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Serum amyloid A inhibits osteoclast differentiation to maintain macrophage function

Jiseon Kim, Jihyun Yang,† Ok-Jin Park,* Seok-Seong Kang,* Cheol-Heui Yun,‡ and Seung Hyun Han*,[1]*

*Department of Oral Microbiology and Immunology, DRI, and BK21 Plus Program, School of Dentistry, Seoul National University, Seoul, Korea; †Infection and Immunity Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea; and ‡Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul, Korea

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ABSTRACT

Serum amyloid A is an acute phase protein that is elevated under inflammatory conditions. Additionally, the serum levels of serum amyloid A are associated with the progression of inflammatory arthritis; thus, serum amyloid A might be involved in the regulation of osteoclast differentiation. In the present study, we examined the effects of serum amyloid A on osteoclast differentiation and function. When bone marrow-derived macrophages, as osteoclast precursors, were stimulated with serum amyloid A in the presence of M-CSF and receptor activator of nuclear factor-κB ligand, osteoclast differentiation and its bone-resorption activity were substantially inhibited. TLR2 was important in the inhibitory effect of serum amyloid A on osteoclast differentiation, because serum amyloid A stimulated TLR2. The inhibitory effect was absent in bone marrow-derived macrophages obtained from TLR2-deficient mice. Furthermore, serum amyloid A inhibited the expression of c-Fos and nuclear factor of activated T cells c1, which are crucial transcription factors for osteoclast differentiation, but prevented downregulation of IFN regulatory factor-8, a negative regulator of osteoclast differentiation. In contrast, serum amyloid A sustained the endocytic capacity of bone marrow-derived macrophages and their ability to induce the proinflammatory cytokines, IL-6, IL-1β, and TNF-α. Taken together, these results suggest that serum amyloid A, when increased by inflammatory conditions, inhibits differentiation of macrophages to osteoclasts, likely to maintain macrophage function for host defense. *J. Leukoc. Biol.* 99: 595–603; 2016.

Introduction

Bone homeostasis is regulated through the balance of bone resorption and formation mediated by osteoclasts and osteoblasts, respectively. Osteoclasts are differentiated from monocyte/macrophage lineage cells via the action of M-CSF and RANKL [1]. M-CSF supports the survival of macrophages and osteoclasts [2], and RANKL is a crucial factor for osteoclast differentiation and activation [3]. The transcription factors, c-Fos and NFATc1, are required for the induction of the genes responsible for osteoclast differentiation, which are induced through RANKL-mediated activation of MAPK [4–6]. Also, osteoclast differentiation is negatively regulated by the expression of inhibitory transcription factors, such as IRF8, Bcl6, and MafB [7–9].

APPs are a group of proteins whose plasma concentrations are altered shortly after injury, trauma, and infection [10]. Among the APPs, the levels of SAA, together with those of C-reactive protein, are substantially increased by ≤1000-fold during inflammation [11]. APPs are mainly produced by liver hepatocytes but are also secreted from extrahepatic tissues such as the intestine and lung and contribute to the regulation of the immune system [12, 13]. In addition to their capacity for immune regulation, APPs have diverse functions, including complement activation, clot formation, and enzyme neutralization [14]. Recently, APPs were reported to affect bone remodeling by regulating osteoclasts and osteoblasts. For example, plasminogen inhibits osteoclast differentiation by increasing the expression of osteoprotegerin in osteoblasts [15]. In addition, haptoglobin induces osteoclast differentiation via PGE_2 biosynthesis in osteoblasts, and lipocalin-2 overexpression decreases trabecular number and cortical bone thickness [16–18].

SAA is one of the major APPs highly conserved in vertebrates through evolution. SAA is produced by various types of cells, including endothelial cells, macrophages, and epithelial cells [14]. In addition, it has been suggested that SAA is recognized by several receptors, including FPR1, CD36, TLR2, and TLR4 [19–21]. SAA has numerous functions, including the recruitment of neutrophils and monocytes [22, 23], inhibition of neutrophil

Abbreviations: APP = acute phase protein, Bcl6 = B-cell lymphoma 6, BMM = bone marrow-derived macrophage, FPR = formyl peptide receptor, IRF = IFN regulatory factor, MafB = v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B, NFATc1 = nuclear factor of activated T cells c1, RANKL = receptor activator of nuclear factor-κB ligand, SAA = serum amyloid A, TRAP = tartrate-resistant acid phosphatase

1. Correspondence: Department of Oral Microbiology and Immunology, DRI, and BK21 Plus Program, School of Dentistry, Seoul National University, 28 Yongon-Dong, Chongno-Gu, Seoul, Korea. E-mail: shhan-mi@snu.ac.kr

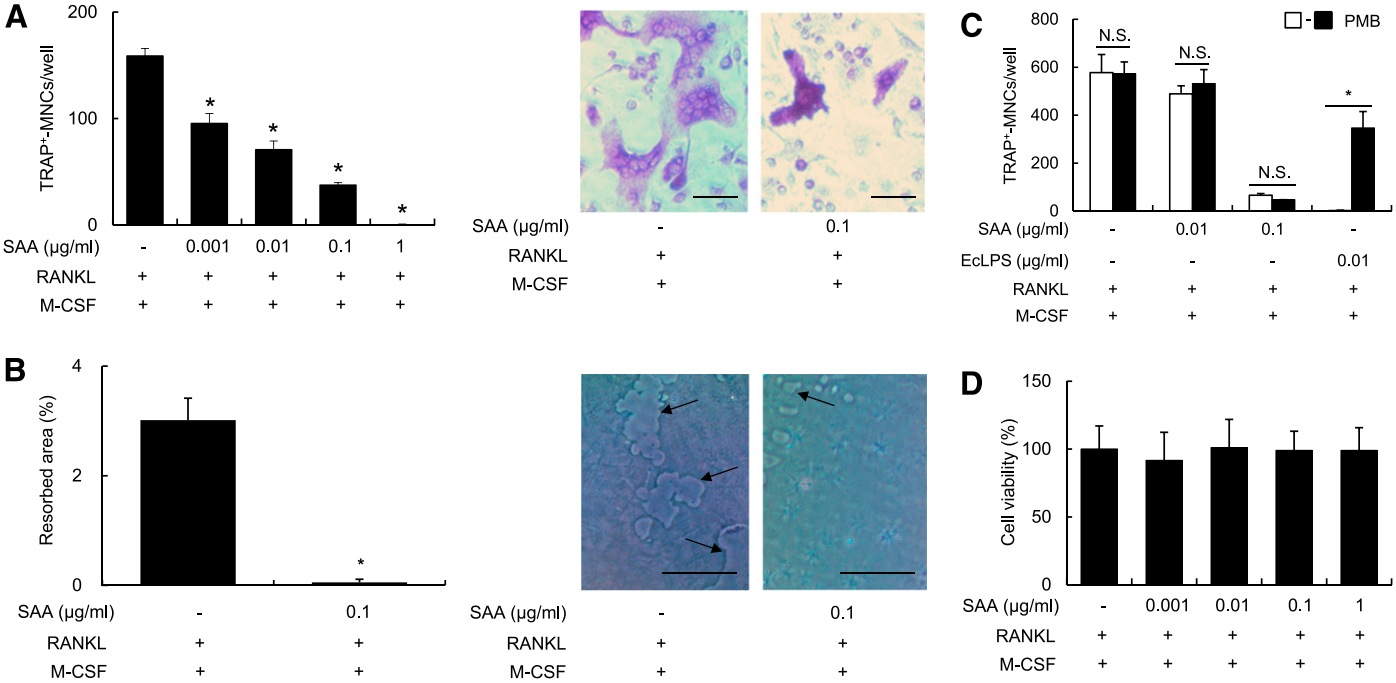

Figure 1. SAA inhibits osteoclast differentiation from BMMs. (A) BMMs plated on 96-well plates were treated with SAA in the presence of M-CSF/RANKL for 3 d. Cells were stained for TRAP, and the number of TRAP$^+$-multinucleated giant cells (MNCs) was counted (left) and photographed using an inverted phase-contrast microscope (right). Scale bars = 50 μm. (B) BMMs plated on a calcium phosphate-coated plate were stimulated with M-CSF/RANKL in the presence or absence of SAA for 5 d. The cells were lysed and the areas of resorption analyzed (left) and photographed (right). Scale bars = 100 μm. (C) BMMs were pretreated with or without polymyxin B (PMB) for 1 h and then stimulated with SAA or *E. coli* LPS (EcLPS) in the presence of M-CSF/RANKL. The cells were stained for TRAP, and TRAP$^+$-MNCs were enumerated. (D) BMMs were stimulated with SAA in the presence of M-CSF/RANKL for 2 d. The live cells were determined using trypan blue staining. Asterisks indicate statistical significance at $P < 0.05$ compared with the control group.

apoptosis [24], opsonization of gram-positive bacteria, and induction of extracellular matrix-degrading enzyme [25]. Furthermore, SAA induces production of proinflammatory cytokines and NO through MAPK activation in macrophages and neutrophils [20, 21]. Because the serum concentrations of APPs reflect inflammatory processes, SAA is considered one of the best markers of inflammatory diseases, including amyloidosis, atherosclerosis, and rheumatoid arthritis [26, 27].

Because increased levels of SAA are related to the severity of early inflammatory arthritis [28] and also because expression of SAA is detected in the bone and bone marrow of patients with osteoarthritis [29], SAA has been presumed to contribute to the regulation of bone metabolism such as osteoclast differentiation. Nevertheless, the effect of SAA on osteoclast differentiation and activation has been poorly studied. Therefore, in the present study, we investigated the effect of SAA on osteoclast differentiation and bone resorption using BMMs as osteoclast precursors, committed osteoclast precursors, an osteoclast-osteoblast coculture system, and a mouse calvarial implantation model.

MATERIALS AND METHODS

Reagents and chemicals

Recombinant human SAA, murine soluble RANKL, and murine M-CSF were purchased from PeproTech (Rocky Hill, NJ, USA). Pam2CSK4 was purchased from EMC Microcollections (Tübingen, Germany). Lipoteichoic acid and lipoproteins were prepared from *Staphylococcus aureus* RN4220, as described previously [30, 31]. *Escherichia coli* LPS (O111:B4), polymyxin B, 1α,25-dihydroxyvitamin D_3, PGE_2, an antibody specific to β-actin, and a leukocyte acid phosphatase staining kit were obtained from Sigma-Aldrich (St. Louis, MO, USA). FITC-conjugated anti-human CD25 antibody and rat anti-mouse CD16/CD32 antibody were obtained from BioLegend (San Diego, CA, USA). Antibodies specific to p38 kinase, phospho-p38 kinase, JNK, phospho-JNK, ERK, phospho-ERK, HRP-conjugated anti-rabbit IgG, and HRP-conjugated anti-mouse IgG were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific to NFATc1, c-Fos, and IRF8 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of osteoclasts and osteoblasts from mice

The institutional animal care and use committee of Seoul National University approved the animal experiments. We obtained 6–12-week-old C57BL/6 mice from Orient Bio (Seongnam, Korea). TLR2-, TLR4-, or MyD88-deficient C57BL/6 mice were provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan). BMMs were prepared as previously described [31]. In brief, bone marrow samples were isolated from mouse femurs and tibiae, and erythrocytes were removed using Red Blood Cell Lysing Buffer (Sigma-Aldrich). The cells were then incubated in α-MEM medium (Gibco-BRL, Paisely, U.K.) supplemented with 10% FBS (Gibco, Grand Island, NY, USA) and 1% Penicillin–Streptomycin Solution (HyClone, Logan, UT, USA) in the presence of M-CSF (5 ng/ml) in a humidified incubator at 37°C with 5% carbon dioxide overnight. Nonadherent cells were collected and incubated with M-CSF (20 ng/ml) for 3 d to differentiate the cells into BMMs, which are osteoclast precursors. The cells were then plated into 96-well culture plates at

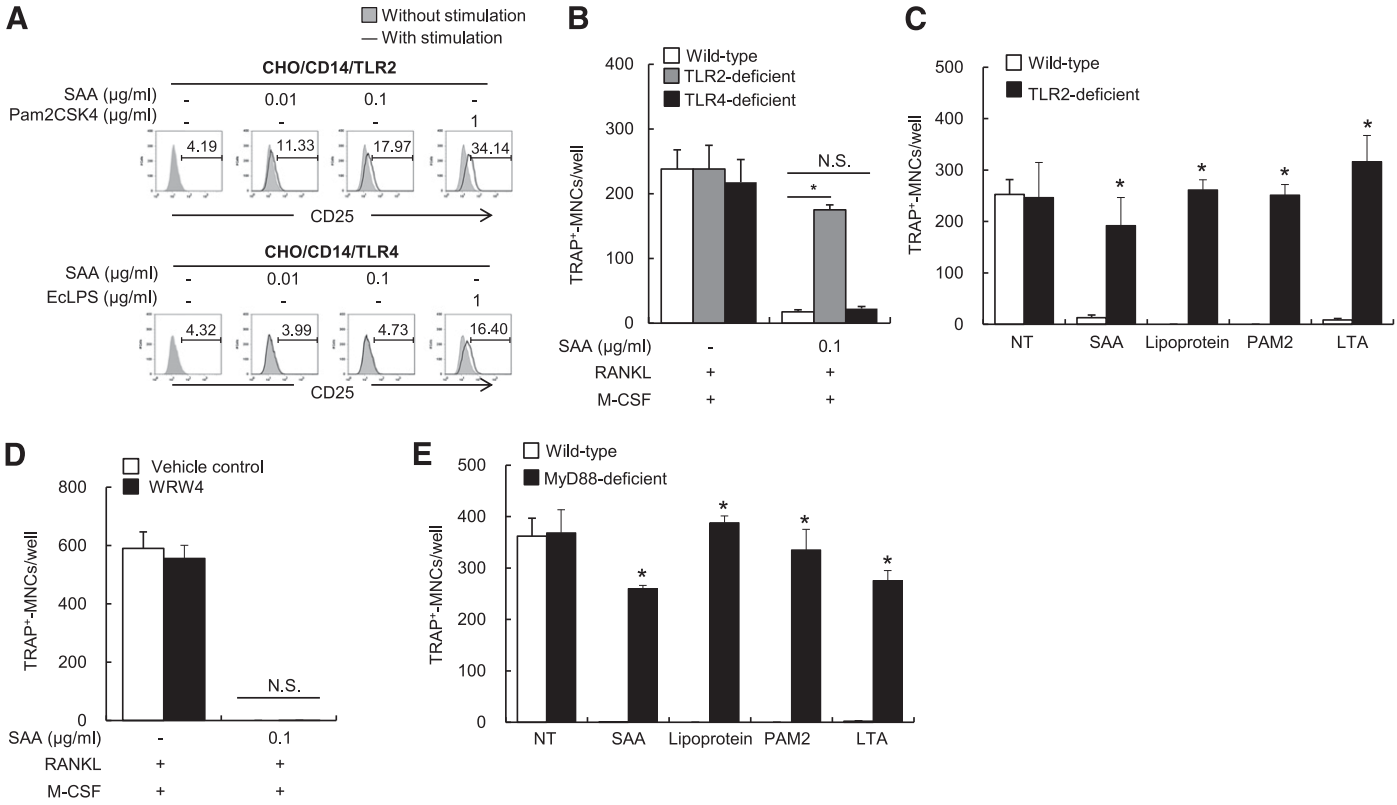

Figure 2. SAA impairs M-CSF/RANKL-induced osteoclast differentiation through TLR2-dependent pathway. (A) CHO/CD14/TLR2 (upper) or CHO/CD14/TLR4 (lower) cells were stimulated with SAA, EcLPS, or Pam2CSK4 (PAM2) for 16 h. The cells were stained with FITC-conjugated anti-CD25 antibody and analyzed by flow cytometry. The number in each panel indicates the percentage of CD25-expressing cells. Pam2CSK4 and EcLPS were used as a positive control. (B) Bone marrow samples obtained from wild-type, TLR2-deficient, or TLR4-deficient mice were differentiated into BMMs with M-CSF for 3 d. Next, the cells were stimulated with M-CSF/RANKL in the presence or absence of SAA for an additional 3 d. The cells were then stained for TRAP to count the number of $TRAP^+$-multinucleated giant cells (MNCs). (C) BMMs were stimulated with SAA (0.1 μg/ml), *S. aureus* lipoprotein (10 μg/ml), Pam2CSK4 (0.1 μg/ml), or *S. aureus* lipoteichoic acid (LTA) (10 μg/ml) in the presence of M-CSF/RANKL for 3 d. The cells were then stained for TRAP to count the number of $TRAP^+$-MNCs. (D) BMMs were pretreated with a FPR antagonist, WRW4 (5 μM) for 1 h. Next, the cells were stimulated with SAA in the presence of M-CSF/RANKL for 3 d. (E) BMMs derived from wild-type and MyD88-deficient mice were stimulated with SAA (0.1 μg/ml), *S. aureus* lipoprotein (10 μg/ml), Pam2CSK4 (0.1 μg/ml), or *S. aureus* lipoteichoic acid (10 μg/ml) in the presence of M-CSF/RANKL for 3 d. After stimulation, the $TRAP^+$-MNCs were enumerated. Asterisks indicate statistical significance at $P < 0.05$ compared with the wild-type control group.

2×10^4 cells/0.2 ml per well and incubated with RANKL (50 ng/ml) and M-CSF (20 ng/ml) in the presence or absence of SAA for 3 d. Mouse osteoblast precursors were isolated from the calvaria of 1-day-old C57BL/6 mice, as previously described [32]. The osteoblast precursors were seeded into 48-well culture plates at 2×10^4 cells per well and incubated with β-glycerophosphate and L-ascorbic acid in the presence or absence of SAA for 6 d. For osteoclast–osteoblast coculture, stroma-free bone marrow samples (1×10^5 cells) and osteoblast precursors (1×10^4 cells) were seeded into 48-well culture plates and incubated with SAA or PGE_2 in the presence of β-glycerophosphate (10 mM), L-ascorbic acid (50 μg/ml), and 1α,25-dihydroxyvitamin D_3 (50 ng/ml) for 8 d.

TRAP staining

The cells were fixed and stained using a leukocyte acid phosphatase-staining kit. The number of TRAP-positive cells with ≥3 nuclei was counted as osteoclasts using an inverted phase-contrast microscope [33].

In vitro resorption assay

Stroma-free bone marrow samples were differentiated into BMMs by incubation with M-CSF (20 ng/ml) for 3 d. The cells were plated at 3×10^4 cells/0.2 ml per well in a 96-well plate coated with calcium phosphate (Corning, Tweksbury, MA, USA) and incubated with RANKL (50 ng/ml) and M-CSF (20 ng/ml) in the presence or absence of SAA (0.1 μg/ml) for 5 d. The cells were lysed with 5% sodium hypochlorite for 10 min and then dried. The resorption pits were photographed, and the resorbed areas were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

In vivo bone resorption assay

A collagen sheet soaked with SAA (10 μg) and/or RANKL (5 μg) was implanted on mouse calvaria. At d 7 after the implantation, the calvarial bone was scanned by micro-computed tomography (Skyscan1172 scanner; Skyscan, Kontich, Belgium), and the resorbed area of the calvarial bone was measured using computed tomography-analyzer software.

Flow cytometric analysis

For the TLR activation assay, the NF-κB reporter cell line CHO/CD14/TLR2 or CHO/CD14/TLR4 was incubated with SAA (0.01 or 0.1 μg/ml), Pam2CSK4 (1 μg/ml), or EcLPS (1 μg/ml) for 16 h and then stained with FITC-conjugated anti-human CD25 antibody for 30 min, as previously

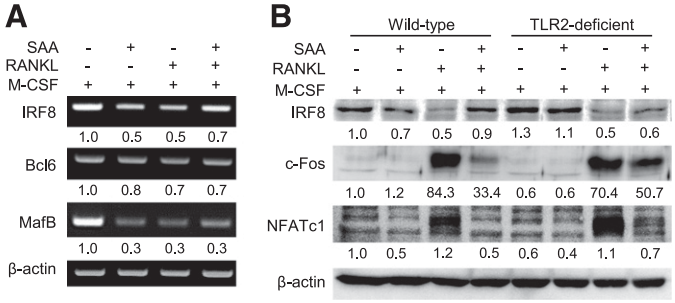

Figure 3. SAA prevents downregulation of IRF8 but inhibits upregulation of c-Fos and NFATc1 during osteoclast differentiation. (A) BMMs were stimulated with SAA and/or RANKL in the presence of M-CSF for 3 h. Total RNA was extracted and subjected to RT-PCR to determine mRNA levels of IRF8, Bcl6, and MafB. β-Actin was used as an internal control. (B) Bone marrow samples isolated from wild-type or TLR2-deficient mice were differentiated into BMMs in the presence of M-CSF for 3 d. BMMs were stimulated with SAA and/or RANKL in the presence of M-CSF for 24 h. Immunoblots were performed with antibodies specific to IRF8, c-Fos, and NFATc1. β-Actin was used as an internal control. The band intensity was measured by densitometry to obtain the relative expression of target molecules normalized by the corresponding β-actin expression. The values indicate the relative expression ratio to that of the control group.

described [34]. For the phagocytosis assay, the osteoclast precursors were incubated with dextran conjugated with FITC (Molecular Probes, Eugene, OR, USA) for 30 min at 37°C or 4°C. Next, the cells were washed 3 times with cold PBS containing 2% FBS and fixed with 1% paraformaldehyde. The cells were then analyzed with a FACSCalibur instrument and CellQuest Pro software (BD Biosciences, San Diego, CA, USA).

Western blot analysis

Western blot analysis was performed as described previously [35]. In brief, the BMMs were serum starved for 3 h and treated with RANKL (50 ng/ml) in the presence or absence of SAA (1 μg/ml) for 5, 15, or 30 min. The samples were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Bedford, MA, USA). After blocking with 5% skim milk in Tris-buffered saline containing 0.05% Tween-20, the membrane was incubated with antibodies specific to MAPKs or phosphorylated MAPKs followed by incubation with HRP-conjugated secondary antibodies. In a separate experiment, BMMs were stimulated with RANKL (50 ng/ml) and/or SAA (0.1 μg/ml) in the presence of M-CSF (20 ng/ml) for 24 h. Immunoblots were prepared as described and incubated with primary antibodies specific to IRF8, c-Fos, and NFATc1. Finally, the immunoblots were incubated with HRP-conjugated secondary antibodies to facilitate detection of the desired immunoreactive bands.

RT-PCR procedure

BMMs were stimulated with SAA (0.1 μg/ml) and/or RANKL (50 ng/ml) in the presence of M-CSF (20 ng/ml) for 3, 6, 12, or 24 h. RT-PCR was performed as described previously [36]. The specific primers used were as follows: IRF8: 5′-GATCGAACAGATCGACAGCA-3′ and 5′-TGGGCTCCTCTTGGTCATAC-3′; Bcl6: 5′-GCACTGGGCAAACACAACAT-3′ and 5′-TCACGGGGAGGTTTAAGTGC-3′; MafB: 5′-CAGGGCTGGTTTGGAATCCT-3′ and 5′-TCCTCTTACTGACCCGCAGA-3′; IL-6: 5′-CCGGAGAGGAGACTTCACAG-3′ and 5′-GGAAATTGGGGTAGGAAGGA-3′; TNF-α: 5′-ATGAGCACAGAAAGCATGATC-3′ and 5′-TACAGGCTTGTCACTCGAATT-3′; IL-1β: 5′-AAGCTCTCACCTCAATGGA-3′ and 5′-TGCTTGAGAGGTGCTGATGT-3′; IL-10: 5′-ATAACTGCACCCACTTCCCA-3′ and 5′- TTTTCACAGGGGAGAAATCG-3′; and β-actin: 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′.

Statistical analysis

The results are reported as the mean value ± SD of triplicate samples from each experiment. Statistical significance was determined using Student's *t* test. An asterisk indicates a significant ($P < 0.05$) difference from the control group.

RESULTS

SAA suppresses differentiation of BMMs into osteoclast

First, we examined the effect of SAA on osteoclast differentiation and bone resorption capacity. SAA inhibited M-CSF/RANKL-induced TRAP-positive multinucleated giant cells from BMMs in a dose-dependent manner (**Fig. 1A**) and decreased the resorbed areas on the calcium phosphate-coated plate (Fig. 1B). High concentrations of SAA (10 μg/ml) also showed the significant inhibition of osteoclast differentiation (data not shown). Because the SAA used in the present study was originated from humans, we also tested whether this SAA inhibits osteoclast differentiation in human primary macrophages. Similar to macrophages from mouse bone marrow, the SAA significantly inhibited the osteoclast differentiation in human primary macrophages (data not shown). To test whether this inhibitory effect was due to endotoxin contamination, the BMMs were pretreated with polymyxin B. As shown in Fig. 1C, the inhibitory effect of SAA was unaffected by polymyxin B treatment, indicating that the inhibitory activity of SAA on osteoclast differentiation was not due to endotoxin contamination. In addition, SAA did not affect cell viability ≤1 μg/ml (Fig. 1D). These findings suggest that SAA suppresses M-CSF/RANKL-induced osteoclast differentiation from BMMs.

SAA inhibition of osteoclast differentiation is mediated through TLR2

Because both TLR2 and TLR4 potentially recognize SAA in macrophages [20, 21], we examined the ability of SAA to stimulate TLR2 and TLR4 using CHO/CD14/TLR2 and CHO/CD14/TLR4 cells, respectively. SAA increased the expression of CD25 on CHO/CD14/TLR2 cells but not on CHO/CD14/TLR4 cells (**Fig. 2A**), indicating that SAA might be recognized by TLR2. To further examine whether TLR2 is involved in the inhibitory effect of SAA on osteoclast differentiation, BMMs derived from wild-type, TLR2-deficient, or TLR4-deficient mice were stimulated with SAA in the presence of M-CSF/RANKL. As shown in Fig. 2B, SAA failed to inhibit osteoclast differentiation of BMMs derived from TLR2-deficient mice, and SAA was inhibitory to the same extent in the BMMs derived from wild-type and TLR4-deficient mice. These results indicate that SAA inhibits osteoclast differentiation through TLR2 signaling. Previous reports have demonstrated that TLR2 ligands such as lipoteichoic acid and lipopeptides inhibit osteoclast differentiation from BMMs [31, 37–39]. Similar to the previous reports, we observed that several TLR2 ligands remarkably inhibited the osteoclast differentiation of BMM from wild-type mice but failed in TLR2-deficienct mice (Fig. 2C), indicating that the

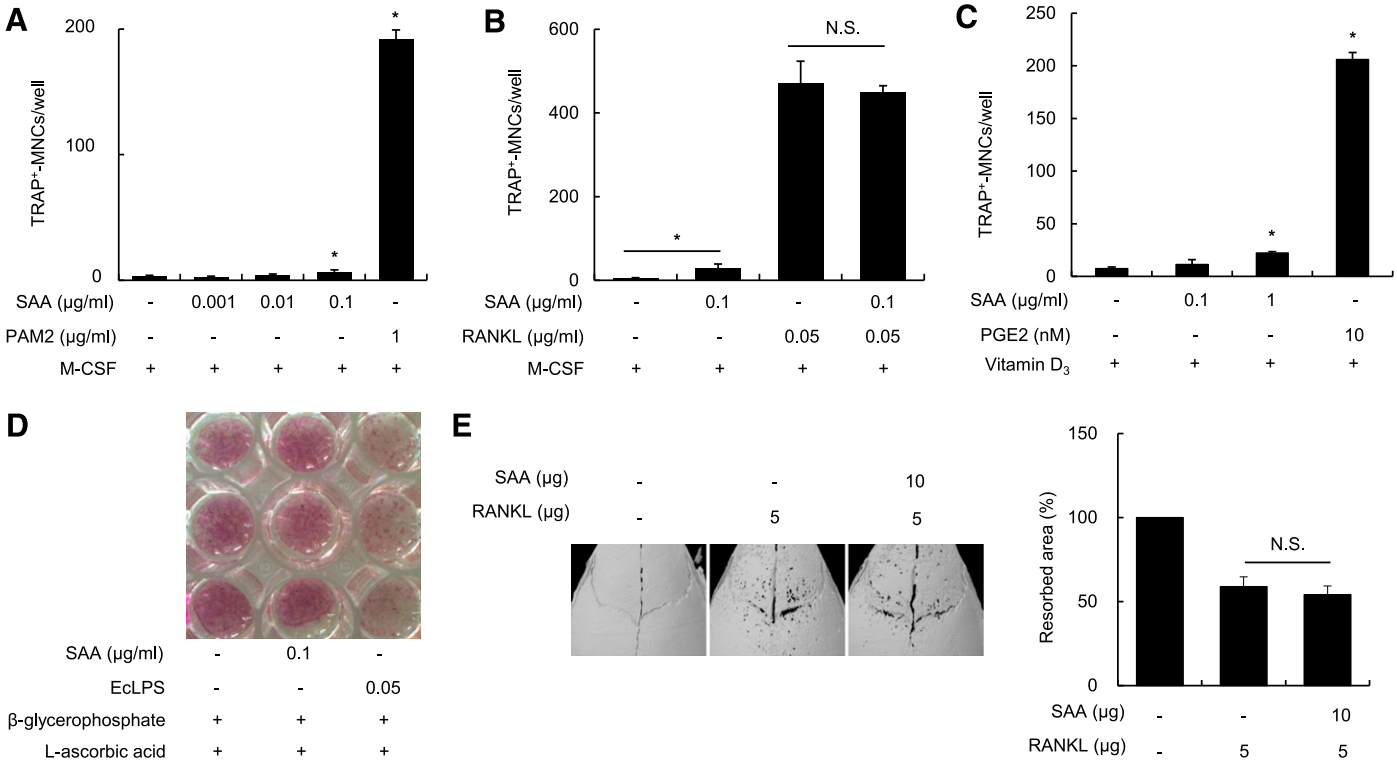

Figure 4. SAA has a negligible effect on differentiation of committed osteoclast precursor cells, osteoclast–osteoblast cocultures, osteoblast differentiation, and calvarial bone resorption. (A) BMMs were incubated with M-CSF/RANKL for 2 d to differentiate BMMs into committed osteoclast precursors. Next, the cells were washed once with fresh culture media and treated with SAA or Pam2CSK4 in the presence of M-CSF for 2 d. The cells were stained for TRAP, and the number of $TRAP^+$-multinucleated giant cells (MNCs) was counted. (B) Committed osteoclast precursors were stimulated with SAA and/or RANKL in the presence of M-CSF for 2 d. The cells were stained for TRAP, and $TRAP^+$-MNCs were enumerated. (C) Mouse calvarial osteoblast precursors were cocultured with stroma-free bone marrow samples in the presence of SAA or PGE_2 in the presence of β-glycerophosphate (10 mM), L-ascorbic acid (50 μg/ml), and 1α,25-dihydroxyvitamin D_3 (50 ng/ml) for 8 d and stained for TRAP, after which $TRAP^+$-MNCs were enumerated. (D) Mouse calvarial osteoblast precursors were stimulated with SAA or EcLPS in the presence of β-glycerophosphate (10 mM) and L-ascorbic acid (50 μg/ml) for 6 d. The cells were stained for alkaline phosphatase and photographed. (E) A collagen sheet soaked with SAA and/or RANKL was implanted on the mouse calvaria for 7 d followed by micro-computed tomography analysis. A representative calvaria scanned image is shown. Asterisks indicate statistical significance at $P < 0.05$ compared with the SAA-untreated control group.

inhibition of osteoclastogenesis by SAA is comparable to the typical effect of TLR2 ligands. However, SAA, an agonist of FPR2, drastically suppressed the osteoclast differentiation even in the presence of WRW4, an antagonist of FPR2 (Fig. 2D), suggesting that G protein-coupled receptors such as FPR2 are not involved in the SAA-mediated inhibition of osteoclast differentiation. Furthermore, SAA was not able to inhibit the osteoclast differentiation of BMMs from MyD88-deficient mice (Fig. 2E), indicating that SAA inhibits osteoclast differentiation through TLR2/MyD88.

SAA interferes with downregulation of IRF8 and activation of c-Fos and NFATc1 induced by treatment with M-CSF and RANKL

IRF8, Bcl6, and MafB are known as negative regulators of osteoclast differentiation [7–9], all of which are downregulated during osteoclast differentiation. Thus, we hypothesized that the inhibitory effect of SAA on osteoclast differentiation results from the sustained expression of negative regulators without downregulation. Concordant with previous studies, the mRNA expression of IRF8, Bcl6, and MafB was diminished by M-CSF/RANKL treatment. In contrast, SAA interfered with the downregulation of IRF8 mRNA under the same conditions (**Fig. 3A**). The expression of c-Fos and NFATc1 was markedly augmented by M-CSF/RANKL. However, a similar increase was not observed in the presence of SAA under the same conditions. The effects of SAA on IRF8, c-Fos, and NFATc1 were confirmed in BMMs derived from wild-type mice but not in cells from TLR2-deficient mice (Fig. 3B). These results suggest that SAA interferes with the downregulation of IRF8 and the activation of c-Fos and NFATc1 induced by M-CSF plus RANKL treatment through a TLR2-dependent signaling mechanism.

SAA is a poor inducer of osteoclast differentiation in committed precursors or an osteoclast-osteoblast coculture system and has no effect on RANKL-induced bone resorption in vivo

We next examined whether SAA also affects osteoclast differentiation of committed osteoclast precursors. Unlike

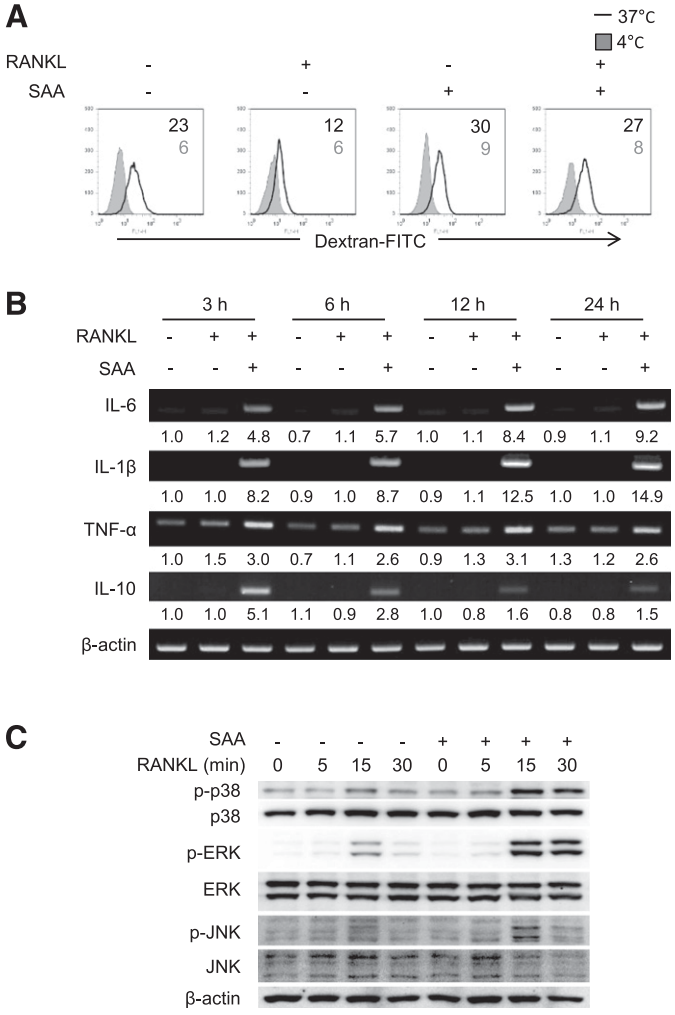

Figure 5. SAA sustains the endocytic capacity and inducibility of proinflammatory cytokines of osteoclast precursors. (A) BMMs were stimulated with SAA and/or RANKL in the presence of M-CSF for 2 d. The cells were then incubated with fresh culture media containing FITC-conjugated dextran for 30 min at 4°C or 37°C, and uptake of dextran-FITC was measured by flow cytometry. The mean fluorescence intensity is shown in each histogram: upper, 37°C; lower, 4°C control. (B) BMMs were stimulated with RANKL and/or SAA in the presence of M-CSF for 3, 6, 12, or 24 h. Total RNAs were extracted and subjected to RT-PCR to determine the mRNA levels of IL-6, IL-1β, TNF-α, and IL-10. β-Actin was used as an internal control. The band intensity was measured by densitometry to obtain the relative expression of cytokines normalized by the corresponding β-actin expression. The values indicate the relative expression ratio to that of the control group. (C) BMMs were serum deprived for 3 h and then stimulated with RANKL in the presence or absence of SAA for 5, 15, or 30 min. Immunoblots were performed with antibodies specific to phosphorylated or nonphosphorylated forms of MAPKs, including ERK, p38 kinase, and JNK.

Pam2CSK4, SAA neither induced TRAP$^+$-multinucleated giant cells in the presence of M-CSF nor enhanced osteoclast differentiation in the presence of M-CSF/RANKL (**Fig. 4A and B**). Moreover, SAA poorly induced osteoclast differentiation in the osteoclast–osteoblast coculture system (Fig. 4C). In contrast, no change was observed in osteoblast differentiation by SAA, and LPS substantially decreased osteoblast differentiation (Fig. 4D). We further examined the effect of SAA on the regulation of bone metabolism in vivo using a mouse calvarial bone resorption model. Concordant with our in vitro results, RANKL-induced bone resorption was not affected by SAA treatment (Fig. 4E). These results suggest that the inhibitory effect of SAA is limited to the differentiation of osteoclast precursor BMMs and does not contribute to RANKL-induced bone destruction in vivo.

SAA enhances endocytic capacity and induces expression of proinflammatory cytokines in osteoclast precursors

As monocyte/macrophage lineage cells differentiate toward osteoclasts, the cells exhibit decreased endocytic capacity and cytokine production [39]. Because SAA inhibited osteoclast differentiation only from BMMs but not from committed osteoclast precursors, we hypothesized that SAA might prevent the reduction of endocytic capacity and the inducibility of proinflammatory cytokines in monocyte/macrophage lineage cells. Uptake of FITC-conjugated dextran was increased when BMMs were differentiated into osteoclasts in the presence of SAA (**Fig. 5A**). Concomitantly, increased mRNA expression of cytokines, including IL-6, IL-1β, TNF-α, and IL-10, was observed (Fig. 5B). Furthermore, phosphorylation of p38 kinase, ERK, and JNK was augmented in BMMs stimulated with SAA compared with those not treated with SAA (Fig. 5C). These results suggest that SAA causes BMMs to maintain their endocytic function by decreasing their capacity for osteoclast differentiation.

DISCUSSION

Serum concentrations of APPs such as SAA can increase rapidly under inflammatory conditions [27]. As summarized in **Fig. 6**, we have shown that SAA inhibited the differentiation of BMMs into osteoclasts through TLR2/MyD88, in which IRF8 might play an important role. Instead, osteoclast precursors maintained their endocytic functions and ability to induce proinflammatory cytokines as macrophages in the presence of SAA, even under the conditions of osteoclast differentiation. Therefore, we suggest that increased SAA expression under inflammatory conditions inhibits differentiation of macrophages to osteoclasts, the reason for which is probably to maintain the function of monocyte/macrophage lineage cells for host defense.

In the present study, we found that SAA inhibited osteoclast differentiation from BMMs. The regulation of bone metabolism by other APPs has also been observed. For example, treatment with plasminogen inhibits osteoclast differentiation in vitro and, in contrast, plasminogen deficiency decreases bone mineral density [15]. Likewise, haptoglobin and lipocalin-2 induce bone destruction by stimulating PGE_2 biosynthesis in osteoblasts [16, 17] and inducing RANKL expression in osteoblasts [18]. However, despite the inhibitory effects of SAA on osteoclast differentiation, SAA was a poor inducer of osteoclast differentiation from committed

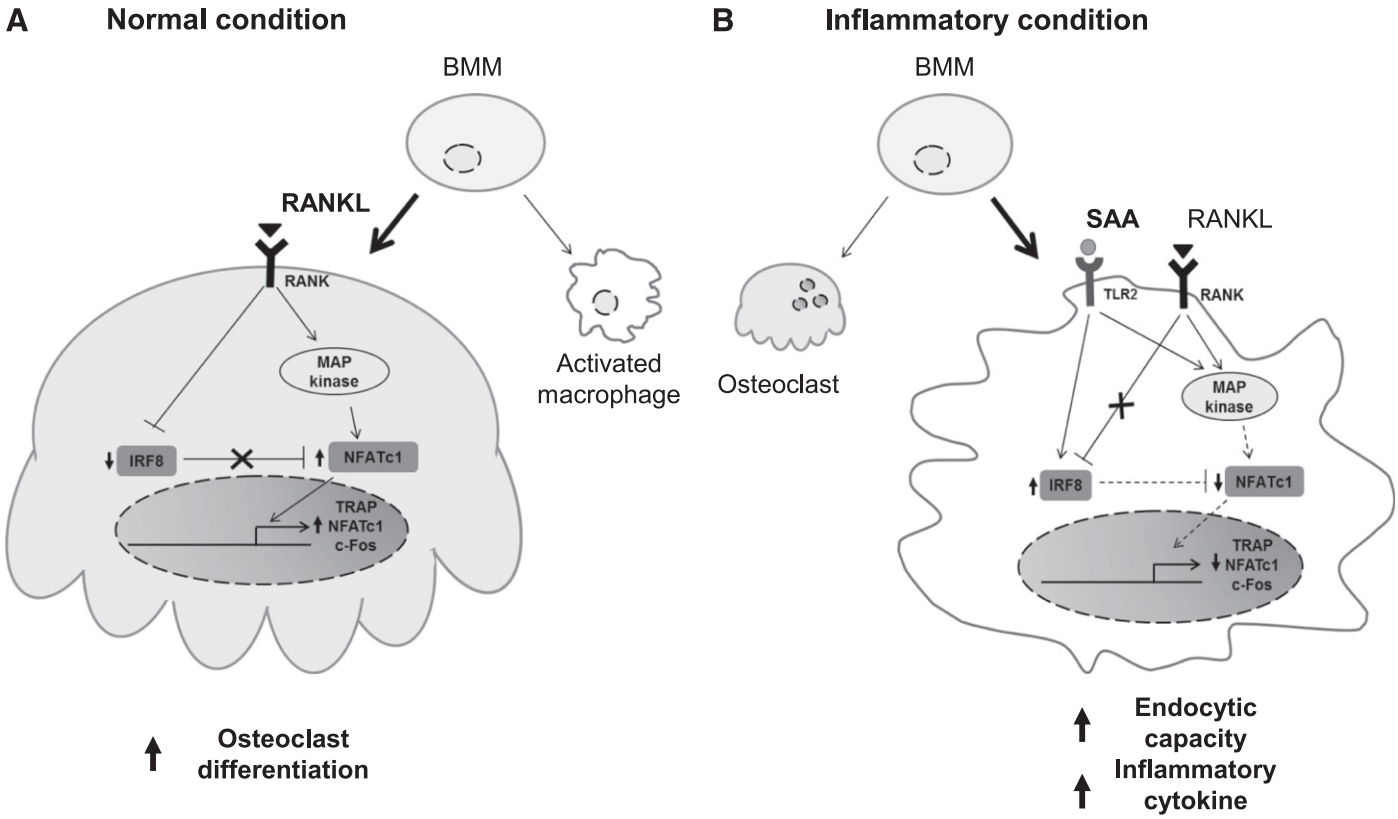

Figure 6. Schematic illustration of the proposed mechanism of action. SAA inhibits osteoclast differentiation by maintaining IRF8 expression. (A) Under normal conditions, RANKL induces osteoclast differentiation from monocytes/macrophages. (B) Under inflammatory conditions, increased SAA expression inhibits RANKL-induced osteoclast differentiation of monocytes/macrophages by preventing downregulation of IRF8 and upregulation of c-Fos/NFATc1. Consequently, it sustains the endocytic capacity and inducibility of proinflammatory cytokines.

osteoclast precursors and osteoclasts in an osteoclast–osteoblast coculture system. Moreover, SAA did not elicit bone destruction when a collagen sheet soaked with SAA was implanted in the mouse calvarial region. Therefore, our results suggest that SAA is similar to other APPs in that it regulates bone metabolism by influencing osteoclast differentiation, albeit through different mechanisms.

IRF8, together with Bcl6 and MafB, is known as a transcriptional repressor that suppresses the expression of genes such as cathepsin K and TRAP, both of which are required for osteoclast function [7–9]. In the present study, SAA sustained mRNA expression of IRF8. IRF8 has been reported to inhibit NFATc1 transcriptional activity and to physically interact with NFATc1. The physical interaction between NFATc1 and IRF8 suppresses the DNA-binding activity of NFATc1 [9]. In addition, overexpression of IRF8 inhibits osteoclast differentiation [40], and IRF8 deficiency induces an osteoporotic phenotype due to an increased number of osteoclasts [9]. Thus, we propose that the inhibitory effect of SAA on osteoclast differentiation might be due to active maintenance of IRF8-mediated suppression of NFATc1 activity.

Our results showed that SAA induced the expression of cytokines, including IL-6, IL-1β, TNF-α, and IL-10, and an increase in the endocytic capacity of BMMs. These results are consistent with other reports showing that SAA enhances the expression of TNF-α, IL-10, and IL-12 through activation of ERK1/2, p38 kinase, and JNK with induction of IκBα degradation, which is absent in $tlr2^{-/-}$ mouse macrophages [20]. Furthermore, SAA induces NO production with phosphorylation of ERK1/2 and p38 kinase in murine peritoneal macrophages [21]. SAA also induces the expression of pro-IL-1β and can activate caspase-1 to facilitate secretion of mature IL-1β [41]. In addition, SAA enhances the endocytic activity of polymorphonuclear cells and induces the expression of CD11c and CD16, both of which are involved in microbial recognition [42]. A recent report showed that depletion of SAA is not intimately associated with osteoclast differentiation [43]. However, APPs, including SAA, are more likely to contribute to the development of inflammatory function of macrophages by inducing inflammatory cytokines through the activation of MAPK and NF-κB activation [44]. Thus, our results, together with those of other studies, suggest that SAA stimulates macrophages to elicit inflammatory responses.

In conclusion, we have demonstrated the inhibitory effect of SAA on the differentiation of macrophages to osteoclasts. SAA sustained the endocytic capacity of macrophages and their ability to express the proinflammatory cytokines IL-6, IL-1β, and TNF-α. Taken together, these results suggest that the increased expression of SAA under inflammatory condition inhibits differentiation of macrophages to osteoclasts, the purpose of

which is likely to prevent a decrease in macrophage function to maintain host defense.

AUTHORSHIP

S.H.H. conceived the research, designed the experiments, and contributed to the discussion of the results followed by writing and reviewing the manuscript. J.K., J.Y., and O.J.P. performed the experiments and contributed to the discussion of the results followed by writing and reviewing the manuscript. J.K., J.Y., O.J.P., S.S.K., C.H.Y., and S.H.H. analyzed and/or interpreted the data. S.S.K. contributed to the discussion of the results followed by writing and reviewing the manuscript. C.H.Y. provided critical comments and contributed to the discussion of the results followed by writing and reviewing the manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

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