Serum amyloid A 1 protein isoforms induce Rheumatoid Arthritis

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Abstract

**Background:** SAA1 in RA pathogenesis and its complications remains unknown, making early diagnosis and risk prevention difficult. This study is to determine the pathogenetic mechanisms of three different SAA1 protein isoforms in RA progression.

**Methods:** We modified an experimental adenovirus infection protocol in order to successfully introduce SAA1.2, SAA1.3, SAA1.5 gene alleles into the rear knee joints of C57BL/6 mice. Micro-computed tomography (micro-CT) analysis was applied to determine changes in bone morphology and density. Immunohistochemistry (IHC), flow cytometry, ELISA and real-time PCR were used to investigate disease progression and cytokine alterations in the course of adenoviral SAA-induced knee joint inflammation and bone destruction.

**Results:** The pathogenetic functions of SAA1.2, SAA1.3 and SAA1.5 protein isoforms in promoting the initiation and progression of RA were determined. We established that SAA1.2 was the most aggressive factor in RA induction and progression. Mechanistically, we found that the arthritis-inducing effect of SAA1.2 transcription in the knee joints and mutant SAA1 protein secretion in blood results in stimulation of immune responses, leading to CD8^+^ T cell and pro-inflammatory cytokine elevation, with subsequent synovial inflammation and bone destruction.

**Conclusions:** These findings indicate that SAA1 protein isoforms, particularly SAA1.2, play a significant role in the induction and progression of RA and may have potential value in the early diagnosis and severity prediction for RA.

**Background**

Secondary amyloid A (AA) amyloidosis is a systemic metabolic disease, which is a complication induced by serum accumulation of serum amyloid A 1 (SAA1) protein over-
secreted by hepatocytes in reaction to acute and chronic inflammation [1, 2]. This leads to deposition of AA amyloid fibrils in key organs, resulting in multiple life-threatening human diseases, such as severe chronic infection, tumorigenesis, liver and kidney failure, and serious complication of rheumatoid arthritis (RA) [3-7]. RA is a chronic and systematic autoimmune condition which leads to the development of bone destruction, long-term pain, serious complications, and eventual progressive disability that affect the quality of life of RA patients and create significant social burden to our society [8]. Genetic factors play a crucial role in the initiation of RA. Around 60% of RA twin patients are related to genetic inheritance [9]. However, causative genes leading to the pathogenesis of RA, as well as those that contribute to the severe life-threatening complications of RA, remain unidentified, making early diagnosis and effective treatment of RA difficult. When RA coexists with serum amyloid A (AA) amyloidosis, lethal outcomes have been reported [1, 10], with infection and renal failure the commonest causes of death [7]. However, monitoring SAA1 serum levels is not yet a routine practice in RA patients, so the risk of SAA1 dysregulation in RA patients is still largely unknown.

Previously, using the whole exome sequencing method (WES), we identified multiple novel genes that were enriched with deleterious variants in 58 RA patients’ peripheral blood mononuclear cells (PBMC) [11]. One novel deleterious mutation was in the SAA1 gene, creating an amino acid change (G90D) in the SAA1.2 protein isoform. Five different isoforms of the SAA1 gene have been reported [12], as a result of extensive genome wide association studies (GWAS). Little is known about the SAA1.1 and SAA1.4 isoforms, however previous studies have reported that SAA1.3 and SAA1.5 have been associated with RA in several clinical cohorts [2, 13, 14]. The SAA1.3 isoform was reported closely related to AA amyloidosis and disease onset as well as poor prognosis in a Japanese RA patient’s cohort [7]. In contrast, SAA1.5 was reported to have low ability to induce
inflammation due to lower secreting ability of pro-inflammatory cytokines [15]. To our knowledge, the biological roles of these SAA1 isoforms including our newly identified SAA1.2 mutation have not yet been comprehensively investigated.

Here, we aim to clarify the role of the SAA 1.2, SAA1.3 and SAA 1.5 alleles as a pathogenesis factor or an inhibitory factor in the development and outcome of RA. We optimized an experimental adenovirus infection protocol to successfully transfer gene constructs to the bone cartilage genome of C57BL/6 mice. We then examined the biological and functional roles of these three SAA1 isoforms as a function of time. The results indicate that the SAA1.2 protein isoform expression most aggressively induces both the initiation and progression of RA, and thus may provide a novel diagnostic marker for risk evaluation of RA patients.

Material And Methods

1. Adenovirus vectors and animal experimental protocol

Three recombinant adenoviruses harboring the full-length cDNA of three different human SAA isoforms, SAA1.2, SAA1.3 and SAA1.5 were cloned in front of the EGFP reporter gene, and adenoviruses were generated with constitutive expression under the control of the CMV promoter (VectorBuilder, USA). The vector maps of the three adenoviral SAA1 alleles, as well as the amino acid sequences of all isoforms, are shown in Fig 6a-d. Female C57BL/6 mice (10-14 weeks old) was used in our studies, after one weeks adaptive feeding, all mice were randomly and evenly divided into 4 groups (10 mice each), including SAA1.2, SAA1.3, SAA1.5 adenovirus injection groups and the EGFP control group. The rear right knee joints were injected laterally with $1 \times 10^8$ pfu of adenovirus containing either EGFP and or one of the three SAA1 isoforms. The animals were sacrificed at 1, 2, 4, or 6 weeks after injection. The study protocol was approved by the institutional committee.
for animal welfare. All animals included in our studies were housed in a 12 hr light/dark cycle, and provided free access to standard rodent food and pure water.

2. Micro-computed tomography (micro-CT) analysis

Mice were anaesthetized and gently tied on CT scanning groove with knee joints scanned by micro-CT scanner (Bruker, Belgium). Scans were conducted using a voxel size of 9 μm. The X-ray tube current was 441 μA and the voltage was 43 KV. The severity of bone erosion was ranked with the radiological score system according to the method described previously [16]. Moreover, 3D micro-structural properties of bone were calculated using CT-Analyzer, a software supplied by the manufacturer. We also developed a new evaluation method to assess bone destruction, called the micro-CT score. This score contained 5 disease-related indexes of the micro-CT analysis results for calcaneus including trabecular bone mineral density (BMD), tissue mineral density (TMD), trabecular number (Tb.N), porosity percent (TOT) and the trabecular volume rate (BV/TV). We determined that all the five indexes changed synchronously with the development of arthritis, but to different extent. Therefore, we modified and normalized the formula as follows: (X-min)/(max-min) or 1-(X-min)/(max-min). Then we averaged the five items to get the final micro-CT score exhibited all the five aspect of bones. Additionally, we set a bone destruction level on the basis of micro-CT score to evaluate the extent of damage more visually and directly.

3. Histological grading

Knee samples were fixed in 4% paraformaldehyde, demineralized in 10% EDTA for 28 days, dehydrated, embedded in paraffin, sectioned sagittally, and stained with haematoxylin and eosin. To assess synovial inflammation, histological scoring based on the reported critical criteria was modified [17]. Synovial hyperplasia, fibrosis, inflammation, articular cartilage destruction and bone erosion damage were used as
criteria for scoring, and this qualitative grading system was modified from Aletaha D et.
al. (Table 1).

4. Immunohistochemical (IHC)

Paraffin-embedded samples were cut into 5 μm sections. The distribution of SAA1 was studied with antibodies prepared against human and murine SAA1, which also detected human SAA1 (Abcam, UK). After washing, the slides were incubated with streptavidin-conjugated horseradish peroxidase for 10 minutes (Histo-Mouse kit, CA). Colour was developed with diaminobenzidine, and the sections were counterstained with hematoxylin.

5. Flow cytometric analysis

Examination of the change in T cell percentages was investigated by a CD4 and CD8 staining assay according to the manufacturer’s protocol [18]. The indicated number of mouse spleen and PBMC cells were resuspended in 100 μL of 1× staining buffer, and anti-CD4 and anti-CD8 fluorescence-tagged antibodies were added to the cell suspensions. At least 100,000 events were collected for quantitative analysis by flow cytometry (BD FACSCalibur, USA).

6. Real-time PCR

Knee samples were dissected 5 mm from the femoral head and 5 mm from the tibial head and prepared free of surrounding muscles. The samples were pulverized under liquid nitrogen. RNA was extracted using TRIzol reagent (Invitrogen, USA). The concentration of RNA was examined by a NanoDrop 2000 system (Thermo Fisher, USA). cDNA synthesis was performed with cDNA synthesis kits (Roche, Germany). The primers used for cDNA amplification were listed in Table 2 (primers from GenePharma, China). CT values were obtained using a ViiATM7 High-Productivity Real-Time Quantitative PCR system (Life Technologies, USA).

7. Enzyme-linked immunosorbent assay (ELISA)
The concentrations of the SAA, and the inflammatory factors IL-6, IL-22, MMP-3, MMP-9, IL-17, IL-23, and G-CSF were measured in mouse serum using commercially available ELISA kits (R&D, USA). According to the manufacturer’s instructions, the blood samples were allowed to clot for 2 h at room temperature before being centrifuged for 20 min at 2000 x g. The serum was removed and assayed immediately or after storage at ≤-20°C. Both samples and standards were run in triplicate.

8. Western blot analysis

Liver, kidney and lung tissues were lysed with ice-cold RIPA lysis buffer. The protein samples were electrophoretically separated and transferred to nitrocellulose (NC) membranes. The membranes were incubated with primary antibodies at 4°C overnight and incubated with a secondary fluorescent antibody for 1 hour at room temperature. Bands were visualized on a LI-COR Odyssey scanner (Belfast, USA). Primary antibodies human SAA1 was purchased from Abcam (Abcam, UK) and GAPDH from Cell Signaling Technology (Danvers, USA).

9. Statistical analysis

All the data are presented as the mean ± SD or SEM (indicated in each figure) of 3 individual experiments. Differences were analyzed by one-way ANOVA using Graph Pad Prism 5.

Results

1. Adenoviral expression of three SAA1 isoforms induced bone destruction of knee joints in C57BL/6 mice

To identify the roles of these three SAA1 isoforms in RA, we created a timeline for animal experiments (Fig 1a). Adenoviruses carrying SAA1 alleles and an EGFP control were injected in a single dose into the articular cavity of the right knee in mice; computed tomography CT scores were monitored, and tissues were collected at 4 time points (the
1st, 2nd, 4th and 6th weeks). We observed no differences in body weight among the groups during this 6-week period, suggesting that adenovirus infection did not induce toxicity (Fig S1a). RT-PCR confirmed successful gene integration and transcription in the bone cartilage throughout the experimental period; SAA1 mRNA levels were elevated in week 1 and gradually decreased from weeks 2 to 6, but the levels remained significantly higher than those of EGFP-treated animals (Fig 1b). An increase in secreted-SAA levels in serum was also observed (fig S6a).

Then, we analyzed changes in including trabecular bone mineral density (BMD), tissue mineral density (TMD), trabecular number (Tb.N), porosity percent (TOT) and the trabecular volume rate (BV/TV) using axial micro-CT in both legs. In the right leg, bone destruction occurred from weeks 2 to 4 and gradually recovered by week 6; the CT scores of the SAA1.2, SAA1.3, and SAA1.5 groups decreased by 27.8%, 26.7% and 10.9% in week 2 and were restored to decreases of 8.7%, 15.8% and 5.8%, respectively, in week 6, compared to those of the EGFP group. For BMD, SAA1.2 showed an 8.7% decrease in week 2, and SAA1.2 and SAA1.3 decreased by 12.3% and 11.3%, respectively, in week 4 and were restored to normal values in week 6. Other parameters also showed the most significant change in week 2, except for TMD (Fig 1c-d, Fig S1b). To visualize bone destruction, we reconstructed a 3D model of the mouse tibia and observed remarkable attenuation of the trabecular bone in the SAA1 groups, especially in the SAA1.2 group in week 4 (Fig 1e); the bone destruction recovered by week 6, which was consistent with a decrease in the SAA1 level, indicating that stress reaction recovery had occurred.

Surprisingly, bone destruction also occurred in the left leg, although no injection procedures were performed. BMD showed significant decreases of 13.2% and 14.7% in the SAA1.2 and SAA1.3 groups, respectively, in week 4, and these values were restored to normal in week 6, suggesting that blood circulation of the SAA1 protein induces
systematic inflammatory responses, as reported for human RA (Fig 5a & S7).

2. Three SAA1 isoforms induced synovial and systemic inflammation

Histological scoring [19] and qualitative scoring of the degree of synovitis were used to assess synovial inflammation (Table 1). The arthritis scores increased in week 2 in SAA1.2 and SAA1.3 groups. In week 4, only SAA1.2 remained significantly different, suggesting that SAA1.2 may dominate human RA progression. After week 6, the arthritis scores were returned almost to normal in all groups of animals (Fig 2a-d). Only 1 week after adenovirus infection, all SAA1 induction groups exhibited inflammatory responses in the articular cartilage, as illustrated by increases of the mononuclear cell infiltration and synovial cell expansion (H&E staining, Fig S2-4). In week 4, superficial synovial erosion appeared in the SAA1.2 group, with no significant change in EGFP-treated animals (Fig 1e-h). On the left knee joints biopsy, we did not observe significant bone erosion (Fig S9, 10). IHC analysis confirmed SAA1 protein expression from week 1 until week 4 in the synovium, growth plate, and even deep into the bone (Fig 2e-h, Fig S2-4). Interestingly, all three SAA1 isoforms protein expression were also found in left knee joints on 1st week, and the protein expression remained high until the 4th week (Fig S9, 10).

3. Three SAA1 isoforms induced T cell mediated immune response and infiltration into articular joints and release of pro-inflammatory cytokines

RA is characterized by T cell infiltration in the synovial membranes [20]. Flow cytometric analysis of mouse spleens in week 2 showed that SAA1.2 induced a 10.9% decrease in CD8+ T cells and an 11.3% increase in CD4+ T cells (Fig 3a, b), resulting in an increase in the CD4+/CD8+ ratio. No changes in CD8+ or CD4+ T cells were observed in the spleen in other groups throughout the 6-week study period. On the other hand, compared to the EGFP-treated mice, all SAA1 isoforms induced changes in CD4+ and CD8+ T cells in the
blood in week 4 (Fig 3c, d).

RT-PCR and ELISA results showed that interleukin (IL)-6 and IL-22 expression was remarkably stimulated in week 1 at right leg and blood circulation of mice with SAA1 isoform transfected (Fig 4a, b), consistent with the recent report that robust production of IL-22 induces SAA1 production and inflammation [21], further explaining why SAA1.2 caused the most serious bone damage (Fig 1c & d). Previous reports demonstrated that these cytokines can polarize T cells into different subsets of T helper cells [22], which further produce different cytokines [23]. Our results also showed upregulation of IL-17, IL-23, tumour necrosis factor (TNF)-α, IL-1β, granulocyte-colony stimulating factor (G-CSF), and interferon (IFN)-γ (Fig S5 a-d, Fig 6b-d). Increased TNF-α or IL-17 could enhance matrix metalloproteinases (MMPs) production result in articular bone degradation, which ultimately results in destruction of the joint structure [24-26]. In line with this finding, intra-articular secreted and blood circulated MMP-3 and MMP-9 were activated after SAA1 adenovirus injection (Fig 4g, h). At the same time, the MMP inhibitor TIMP-1 (tissue inhibitor of metalloproteinases 1) exhibited trends opposite to those of the MMPs level (Fig S5e). Interestingly, we observed SAA1 protein accumulation in key organs, including liver, lung and kidney (Fig 5c, d). In addition, similar cytokines elevation was also observed at the left leg (Fig 5b, S8 & S9), further confirming that SAA1 is circulating in blood to left leg after expression at the right knee and induce arthritis condition.

Discussion

RA is a complex and difficult-to-treat autoimmune disease due to unknown pathogenesis and the lack of a valid drug target [27]. Subclinical amyloidosis was found common in RA and other inflammatory rheumatic diseases, which may lead to serious complications [28]. The prevalence rates of secondary amyloidosis in RA patients range from 7-26% [29], but so far the pathogenesis and consequence of amyloidosis with RA were largely unknown.
Several SAA alleles were reported previously associated with RA, however their pathological roles have not yet been clarified. SAA1.2 was first identified by us in RA patients using whole exome sequencing. The SAA1.3 allele has been reported to be a risk factor for the association of AA amyloidosis that adversely influences the outcome in RA patients. Lethal cases were reported in patients with SAA1.3 allele in a Japanese cohort, suggesting the potential importance of SAA1 single nucleotide polymorphisms [7]. SAA1.5 has been shown to exhibit slower serum SAA clearance compared to the other alleles, which indicates it is less amyloid genesis [15]. At the same time, SAA1.5 also has lower ability to induce inflammatory cytokines than the other alleles, which may weaken the inflammatory response [30]. Here, in this study, we have successfully clarified the role of these three SAA1 allele products to induce RA symptoms and SAA1.2 was demonstrated to be the most aggressive.

Classic adjuvant-induced arthritis (AIA) and collagen-induced arthritis (CIA) models are unable to investigate the pathological role of a single gene [31]. In this study, we applied adenovirus infection methods by injection of virus carrying mutant human gene SAA1.2 to the bone cartridge of the right leg of C57BL/6 mice, causing direct gene integration into the mouse genome. Up to date, only a few successful cases of adenovirus-mediated single gene transfer have been reported [32, 33] because this model requires strong injection skills and a high virus titre. Here, for the first time, we report an optimized experimental protocol with a 6-week time course, with optimal times between 2-4 weeks. This investigation strategy can more truly reflect the pathogenic function of a gene during RA initiation and progression, and can scientifically provide evidence of the negative feedback mechanism of RA induction model. At time point of 6th week, the target gene SAA1 was still highly translated which could be due to the reacted feedback response of articular inflammation, further enhancing the activity of inflammation [34].
Synovitis occurs as a consequence of leukocyte infiltration into the synovium. The accumulation of leukocytes in the synovium does not result from local cellular proliferation but rather from migration of leukocytes from distant sites of formation in response to expression of adhesion molecules and chemokines by activated endothelial cells of synovial micro vessels [35]. In general, there is a 2-week time lag in the immune responses in blood compared with that in the spleen, suggesting that T cell differentiation first occurs in the spleen and active T cells then circulate in the blood. The synovitis, swelling and joint damage that characterize active RA are the end results of complex autoimmune and inflammatory processes that involve components of both the innate and adaptive immune systems [36]. The innate immune system involves the interaction of fibroblast-like synovial-cytes with other cells of the innate immune system. The intra-articular cellular infiltrate located in the synovial membrane includes granulocytes, monocytes/macrophages, NK cells, B cells, and especially CD4+ and CD8+ T cell, all leading to the production of large amounts of chemokines and pro-inflammatory cytokines [20, 22, 37]. In RA, there is abundant evidence that the innate immune system is persistently activated [38]. In this study, we observed up-regulated expression of inflammatory cytokines, such as TNF-α, IL-22 and G-CSF, which leads to the intra-articular immune activity and aggravate joints inflammation.

Previously, we have applied molecular docking to compare the protein structures of wild-type SAA1 and SAA1.2 [11]. The SAA1.2 mutation occurred in a-helix 3, and this mutation resulted in a protein conformation change, with a more compressed and condensed protein structure. This mutant may cause more severe inflammatory induction due to the higher potential for aggregation. Other SAA1 isoforms only deviate from wild-type SAA1 by one amino acid but also exhibit severe inflammatory conditions, highlighting the importance of discovering new SAA1 isoforms or mutants in patients in the future.
Conclusions

We have determined the roles of SAA1.2, SAA1.3 and SAA1.5 in the pathogenesis of RA, as well as the downstream mechanisms by which they promote inflammation. While all three have pathologic effects, SAA1.2 induces RA most aggressively. The progression of RA involves multiple organs. SAA1 protein expression started in synovial fibroblasts and cartilage in the right knee and then circulated in the bloodstream and arrived in the spleen, triggering naïve T cell differentiation and mature T cell activation. Active cytotoxic CD8\(^+\) T cells circulate in the blood and secrete multiple pro-inflammatory cytokines, resulting in bone destruction (Fig 6). Fig 6 illustrates the signalling pathway involved in this scenario. Collectively, this study highlights the need to detect and study SAA1 isoforms in RA patients to facilitate early diagnosis and avoid serious complications. Our discovery also provides clues for the identification of new therapeutic targets for RA drug development in the future.

Abbreviations

SAA1: serum amyloid A 1;
RA: rheumatoid arthritis;
WES: whole exome sequencing method;
PBMC: peripheral blood mononuclear cells;
GWAS: genome wide association studies;
micro-CT: Micro-computed tomography;
BMD: trabecular bone mineral density;
TMD: tissue mineral density;
Tb.N: trabecular number;
TOT: porosity percent;
BV/TV: trabecular volume rate;

IHC: Immunohistochemical;

IL-6: interleukin -6;

TNF: tumour necrosis factor;

G-CSF: granulocyte-colony stimulating factor;

IFN-γ: interferon -γ;

Declarations

**Ethics approval and consent to participate**

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Macau University of Science and Technology (NO. MUST-NSFC-20190330007).

**Consent for publication**

Not applicable.

**Availability of data and materials**

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

**Conflict of interests**

The authors declare no competing interests.

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**Authors' contributions**

L.L. and E.L.H.L conceived the research and led the project. E.L.H.L., H.L.L. and R.Z.L. wrote the manuscript. L.L. and E.L.H.L. revised and final proof the manuscript. H.L.L.,
R.Z.L., H.D.P., Z.B.J., Y.L., F.G.D., J.H.X., Y.Z.Z., C.L.W., A.X.S., F.Y.Z., and X.J.Y. carried out the experiments, analyzed the data and prepared the figures. All authors reviewed the manuscript.

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Tables
Table 1 qualitative grading system used to assess the degree of synovitis in the mouse knee joint, modified from Aletaha D et al.
| I Synovial lining cell hyperplasia | 0 | Absent |
|----------------------------------|---|--------|
|                                  | 1 | <50% of the synovial lining shows change |
|                                  | 2 | >50% of the synovial lining shows change |
| II Fibrosis                      | 0 | Absent |
|                                  | 1 | Histiocytes |
|                                  | 2 | Intermediary |
|                                  | 3 | Marked |
| III Inflammation                 | 0 | Absent |
|                                  | 1 | Histiocytes |
|                                  | 2 | Intermediary |
|                                  | 3 | Marked |
| IV Articular cartilage destruction | 0 | Absent |
|                                  | 1 | <1/3 Small focal damage |
|                                  | 2 | <1/2 Small focal damage |
|                                  | 3 | <2/3 Small focal damage |
|                                  | 4 | Small stove-like destruction |
| V Bone erosion damage            | 0 | Absent |
|                                  | 1 | Superficial destruction |
|                                  | 2 | 1/2 of the deep cartilage layer |
|                                  | 3 | Deep cartilage |

Table 2. Primer design for RT-PCR studies

| ID | Primer      | Sequence (5'to3') |
|----|-------------|-------------------|
| 1  | H-SSA-1 F   | TTTCTGCTCCTTGTCCT |
| Figure | Gene | Primer Name | Sequence |
|--------|------|-------------|----------|
| 2 | H-SSA-1 | R | CAGGTCGGAAGTGATTGG |
| 3 | M-TNF-α | F | AGCGAGGACAGCAAGGGA |
| 4 | M-TNF-α | R | TCTTTTCTGGAGGGAGTGG |
| 5 | M-IL-1β | F | TTGACGGACCCCCAAAAGATG |
| 6 | M-IL-1β | R | AGAAGGTGCTCATGTCTCTCA |
| 7 | M-IL-6 | F | GGTGACAACCACGGCCTTCCC |
| 8 | M-IL-6 | R | AAGCCTCCGACTTGGAAGTG |
| 11 | M-IL-22 | F | AGCAATCAGCTCAGCTCTG |
| 12 | M-IL-22 | R | CTTCTTCTCGCTCAGACGCA |
| 13 | M-IFN-γ | F | GCCACGGCACAGTCATTGA |
| 14 | M-IFN-γ | R | TGCTGATGGCCTGATTGTCTT |
| 15 | M-MMP-3 | F | ACATGGAGACTTTGTCCCTTT |
| 16 | M-MMP-3 | R | TTGGCTGAGTGGTAGAGTCCC |
| 17 | M-MMP-9 | F | TGAATCAGCTGGCTTTTGTG |
| 18 | M-MMP-9 | R | ACCTTCCAGTAGGGGCAACT |
| 19 | M-G-CSF | F | CCCACCTTGGACTTTGCTCAG |
| 20 | M-G-CSF | R | AGGTACGAAATGGCCAGGAC |
| 21 | M-TIMP-1 | F | CTGGGTCTCCCTGGGCCTACTC |
| 22 | M-TIMP-1 | R | ACCTGATCCGTCCACAAACAG |
| 23 | M-GAPDH | F | TTTGTCGTCCTTGTCAACAGC |
| 24 | M-GAPDH | R | CTGGGGAGTTTCAGGTCCCT |

**Figures**
Adenoviral expression of three SAA1 isoforms induces bone destruction in the knee joints of C57BL/6 mice. a, Timeline of in vivo experiments in this study. b, Relative mRNA level of SAA1 extracted from liquid nitrogen pulverized knee joints.
in TRIzol after infection with $1 \times 108$ pfu/mice of adSAA1 isoforms. (n=5~6 in each group). c, Trend changes of CT scores over timecourse for three SAA1 isoforms compared with the EGFP control group. (n=8 in each group). d, BMD values of right (the injected side) knee joints in all groups over the timecourse. (n=8 in each group). e, Micro-CT reconstructed bone images of knee joints for three SAA1 isoforms and the EGFP control group. *: Represents the p-value for the comparison of each SAA1 allele to the EGFP control group. Values are presented as the mean (SEM). #: Represents the p-value for the change in each SAA1 allele over time. (* & # p<0.05, ** & ## p<0.01, *** & ### p<0.001).

Figure 2

Overexpression of SAA1 isoforms induces synovial inflammation, and adSAA1 infects multiple tissues in the knee joints. a-d, Qualitative grading results for synovitis in mouse knee joints according to synovial cell hyperplasia, fibrosis,
inflammation, articular cartilage destruction and bone erosion in all groups at the indicated time points (n=5 in each group). Values are presented as the mean (SD). e-h, Analysis of H&E and Immunohistochemical images of sections of knee joints from the EGFP, SAA1.2, SAA1.3, SAA1.5 groups after 4 weeks of infection. The black arrow indicates superficial erosion of articular cartilage in the SAA1.2 group. The red arrow indicates positive expression of SAA1 in synovial cells, the yellow arrow indicates expression of SAA1 in the growth plate, and the blue arrow represents expression in bone tissue. *: Represents the p-value for the comparison of the qualitative grading of each SAA1 allele to the EGFP control group. (n=5 in each group). Values are presented as the mean (SEM). (* p<0.05).
Figure 3

SAA1 isoforms induced T cell-mediated immune responses. a, c, Flow cytometric analysis of the percentages of CD4+ and CD8+ T cells and the fold changes of the
CD8+/CD4+ ratio in the spleen and blood in the SAA1 groups compared with the EGFP group at 4 different time points. b, d, Flow cytometry analysis of the percentage of CD4+ and CD8+ T cell of blood and spleen in three SAA 1 groups compared with EGFP group at four different time points. *: Represents the p-value for the comparison of each SAA1 allele to the EGFP control group. (n=8 in each group). Values are presented as the mean (SEM). (*p<0.05, ** p<0.01)
Figure 4
SAA1 isoforms induced pro-inflammatory cytokines release in articular joints and blood circulation. a-d, RT-PCR analysis of the expression of cytokines IL-22, IL-6, MMP-3, and MMP-9 in pulverized knee joints 1, 2, 4, and 6 weeks after SAA1 adenoviral infection compared with the EGFP group. (n=5-6 in each group). e-h, ELISA analysis of the secretion of cytokines, IL-6, IL-22, MMP-3, and MMP-9 in serum 1, and 4 weeks after SAA1 adenoviral infection compared with the EGFP group. (n=6-8 in each group) *: Represents the p-value for the comparison of value of each SAA1 allele to the EGFP group. Values are presented as the mean (SEM). #: Represents the p-value for the value change of each SAA1 allele over time. (* & # p<0.05, ** & ## p<0.01, *** & ### p<0.001)
Figure 5

Systemic effect induced by SAA1 isoforms. a, Trend changes of CT scores and BMD values of left knee joints over the time course for three SAA1 isoforms compared with the EGFP control group. (n=8 in each group). b, RT-PCR analysis of
the expression of SAA1 and cytokines IL-22, IL-1β and MMP-3 in left knee joints 1, 2, 4, and 6 weeks after SAA1 adenoviral infection compared with the EGFP group. (n=5 in each group). c, Immunoblots for SAA1 expression in liver, kidney and lung form mice of EGFP control group, SAA1.2, SAA1.3 SAA1.5 groups. d, quantification of the SAA1 level of liver, kidney and lung determined by western blot analysis. (n=5-6 in each group). *: Represents the p-value for the comparison each SAA1 allele to the EGFP control group. Values are presented as the mean (SEM). #: Represents the p-value for the change in each SAA1 allele over time. (* & # p<0.05, ** & ## p<0.01, *** & ### p<0.001)
Vector construction map of adSAA1.2, adSAA1.3 and adSAA1.5, and the schematic diagram of arthritis induction by SAA1 isoforms. a, The protein sequence
alignments of SAA1.1, SAA1.2, SAA1.3, SAA1.4 and SAA1.5 encode a few amino-acid differences in the protein structure. 

b, Vector construction map of adSAA1.2. 
c, Vector construction map of adSAA1.3. 
d, Vector construction map of adSAA1.5. 
e, After intra-articular injection into the knee joint, SAA1 is first expressed by synovial cells and is presented to naïve T cells by antigen-presenting cells. Then, T cells become activated and differentiate into Th1, Th17, Th22 or CD8+ T cells, resulting in the release of cytokines like IL-6 and IL-22, which can activate macrophages. In addition, adSAA1 transfects into macrophages and induces SAA1 secretion into the synovium, thus activating immune cells to secrete proinflammatory cytokines, such as TNF-α, IL-6, IL-17, and IL-22, etc. Under these conditions, fibroblasts that express receptor activators of TNF-α, IL-1β or IL-6 can activate macrophages to differentiate via preosteoclasts into osteoclasts that resorb bone from the synovium. These cytokines also activate chondrocytes to secrete MMPs that degrade cartilage. This process starts at the junction between cartilage and bone. Moreover, adSAA1 transfects into the blood vessels of the synovium, which promotes endothelial cell secretion of SAA1 and consequently activates T cells in the spleen. Finally, T cell activation and differentiation can produce lymphokines that further promote inflammation in the knee joints via the circulation.

Supplementary Files

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supplemental data.pdf

33