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Human Serum Albumin-TRAIL Conjugate for the Treatment of Rheumatoid Arthritis

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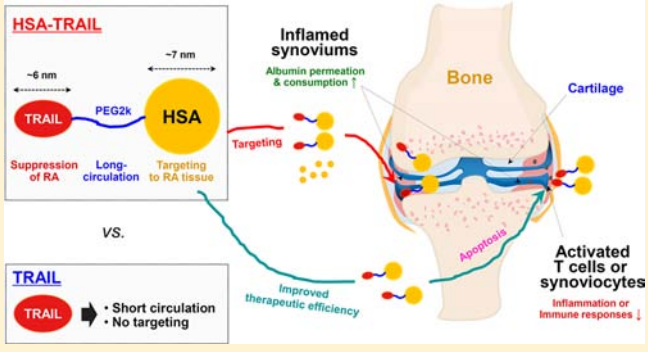
ABSTRACT: Albumin conjugation is viewed as an effective means of protracting short in vivo lifespans of proteins and targeting rheumatoid arthritis (RA). In this study, we present a human serum albumin (HSA) conjugate linked with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) via a bifunctional PEG derivative (HSA-TRAIL). Prepared HSA-TRAIL was found to have a larger molecular size (~240 kDa, 15.4 nm) than TRAIL (~66 kDa, 6.2 nm), and its bioactivity (apoptosis, cytotoxicity, and antiproliferation) was well preserved in Mia Paca-2 cells and mouse splenocytes. The enhanced therapeutic efficacy of HSA-TRAIL was demonstrated in collagen-induced arthritis (CIA) mice. The incidence and clinical scores, expressed as degree of erythema and swelling in HSA-TRAIL-treated mice, were remarkably lower than those of TRAIL-treated mice. The serum levels of pro-inflammatory cytokines IFN-γ, TNF-α, IL-1β, and IL-2 in HSA-TRAIL-treated mice were significantly lower than those of TRAIL-treated mice. Furthermore, HSA-TRAIL accumulated in the hind paws of CIA mice, not in naïve TRAIL mice. Pharmacokinetic profiles of HSA-TRAIL were greatly improved in comparison to those of TRAIL (AUC_{inf}: 844.1 ± 130.0 vs 36.0 ± 1.2 ng·h/mL; $t_{1/2}$: 6.20 ± 0.72 vs 0.23 ± 0.01 h, respectively). The HSA-TRAIL conjugate, which presents clear advantages of targeting RA and long systemic circulation by HSA and unique anti-inflammatory efficacy by TRAIL, has potential as a novel treatment for rheumatoid arthritis.



INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory and destructive joint disease that affects ~0.8% of the adult population worldwide.[1−3] Ongoing joint inflammation causes synovial hyperplasia and destroys cartilage, bone, and tendons.[4] Active RA causes disability and decreases quality of life, accompanied by high therapeutic costs.[4,5] Nonsteroidal anti-inflammatory drugs (NSAIDs) or disease-modifying anti-rheumatic drugs (DMARDs) have been used as a primary choice of treatment. However, these drugs are restricted to temporary improvement of symptoms or disease process in RA and pose potential adverse effects.[1,4] Therefore, new therapeutics that play a specific role in inhibiting the cause of inflammation by mechanism are required for the successful treatment of RA.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane protein of the TNF family.[6] Trimeric TRAIL specifically binds to tripartite death receptors, such as DR4 and DR5, which are highly expressed in malignant or inflamed tissue cells in cancers or arthritis.[7−9] The binding of TRAIL to death receptors leads to receptor oligomerization, followed by caspase-8 or caspase-3-dependent programmed cell death.[8] Therefore, this protein displays prominent cytotoxicity to abnormal cells, with negligible damage to normal cells.[7,8] TRAIL has been reported to be effective for treating rheumatoid arthritis because it exerts an apoptotic and anti-inflammatory effect on arthritis-related cells.[9,10] Studies have shown remarkable therapeutic efficacy of TRAIL in arthritis animal models.[11−13]

Albumin is viewed as a protein carrier with the ability to protract the circulating lifespan of short-life proteins and drug targeting.[14] Albumin has considerable pharmaceutical utility because the protein is abundant in serum, physicochemically stable, and biocompatible.[14,15] First, it is used as a conjugation counterpart of therapeutic proteins, and physically/chemically associated or genetically fused albumin-proteins have markedly extended therapeutic durations with greatly reduced glomerular filtration.[16−18] Second, albumin accumulates in tumor or

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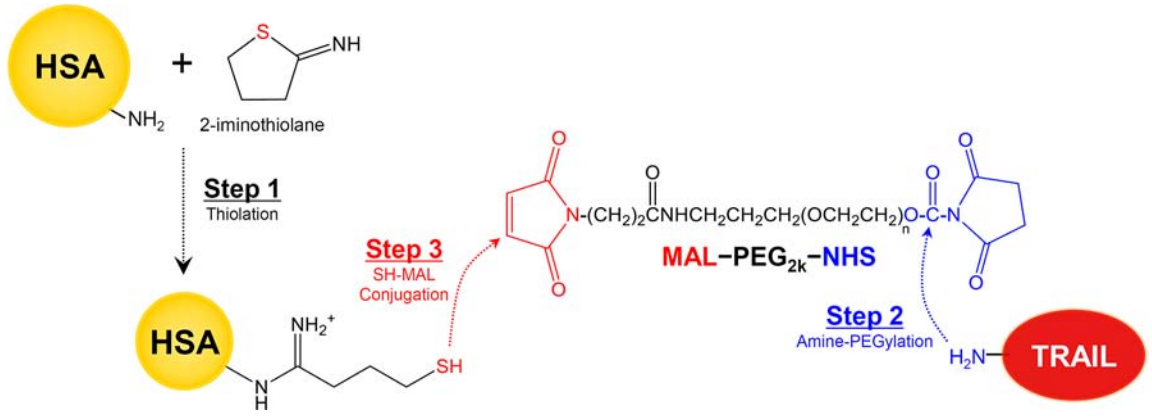
Scheme 1. Synthetic Scheme of HSA-TRAIL Conjugate



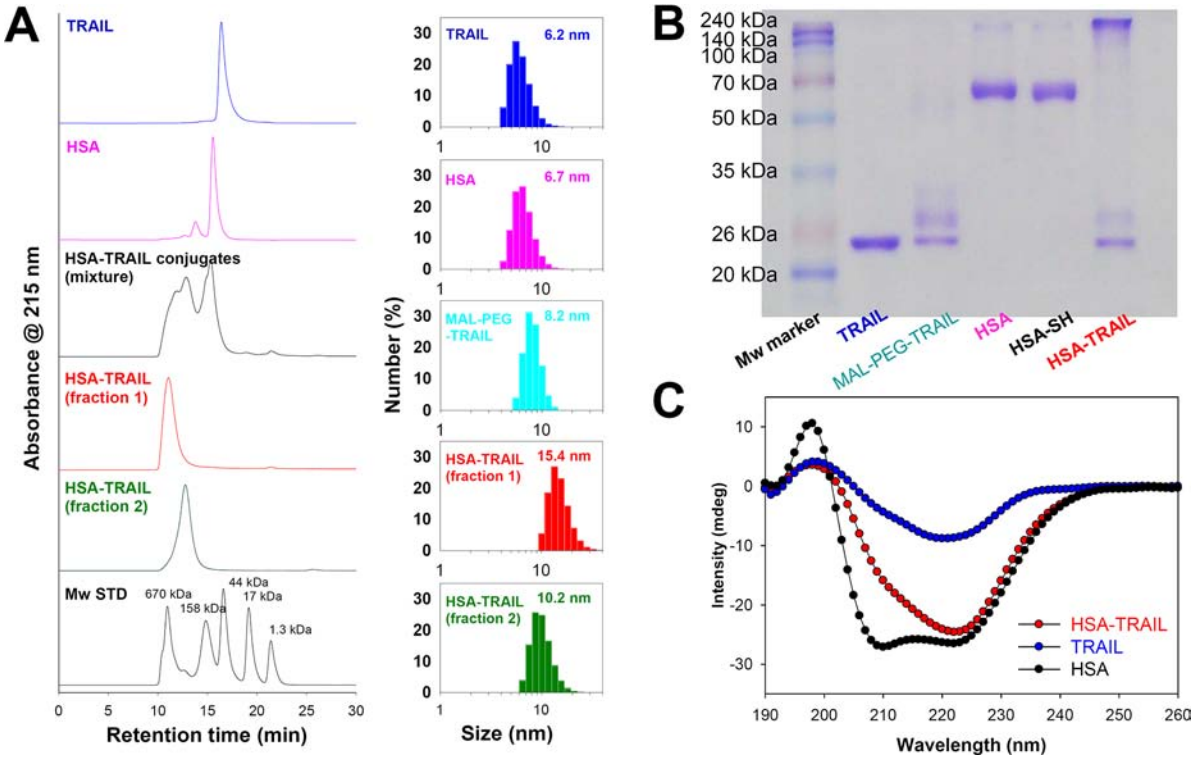


Figure 1. Characterization of HSA-TRAIL conjugates. (A) Size-exclusion chromatograms (left) and particle size histograms (right) of TRAIL, HSA, and HSA-TRAIL conjugates and molecular weight standards. (B) SDS-PAGE analysis of TRAIL, HSA, MAL-PEG-TRAIL, and HSA-TRAIL (fraction 1). (C) Far-UV circular dichroism spectra of TRAIL, HSA, and HSA-TRAIL (fraction 1).

inflamed tissues due to altered physiology and metabolism of tissues.[15,19] It is well-known that RA patients develop hypoalbuminemia, caused by high albumin consumption at inflamed sites,[20,21] and the blood-joint barrier increases albumin permeability to inflamed joints.[22] Accordingly, clinical advantages of albumin are useful for treating RA, when combined with therapeutic efficacy of relevant drugs with a specific anti-RA mechanism.

We developed a new conjugate of human serum albumin (HSA)-TRAIL linked by polyethylene glycol (PEG), which has a clear therapeutic efficacy for rheumatoid arthritis. Two principal parts of this conjugate, HSA and TRAIL, are believed to play respective critical roles in targeting arthritis tissues and prolonging systemic circulation, and suppressing inflammation in arthritis tissues. Further, the additional PEG part was considered to help extend therapeutic duration and in vivo stability of the HSA-TRAIL conjugate. This study examined physicochemical properties, in vitro bioactivity in mouse splenocytes, pharmacokinetic behavior, and targetability and anti-arthritis efficacy of this HSA-TRAIL conjugate in a collagen-induced arthritis mouse model.

RESULTS

Characterization of HSA-TRAIL Conjugates. HSA-TRAIL conjugates were prepared as described in Scheme 1. Size-exclusion chromatography showed that HSA-TRAIL conjugates consisted of two major peaks corresponding to HSA-TRAIL fraction 1 and 2, respectively. The apparent molecular sizes of both fractions of HSA-TRAIL conjugates were found to be greater (>300 kDa) than those of unmodified HSA and TRAIL (Figure 1A, left). Further, the SEC chromatograms of HSA-TRAIL fractions 1 and 2 revealed

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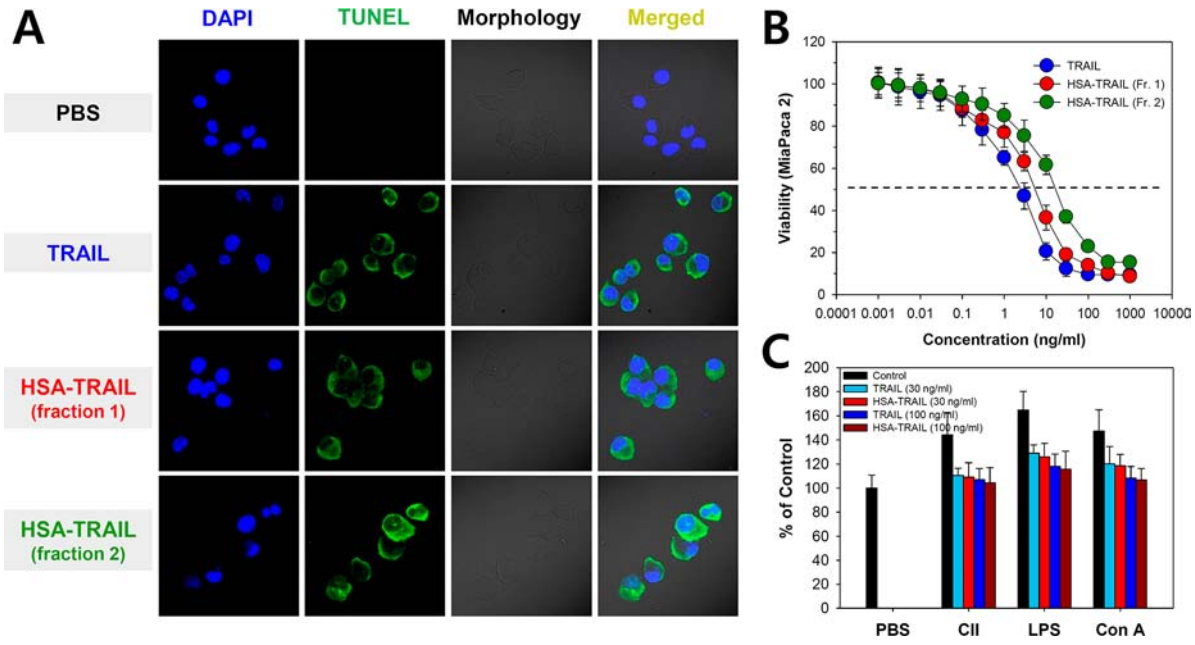

Figure 2. Bioactivity evaluation of HSA-TRAIL conjugates. (A) CLSM images of MiaPaca 2 cells treated with TRAIL (10 ng/mL), HSA-TRAIL (fraction 1, 10 ng/mL), and HSA-TRAIL (fraction 2, 10 ng/mL) (nuclei are stained blue and apoptotic cells green, 400×). (B) Cytotoxicity of TRAIL, HSA-TRAIL (fraction 1), and HSA-TRAIL (fraction 2). (C) Antiproliferative activity of TRAIL and HSA-TRAIL (fraction 1) in mouse splenocytes (n = 6).

high purity and homogeneity, free of other significant impurities. However, the overall preparation yield for HSA-TRAIL (fraction 1) was low at ∼12%.

Molecular sizes of TRAIL, HSA and HSA-TRAIL fraction 1 and 2 were determined to be 6.2 ± 0.2, 6.7 ± 0.4, 15.4 ± 0.1, and 10.2 ± 0.2 nm, respectively. The molecular size of HSA-TRAIL (fraction 1) was significantly greater than those of HSA and HSA-TRAIL fraction 2 (Figure 1A, right). The apparent molecular weights of HSA, TRAIL, and HSA-TRAIL (fraction 1) were determined by SDS-PAGE analysis (Figure 1B). The band corresponding to HSA-TRAIL (fraction 1) was at a higher position (>240 kDa) than unmodified HSA (∼67 kDa) and TRAIL monomer (∼22 kDa).

CD spectra for TRAIL and HSA across the near-to-far-UV showed unique secondary structures (Figure 1C). The secondary structure was determined using the CDNN software for protein structural analysis. The secondary conformation of HSA-TRAIL was found to be 41.3% α-helix, 28.1% β-sheet (including parallel and antiparallel), and 30.6% random coil, which were changed by the conjugation of HSA and TRAIL [vs 39.3% and 19.3% of α-helix, 33.9% and 44.0% of β-sheet, and 26.8% and 36.7% of random coil for HSA and TRAIL, respectively]. Overall, the CD spectrum for HSA-TRAIL appeared to be merged by the respective spectra of TRAIL and HSA, maintaining their unique pattern of secondary structure.

The compositions of HSA-TRAIL conjugates were determined by using the fluorescein-tagging method. The approximate ratio of HSA and TRAIL in each HSA-TRAIL conjugate (fractions 1 and 2) was found to be 1:1.2 and 1:0.7, respectively. Especially, HSA-TRAIL (fraction 1) seemed to be the approximate 1:1 conjugate of the two molecules.

Bioactivities of HSA-TRAIL. Bioactivities of HSA-TRAIL conjugates (fractions 1 and 2) in terms of apoptotic activity, cytotoxicity, and antiproliferative activity were determined using different unit experiments. Apoptosis induced by HSA-TRAIL (fractions 1 and 2) (10 ng/mL in 10 mM PBS, pH 7.4) was visualized using a TUNEL assay. Mia Paca-2 cells treated with both HSA-TRAILs (fractions 1 and 2) and naïve TRAIL displayed a strong green coloration, indicating clear late apoptosis, while Mia Paca-2 cells treated with PBS were not colored (Figure 2A). Furthermore, green-colored and blue-colored DAPI stained cells were superimposable. Cytotoxicity by HSA-TRAIL (fractions 1 and 2) was measured by using a MTT assay in Mia Paca-2 cells. IC_{50} values were determined to be 5.4 ± 0.2 ng/mL and 16.4 ± 0.3 ng/mL, respectively, which were significantly higher than that of TRAIL (2.5 ± 0.1 ng/mL) (Figure 2B). The proliferative responses of splenocytes cultured with CII (500 μg/mL), LPS (20 μg/mL), or Con A (5 μg/mL) in the presence of HSA-TRAIL at 100 ng/mL were 39.7%, 50.0%, and 40.5% reduced versus PBS-treated controls, which were comparable to naïve TRAIL (Figure 2C).

Pharmacokinetics of TRAIL or HSA-TRAIL in Normal Rats. Blood concentration vs time curves were plotted for TRAIL and HSA-TRAIL in normal SD rats after i.v. injection. TRAIL rapidly exited circulation with a $t_{1/2}$ of 0.23 ± 0.01 h, while HSA-TRAIL had a substantially greater $t_{1/2}$ of 6.20 ± 0.72 h (Figure 3 and Table 1). Furthermore, an AUC_{inf} value (844.1 ± 129.7 ng·h/mL) of HSA-TRAIL was 23.4 times greater than TRAIL at 36.0 ± 1.2 ng·h/mL. In contrast, the clearance (CL) value of HSA-TRAIL (12.1 ± 1.9 mL/h) was lower than that of naïve TRAIL (277.9 ± 9.5 mL/h), and volume of distribution (V_d) was insignificant for both drugs.

Anti-Arthritis Efficacy of HSA-TRAIL in CIA Mouse. Collagen-induced arthritis developed rapidly in mice immunized with CII. Clinical signs of arthritis (incidence: 85.7%) appeared in the hind paws of all vehicle-treated mice at 23 days post-collagen induction (CI). Clinical incidences in TRAIL-treated mice (30, 100 μg/mouse) decreased to 71.4% and 42.9%, respectively, at 23 days (Figure 4A).

The incidence value was 26.8% in HSA-TRAIL-treated mice (30 μg/mouse), and HSA-TRAIL-treated mice (100 μg/

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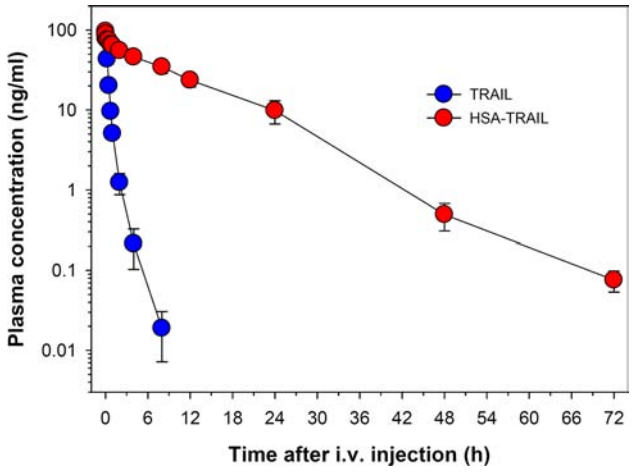

Figure 3. Blood clearance profiles of TRAIL and HSA-TRAIL in normal rats after i.v. injections at a dose of 10 μg.

Table 1. Pharmacokinetic Parameters of TRAIL and HSA-TRAIL in Normal Rats[a]

PK parameters	TRAIL	HSA-TRAIL
AUC_{inf} (ng·h/mL)	36.0 ± 1.2	844.1 ± 130.0
$t_{1/2}$ (h, β)	0.23 ± 0.01	6.20 ± 0.72
CL (mL/h)	277.9 ± 9.5	12.1 ± 1.9
V_d (mL)	92.7 ± 6.0	106.4 ± 9.3

[a]Data represent four individual animals and are presented as means ± SDs.

mouse) showed no significant clinical signs of arthritis at 23 days post-CI. Hind-paw erythema and swelling increased in frequency and severity in a time-dependent manner, and a mean maximum clinical score was reached ~10.0 at 41 days post-CI by vehicle-treated mice (Figure 4B). However, clinical scores were significantly reduced by treatment with TRAIL or HSA-TRAIL (30 and 100 μg/mouse), with clinical scores only 0.4−1.2 at 41 days post-CI by HSA-TRAIL (30 and 100 μg/mouse). The degree of hind-paw erythema and swelling in HSA-TRAIL-treated mice appeared to be negligible and similar to that of normal mice without CIA, which was lower than that of nontreated or TRAIL-treated mice (Figure 5).

Histological Evaluation of CIA Mouse Treated with TRAIL or HSA-TRAIL. To investigate the effects of HSA-TRAIL on pathological changes of inflamed joints, H&E staining was performed on the joint tissue of hind paws obtained from the treated or nontreated CIA mice. Histological evaluations demonstrated that HSA-TRAIL treatment markedly suppressed arthritis development in joint tissues (Figure 6). Images of H&E-stained joint tissue sections from nontreated or TRAIL (30 μg/mouse)-treated mice revealed severe signs of arthritis, such as infiltration of inflammatory cells, extensive synovitis and pannus formation, destruction of articular cartilage, and bone erosion. In contrast, no histological signs except mild inflammatory cell infiltration were present in H&E-stained joint tissue sections of HSA-TRAIL-treated mice (30 μg/mouse).

In Vivo Cytokine Levels of CIA Mouse Treated by TRAIL or HSA-TRAIL. To investigate whether TRAIL modulates the inflammatory process by regulating secretions of cytokines, the serum levels of proinflammatory cytokines IFN-γ, TNF-α, IL-1β, and IL-2 were determined. Significant increases in proinflammatory cytokine levels were found in

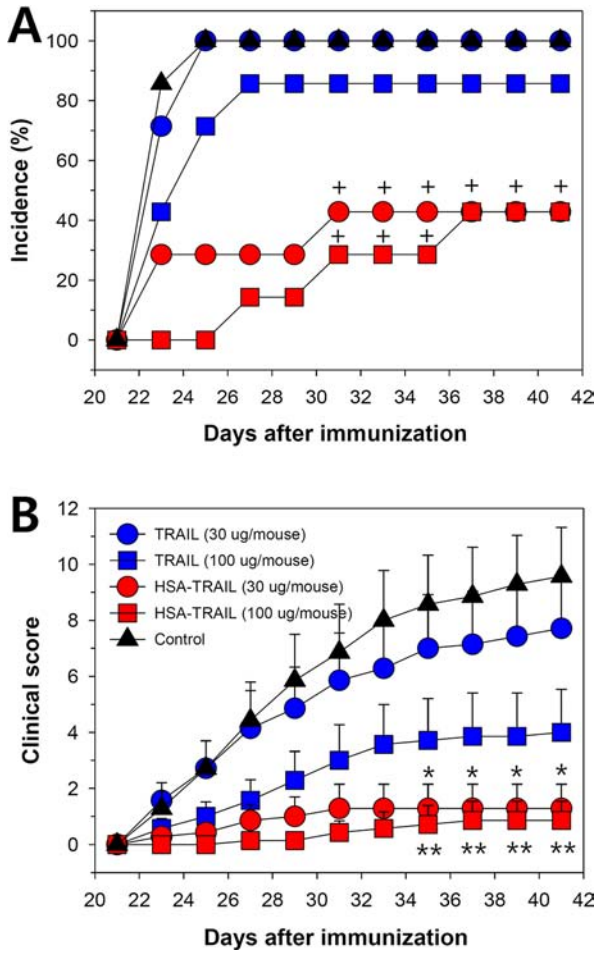

Figure 4. Incidence (A) and clinical score (B) of CIA after booster immunization and/or treatment with TRAIL and HSA-TRAIL (30 or 100 μg/mouse). Error bars represent standard deviation. $^{+}P < 0.001$ TRAIL (100 μg/mouse), $^{*}P < 0.01$ and $^{**}P < 0.05$ over TRAIL (100 μg/mouse), respectively ($n = 7$).

serum samples of vehicle-treated mice at day 41 post-CI (Figure 7). In contrast, all cytokine levels were significantly and dose-dependently lower in TRAIL- or HSA-TRAIL-treated mice than in vehicle-treated mice. HSA-TRAIL (30 μg/mouse) dramatically reduced serum of IFN-γ, TNF-α, IL-1β, and IL-2 to levels similar to normal mice.

Inflamed Tissue Localizations of TRAIL or HSA-TRAIL in CIA Mouse. Both TRAIL and HSA-TRAIL were mainly localized in the livers of normal mice at 3 h postinjection (Figure 8A). However, different biodistributions of TRAIL and HSA-TRAIL were found in CIA mice, with localizations of both drugs reduced in livers. HSA-TRAIL accumulated in inflamed hind paws of CIA mice, while accumulation of naïve TRAIL appeared negligible. Further, the localization of HSA-TRAIL in inflamed hind paws continued for 3 days, while naïve TRAIL disappeared within 1 day of systemic circulation (Figure 8B).

DISCUSSION

Rheumatoid arthritis remains difficult to treat despite the existence of effective therapies, due to various physiological or mechanism-based causes related to inflammation and autoimmunity. A series of disease-modifying biologics, such as therapeutic antibodies and fusion proteins, have displayed remarkable clinical success, compared to orally administered

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Figure 5. Photographs of hind paws (bottom side) of normal or nontreated mice or mice treated with TRAIL or HSA-TRAIL (30 μg/mouse) at day 21, 28, 35, and 41 after first immunization (left). Photographs of representative hind paws of each mouse (top side) at day 41 (right).

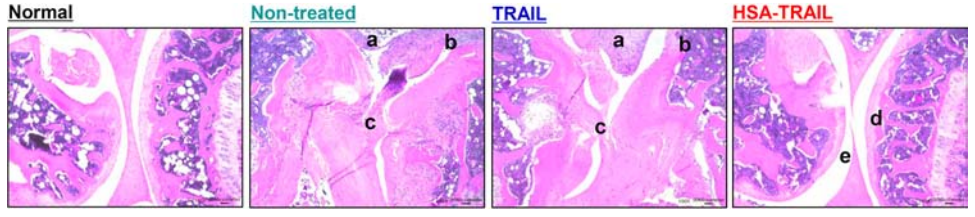

Figure 6. Histopathologic investigation (H&E staining) in knee joints of normal or CIA mice, with or without TRAIL or HSA-TRAIL treatment (30 μg/mouse). Knee joints of CIA mice were demonstrated pathological features, including inflammatory cell infiltration (a), synovial hyperplasia (b), and cartilage and bone erosions (c). CIA mice treated with HSA-TRAIL showed smooth articular surface (d) and wider articular space (e).

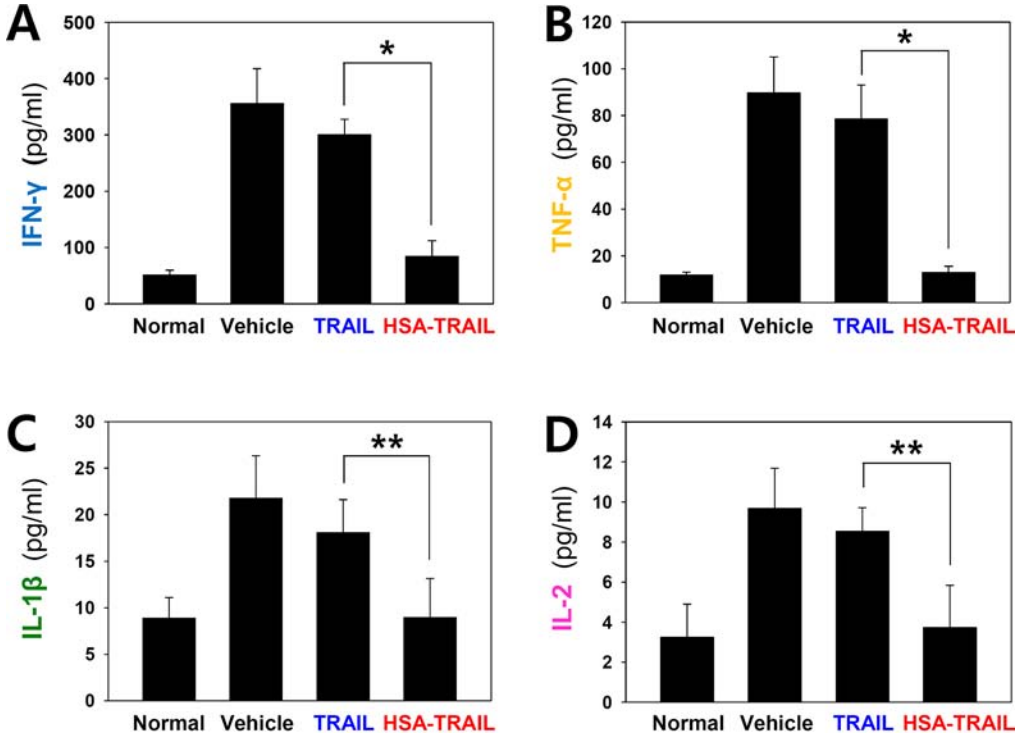

Figure 7. Effect of TRAIL and HSA-TRAIL (30 μg/mouse) on the productions of pro-inflammatory cytokines, such as (A) IFN-γ, (B) TNF-α, (C) IL-1β, and (D) IL-2 in sera of CIA mice. *P < 0.01 and **P < 0.05 over TRAIL, respectively (n = 7).

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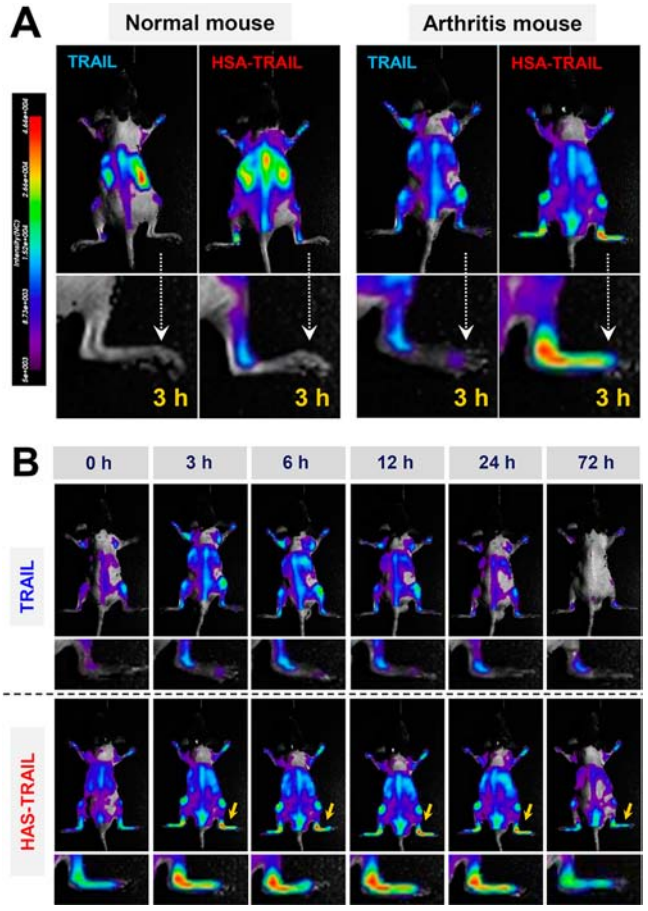

Figure 8. (A) Biodistribution of TRAIL and HSA-TRAIL (30 μg/mouse) in normal or CIA mice at 3 h after i.v. injections. (B) Monitoring of uptake of TRAIL and HSA-TRAIL in the hind paws of CIA mice until 72 h.

chemical drugs.[4] TRAIL, a unique protein biologic viewed as a new way of treating arthritis, has displayed promising anti-inflammatory efficacy in animals with RA.[11,12] However, TRAIL is inefficient for use as an RA treatment because of a short therapeutic duration.[23] Therefore, a new prototype of TRAIL with better pharmacokinetic behavior and pharmacologic efficacy appeared inevitable. To achieve this, we designed and prepared an albumin-conjugated TRAIL (HSA-TRAIL) with a clear targetability to RA tissue and a long circulation for the treatment of arthritis.

TRAIL suppresses the development of rheumatoid arthritis. However, its mechanism-based role in RA treatment remains unclear. Studies have shown that DR4 and/or DR5 are expressed in fibroblast-like synoviocytes (FLS) from RA patients, which play a pivotal role in initiating and perpetuating RA.[24−26] TRAIL was reported to downregulate self-antigens in RA on the basis of apoptosis induction on activated T cells and synoviocytes.[10] Due to this unique pharmacologic advantage, TRAIL has demonstrated anti-inflammatory efficacy in arthritis animal models and the potential to treat RA.

To prepare HSA-TRAIL conjugates, we used a bifunctional MAL-PEG-NHS (Mw 2 kDa) that reacts with amines in TRAIL and thiols in HSA modified by 2-iminothiolane. The use of a linker PEG between HSA and TRAIL appeared to result in two advantages: in vivo stability and reaction control. First, PEGylation of proteins is well documented to increase circulation duration and decrease immnunogencity.[17,23,27] Despite the low molecular weight of 2 kDa, MAL-PEG-NHS may have a theoretical 130 Å size, and appeared to play a meaningful role in increasing overall molecular size and prolonging the pharmacologic action of HSA-TRAIL. Second, the linear strand of PEG protects HSA-TRAIL from severe attack by various antibodies and proteolytic enzymes. We reported that the use of linker PEG between HSA and anti-diabetic exendin-4 peptide significantly helped protract its circulation and hypoglycemic efficacy in plasma, compared to the HSA-exendin-4 conjugate without linker PEG.[17] However, the overall PEGylation effect was not dominant because the size of PEG is relatively small in comparison to HSA and TRAIL proteins. In contrast, the use of linker PEG appeared to result in an approximate 1:1 controlled reaction between HSA and TRAIL, as it provides considerable spatial distance between them to prevent random conjugations. HSA and TRAIL were randomly and mutually conjugated in the absence of PEG, and the reaction was difficult to control in repeated experiments (data not shown). Consequently, two major fractions (fraction 1 and 2) were produced using a conjugation method. The first fraction of HSA-TRAIL conjugates (fraction 1: ∼15.4 nm; ∼240 kDa), separated by SEC and designated HSA-TRAIL, was chosen for exhibiting better-preserved bioactivity than HSA-TRAIL conjugate fraction 2 in terms of apoptosis, cytotoxicity, and antiproliferation. This is probably due to the higher composition of TRAIL in the HSA-TRAIL conjugate fraction 1 (HSA:TRAIL = 1:1.2) than fraction 2 (1:0.7). Furthermore, such a composition difference in the HSA-TRAIL conjugates seems to be responsible for the different biological activities of two conjugates (apoptotic activity, cytotoxicity, and antiproliferative activity).

The murine CIA model has been widely used to investigate the pathogenesis of autoimmune arthritis and the potential anti-inflammatory efficacy of anti-RA drugs. In this study, erythema and swelling were observed in the hind paws of CIA mice from day 23. Similar to previous results,[11,12] the serum levels of anticollagen CII antibodies (IgG2a) were elevated in control CIA mice, but the serum IgG2a level in HSA-TRAIL-treated CIA mice (30 μg/mouse) appeared to be normalized (∼6000 pg/mL) (data not shown). Further, it is well documented that the imbalance between pro- and anti-inflammatory cytokine activity causes chronic inflammation and joint/cartilage destruction due to abnormality in the production of pro-inflammatory cytokines.[11] Serum levels of IFN-γ, TNF-α, IL-1β, and IL-2 were abnormally elevated in nontreated CIA mice, while levels by CIA were normalized after HSA-TRAIL treatment (30 μg/mouse) (Figure 7). Cytokine and CII antibody levels are known to be closely related with the progress/development of RA. Therefore, decreased levels indicated that HSA-TRAIL suppressed the progress of arthritis and ameliorated clinical signs of inflammation. HSA-TRAIL decreased clinical scores in CIA mice as reflected by the degree of erythema and swelling in inflamed paws (Figure 5), and significantly suppressed the deformation of joint tissue involved in inflammatory cell infiltration, synovitis and pannus formation, and destruction of articular cartilage (Figure 6).

The strategy for HSA-TRAIL in treating RA was based on targeting behavior of HSA to inflamed tissues in RA. Inflamed synovial cells in RA are highly upregulated for catabolism and necessitate more nitrogen than normal, likely from albumin, as a high energy source.[14,20,21] Fluorescein-labeled HSA and ^{111}In-labeled HSA had significantly accumulated in inflamed paws of CIA mice, compared to mice without arthritis induction.[21]

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HSA-TRAIL had accumulated in the paws of CIA mice, but not in paws of normal mice (Figure 8). This altered distribution of HSA-TRAIL is notable as both TRAIL and HSA-TRAIL were primarily located in livers of normal mice. Therefore, the enhanced accumulation of HSA-TRAIL via the targeting role of HSA appeared to ameliorate TRAIL in efficiently suppressing inflammation in relevant activated T cells or synoviocytes of arthritis tissues.

Another advantage of albumin conjugation is the increased circulating lifespan of TRAIL. TRAIL was reported to have a 20 min circulating $t_{1/2}$ in rat or mice,[23] which restricts therapeutic efficacy in vivo despite potent anti-inflammatory activity. TRAIL appeared not to exert pharmacological action at 3 h after i.v. injection, as assumed by the pharmacokinetic profile. HSA has the ability to bypass glomerular filtration, as the molecular size (~6−7 nm) of HSA is similar to the pore size of kidney glomerulus. This may have application to the clinical usage of HSA for short-life peptides and proteins, such as GLP-1, exendin-4, insulin, and interferon.[14] This study found that the HSA part of HSA-TRAIL contributed to extending the circulating half-life of TRAIL in normal rats. However, HSA-TRAIL is expected to have limited residence in plasma of RA patients because HSA-TRAIL significantly accumulated in RA tissue. Nonetheless, with the potential of longer circulation, HSA-TRAIL could significantly improve therapeutic efficacy and in vivo stability in RA patients.

■ CONCLUSION

We described an HSA-TRAIL conjugate that shows enhanced therapeutic efficacy for the treatment of rheumatoid arthritis. We sought to take full advantage of albumin and TRAIL for treating RA in terms of targetability and long-circulation, and anti-inflammatory efficacy. In this respect, the direct conjugation of both proteins was considered to be the best approach. HSA-TRAIL was found to be more targeted in inflamed tissues of CIA mice than naïve TRAIL, unlike normal mice, and displayed a circulating half-life >26 times longer than TRAIL. Further, HSA-TRAIL showed enhanced anti-inflammatory efficacy in CIA mice on the basis of RA incidence and clinical scores, compared to TRAIL. This result could be attributed to increased lifetime in plasma and efficient targeting to RA tissue from the HSA part, and unique apoptosis-based anti-RA therapy mechanism from TRAIL. We believe that the HSA-TRAIL conjugate has promising potential as a therapeutic agent for the treatment of rheumatoid arthritis.

■ EXPERIMENTAL PROCEDURES

Materials. TRAIL(114−281) that has a trimer-forming domain was produced from *Escherichia coli*, as previously described.[27] Human serum albumin (HSA, ~99%) and 2-iminothiolane hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bifunctional PEG activated by *N*-hydroxysuccinimide (NHS) and maleimide (MAL-PEG-NHS, Mw 2000 Da) was obtained from the NOF Corporation (Tokyo, Japan). Cy5.5-NHS ester dye and TRAIL ELISA kits were purchased from GE Healthcare (Piscataway, NJ, USA) and Abcam (Cambridge, MA, USA), respectively. In situ cell death detection kits and fluorescein-NHS were purchased from Roche Diagnostics (Mannheim, Germany) and Pierce (Rockford, IL, USA), respectively. All other reagents and chemicals, unless otherwise specified, were purchased from Sigma-Aldrich.

Animals. DBA/1 mice (males, 7−8 weeks old) and Sprague−Dawley (SD) rats (males, 6 weeks old) were purchased from the Hanlim Experimental Animal Laboratory (Seoul, Korea). All experiments related to animals were conducted according to the guidelines of the National Institute of Health (NIH) on the use of laboratory animals (NIH publication 80−23, revised in 1996). Animals were acclimated for a week before use and housed with free access to feed and water. This animal study was approved by the Ethical Committee on Animal Experimentation of Sungkyunkwan University.

Preparation of HSA-TRAIL Conjugates. HSA-TRAIL conjugates were prepared using a modification of previously described procedures.[17,28−30] Primary amines of HSA were thiolated. Briefly, a 930 μL aliquot of 2-iminothiolane (Traut's Reagent; 1 mg/mL, 6.75 μmol, approximately 9 equiv to an HSA molecule) was mixed with 70 μL of HSA (50 mg, 0.75 μmol) in 10 mM of phosphate buffered saline (PBS, pH 7.4) and allowed to react for 30 min. Five milligrams of glycine (67.5 μmol) was added to the mixture to inactivate the reactivity of unreacted 2-iminothilane and stirred for 30 min. Separately, 13.8 mg of MAL-PEG-NHS (6.84 μmol) was mixed with 50 mg of TRAIL (0.76 μmol) in 50 mL of 10 mM of PBS (pH 7.4) at room temperature for 1 h. The reaction was terminated by adding 5 mg of glycine, and buffer media in this mixture was changed to PBS (pH 7.4) by desalting using a Sephadex G-25 column, then concentrated using a Centricon-30 concentrator (MWCO 30 kDa; Millipore, Beverly, MA, USA). Finally, an aliquot (0.9 mL) of thiolated HSA (50 mg/mL, 0.68 μmol) was quickly transferred to 22.5 mL of TRAIL-PEG-MAL (2 mg/mL, 0.68 μmol) in PBS (pH 7.4), and the reaction was allowed to continue at room temperature for 3 h. The resulting mixture was then applied to a Superdex 200 10/300 GL column (300 × 10 mm; GE Healthcare Life Sciences, Piscataway, NJ) and eluted with PBS at a flow rate of 0.6 mL/min, and fractions corresponding to each HSA-TRAIL conjugate were collected. Final HSA-TRAIL conjugates (fraction 1 and 2) were concentrated to 1 mg/mL and stored at −70 °C until needed.

Composition Determination of HSA-TRAIL Conjugates. Concentration and composition of HSA-TRAIL conjugates were investigated using a modification of previously described procedures.[17,27,31] Five milligrams of TRAIL (0.08 μmol) in 5 mL PBS (pH 7.4) was consecutively mixed with 1.4 mg of MAL-PEG-NHS (0.68 μmol) and then with 360 μg of fluorescein-NHS (0.76 μmol), and the reactions were allowed to continue at room temperature for 1 h. After termination of the reaction by adding glycine (0.5 mg), the resulting mixture was desalted using a Sephadex G-25 column and then concentrated to 2 mg/mL (using a BSA standard). An aliquot of fluorescein-tagged MAL-PEG-TRAIL was kept for a calibration curve of fluorescence. Fluorescein-tagged MAL-PEG-TRAIL was mixed with thiolated HSA at a molar ratio of 1:1 as described above, and fluorescein-tagged HSA-TRAIL fractions were collected using a Superdex 200 10/300 GL column. Separately, the concentrations of HSA, TRAIL, and fluorescein-tagged HSA-TRAIL conjugates were determined using a BCA protein assay (a BSA standard), and their fluorescence intensity (excitation and emission wavelengths: 490 and 520 nm, respectively) was also measured using a Tecan Infinite M200 plate reader. On the basis of a correlation between protein concentrations of each part (HSA or TRAIL) and fluorescence intensity from a TRAIL part in the conjugates,

the conjugation HSA:TRAIL ratios in HSA-TRAIL conjugates were calculated.

Characterization of HSA-TRAIL Conjugates. A series of HSA-TRAIL conjugates [TRAIL, HSA, MAL-PEG-TRAIL, or HSA-TRAIL conjugates (fraction 1: hereafter referred to as HSA-TRAIL and fraction 2)] were characterized using a modification of previously described procedures.[17,23,28] HSA-TRAIL conjugates were subjected to size-exclusion chromatography (SEC) on a TSKgel G3000SWXL column (7.8 × 300 mm, Tosoh, Tokyo, Japan) and eluted with PBS (pH 7.4) at a flow rate of 0.6 mL/min. Chromatograms were detected by UV at 215 nm. A calibration based on molecular weights (Mws) and retention time of protein standards was used to estimate the apparent Mws of HSA-TRAIL conjugate. Separately, the number-based particle sizes of HSA-TRAIL conjugates were measured by a Zetasizer Nano-S90 (Malvern Instruments, MA, USA). HSA-TRAIL conjugates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12%). Circular dichroism (CD) spectra from HSA, TRAIL, and HSA-TRAIL (fraction 1) were recorded over the far-UV range between 190 and 260 nm at a concentration of 0.5 mg/mL in PBS using a Chirascan-plus spectrometer (Applied Photophysics, UK). The secondary structure was determined using the CDNN software for protein secondary structure analysis.

Antibody-Binding Affinity of HSA-TRAIL. The antibody-binding affinity and concentration of TRAIL and HSA-TRAIL were determined using enzyme-linked immunosorbent assay kits (Abcam, Cambridge, MA, USA).[27] For the ELISA assay, a naïve TRAIL standard of exactly known concentration was utilized for a calibration curve (absorbance vs concentration). Absorbance from various samples was measured at 450 nm using a microplate reader (VERSAmax, Molecular Devices Corp., Sunnyvale, CA, USA). Data are presented as means ± SDs (n = 3).

Bioactivity of HSA-TRAIL. Bioactivities of HSA-TRAIL conjugates (fraction 1, 2) on the basis of cytotoxicity and apoptotic activity were investigated in Mia Paca-2 cells, which were cultivated with a slight modification of previously described methods.[32,33] After a 24 h incubation in a 96-well plates (10 000 cells/well), the cytotoxicities of HSA-TRAIL conjugates over the concentration range of 0.001−1000 ng/mL were determined using a MTT assay. Viabilities (%) versus controls were calculated, and inhibitory concentrations (IC_{50}) were calculated (n = 6). Separately, apoptotic activities of HSA-TRAIL conjugates were observed from terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay (in situ cell death detection kit, Roche, Diagnostics, Mannheim, Germany), with the same method as previously described.[32] Briefly, glass slides of cells reacted with a series of TUNEL reagents were rinsed three times with PBS and reacted with a DAPI solution, and further cells were visualized by confocal laser scanning microscopy (CLSM).

Antiproliferative Activity of HSA-TRAIL in Mouse Splenocyte. Antiproliferative activity of TRAIL and HSA-TRAIL was investigated using a slight modification of previously described methods.[11,34] Splenocytes were isolated from the spleen of DBA/1 mice sacrificed at 41 days after first immunization, using a fine nylon cell strainer (BD Falcon, USA) to obtain a homogeneous cell suspension. Erythrocytes were lysed by the addition of a red blood cell lysing buffer (Sigma-Aldrich, USA) at 37 °C for 2 min. After centrifugation, collected cells were washed three times with PBS and resuspended in RPMI 1640 (Corning, USA) containing 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin, and seeded in a 96-well plate (4×10^5 cells/well). The stimulators (each 10 μL) of 500 μg/mL heat-denatured bovine collagen type II (CII), 20 μg/mL lipopolysaccharide (LPS), or 5 μg/mL concanavalin A (Con A) were added to cells (80 μL media/well), which were then treated with aliquots (10 μL) of TRAIL or HSA-TRAIL at predetermined final concentrations. After 2 days, cell viability was examined using a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies Inc., Gaithersburg, MD, USA).

Blood Clearance of HSA-TRAIL. The right jugular vein and left femoral vein of SD rats weighing 220−230 g were cannulated with PE tubing for the injection and sampling. After a 2 day recovery period, TRAIL and HSA-TRAIL (10 μg/rat) were administered to the rats (n = 3 each) by i.v. injection into the femoral venous catheter. Serial blood samples (approximately 0.3 mL each) were withdrawn from the jugular vein at 0, 3, 10, 15, and 30 min and 1, 2, 3, 6, 12, 24, 48, and 72 h, and an equal volume of sterilized saline was replaced after each sampling. The resulting plasma concentrations were determined using a TRAIL ELISA kit. Pharmacokinetic parameters were calculated by two-compartmental analysis using WinNolin, v 1.1 (Scientific Consulting, Inc., Cary, NC). AUC_{inf} values and circulating half-lives ($t_{1/2}$) were obtained by calculating areas under the curves from zero to infinity using the trapezoidal rule.[23]

Induction of Collagen-Induced Arthritis in Mouse and Treatment by HSA-TRAIL. Collagen-induced arthritis (CIA) was induced using a modification of previously described procedures.[9,11,12] Briefly, 50 μL of bovine type-II collagen (CII; Chondrex, Redmond, USA) was emulsified with 50 μL of complete Freund's adjuvant (CFA). DBA/1 mice were subcutaneously injected at the base of the tail with 100 μL of emulsion. On day 21 after primary immunization, mice received an s.c. booster injection (100 μL of CII in incomplete Freund's adjuvant (IFA) without CFA). Animals CIA-induced were randomly divided into five treatment groups (n = 7 each) according to samples of different doses: sterilized PBS, TRAIL (30 or 100 μg/mouse), or HSA-TRAIL (30 or 100 μg/mouse). Mice were given intravenous injections of each sample solution (100 μL) every 3 days for 3 weeks (21, 24, 27, 30, 33, 36, and 39 days after primary immunization). Clinical severities of arthritis were carefully evaluated as follows: 0, normal; 1, erythema and slight swelling confined to the tarsals or ankle joint; 2, erythema and mild swelling extending from the ankle to the tarsals; 3, erythema and moderate swelling extending from the ankle to the metatarsal joints; 4, erythema and severe swelling encompass the ankle and digits. Mice were monitored every other day, and each limb was scored on a scale of 0−4 (maximum score, 16 per mouse).

Cytokine and Antibody Determinations. Retro-orbital blood samples were aspirated and collected from mice sacrificed at 41 days after first immunization.[11] Levels of TNF-α, IFN-γ, IL-1β, and IL-2 in the mice serum were measured using Milliplex MAP mouse cytokine/chemokine magnetic bead panel (Millipore, Billerica, MA). Separately, collagen-specific auto antibody (IgG1 or IgG2a) levels in the mice serum were determined using Milliplex MAP mouse immunoglobulin isotyping magnetic bead panel (Millipore, Billerica, MA).

Histologies of CIA Joints Treated with HSA-TRAIL. On day 41, mice were sacrificed by cervical dislocation.[11,12,35] Knee

joints were obtained from mice and quickly fixed in 10% neutral buffered formalin solution for 24 h and embedded in paraffin. Cross-sliced tissues of the joint were deparaffinized in xylene, rehydrated through a graded alcohol/water mixture, and stained with H&E (hematoxylin and eosin). Stained samples were observed under an optical microscope.

Targeting of HSA-TRAIL to Inflamed Tissues in CIA Mouse. Time-dependent localizations of TRAIL and HSA-TRAIL were monitored in collagen-induced arthritis mice. TRAIL and HSA-TRAIL were modified with Cy5.5-NHS as previously described,[36] and unreacted Cy5.5-NHS was removed from the reaction mixture by desalting using a Sephadex G-25 column. A portion (100 μg in 0.1 mL PBS) of Cy5.5-labeled TRAIL and HSA-TRAIL was intravenously injected into CIA mouse and normal mouse, respectively. Systemic distribution of TRAIL and HSA-TRAIL was monitored using the Optix MX3 (Advanced Research Technologies Inc., Montreal, Canada) for 3 days.

Statistical Analysis. Data are presented as means ± SDs. The significance of differences was determined using a Student's *t*-test. *P*-values <0.05 were considered statistically significant.

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Notes

The authors declare no competing financial interest.

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