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Serum Amyloid A Contributes to Chronic Apical Periodontitis via TLR2 and TLR4

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Abstract

In the current concept of bacterial infections, pathogen-associated molecular patterns (PAMPs) derived from pathogens and damage-associated molecular patterns (DAMPs) released from damaged/necrotic host cells are crucial factors in induction of innate immune responses. However, the implication of DAMPs in apical and marginal periodontitis is unknown. Serum amyloid A (SAA) is a DAMP that is involved in the development of various chronic inflammatory diseases, such as rheumatoid arthritis. In the present study, we tested whether SAA is involved in the pathogenesis of periapical lesions, using human periapical surgical specimens and mice deficient in SAA and Toll-like receptors (TLR). SAA1/2 was locally expressed in human periapical lesions at the mRNA and protein levels. The level of SAA protein appeared to be positively associated with the inflammatory status of the lesions. In the development of mouse periapical inflammation, SAA1.1/2.1 was elevated locally and systemically in wild-type (WT) mice. Although SAA1.1/2.1 double-knockout and SAA3 knockout mice had redundant attenuation of the extent of periapical lesions, these animals showed strikingly improved inflammatory cell infiltration versus WT. Recombinant human SAA1 (rhSAA1) directly induced chemotaxis of WT neutrophils in a dose-dependent manner in vitro. In addition, rhSAA1 stimulation significantly prolonged the survival of WT neutrophils as compared with nonstimulated neutrophils. Furthermore, rhSAA1 activated the NF-κB pathway and subsequent IL-1α production in macrophages in a dose-dependent manner. However, TLR2/TLR4 double deficiency substantially diminished these SAA-mediated proinflammatory responses. Taken together, the SAA-TLR axis plays an important role in the chronicity of periapical inflammation via induction of inflammatory cell infiltration and prolonged cell survival. The interactions of PAMPs and DAMPs require further investigation in dental/oral inflammation.

Keywords: endodontics, alarmins, inflammation, innate immunity, pattern recognition receptors, chemotaxis

Introduction

The discovery of a causal relationship between bacterial infection and apical periodontitis (periapical lesions; Kakehashi et al. 1965) has led to numerous studies on host-bacteria interactions. Activation of a cascade of inflammatory events in response to endodontic infection contributes to the killing and containment of pathogens. Thus, inflammation is basically a protective response. However, failure of bacteria clearance will result in a chronic inflammatory state, leading to the unwanted destruction of periapical tissue (Stashenko et al. 1998; Kawashima and Stashenko 1999). In these processes, pathogen-associated molecular patterns trigger the inflammatory cascade via Toll-like receptors (TLRs) and other pattern recognition receptors (Martinon and Tschopp 2005). However, there is increasing interest in damage-associated molecular patterns, including HMGB1 (high-mobility group box 1), S100A8, S100A9, and SAA (serum amyloid A), which are endogenous molecules released by damaged/necrotic host cells (Bianchi 2007; Rubartelli and Lotze 2007). When damage-associated molecular patterns are persistently released, inflammation fails to resolve, and chronic diseases and fibrosis/granulation tissue develop (Rubartelli and Lotze 2007).

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A supplemental appendix to this article is available online.

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The SAA family comprises 4 members. Humans have 2 SAA isoforms, SAA1 and SAA2, that are concomitantly strongly induced in the liver but are also expressed extrahepatically. Plasma levels of SAA1 and SAA2, as acute-phase proteins, can increase ≥1,000-fold (>700 μg/mL) during acute inflammation, making up the bulk of SAA associated with high-density lipoproteins (HDL; Coetzee et al. 1986; Casl et al. 1995; Yamada 1999). The mouse homologs SAA1.1 and SAA2.1 are the major acute-phase SAAs produced in the liver. Mice express a third acute-phase SAA isoform, SAA3, which is predominantly produced by extrahepatic tissues and is not present in humans due to a premature stop codon (Kluve-Beckerman et al. 1991). SAA3 is induced in the liver as well as extrahepatic sites. Plasma SAA3 levels constitute ~20% of SAA1.1/2.1 levels. The majority of SAA3 is associated with HDL, with ~15% in a lipid-poor/free form (Tannock et al. 2018). The fourth SAA isoform, SAA4, is constitutively expressed at low levels in mice and humans (Whitehead et al. 1992).

As a component of the innate immune system, SAA acts in a proinflammatory way when it is not associated with HDL (Kim et al. 2013; Shridas et al. 2018). In modern chronic inflammatory diseases, including rheumatoid arthritis, metabolic syndrome, diabetes, and cardiovascular diseases, SAA remains elevated at low levels over extended periods, and its proinflammatory action becomes noxious (O'Hara et al. 2000; King et al. 2011; Marzi 2013; Yang et al. 2016).

In preliminary studies, we found that the *SAA3* gene is highly upregulated in wild-type (WT) mouse periapical lesions (>90-fold vs. negative controls). Also, 2 recent studies reported that periodontitis elevates the systemic level of SAA, which correlated with the severity of disease (Ardila and Guzmán 2015; Türer et al. 2017). However, the exact role of SAA in the development of oral inflammatory diseases has not been determined. In the present study, we investigated the role of SAA in the pathogenesis of periapical lesions using human radicular granuloma samples and SAA- and TLR-deficient mice.

Materials and Methods

Human Periapical Surgical Specimens

Specimens ($n = 8$) of human periapical lesions diagnosed by a pathologist as radicular granuloma were collected during endodontic surgery. Samples were divided for total RNA extraction and histologic examination and appropriately stored for each purpose. All patients signed an informed consent form for participation in the study and for the use of their biological tissues. A summary of clinical samples is available in the Appendix. The study was reviewed and approved by the institutional ethics committee of Tokyo Medical and Dental University (approval D2014-039).

Animals

SAA1.1/2.1 double knockout (dKO; de Beer et al. 2010), SAA3 knockout (KO), TLR2 KO, TLR4 KO (Hoshino et al. 1999), and TLR2/4 dKO (Rider et al. 2016) mice were employed in this study. Frozen sperm of the SAA3 KO strain and breeding pairs of TLR2 KO mice (B6.129-*Tlr2*^{tm1Kir}/J) were purchased from the Knockout Mouse Project at the University of California–Davis and The Jackson Laboratory, respectively. C57BL/6NTac (Taconic Bioscience) and C57BL/6J (The Jackson Laboratory) served as the WT controls. Mice were maintained at either The Forsyth Institute Animal Facility or the University of Michigan Laboratory Animal Facility under specific pathogen-free conditions. All experimental protocols were approved by the Institutional Animal Care and Use Committees at The Forsyth Institute and the University of Michigan.

Reverse Transcription Polymerase Chain Reaction

To examine human *SAA1* and *SAA2* expressions, total RNA samples isolated from human surgical specimens were reverse transcribed and subjected to reverse transcription polymerase chain reaction (see Appendix Materials and Methods for further details). The *ACTB* (actin beta) gene served as a reference gene.

Induction of Mouse Periapical Lesions

Age-matched SAA1.1/2.1 dKO, SAA3 KO, and WT mice (8 wk of age) were subjected to pulpal infection as previously described (Sasaki et al. 2000). Mice were sacrificed on days 0 (noninfected baseline control), 10, 21, 28, and 56 after infection. The sample size was at least 4 per group (Figs. 2, 3) per a power analysis based on our previous studies. After euthanasia, mandibles were isolated and hemisected. One hemimandible was fixed in 4% paraformaldehyde in phosphate-buffered saline and subjected to micro–computed tomography and histology. The other hemimandible was immediately frozen and kept in −80 °C until total protein extraction for enzyme-linked immunosorbent assays (ELISAs). In addition, serum samples were obtained by tail vein bleeding at the end of the indicated observation periods.

Micro–computed tomography and Histology

The extent of mouse periapical lesions was quantified with micro–computed tomography and expressed as millimeters squared (mm^2) as previously described (AlShwaimi et al. 2013). The mouse hemimandibles and human surgical specimens were subjected to histology, including immunohistochemistry for human SAA, Ly-6G (neutrophils), and Mac2 (macrophages; see Appendix Materials and Methods).

Isolation of Primary Myeloid Cells

Mouse resident peritoneal neutrophils and macrophages were isolated as previously described (Sasaki et al. 2000; Swamydas et al. 2015). The cells were cultured in RPMI 1640 (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum

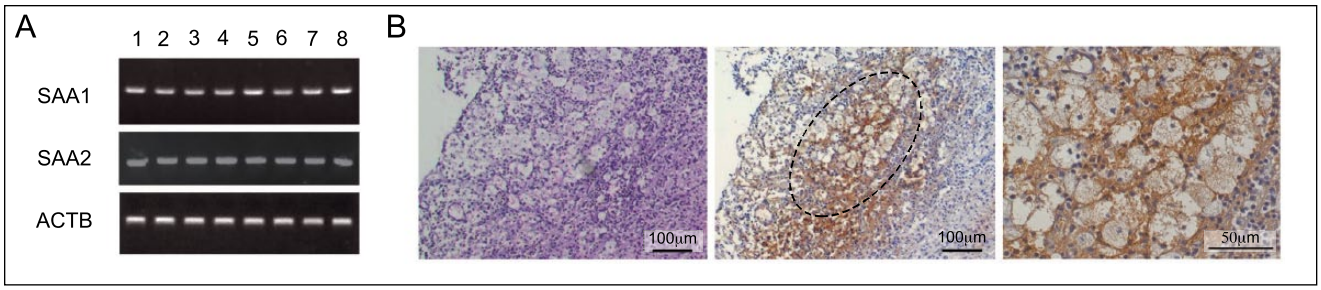

Figure 1. Serum amyloid A (SAA) was expressed and is present in human periapical lesions. (**A**) *SAA1* and *SAA2* gene expressions in 8 human radicular granuloma samples by reverse transcription polymerase chain reaction. (**B**) Histologic analysis for the localization of SAA1/2. Left panel, representative images of hematoxylin and eosin staining; middle and right panels, immunohistochemistry for SAA (×100). The circle in the middle panel specifies the area for high-power observation (×200) of SAA-positive cells (right panel).

(Biowest USA); stimulated with recombinant human SAA1 (rhSAA1; PeproTech); and subjected to ELISA, chemotaxis, and cell viability assays.

Enzyme-Linked Immunosorbent Assay

Expression of mouse SAA1.1/2.1, SAA3, and IL-1α in mouse serum samples, periapical lesions, and cell culture supernatants was determined by ELISA with commercially available kits (Mouse SAA/SAA1 PicoKine ELISA Kit and Mouse SAA3 ELISA Kit, MyBiosource; Mouse IL-1α DuoSet ELISA, R&D Systems) following the manufacturers' instructions. For ELISA, periapical tissue samples were disrupted with FastPrep-24 with matrix A (MP Biomedicals) in a cell lysis buffer (Cell Signaling Technology) supplemented with Halt protease inhibitor cocktail (Thermo Fisher Scientific). Culture supernatant samples were harvested after stimulation with rhSAA1 (10 and 100 μg/mL), LPS (lipopolysaccharide, *Escherichia coli* serotype 0111:B4 [TLR ligand tested], 0.1 μg/mL; Sigma-Aldrich), and Pam2CSK4 (0.1 μg/mL; InvivoGen) for 24 h. Medium alone served as a control. Results were expressed as cytokine/periapical tissue (ng/mg) or cytokine/supernatant (ng/mL).

Chemotaxis Assay

SAA-induced chemotaxis of neutrophils was quantified with a multichannel module, μ-Slide Chemotaxis chamber (ibidi), according to the manufacturer's instructions. Time-lapse images of chemotactic movement were captured with a BZ-X700 microscope (Keyence) at 2-min intervals for 3 h. ImageJ (National Institutes of Health) and the Chemotaxis and Migration Tool Software (ibidi) were used for data analyses. The x-axis forward migration indices were used for statistical evaluation of migration directionality in response to rhSAA1 (Foxman et al. 1999).

Cell Viability Assay

Cell viability of cultured neutrophils in the presence or absence of rhSAA1 (10 and 100 μg/mL) was determined at 0, 6, 12, and 18 h after stimulation with the LIVE/DEAD Viability/Cytotoxicity Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Results were expressed as percentage of viable cells/total cells.

NF-κB Reporter Assay

The effect of SAA on NF-κB activity was assessed with NF-κB Luciferase Stable RAW264.7 cells (NF-κB RAW; Applied Biological Materials), in which luciferase expression depends on NF-κB promoter activation.

Statistics

Statistical analyses were performed by 1- or 2-way analysis of variance with Bonferroni post hoc test. Additional details of experimental procedures are provided in the Appendix Materials and Methods. The in vivo experiment was performed once. All in vitro assays were repeated at least 2 times; no significant variability was seen in any experiment.

Results

SAA1/2 Expression in Human Radicular Granulomas

In human radicular granulomas, the *SAA1* and *SAA2* genes were expressed in all samples (Fig. 1A). In 6 of 8 lesions, the presence of SAA proteins was assessed immunohistochemically (Fig. 1B, Appendix Table 1); there was a trend toward higher SAA levels in lesions that correlated with the level of inflammatory cell infiltration. Consistent with previous studies (Meek et al. 1992; Yang et al. 2016), strongly SAA-positive cells were macrophages by morphology (Fig. 1B).

Endodontic Infection Elevates Local and Systemic Levels of SAA1.1/2.1 in Mice

The function of SAA was subsequently investigated in a well-established mouse periapical lesion model with SAA1.1/2.1 dKO, SAA3 KO, and corresponding WT mice. We first examined the effect of endodontic infection on systemic and local

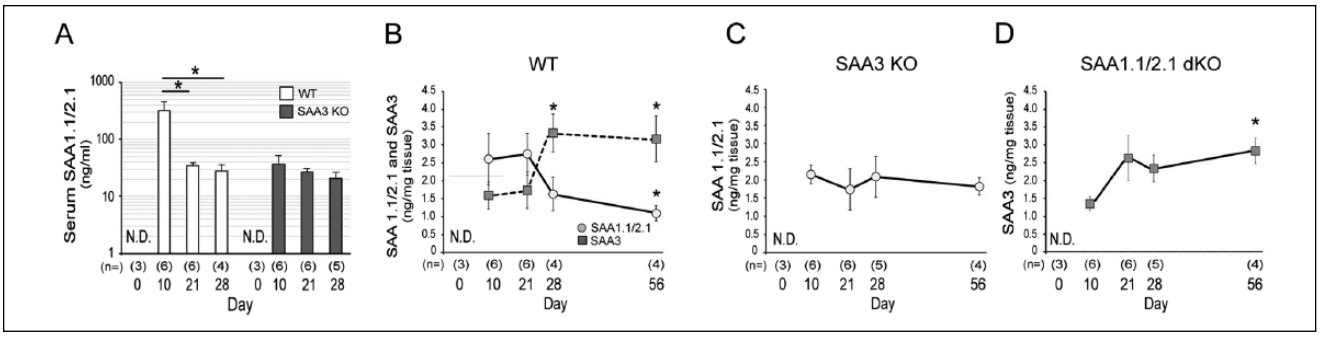

Figure 2. Endodontic infection elevated local and systemic levels of SAA in mice as determined by ELISA. (**A**) Serum levels of SAA1.1/2.1 in WT and SAA 3 KO mice after pulpal infection. *$P < 0.05$. (**B**) Local production of SAA in WT periapical lesions. *$P < 0.05$ vs. day 10. (**C**) Local production of SAA1.1/2.1 in SAA3 KO lesions. (**D**) Local production of SAA3 in SAA1.1/2.1 dKO lesions. *$P < 0.05$ vs. day 10. The level of SAA proteins was expressed as SAA/serum (ng/mL) or SAA/periapical tissue (ng/mg). Error bars indicate SD. dKO, double knockout; KO, knockout; ND, not detected; SAA, serum amyloid A; WT, wild type.

SAA protein levels. In WT mice, pulpal infection induced a sharp SAA1.1/2.1 spike in serum on day 10 after pulpal infection, with levels declining rapidly thereafter to approximately 10% in the chronic phase after day 21 (Fig. 2A). In SAA3 KO mice, a low level of serum SAA1.1/2.1 was detected throughout the experiment; however, no clear SAA spike was observed (Fig. 2A). Locally, pulpal infection clearly induced SAA1.1/2.1 in WT lesions on day 10 (Fig. 2B), and the level was maintained up to day 21, declining thereafter by about 50% versus the initial induction. The pattern of SAA3 production in WT lesions was distinct, showing a modest induction by day 10 and peaking later (days 28 and 56). In infected SAA3 KO mice, SAA1.1/2.1 was expressed at a constant level in periapical tissue (Fig. 2C). However, local SAA3 gradually increased in SAA1.1/2.1 dKO mice and reached a peak on day 56 (Fig. 2D).

SAA Modulates Periapical Inflammatory Cell Infiltration but Not Lesion Size

The impact of SAA deficiencies on the development of periapical lesions was examined (Fig. 3A). Within each observation period, no genotype effect was observed on lesion size except for day 28 (WT vs. SAA1.1/2.1 dKO), indicating that SAA deficiencies had no significant overall effect on the extent of periapical lesions. In contrast, a striking histologic difference was observed between WT and SAA KO strains (Fig. 3B, C). Although all genotypes exhibited mild-moderate periapical inflammation on day 10 (data not shown), inflammatory cell infiltration by myeloid cells was dramatically reduced in both SAA1.1/2.1 dKO and SAA3 KO mice as compared with WT controls after day 21. Whereas widely diffused infiltration of inflammatory myeloid cells was observed in WT lesions, SAA KO mice exhibited very localized infiltration centered on the apical foramen.

SAA Regulates Chemotaxis and Survival of Neutrophils via TLR2 and TLR4

Since SAA deficiencies attenuated periapical inflammatory cell infiltration, we analyzed SAA-mediated neutrophil chemotaxis in vitro. rhSAA1 directly induced directional chemotaxis of WT neutrophils in a dose-dependent manner, which was statistically significant at 100 μg/mL (Fig. 4A, B). rhSAA1 (10 μg/mL) stimulated nondirectional neutrophil migration (Appendix Fig. 1). Next, we determined if TLR2 and TLR4 are involved in the SAA-stimulated chemotaxis with neutrophils isolated from TLR2 KO, TLR4 KO, and TLR2/TLR4 dKO mice. TLR2 and TLR4 deficiency resulted in reduced migration directionality per x-axis forward migration indices (57% and 63% vs. WT cells, respectively; Fig. 4C, D). In contrast, TLR2/TLR4 dKO neutrophils failed to respond to rhSAA1 as compared with WT cells ($P < 0.05$), suggesting that SAA utilizes TLR2 and TLR4 similarly for directional chemotaxis of neutrophils. In contrast, TLR4 appeared to be important in the SAA-stimulated nondirectional migration of neutrophils (Fig. 1, Appendix). Furthermore, rhSAA1 significantly enhanced the viability of WT neutrophils in vitro (Fig. 4E). This effect was totally abolished in TLR2/TLR4-deficient neutrophils (Fig. 4F).

Recombinant Human SAA1 Stimulates Macrophages via TLR2 and TLR4

Because macrophages play a prominent role in periapical inflammation (Wang and Stashenko 1993) and are a source of extrahepatic SAA3 (Meek et al. 1992), we assessed the in vitro stimulation of this cell type by rhSAA1. As shown in Figure 5A, only LPS, a major ligand for TLR4, triggered macrophage SAA3 production, whereas the synthetic TLR2 ligand Pam2CSK4 had no effect. SAA1.1/2.1 was not clearly induced by either of these 2 pathogen-associated molecular patterns (data not shown). We next examined if SAA activates NF-κB via TLRs. As shown in Figure 5B, rhSAA1 alone significantly activated the NF-κB promoter in a dose-dependent fashion (range, 0.1 to 10 μg/mL; $P < 0.05$). The level of NF-κB promoter activity induced by 10 μg/mL of rhSAA1 was comparable to that induced by 0.1 μg/mL of LPS and 0.1 μg/mL of Pam2CSK4. Using siRNAs, we showed that knockdown of TLR2 and TLR4 reduced SAA-stimulated luciferase activity by 79% and 64% versus scrambled siRNA control, respectively (Fig. 5C).

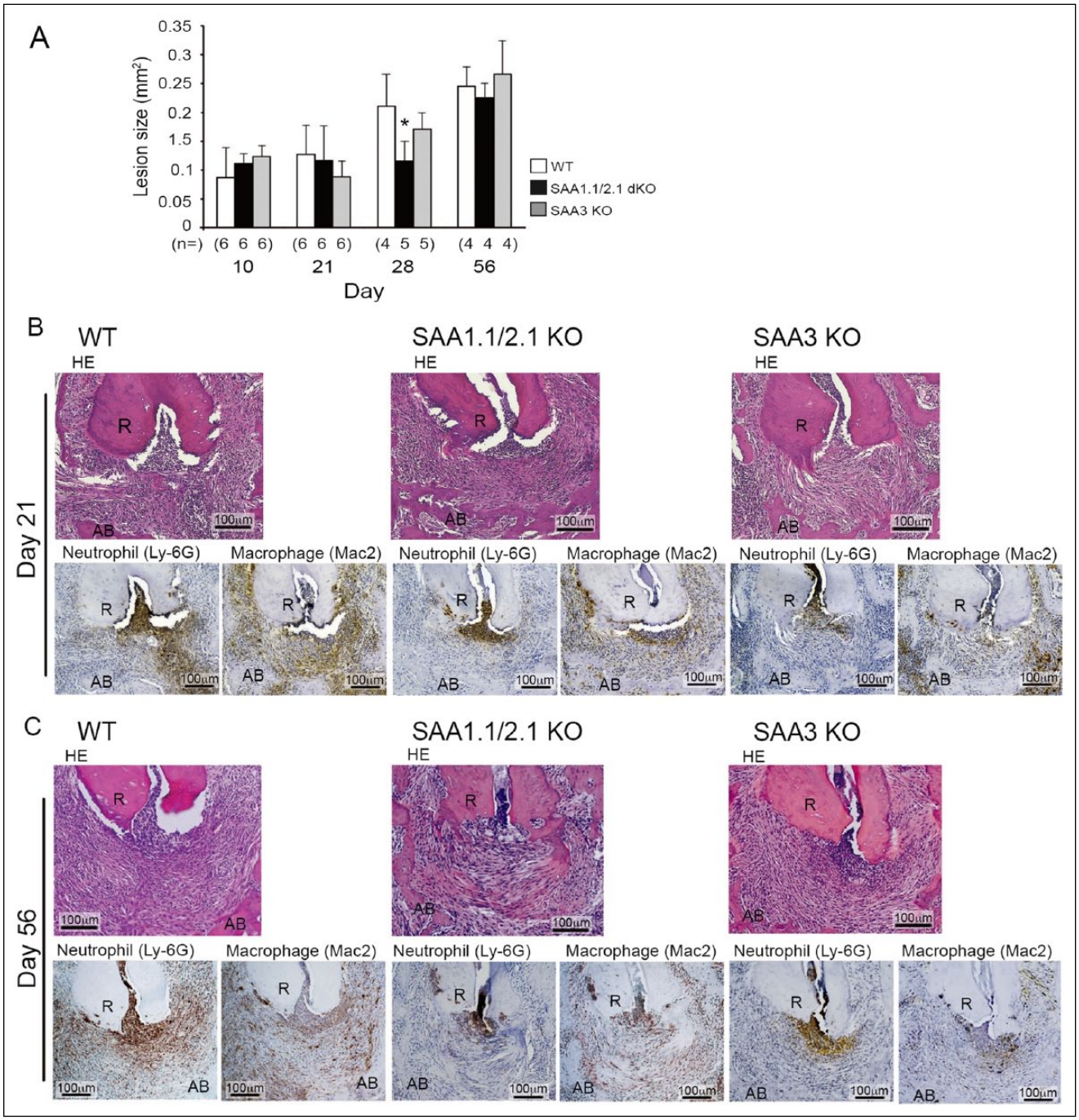

Figure 3. SAA deficiencies attenuate periapical inflammatory cell infiltration but not lesion size. (**A**) Kinetics of the extent of periapical lesion development in SAA1.1/2.1 dKO, SAA3 KO, and WT mice. The lesion size was obtained by subtraction of an averaged normal periodontal ligament space in baseline controls from the total periapical radiolucent area. Error bar indicates SD. *$P < 0.05$ vs. WT on day 28. Histology of periapical lesions on (**B**) day 21 and (**C**) day 56 after pulp exposure. Original magnification: ×100. In each panel, a representative image of hematoxylin and eosin (HE) staining and immunohistochemistry for neutrophils (Ly-6G) and macrophages (Mac2) is shown. AB, alveolar bone; dKO, double knockout; KO, knockout; R, dental root; SAA, serum amyloid A; WT, wild type.

Because most bone-resorptive activity was attributable to the activity of IL-1α (Wang and Stashenko 1993), we lastly tested the impact of rhSAA1 on IL-1α production by macrophages derived from WT- and TLR-deficient mice. As shown in Figure 5D, rhSAA1 significantly induced IL-1α production by WT macrophages in a dose-dependent manner ($P < 0.05$ vs. control). However, SAA-mediated IL-1α production was markedly lower in TLR2 KO and TLR4 KO macrophages (47% and

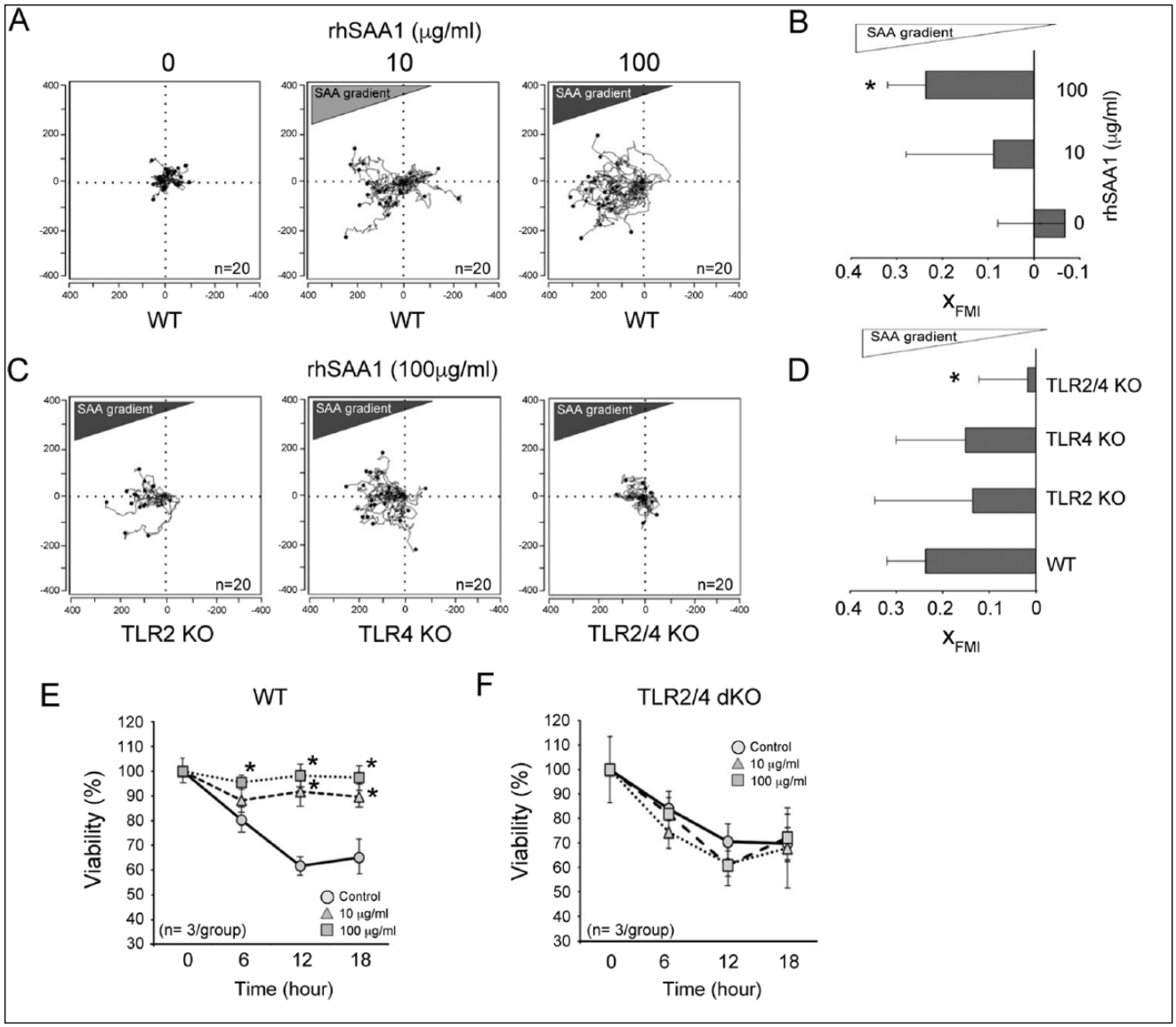

Figure 4. SAA directly induces chemotaxis and survival of neutrophils via TLR2 and TLR4 signaling. (**A**) Trajectories of WT neutrophils stimulated dose dependently with rhSAA1. (**B**) Dose-effect of SAA on WT neutrophil chemotaxis. X-axis forward migration index (x_{FMI}) represents the level of the migration directionality. *$P < 0.05$ vs. nonstimulated cells. (**C**) Trajectories of TLR-deficient neutrophils stimulated with 100 μg/mL of rhSAA1. (**D**) Effect of TLR deficiencies on SAA-mediated (100 μg/mL) neutrophil chemotaxis. *$P < 0.05$ vs. WT. (**E**) Kinetics of WT neutrophils viability during rhSAA1 stimulation for 18 h. Triplicates per condition. *$P < 0.05$ vs. control medium. (**F**) Kinetics of TLR2/4 dKO neutrophils viability during rhSAA1 stimulation for 18 h. Note that in each graph presented in panels A and C, trajectories of 20 randomly selected neutrophils were plotted after correcting the origin. $n = 3$ per condition in cell viability assays in panels E and F. Error bars in panels B, D, E, and F indicate SD. dKO, double knockout; rhSAA1, recombinant human SAA1; SAA, serum amyloid A; TLR, Toll-like receptor; WT, wild type.

53% of that in WT macrophages, respectively) and was almost completely absent in TLR2/TLR4 dKO macrophages (Fig. 5E).

Discussion

In this study, we demonstrate the expression of SAA, which is an acute-phase protein and a damage-associated molecular pattern, in human periapical lesions. The level of SAA expression appeared to be associated with the intensity of inflammatory cell infiltrate in lesions. We also confirmed that macrophages are an important source of SAA in inflamed tissue as previously reported (Meek et al. 1992; Yang et al. 2016). To elucidate the functional consequences of the association between SAA and periapical inflammation, we determined the role of SAA in the development of periapical inflammation in vivo and in vitro. Since SAA4 is not hyperinducible, we focused on the other 3 isoforms and employed SAA1.1/2.1 dKO, SAA3 KO, and corresponding WT mice.

In the course of experimental periapical inflammation, acute-phase SAA expression (SAA1.1, SAA2.1, and SAA3) was observed locally and systemically in WT mice. Interestingly, SAA3 deficiency resulted in a lower and consistent expression of SAA1.1/2.1 without an SAA spike. Although proinflammatory cytokines such as IL-1 and TNFα are crucial for induction of SAA (Raynes et al. 1991; Thorn et al. 2004), SAA deficiencies did not alter the level of proinflammatory cytokines in periapical lesions as compared with WT (data not shown). Our findings therefore suggest that locally produced SAA3 is essential for the SAA serum spike in response to endodontic infection. Importantly, no genotype effect was observed in local SAA3 production between WT and SAA1.1/2.1 dKO mice, indicating that infection-induced local SAA3 expression is SAA1.1/SAA2.1 independent.

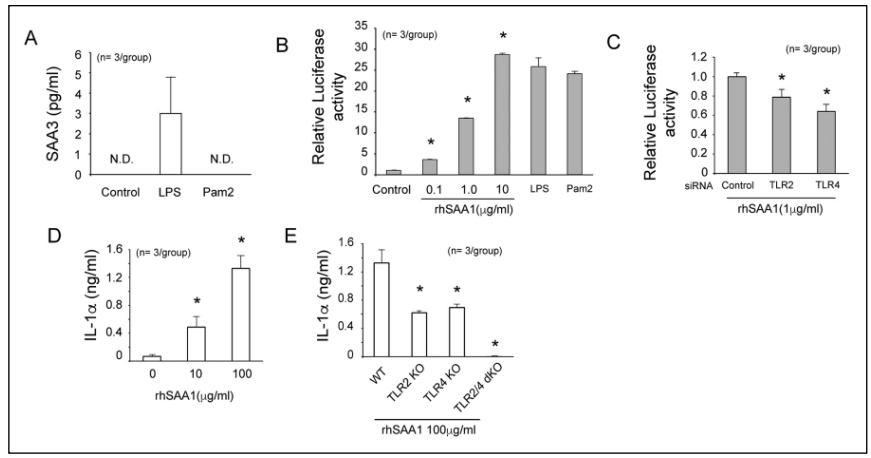

Figure 5. rhSAA1 directly stimulates macrophages via TLR2 and TLR4 signaling. (**A**) SAA3 production by PAMP-stimulated WT macrophages. As macrophages are a major source of extrahepatic SAA3, the effect of PAMPs on SAA3 production was assessed in WT macrophages. SAA3 was induced by activation of LPS-mediated TLR4 signaling but not Pam2CSK4-mediated TLR2 signaling. (**B**) Dose-effect of rhSAA1 on NF-κB transcriptional activity in NF-κB RAW cells. A mouse macrophage-like cell line, NF-κB RAW cells, was stimulated with rhSAA1 (0.1 to 10 μg/mL), LPS (0.1 μg/mL), Pam2CSK4 (0.1 μg/mL), or control medium. rhSAA1 dose dependently stimulated NF-κB promoter activity, reaching levels comparable to the NF-κB transcriptional activity of LPS (0.1 μg/mL) and Pam2CSK4 (0.1 μg/mL). *$P < 0.05$ vs. control medium. (**C**) Effect of TLR2 and TLR4 knockdown on rhSAA1-mediated NF-κB transcriptional activity. Luciferase activity was measured in NF-κB RAW cells treated for 48 h with a scrambled or TLR2- and TLR4-specific siRNA, prior to 6-h treatment with rhSAA1 (1 μg/mL). *$P < 0.05$ vs. scrambled control siRNA. (**D**) Dose-effect of rhSAA1 on IL-1α production by WT macrophages. *$P < 0.05$ vs. control medium. (**E**) Effect of TLR deficiencies on macrophage IL-1α production stimulated by 100 μg/mL of rhSAA1. $n = 3$ in all assays. *$P < 0.05$ vs. WT. Error bars indicate SD. dKO, double knockout; KO, knockout; LPS, lipopolysaccharide; ND, not detected; PAMP, pathogen-associated molecular pattern; rhSAA1, recombinant human SAA1; SAA, serum amyloid A; TLR, Toll-like receptor; WT, wild type.

Apical periodontitis is an infection-induced inflammation that occurs with periapical bone destruction. Our in vitro data indicating that rhSAA1 stimulates NF-κB activation and subsequent IL-1α production (Fig. 5) suggested that SAA may stimulate periapical bone destruction by osteoclasts. Unexpectedly, the extent of periapical lesions and the level of proinflammatory cytokines were not altered in SAA KO mice. However, our histologic observations suggest that the central role of SAA in the development of periapical lesions may be to regulate inflammatory cell infiltration, which was markedly reduced in SAA KO animals. We also confirmed that rhSAA1 directly induces directional chemotaxis of neutrophils and prolonged survival of neutrophils (Fig. 4E) and macrophages in vitro (data not shown). In addition to direct chemotaxis, SAA can activate the chemotaxis cascade by induction of chemokines from neutrophils, dendritic cells, and macrophages via FPR2 (formyl peptide receptor 2) signaling (De Buck et al. 2015; Gouwy et al. 2015). SAA competes with lipoxin A4, which is a proresolving lipid mediator, for a common receptor, FPR2 (Bozinovski et al. 2012), suggesting that SAA may interfere with natural mechanisms for resolution of inflammation.

Regarding the lack of effect on periapical bone destruction in SAA KO mice, we speculate that impairment of antibacterial immunity via a reduction in inflammatory cell infiltration and absent SAA-mediated TLR signaling would tend to increase the extent of periapical lesions, similar to periapical bone destruction in P-/E-selectin KO mice with decreased neutrophil infiltration (Kawashima et al. 1999). The lack of SAA in KO mice could concomitantly reduce osteoclast differentiation, activation, and survival, which are known to be NF-κB dependent. Thus, the reduced myeloid cell infiltration and activity could be counterbalanced by decreased osteoclast activity. Although osteoclasts were not directly assessed in the present study, the RAW264.7 cells used in our reporter assays represent preosteoclasts. Collectively, in periapical lesions, SAA appears to be a key damage-associated molecular pattern for the induction and prolongation of inflammatory cell infiltration rather than the elevation of bone destruction.

Pattern recognition receptors, in particular TLRs, are crucial in recognition of pathogen- and damage-associated molecular patterns (Martinon and Tschopp 2005; Yu et al. 2015), and SAA was proposed as a ligand for TLR2 and TLR4 (Erridge 2010). Since TLR2 and TLR4 form a complex network in the regulation of periapical lesions (Hou et al. 2000; Rider et al. 2016), we examined how SAA interacts with TLR2 and TLR4 with primary cells derived from TLR-deficient mice. Our data show that rhSAA1 utilizes TLR2 and TLR4 signaling similarly for its biological functions, including macrophage activation, chemotaxis, and prolonged survival of neutrophils.

Although this study focused on the pathogenicity of SAA in periapical lesions, the effect of potential molecular complexes of SAA with SAA itself, other host molecules (e.g., serum albumin), and bacteria/pathogen-associated molecular patterns on the outcome of TLR signaling needs to be further investigated in development of infectious diseases. Anti-inflammatory properties of SAA have been reported as well. SAA is involved

in production of anti-inflammatory cytokines (including IL-10), activation of M2 macrophages, and improved efferocytosis of apoptotic neutrophils (Anthony et al. 2014; Sun et al. 2015). These findings suggest that SAA has regulatory roles in the resolution of inflammation. However, these SAA-mediated proresolving responses are understudied in oral inflammation.

In follow-up studies, the role of circulating SAA in the relationship between local and systemic inflammation is an important issue still to be resolved. Given that SAA family members differ between mice and humans, the expression of SAA in human periapical lesions and its impact on circulating SAA should also be carefully examined. Finally, the effect of SAA on the bactericidal activity and clearance of bacteria by phagocytes remains to be resolved.

In conclusion, we demonstrate for the first time that SAA is locally expressed in human and mouse periapical lesions and that endodontic infection elevates circulating SAA in mice. Our functional data indicate the importance of the SAA-TLR2/TLR4 axis in regulating the infiltration of inflammatory cells and their survival and possibly in modulating osteoclast activation. Thus, SAA can be considered a key damage-associated molecular pattern in the development of periapical lesions.

Author Contributions

K. Hirai, contributed to design, data acquisition, analysis, and interpretation, drafted the manuscript; H. Furusho, contributed to data acquisition, analysis, and interpretation, drafted the manuscript; N. Kawashima, contributed to design, data acquisition, analysis, and interpretation, critically revised the manuscript; S. Xu, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; M.C. de Beer, R. Battaglino, contributed to design and data interpretation, critically revised the manuscript; T. Van Dyke, P. Stashenko, contributed to conception and data interpretation, critically revised the manuscript; H. Sasaki, contributed to conception, design, and data interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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