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

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APPENDIX

Appendix – List of abbreviations in alphabetical order

ACTB – actin beta; ATCC – the American Type Culture Collection; ANOVA – analysis of valiance; CMC – carboxymethyl cellulose; DAMP – damage-associated molecular pattern; FBS – fetal bovine serum; FPR2 – formyl peptide receptor 2; HE – hematoxylin and eosin; HDL – high density lipoproteins; HMGB1 – high mobility group box-1; i.p. – intra-peritoneal; KO – knockout; LPS – lipopolysaccharide; μCT – micro computed tomography; n – sample number; NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells; NF-κB RAW – NF-κB Luciferase Stable RAW264.7 cells; PBS – phosphate buffered saline; PAMP – pathogen-associated molecular pattern; PRR – pattern recognition receptor; rhSAA1 – recombinant human serum amyloid A1; RT-PCR – reverse transcription-polymerase chain reaction; SAA – serum amyloid A; siRNA – small interfering ribonucleic acid; TLR – Toll-like receptor; WT – wild-type; x_{FMI} – x-axis forward migration indices.

Appendix – MATERIALS AND METHODS

Human periapical endodontic surgical specimens

No additional information.

Animals

According to the Mouse Genome Informatics at the Jackson Laboratory (http://www.informatics.jax.org/allele/summary?phenotype=&nomen=SAA&chromosome=any&cm=&coordinate=&coordUnit=bp), there are no considerable confounding factors in SAA1.1/1.2 dKO and SAA3 KO mice compared to WT mice.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from human periapical endodontic surgical specimens (a total 30-50 mg tissue/case) using the TRIzol Reagent (Thermo Fisher Scientific, Inc.) and a motor-driven tissue grinder (Pellet Pestles, Sigma-Aldrich). The TRIzol reagent was used following the manufacturer's instruction. The RNA samples were subjected to DNase I treatment (RQ1, Promega, Madison, WI, U.S.A.) on an ultra-thin polymer membrane (QuickGene, Kurabo Industries Ltd, Osaka, Japan). Following the DNase I treatment, RNA was resuspended in nuclease-free water. cDNA was reversely transcribed from 300 ng of total RNA using PrimeScript RT Reagent Kit (Perfect Real Time, Takara Bio Company, Kyoto, Japan) following the manufacturer's instruction. PCR was performed using Taq polymerase (GoTaq DNA Polymerase, Promega) and gene specific primers. The *ACTB* (actin beta) gene served as a reference gene. The primer sequences for human *SAA1, SAA2,* and *ACTB* are following: *SAA1* forward 5'-CAG ACA AAT ACT TCC ATG CT-3'; reverse 5'-ATT GTG TAC CCT CTC CCC-3', *SAA2* forward 5'-CAG ACA AAT ACT TCC ATG CT-3'; reverse 5'- ATT ATA TGC CAT ATC TCA GC-3' and *ACTB* forward 5'-GTA GCA CAG CTT CTC CTT AAT GTC A-3'; reverse 5'-CTG ACT GAC TAC CTC ATG AAG ATC C-3. The condition of thermal cycling was 94°C for 30 s (denaturing), 58°C for 30 s (annealing), and 72°C for 30 s (extension) and applied 35 cycles for *SAA1 and SAA2* and 25 cycles for *ACTB*. The PCR products were visualized on 1.2% agarose gels under UV illumination after ethidium bromide staining.

Induction of periapical lesions

Age-matched SAA1.1/2.1 dKO, SAA3 KO and WT mice (8 weeks of age) were subjected to pulpal infection as previously described (Hou et al., 2000; Sasaki et al., 2000).

On day 0, mice were anesthetized by intra-peritoneal (i.p.) injection of 62.5 mg/kg ketamine-HCl and 12.5 mg/kg xylazine in sterile PBS and were placed on a jaw retraction board. The dental pulps of the mandibular first molars were exposed using a slow-speed electric dental hand piece (Aseptico, Woodinville, WA, U.S.A.) and a no. 1/4 round bur (SS White, Lakewood, NJ, U.S.A.) under a surgical microscope (Evolution xR6; Seiler, St. Louis, MO, U.S.A.). Common human endodontic pathogens (*Parvimonas micra*; ATCC 33270, *Streptococcus intermedius*; ATCC 27335, *Prevotella intermedia*; ATCC 25611, and *Fusobacterium nucleatum*; ATCC 25586) were anaerobically cultured, harvested, and equally mixed to yield 10^{10} cells/ml of PBS containing 2% carboxymethyl cellulose (CMC; Sigma-Aldrich). A total 10 μl of the mixture per tooth was

directly inoculated into the pulp chamber and root canals with #6 endodontic file. The access cavity was not closed.

In WT mice, acute inflammatory response (inflammatory cell infiltration) typically occurs within 1-3 days after pulpal infection. Inflammation shifts to chronic phase after day 10 and becomes chronic after day 21 post pulpal infection.

Micro computed tomography (μCT) and histology

Hemimandibles were scanned using a compact fan beam-type tomograph (μCT40; Scanco Medical, Bassersdorf, Switzerland) providing a 10-μm nominal resolution. The most centrally located section, which included the distal root canal of the mandibular first molar and that exhibited a patent root canal apex in anterior-posterior direction, was selected for quantitation. The cross-sectional area of periapical lesions was selected with Adobe Photoshop CS6 (Adobe Systems, San Jose, CA) and measured with ImageJ (National Institutes of Health, Bethesda, MD). In each strain, the lesion size was calculated by subtraction of an averaged normal periodontal ligament space in baseline controls from a total periapical radiolucent area in each infected sample and expressed in square millimeter.

Human endodontic specimens and mouse mandible samples after μCT analysis were subjected to histology. Fixed hemimandibles were decalcified, embedded in paraffin and sectioned at six μm thickness following general histology protocol. Hematoxylin and eosin (HE) staining was used for initial histological examination. Upon the initial observation, pivotal sections containing patent root canal with localized periapical lesion were immunohistochemically stained for neutrophils (Purified anti-Ly-6G; dilution 1:500; BioLegend, Inc., San Diego, CA, U.S.A.), macrophages (Purified anti-Mac2 protein; dilution 1:500; BioLegend, Inc.) and SAA (Anti-serum Amyloid A antibody; dilution 1:500; Abcam Inc., Cambridge, MA, U.S.A.). Primary antibodies were detected using the Liquid DAB+ Substrate Chromogen System (DAKO Denmark A/S, Denmark) following the manufacturer's instructions.

Isolation of primary myeloid cells

Mouse resident peritoneal macrophages and thioglycolate-elicited neutrophils were isolated from WT, TLR2 KO, TLR4 KO, and TLR2/4 dKO mice as previously described (Sasaki et al., 2000; Swamydas et al., 2015). For neutrophil isolation, 3% thioglycolate broth (Thermo

Fisher Scientific, Inc.) was intraperitoneally injected 12 hours prior to the cell isolation. After euthanasia, a total of 5 ml cold complete culture medium, consisting of RPMI 1640 supplemented with 10% FBS (fetal bovine serum), was injected into the peritoneal cavity and collected with constituent peritoneal cells under sterile conditions. Macrophages were enriched by removal of non-adherent cells after at least 2-hour pre-incubation. Neutrophils were enriched by a discontinuous density gradient centrifugation using 63% Percoll (GE Healthcare, Chicago, U.S.A.). All cells were subjected to proposed assays after washing three times with warm complete medium.

Enzyme-linked immunosorbent assay (ELISA)

In mouse experiments, the periapical tissues surrounding the mesial and distal root apices were extracted, together with surrounding bone in a block specimen under a surgical microscope. Periapical tissues were rinsed in PBS, freed of clots, weighed, and immediately frozen at −70°C until protein extraction.

Note that the SAA/SAA1 ELISA kit (Mouse SAA/SAA1 PicoKine^{TM} ELISA Kit, MyBiosource, San Diego, CA, U.S.A) detects both SAA1.1 and SAA2.1 due to highly homologous amino acid sequences (95%).

Chemotaxis assay

A multi-channel chemotaxis assay module, μ-slide chemotaxis (ibidi, Am Klopferspitz, Martinsried, Germany) was employed and used according to the manufacturer's instruction. For development of a SAA gradient, one reservoir (SAA positive side) was filled with FBS-free RPMI 1640 containing rhSAA1 (0 (vehicle control), 10 and 100 μg/ml), and the other reservoir in SAA-blank side was filled with RPMI 1640 alone. Primary neutrophils isolated from WT and TLR KO mice were seeded in the observation area in density 5 x 10^6 cells/ml of 20% of collagen I gel (Corning). The chamber was incubated in an on-stage incubator in the BZ-X700 microscope (Keyence) at 37ºC and 95% air/5% CO_2 atmosphere. Time-lapse images were taken at two-minute intervals for three hours by the BZ-X700. 20 neutrophils were randomly selected, and the coordinates of the cells in each image were tracked using the manual-tracking tool of ImageJ. Cell trajectories were analyzed using the Chemotaxis and Migration Tool Software (ibidi). The x-axis forward migration indices (x_{FMI}) and cell moving velocities were used for the statistical evaluation of migration directionality and activities (Foxman et al., 1999). The x_{FMI}

indicates the net distance moved in the direction to the gradient divided by total path length. Therefore the x_{FMI} represents the efficiency of forward migration of cells (parallel to the gradient).

Cell viability assay

Neutrophils were cultured in absence or presence of rhSAA1 (10 and 100 μg/ml FBS-free RPMI 1640). Medium alone served as vehicle control. The effect of rhSAA1 stimulation on neutrophil survival was assessed using LIVE/DEAD® Viability/Cytotoxicity Kit (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. The viability was determined at 0, 6, 12 and 18 hours after the stimulation. Live cells (Calcein AM; Excitation/Emission 494/517 nm) and dead cells (ethidium homodimer-1; Excitation/Emission 528/617 nm) were separately counted using hemocytometer under the fluorescent microscope BZ-X700 (Keyence). Results were expressed as a percentage of viable cells in total cells.

Note that, in chemotaxis and cell viability assays, FBS-free RPMI 1640 was employed, because the use of FBS led to inconsistent outcomes in preliminary assays.

NF-κB reporter assay

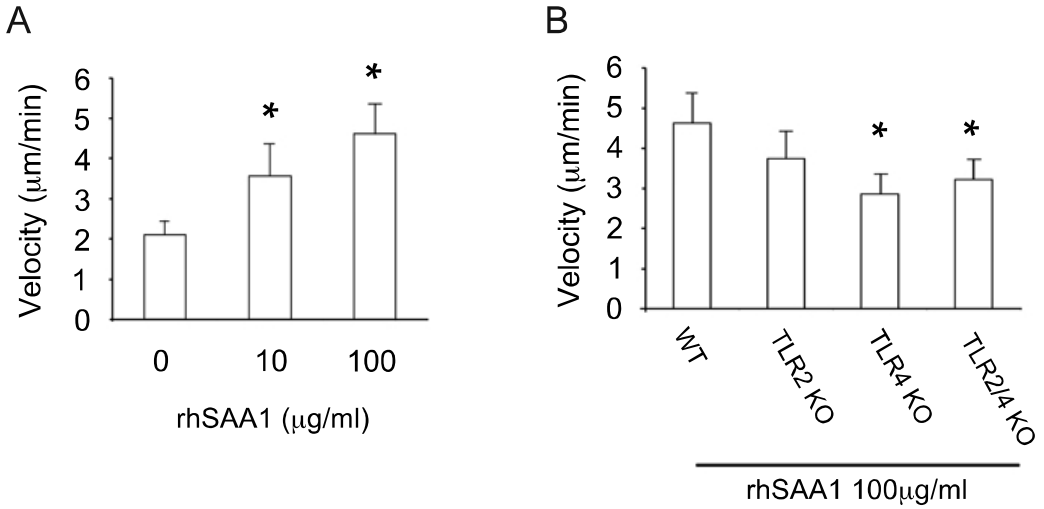
The effect of rhSAA1 on NF-κB activity was assessed by a reporter assay using NF-κB Luciferase Stable RAW264.7 cells (NF-κB RAW; Applied Biological Materials Inc, BC, Canada). NF-κB RAW is a mouse macrophage-like cell line stably transfected with a construct expressing firefly luciferase driven by a promoter containing NF-κB response element. NF-κB RAW cells were seeded at 1.5 x 10^5 cells/well in 48-well plates (Corning) and pre-cultured for six hours in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and G418 (100 μg/ml, Sigma-Aldrich). The cells were stimulated with of rhSAA1 (0.1, 1, and 10 μg/ml), *E. coil* LPS (lipopolysaccharide; *E. coli* serotype 0111:B4 (TLR ligand tested), 0.1 μg/ml; Sigma-Aldrich), and Pam2CSK4 (0.1 μg/ml; InvivoGen, San Diego, CA, U.S.A.). Medium alone served as vehicle control. To determine the role of TLR2 and TLR4 in SAA-mediated NF-κB activation, TLR2 and TLR4 were knocked down using specific siRNA (25 nM; Qiagen, Germantown, MD, U.S.A.) Scrambled siRNA controls was purchased from Thermo Fisher Scientific, Inc. Transfection of siRNA was carried out using Viromer Blue (Lipocalyx Halle, Germany) according to the manufacturer's instructions. We preliminarily confirmed that, at 48-h

after transfection, expression of TLR2 and TLR4 was suppressed approximately 58% and 54%, respectively. Then, the transfected cells were subjected to rhSAA stimulation (1 μg/ml) for six hours. Luciferase activity was determined using Luciferase Assay System (Promega) and a multimode microplate reader (Synergy HT, BioTek U.S., Winooski, VT, U.S.A.). The luminescence was measured for 5 seconds per sample. Results were expressed as a ratio of experimental group luminescence divided by control group luminescence (relative luciferase activity).

Appendix Table. Summary of histological examination of human samples.

Sample	Clinical Diagnosis	SAA gene expression (RT-PCR)	Inflammatory cells infiltration	Granulation tissue	SAA protein production	Histopathological characteristics
1	Radicular granuloma	Yes	++	+	–	Fragments of cystic capsule lined with stratified squamous epithelium was observed
2	Radicular granuloma	Yes	++	++	++	Fragments of cystic capsule lined with stratified squamous epithelium was observed
3	Radicular granuloma	Yes	+++	+++	+++	Fragments of cystic capsule lined with stratified squamous epithelium was observed
4	Radicular granuloma	Yes	++	++	+	Fibrous connective tissue was observed in the outer layer
5	Radicular granuloma	Yes	++	+++	+++	Fibrous connective tissue was observed in the outer layer
6	Radicular granuloma	Yes	++	++	+	Fragments of cystic capsule lined with stratified squamous epithelium was observed

Human endodontic surgical specimens were assessed by RT-PCR and histology.
–: Not detected, +: Low, ++: Moderate, +++: High.



Appendix Figure. SAA directly stimulated migration velocity of neutrophils via TLR2 and TLR4. The effect of rhSAA1 on chemotaxis was analyzed by ibidi chemotaxis chamber. **(A)** rhSAA1 stimulated migration velocity of neutrophils in a dose-dependent manner. *: $p<0.05$ compared to control medium. **(B)** rhSAA-stimulated neutrophil migration was in part mediated by TLR2 and TLR4. Neutrophils isolated from WT, TLR2 KO, TLR4 KO and TLR2/4 dKO mice were stimulated with 100 μg/ml of rhSAA1. *: $p<0.05$ compared to WT. Error bars: SD in both graphs.