicle group. $^{\#\#}$P<0.01 compared with the same concentration of lipid A alone. ALN, alendronate; TLR4, Toll-like receptor 4; KO-CASP11, caspase-11-deficient RAW264.7 cells.

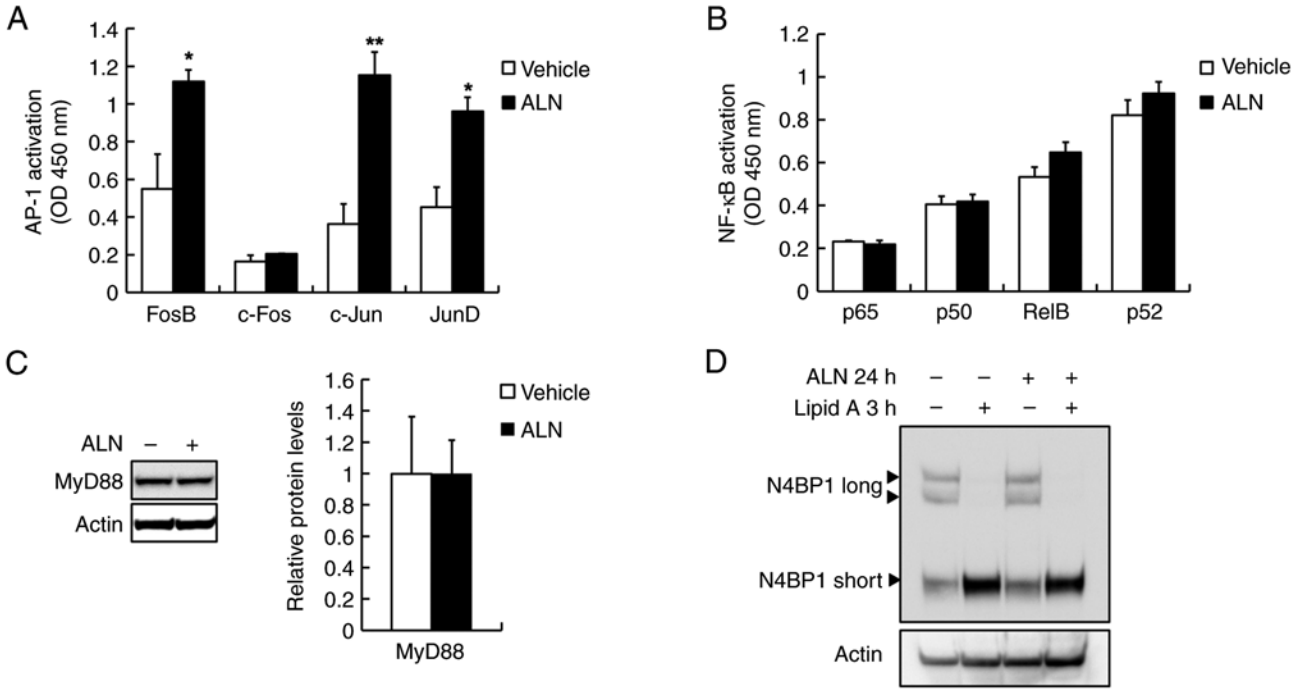

Figure 3. Effects of ALN on activation of AP-1 or NF-κB in RAW264 cells. Cells were treated with vehicle (white bars) or 100 μM ALN (black bars) for 24 h. (A) AP-1 activation. (B) NF-κB activation. (C) Western blotting of MyD88 in the cytosol of RAW264 cells. (D) Western blotting of N4BP1 in the nuclear extracts of RAW264 cells. *P<0.05 and **P<0.01 compared with respective vehicle groups. ALN, alendronate; AP-1, activator protein 1; MyD88, myeloid differentiation factor 88; N4BP1, NEDD4-binding protein 1.

augmented 100 ng/ml lipid A-induced IL-1β release compared with lipid A alone (P<0.01; Fig. 1B). These results suggest that ALN and lipid A can significantly augment the release of IL-1β by RAW264 cells even in the absence of ASC. Pretreatment with 100 μM ALN also induced cell membrane damage compared with the same concentration of lipid A alone, although the number of cells was not significantly reduced by the pretreatment (Fig. 1C and D). The release of

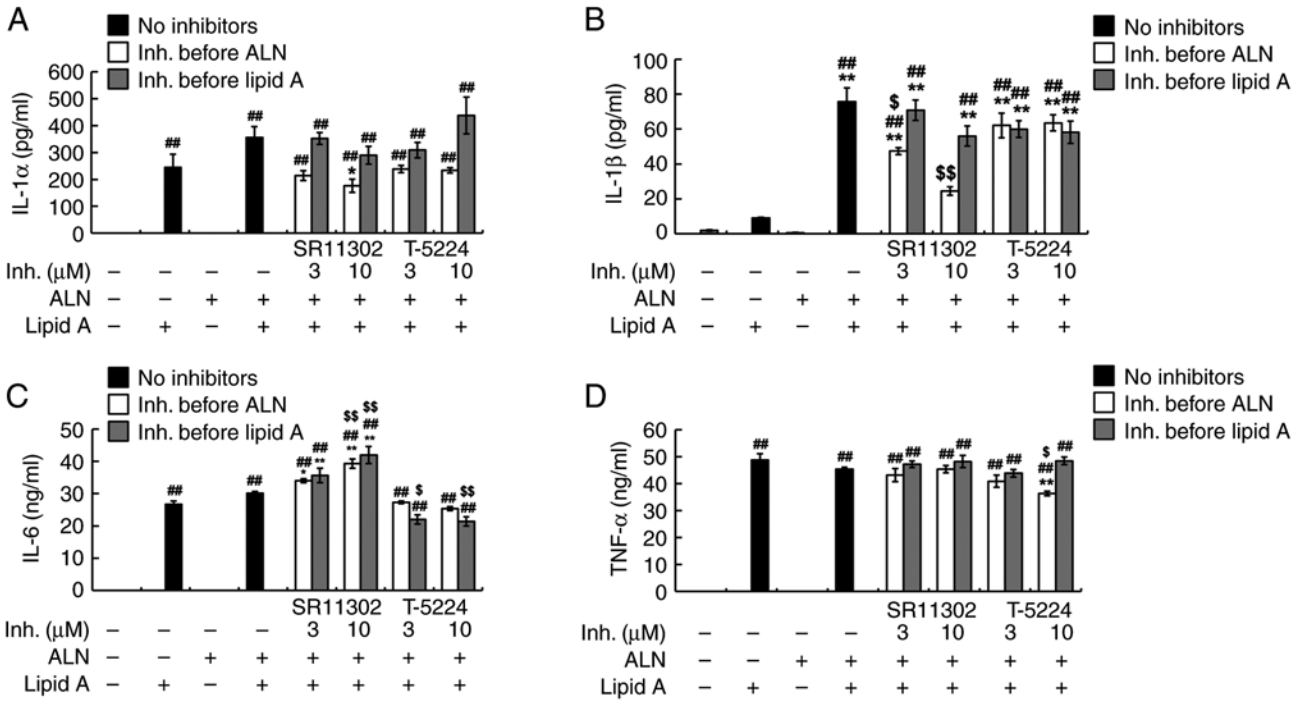

Figure 4. Pretreatment of RAW264 cells with ALN augments lipid A-induced IL-1β release via AP-1. RAW264 cells were incubated in medium containing the inhibitors indicated or DMSO for 1 h, followed by treatment with vehicle or 100 μM ALN for 24 h. Cells were then washed, incubated in medium containing the inhibitors indicated or DMSO for 1 h, and treated with or without 100 ng/ml lipid A for 24 h. Expression levels of (A) IL-1α, (B) IL-1β, (C) IL-6 and (D) TNF-α. *P<0.05 and **P<0.01 compared with lipid A alone. $^{\#\#}$P<0.01 compared with ALN alone. $^{\$}$P<0.05 and $^{\$\$}$P<0.01 compared with ALN and lipid A treatment without inhibitors. ALN, alendronate; IL, interleukin; Inh., inhibitor.

LDH from cells is considered an indicator of cell damage, but not cell death.

ALN does not upregulate the expression of TLR4 and caspase-11 and augments IL-1β release by lipid A-treated caspase-11 deficient RAW264.7 cells. The present study next investigated whether ALN could upregulate the expression of TLR4 and caspase-11 in RAW264 cells. Treatment of cells with ALN even at 100 μM for 24 h did not markedly upregulate the expression of TLR4 and caspase-11 (Fig. 2A and B). In addition, pretreatment with 100 μM ALN significantly upregulated 10 or 100 ng/ml lipid A-induced IL-1β release by caspase-11 deficient RAW264.7 cells compared with the same concentration of lipid A alone (P<0.01; Fig. 2C). These results suggest that pretreatment with ALN does not augment lipid A-induced IL-1β release via upregulation of the expression of LPS receptors, TLR4 and caspase-11.

ALN induces the translocation of FosB, c-Jun and JunD, but not c-Fos or NF-κB, in RAW264 cells. Whether ALN activates AP-1 or NF-κB in RAW264 cells was next examined. While 100 μM ALN alone did not induce c-Fos activation, treatment with 100 μM ALN for 24 h significantly augmented the translocation of FosB, c-Jun and JunD in RAW264 cells (P<0.05; Fig. 3A). However, treatment with 100 μM ALN did not significantly upregulate the translocation of p65, p50, RelB or p52 (Fig. 3B). In addition, treatment with 100 μM ALN did not upregulate the expression of MyD88, which is upstream of AP-1 and NF-κB (Fig. 3C). The present study also investigated whether ALN degrades N4BP1 in RAW264 cells. Even at 100 μM, ALN did not upregulate N4BP1 degradation,

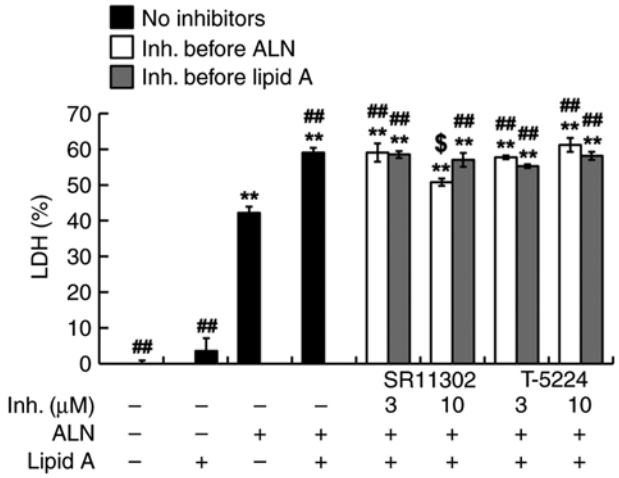

Figure 5. Pretreatment of RAW264 cells with SR11302 before addition of ALN inhibits ALN-augmented cell membrane damage. LDH levels in culture supernatants were measured to evaluate cell membrane damage. **P<0.01 compared with lipid A alone. $^{\#\#}$P<0.01 compared with ALN alone. $^{\$}$P<0.05 compared with ALN and lipid A treatment without inhibitors. ALN, alendronate; LDH, lactate dehydrogenase; IL, interleukin; Inh., inhibitor.

although treatment with lipid A led to the complete degradation of N4BP1 in RAW264 cells (Fig. 3D). These results suggest that ALN does not activate AP-1 via MyD88 or the degradation of N4BP1 in RAW264 cells.

ALN augments lipid A-induced IL-1β release by RAW264 cells via activation of AP-1, but not c-Fos. Whether AP-1 activation is required for ALN-augmented IL-1β release by RAW264

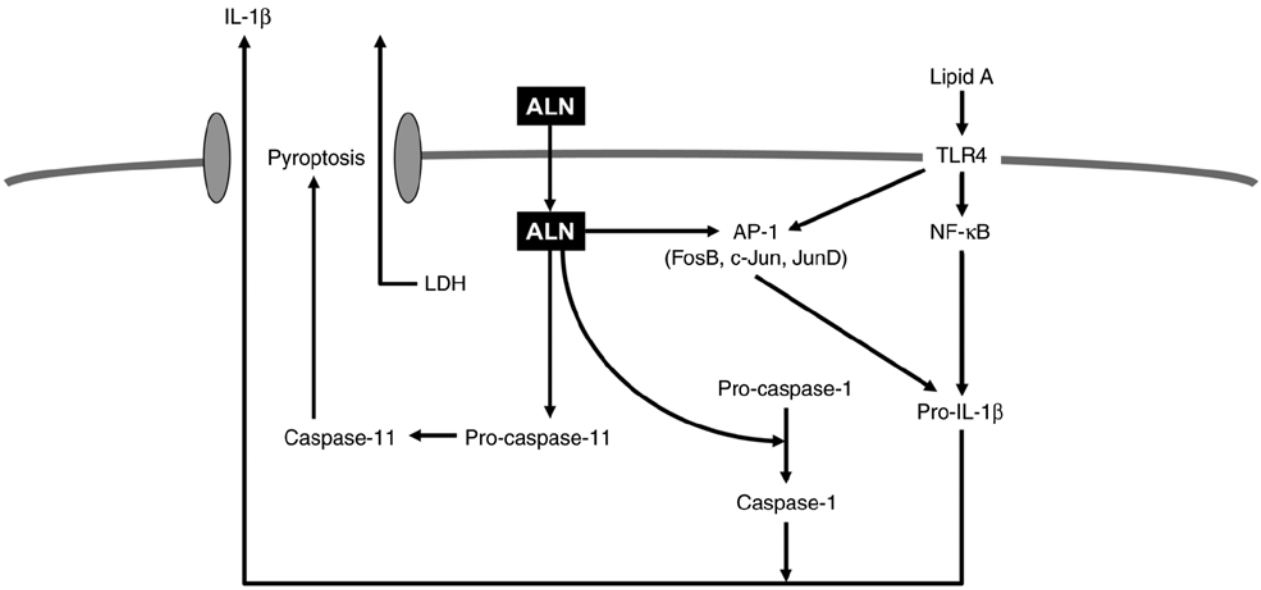

Figure 6. Mechanism of ALN-augmented cell membrane damage and IL-1β release via AP-1 activation. ALN, alendronate; LDH, lactate dehydrogenase; IL, interleukin; TLR4, Toll-like receptor 4; AP-1, activator protein-1.

cells was investigated using various inhibitors. Pretreatment with 10 μM SR11302, an AP-1 inhibitor, prior to the addition of 100 μM ALN, inhibited ALN-upregulated, 100 ng/ml lipid A-induced IL-1β release compared with in cells treated with ALN and lipid A without inhibitors ($P<0.01$; Fig. 4B). However, pretreatment with T-5224, an inhibitor of c-Fos, prior to the addition of 100 μM ALN, did not inhibit ALN-augmented lipid A-induced IL-1β release compared with in cells treated with ALN and lipid A without inhibitors (Fig. 4B). These results are consistent with AP-1 activation by ALN, independently of c-Fos. Moreover, pretreatment with 100 μM ALN did not upregulate lipid A-induced production of IL-1α, IL-6 or TNF-α compared with lipid A alone (Fig. 4A, C and D). Pretreatment with T-5224 before the addition of 100 ng/ml lipid A, but not ALN, significantly inhibited lipid A-induced IL-6 release compared with ALN and lipid A treatment without inhibitors group (Fig. 4C). Pretreatment with SR11302 or T-5224 before the addition of 100 μM ALN or 100 ng/ml lipid A did not inhibit lipid A-induced IL-1α production compared with in cells treated with ALN and lipid A without inhibitors. These results suggest that ALN-augmented lipid A-induced release of IL-1β, but not IL-1α, IL-6 or TNF-α, depends on AP-1 activation, independently of c-Fos.

ALN induces cell membrane damage in RAW264 cells via activation of AP-1, but not c-Fos. Whether AP-1 activation is required for ALN-augmented cell membrane damage in RAW264 cells was next examined. LDH assays are commonly used to assess the progression of cell damage. Pretreatment with 10 μM SR11302 prior to the addition of 100 μM ALN inhibited ALN-augmented cell membrane damage compared with in cells treated with ALN and lipid A without inhibitors ($P<0.05$; Fig. 5). However, pretreatment with 10 μM T-5224 prior to the addition of 100 μM ALN did not inhibit ALN-augmented cell membrane damage compared with in cells treated with ALN and lipid A without inhibitors. These results suggest that ALN induces AP-1-mediated cell membrane damage, independently of c-Fos. These results collectively suggest that ALN augments cell membrane damage in and lipid A-induced IL-1β release by RAW264 cells (Fig. 6).

Discussion

ASC is a small molecule that constitutes an inflammasome with nucleotide-binding oligomerization domain (NOD)-like receptor and caspase-1 (15). In a previous study, pretreatment with an anti-ASC antibody prior to the addition of ALN inhibited the ALN-augmented release of IL-1β, caspase-1 and LDH by J774.1 cells (10). LPS does not significantly induce mature IL-1β release by RAW264 cells because RAW 264 cells do not express ASC, which plays an important role in IL-1β release via caspase-1 activation (25). In the present study, lipid A (a moiety of LPS) and ALN significantly induced IL-1β release by RAW 264 cells, although ALN did not upregulate the expression of TLR4 or MyD88, which are required for lipid A-induced proinflammatory cytokine production (13). Lipid A is recognized not only by TLR4 but also by caspase-11, an intracellular LPS receptor (14). Caspase-11 is defined as a non-canonical NLRP3 inflammasome because it not only activates the canonical NLRP3 inflammasome but also induces pyroptosis (gasdermin D-dependent cell death) independent of NLRP3 (27,28). The present study revealed that ALN did not upregulate caspase-11 expression in RAW264 cells but di augment lipid A-induced IL-1β release by caspase-11 knockout RAW264.7 cells. Thus, ALN may augment lipid A-induced IL-1β release even in the absence of caspase-11 or ASC.

IL-1β production requires the activation of transcription factors, such as AP-1 and NF-κB (29). AP-1 contains homodimers or heterodimers of c-Fos, FosB, JunD and c-Jun. c-Jun is crucial for NLRP3 inflammasome activation by macrophages and vascular endothelial cells and also induces apoptosis (30,31). In the present study, ALN activated AP-1, FosB, c-Jun and JunD, but not c-Fos or NF-κB, even in ASC-deficient mouse macrophage-like RAW264 cells likely

because ALN activates caspase-8, which in turn mediates AP-1 activation (10,32). Moreover, 100 μM ALN did not upregulate MyD88 expression and N4BP1 degradation, suggesting that ALN may directly activate AP-1, but not NF-κB, in RAW264 cells. ALN could activate ERK, which is upstream of AP-1 and prevents apoptosis of osteocytes and osteoblasts (33). Thus, ALN might not reduce the viability of RAW264 cells.

AP-1 is also necessary to induce the production of IL-1α, IL-6 and TNF-α, although SR11302, an AP-1 inhibitor, has been reported to inhibit the mRNA expression of IL-1β, but not TNF-α, in microglia (29). In the present study, pretreatment with ALN did not upregulate lipid A-induced IL-1α, IL-6 or TNF-α production by RAW264 cells. Mature IL-1β release, but not the production of IL-1α, IL-6 or TNF-α, requires the activation of caspase-1, which is involved in inflammasomes including NOD-like receptors, such as NLRP3 and NLRC4 (14). ALN upregulates caspase-1 activation because inhibition of the mevalonate pathway by NBPs results in NLRP3 activation by macrophages (7,9,10). Furthermore, overexpression of c-Jun and JunD augments activation of the NLRP3 inflammasome and release of IL-1β (30,34). Thus, in the present study, pretreatment of RAW264 cells with ALN upregulated the lipid A-induced release of IL-1β, but not IL-1α, IL-6 or TNF-α and pretreatment with SR11302 before the addition of ALN inhibited ALN-augmented IL-1β release.

ALN-augmented cell membrane damage is caused by the progression of pyroptosis, apoptosis and necroptosis (mixed lineage kinase domain like pseudokinase-dependent cell death) in J774.1 cells (4). In the present study, ALN and lipid A also significantly upregulated LDH release by RAW 264 cells. However, the number of cells was not reduced by ALN and lipid A because RAW 264 cells do not have ASC, which contributes to cell death (15). Moreover, pretreatment with SR11302 prior to the addition of ALN inhibited ALN-augmented LDH release by RAW264 cells, suggesting that ALN might augment cell membrane damage via AP-1 activation. This result is consistent with previous reports showing that activation of AP-1 induced the pyroptosis of hepatocytes, apoptosis of cardiomyocytes and necroptosis of acinar cells (35-37). SR11302 is a synthetic retinoid that inhibits AP-1 activity without activating transcription through the retinoic acid response element, although retinoic acid has also been reported to attenuate expression of the NLRP3 inflammasome in macrophages (38,39). The results of the present study suggest the possibility that SR11302 may serve as a palliative agent for necrotizing inflammatory diseases.

Pretreatment with T-5224 prior to the addition of 100 μM ALN did not inhibit ALN-augmented lipid A-induced release of IL-1β by RAW264 cells. T-5224 is known to inhibit the DNA binding activity of c-Fos, although the inhibitor binds to the DNA binding domain of both c-Fos and c-Jun (20,40). However, SR11302 binds to the 12-O-tetradecanoylphorbol-13-acetate response element, which is also bound to AP-1 proteins such as FosB and JunD (20). Thus, pretreatment with SR11302, but not T-5224, could inhibit ALN-augmented release of IL-1β and LDH by RAW264 cells.

NBPs are also used to prevent hypercalcemia caused by cancer-induced bone diseases (19). In the present study, ALN directly upregulated AP-1 activation by ASC-deficient RAW264 cells. The activation of AP-1 is inhibited by Nur77, a transcription factor. Nur77, also named NR4A1, suppresses cytotoxic T cell function and exhausts chimeric antigen receptor T cells, which are engineered to eliminate tumor cells (41). Therefore, NBPs might also contribute to the reactivation of T cells through AP-1 activation.

In addition to LPS, TLR4 recognizes surface components of fungi and viruses, such as mannan of fungi cell walls and the S protein of SARS-CoV-2 (42,43). Thus, infection might be more risky for NBP-treated patients with osteoporosis compared with healthy individuals, as NBP-treated patients with osteoporosis may produce proinflammatory cytokines more compared with healthy individuals (44). SR11302 may be effective for controlling ALN-augmented IL-1β release.

In conclusion, ALN augmented not only the cell membrane damage of, but also lipid A-induced IL-1β release by, ASC-deficient mouse macrophage-like RAW264 cells via activation of AP-1. ASC played an important role in IL-1β release and cell membrane damage but may not be required for ALN-augmented IL-1β release.

Acknowledgements

Not applicable.

Funding

This study was supported by JSPS KAKENHI (grant no. 21K10233) and a Grant-in-Aid for Scientific Research from the Ohu University School of Dentistry, including the President's discretionary expense (grant no. 2022-01).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

NW and RT performed the experiments, data analysis and interpretation. YK contributed to the analysis and interpretation of data. NW, RT and YK provided funding support. RT conceived and supervised the study. RT and YK revised the manuscript. RT and YK confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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