Supplemental Materials

JMolMed

"The N-Glycoform of sRAGE is the Key Determinant for Its Therapeutic Efficacy to Attenuate Injury-elicited Arterial Inflammation and Neointimal Growth"

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Additional materials and methods

Generation of sRAGE(N25T/N81T) mutant

sRAGE (N25T/N81T) mutant was generated using two sets of primers via PCR, each with one of the primers phosphorylated at 5' to facilitate the blunt end ligation:

Set 1: N25T portion:

Forward: 5' GAAGATCT GCT CAA ACC ATC ACA GCC CGG ATT GGC 3'
Bgl II 23
↕
A

Reverse: 5' –Phos-GGG AAG GAC ACG AGC CAC 3'
80

Set 2: N81T portion:

Forward: 5'–Phos-ACC GGC TCC CTC TTC CTT CCG 3'
↕
A
81

Reverse: 5'GCTCTAGA TCA AGT TCC CAG CCC TG 3'
Xba I Stop 340

The numbers underneath the sequences mark the corresponding amino acid number in sRAGE protein. The mutated nucleotide and the introduced restriction sequences are underlined with the original codon AAC (encoding for N) mutated to ACC (encoding for T). The wild-type sRAGE construct was used as the template for both PCR sets. The two amplified fragments were purified with QIAquick PCR purification kit (Qiagen), and ligated with T4 DNA Quick ligase (New England Biolabs). The Quick-ligated mixture was then used as the template for re-amplification by PCR, using the forward primer from the set1 and the reverse primer from the set 2. The re-amplified full-length sRAGE sequence carrying the two mutations was inserted to pJP008 [1] vector via ligation of BamHI/BglII –XbaI sites . The construct was nucleotide-sequenced to confirm the mutations. The single site mutation was generated in a similar way with one of the forward primers carrying the wild-type sequence.

Supplemental figures

Fig. S1

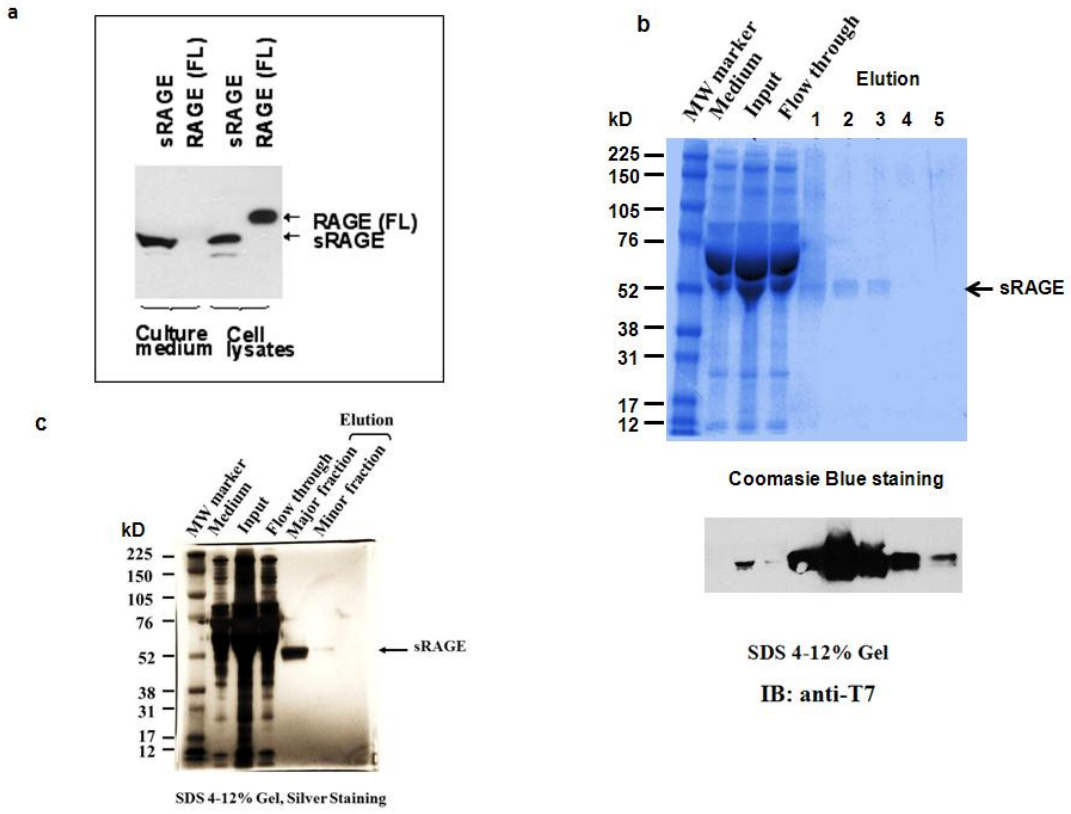

Fig. S1. Expression and purification of T7-tagged human sRAGE. **a** Expression of sRAGE in cell culture medium and lysates. CHO-CD14 cells were transiently transfected with T7-tagged $sRAGE^{CHO}$ and full-length (FL) RAGE constructs. The overnight cell culture medium and cell lysates (10 μg each) were resolved by 4-12% gradient SDS-PAGE, and immunoblotted with anti-T7 antibodies. While RAGE(FL) is expressed in the cell, $sRAGE^{CHO}$ is secreted into the medium and a portion still remains in the cell lysates. **b** Monitoring purification of $sRAGE^{CHO}$ by affinity chromatography. Cell culture medium from untransfected (marked as "medium") and transfected cells (marked as "input") as well as the flow-through and each collected fractions (15 μl of each) were resolved with 4-12% gradient SDS-PAGE and immunoblotted with anti-T7 antibodies. Upper panel: Coomassie Brilliant Blue R-250staining; lower panel: western blotting. **c** Silver staining of $sRAGE^{CHO}$ samples resolved on 4-12% gradient SDS-PAGE. Fraction 2 (major) and 4 (minor) were used. 5 μl of sample was loaded on each lane.

Fig. S2

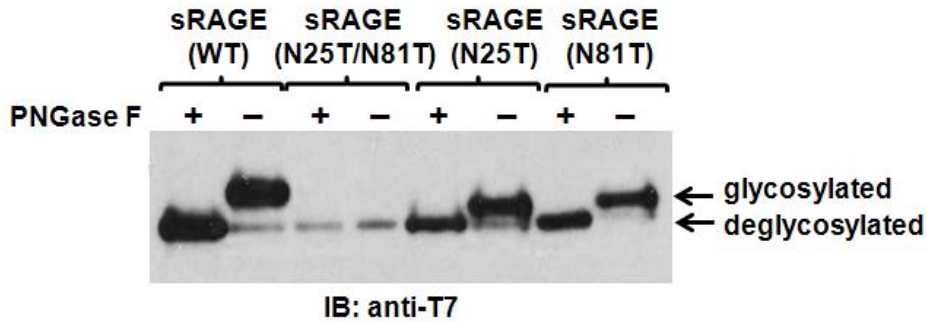

Fig. S2 sRAGE^{CHO} is glycosylated at both N25 and N81 putative sites. Plasmids carrying sRAGE^{CHO} and sRAGE^{CHO} carrying N25T, or N81T, or composite mutations (N25T/N81T) were transfected to CHO-CD14 cells and the overnight cell culture media were collected. Media treated with PNGase F and mock-treated were resolved with SDS 4-12% PAGE and blotted with anti-T7 antibodies.

Fig. S3

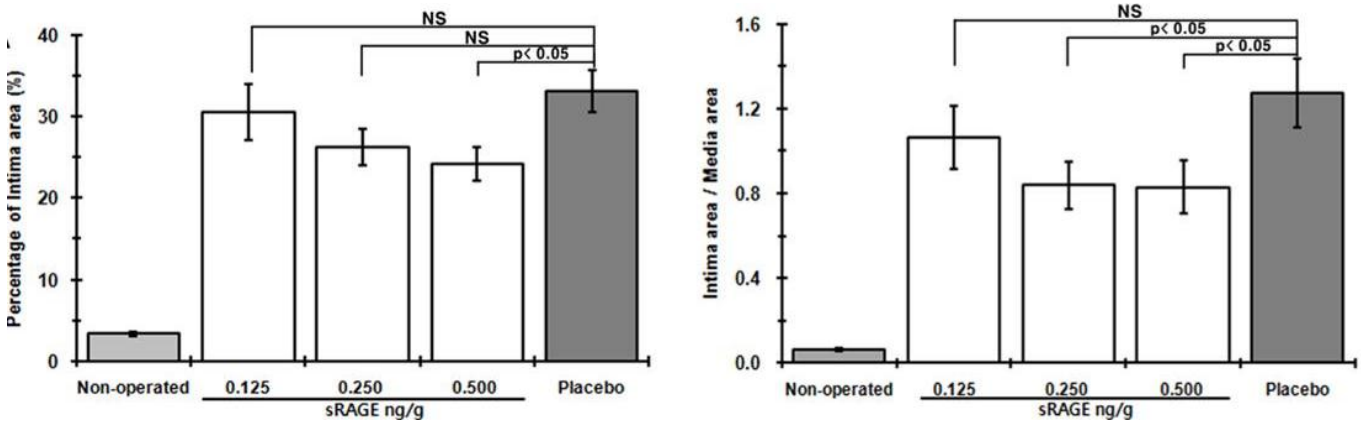

Fig. S3. A low dose of sRAGE^{CHO} sufficiently suppresses neointimal growth in a rat carotid artery balloon injury model. Four groups of rats (n = 8–10/group) were operated and three doses of sRAGE (0.125, 0.25, and 0.5 ng/g body weight) and the placebo (phosphate buffered saline) were administered via i.p. injection. Each dose was injected three times: 24 h prior to the balloon injury procedure, immediately after, and 24 h post-surgery. Histomorphological analyses were performed 2 weeks after surgery. Both percentage of the intima area in the vessel wall (left), and the intima/media ratio (I/M, right) were measured. NS: statistically not significant.

Fig. S4

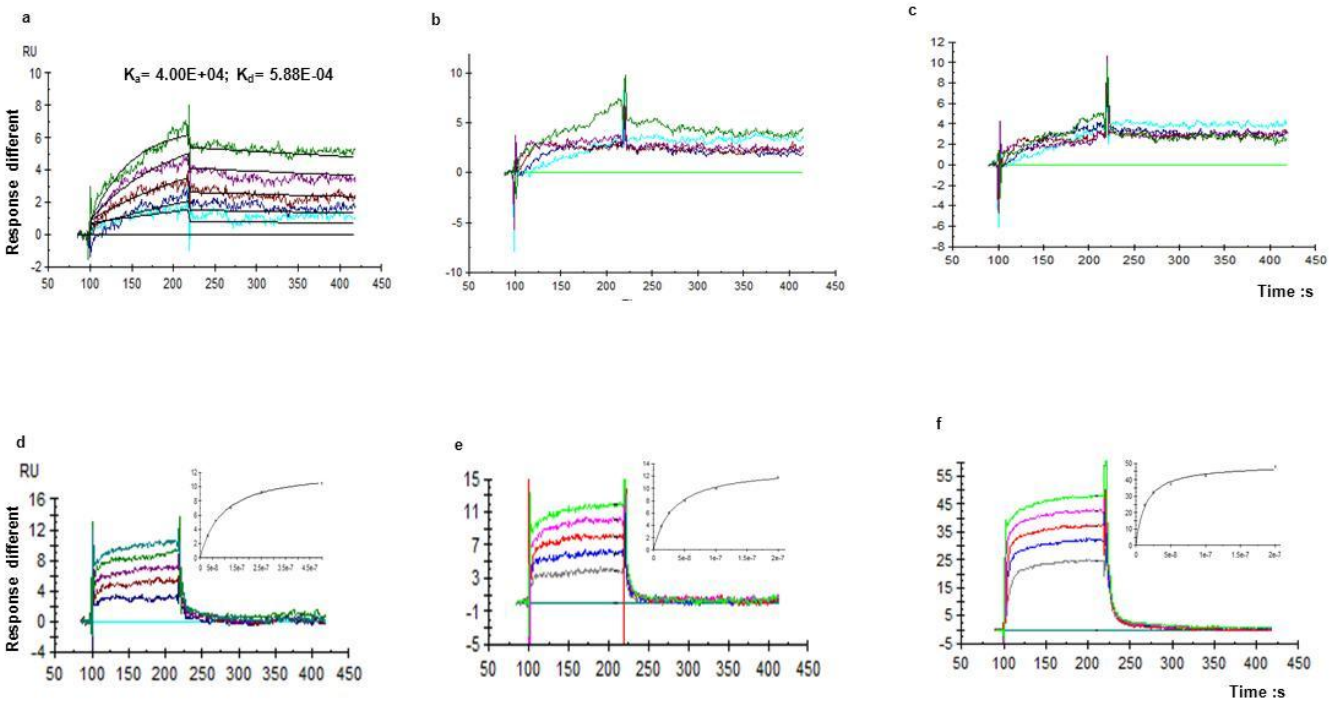

Fig. S4 BIAcore surface plasmon resonance studies of sRAGE-ligand interactions. a-c Sensorgrams of HMGB1 (analyte concentrations used in the study: 31.25, 62.5, 125, 250, 500 nM) **a** $sRAGE^{CHO}$; **b** $sRAGE^{Sf9}$; **c** $sRAGE^{CHO}$(N25T/N81T). **d-f** Sensorgrams of S100B (analyte concentrations used in the study: 12.5, 25, 50, 100, 200 nM). The insets show steady state binding curves with Y axis as response units (RU) and X axis as concentration (nM). **d** $sRAGE^{CHO}$; **e** $sRAGE^{Sf9}$; **f** $sRAGE^{CHO}$(N25T/N81T).

Table S1 Carbohydrate composition of $sRAGE^{CHO}$

	mol/mol protein
Fuc[a]	0.9
GlcNAc[b]	6.6
Gal[a]	3.3
Man[a]	7.8
Neu5Ac[c]	1.1

$RAGE^{CHO}$ sample was hydrolyzed with a: 2 M trifluoroacetic acid at 100ºC for 4 h; b: 4 M HCl at 100ºC for 6 h; c: 20 mM HCl at 55ºC for 30 min. GlcNAc was measured as N-acetylglucosamine after hydrolysis.

Table S2. SPR-measured K_D of sRAGE-ligand interaction

	K_D[a] (HMGB1)	**K_D[b] (s100B)**
sRAGE^{CHO}	13.3 ± 1.4 nM	90.5 nM
sRAGE^{CHO}(desialylation)	ND[c]	25.**3** nM
sRAGE^{Sf9}	ND[c]	32.0 nM
sRAGE^{CHO}(N25T/N81T)	ND[c]	13.6 nM

a: K_D calculated using 1:1 Langmuir model; b: K_D calculated using steady state model; c: no binding.

Table S3. Assessment of organs and body weight in sRAGE–treated rats

		sRAGE treatment[(a)]				
	Placebo[(a)]	0.5ng/g	1.0ng/g	1.5ng/g	3.0ng/g	6.0ng/g
Body weight	459.04±9.09	443.53±16.15	472.19±11.72	461.77±8.21	461.57±14.78	473.15±9.79
Heart /body weight[(b)]	0.31±0.01	0.33±0.01	0.33±0.01	0.30±0.01	0.30±0.01	0.28±0.01
Liver / bodyweight[(b)]	3.83±0.08	3.68±0.12	3.69±0.08	3.76±0.09	3.95±0.10	3.78±0.14
Kidney / bodyweight[(b)]	0.41±0.01	0.43±0.02	0.42±0.01	0.41±0.01	0.39±0.01	0.38±0.01
Spleen / bodyweight[(b)]	0.22±0.01	0.22±0.01	0.21±0.01	0.22±0.01	0.23±0.01	0.23±0.01
Lung / bodyweight[(b)]	0.55±0.02	0.58±0.04	0.56±0.03	0.53±0.03	0.44±0.02	0.46±0.02
Thymus / bodyweight[(b)]	0.12±0.01	0.12±0.01	0.11±0.01	0.11±0.01	0.12±0.01	0.13±0.01
Testis / bodyweight[(b)]	0.40±0.01	0.42±0.01	0.39±0.01	0.41±0.01	0.41±0.01	0.38±0.01

(a) Each treatment or placebo group, *n* = 8–10 and means ± SEM were presented;
(b) The organ/body weight ratio is presented.