SUPPLEMENTARY DATA

Biochemical and Immunological Properties of Engineered Low-Immunogenic Staphylokinases for Next-Generation Thrombolytic Therapy

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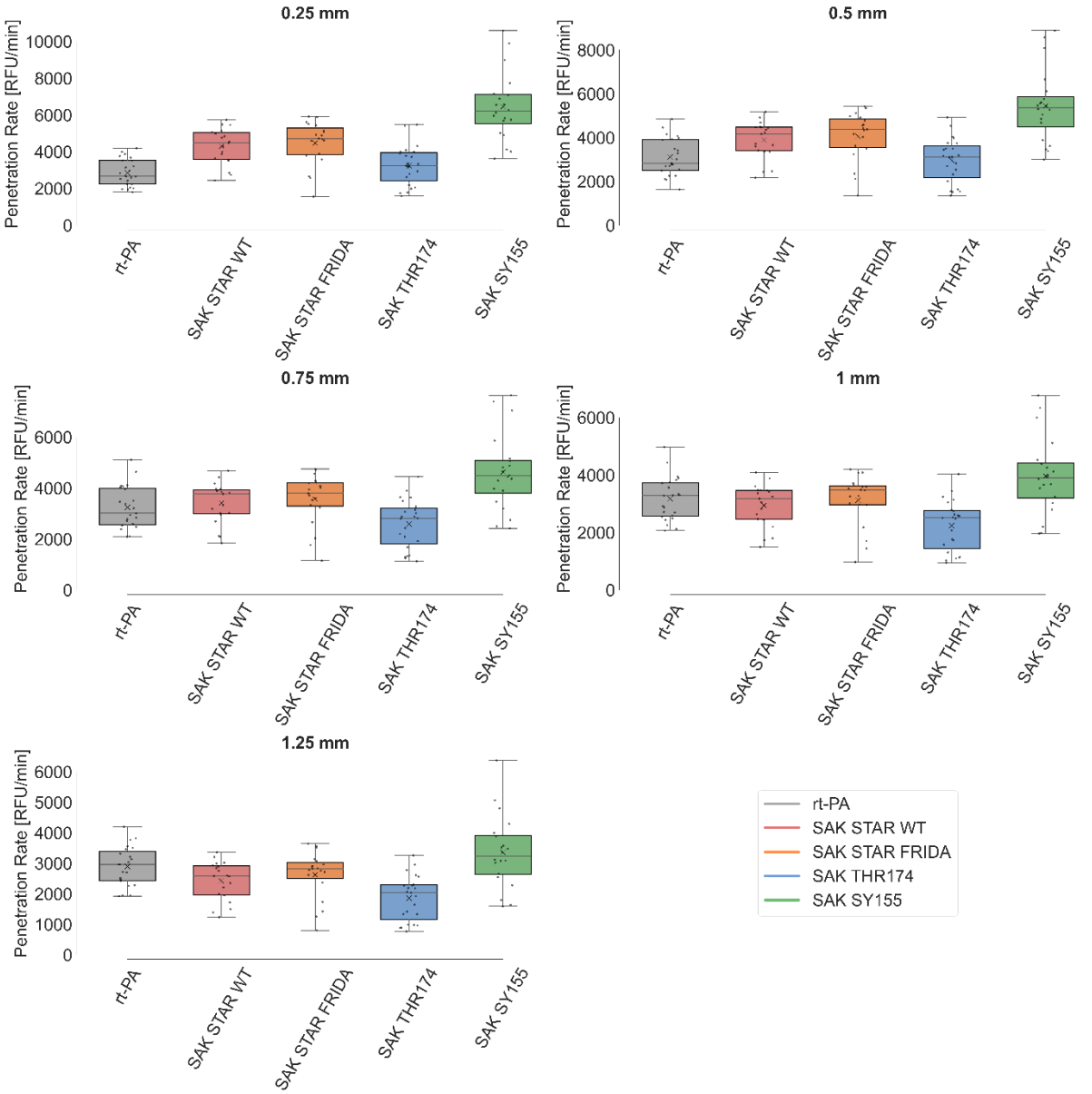


Figure S1 Penetration rates at individual distances. Experiments were conducted at 37 °C for 180 minutes in PBS (pH 7.4), with 19 – 23 repetitions (technical replicates, n) per condition. All agents were used at a concentration of 0.022 µM. The box plots show mean value (X), median (line), interquartile ranges (boxes), and minimum and maximum values (whiskers).

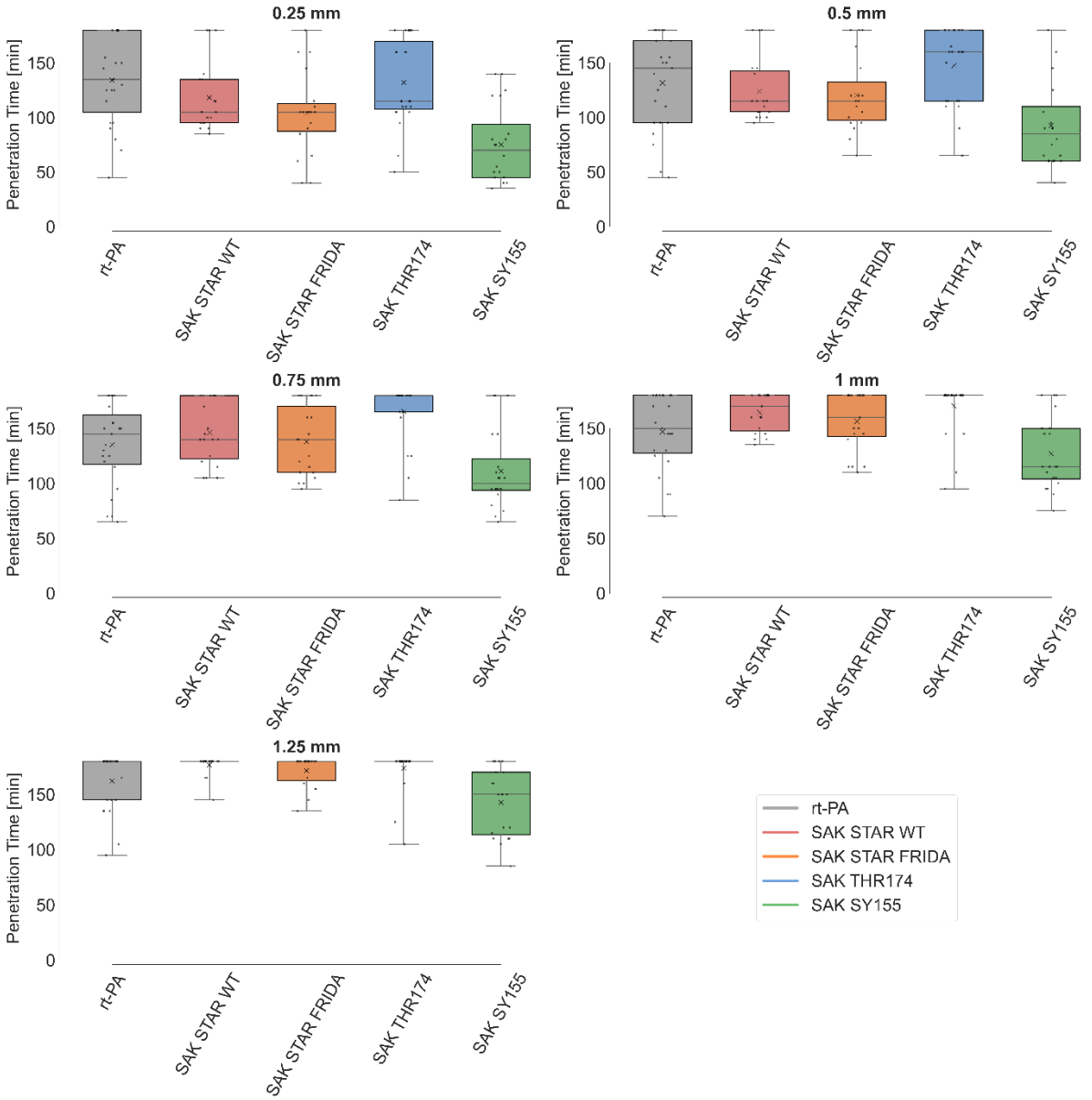


Figure S2 Penetration time at individual distances. Experiments were conducted at 37 °C for 180 minutes in PBS (pH 7.4), with 19 – 23 repetitions (technical replicates, n) per condition. All agents were used at a concentration of 0.022 µM. The box plots show mean value (X), median (line), interquartile ranges (boxes), and minimum and maximum values (whiskers).

Table S1 Static model: descriptive statistics and p-values for clot lysis expressed as relative weight loss. The concentration of all agents was 1.3 µg/mL. The experiment was performed in 0.5 mL of human plasma at 37 °C for 60 minutes. **A)** Descriptive statistics for each group (mean, SD = standard deviation, median, n = number of technical replicates). **B)** Pairwise p-values from the Kruskal-Wallis test with post-hoc Dunn's correction. Statistical significance was set at p<0.05.

A) Clot weight loss: Descriptive statistics						
	Control	**rt-PA**	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
Mean [%]	26.3	61.9	54.1	46.5	50.7	58.5
SD [%]	1.4	6.5	7.0	5.6	8.2	7.4
Median [%]	26.1	62.3	52.5	47.0	48.6	57.4
n	17	18	18	18	18	18

B) Relative clot weight loss: p-values						
	Control	**rt-PA**	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
Control	-	<0.001	<0.001	0.049	0.001	<0.001
rt-PA	-	-	0.485	<0.001	0.020	>0.999
SAK STAR WT	-	-	-	0.495	>0.999	>0.999
SAK STAR FRIDA	-	-	-	-	>0.999	0.008
SAK THR174	-	-	-	-	-	0.255

Table S2 Static model: descriptive statistics and p-values for clot lysis expressed as RBC release. The concentration of all agents was 1.3 µg/mL. The experiment was performed in 0.5 mL of human plasma at 37 °C for 60 minutes. **A)** Descriptive statistics for each group (mean, SD = standard deviation, median, n = number of technical replicates). **B)** Pairwise p-values from Kruskal-Wallis test with post-hoc Dunn's correction. Statistical significance was set at p<0.05.

A) RBC release: Descriptive statistics						
	Control	**rt-PA**	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
Mean [1]	0.06	1.60	1.06	0.62	0.90	1.26
SD [1]	0.02	0.44	0.52	0.36	0.52	0.55
Median [1]	0.07	1.44	1.01	0.55	0.69	1.17
n	18	18	18	18	18	18

B) RBC release: p-values						
	Control	**rt-PA**	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
Control	-	<0.001	<0.001	0.039	<0.001	<0.001
rt-PA	-	-	0.389	<0.001	0.044	>0.999
SAK STAR WT	-	-	-	0.492	>0.999	>0.999
SAK STAR FRIDA	-	-	-	-	>0.999	0.050
SAK THR174	-	-	-	-	-	>0.999

Table S3 Flow model: descriptive statistics and p-values for thrombolytic agent efficacy expressed as recanalisation time. The concentration of all agents was 1.3 µg/mL. The experiment was performed at 37 °C for 120 minutes in human plasma. **A)** Descriptive statistics for each group (mean, SD = standard deviation, median, n = number of technical replicates). **B)** Pairwise p-values from Kruskal-Wallis test with post-hoc Dunn's correction. Statistical significance was set at p<0.05.

A) Recanalisation time: Descriptive statistics						
	Control	**rt-PA**	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
Mean [min]	120	70	63	81	74	89
SD [min]	0	28	22	30	29	31
Median [min]	120	60	55	75	68	90
n	11	12	18	18	18	18

B) Recanalisation time: p-values						
	Control	**rt-PA**	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
Control	-	0.007	<0.001	0.034	0.002	0.221
rt-PA	-	-	>0.999	>0.999	>0.999	>0.999
SAK STAR WT	-	-	-	0.912	>0.999	0.159
SAK STAR FRIDA	-	-	-	-	>0.999	>0.999
SAK THR174	-	-	-	-	-	>0.999

Table S4 Flow model: descriptive statistics and p-values for clot lysis expressed as RBC release. The concentration of all agents was 1.3 µg/mL. The experiment was performed at 37 °C for 120 minutes in human plasma. **A)** Descriptive statistics for each group (mean, SD = standard deviation, median, n = number of technical replicates). **B)** Pairwise p-values from Kruskal-Wallis test with post-hoc Dunn's correction. Statistical significance was set at p<0.05.

A) RBC release: Descriptive statistics						
	Control	**rt-PA**	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
Mean [1]	0.01	0.13	0.13	0.10	0.13	0.10
SD [1]	0.01	0.07	0.05	0.07	0.05	0.05
Median [1]	0.01	0.11	0.13	0.11	0.15	0.08
n	11	12	18	18	18	18

B) RBC release: p-values						
	Control	**rt-PA**	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
Control	-	0.001	<0.001	0.006	<0.001	0.010
rt-PA	-	-	>0.999	>0.999	>0.999	>0.999
SAK STAR WT	-	-	-	>0.999	>0.999	>0.999
SAK STAR FRIDA	-	-	-	-	>0.999	>0.999
SAK THR174	-	-	-	-	-	0.835

Table S5 Flow model: descriptive statistics and p-values for thrombolytic agent efficacy expressed as relative clot reduction. The concentration of all agents was 1.3 µg/mL. The experiment was performed at 37 °C for 120 minutes in human plasma. **A)** Descriptive statistics for each group (mean, SD = standard deviation, median, n = number of technical replicates). **B)** Pairwise p-values from Kruskal-Wallis test with post-hoc Dunn's correction. Statistical significance was set at $p<0.05$.

A) Relative clot reduction: Descriptive statistics						
	Control	**rt-PA**	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
At 60 minutes						
Mean [%]	4.4	65.5	78.5	47.3	56.7	44.9
SD [%]	12.1	41.0	34.4	39.6	42.9	39.8
Median [%]	2.1	88.6	100.0	31.4	60.5	33.2
n	11	12	18	18	18	18
At 120 minutes						
Mean [%]	3.3	87.1	96.7	80.8	86.5	71.9
SD [%]	12.6	30.3	14.1	37.5	31.9	38.8
Median [%]	-0.3	100.0	100.0	100.0	100.0	100.0
n	11	12	18	18	18	18

B) Relative clot reduction: p-values						
	Control	**rt-PA**	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
At 60 minutes						
Control	-	0.004	<0.001	0.044	0.009	0.069
rt-PA	-	-	>0.999	>0.999	>0.999	>0.999
SAK STAR WT	-	-	-	0.520	>0.999	0.348
SAK STAR FRIDA	-	-	-	-	>0.999	>0.999
SAK THR174	-	-	-	-	-	>0.999
At 120 minutes						
Control	-	<0.001	<0.001	<0.001	<0.001	<0.001
rt-PA	-	-	>0.999	>0.999	>0.999	>0.999
SAK STAR WT	-	-	-	>0.999	>0.999	0.780
SAK STAR FRIDA	-	-	-	-	>0.999	>0.999
SAK THR174	-	-	-	-	-	>0.999

Table S6 Flow model: p-values for thrombolytic agent efficacy expressed as recanalisation frequency. The concentration of all agents was 1.3 µg/mL. The experiment was performed at 37 °C for 120 minutes in human plasma. Statistical analysis was performed using Fisher's exact test with post-hoc Holm–Bonferroni correction. Statistical significance was set at $p<0.05$.

Recanalisation frequency: p-values						
	Control	**rt-PA**	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
At 60 minutes						
Control	-	0.089	0.006	0.547	0.122	0.492
rt-PA	-	-	>0.999	>0.999	>0.999	>0.999
SAK STAR WT	-	-	-	0.233	>0.999	0.214
SAK STAR FRIDA	-	-	-	-	>0.999	>0.999
SAK THR174	-	-	-	-	-	>0.999
At 120 minutes						
Control	-	0.001	<0.001	0.001	<0.001	0.011
rt-PA	-	-	>0.999	>0.999	>0.999	>0.999
SAK STAR WT	-	-	-	>0.999	>0.999	0.162
SAK STAR FRIDA	-	-	-	-	>0.999	>0.999
SAK THR174	-	-	-	-	-	>0.999

Table S7 Penetration assay: Spearman's correlation between penetration rate and depth for different thrombolytic agents. Experiments were conducted at 37 °C for 180 minutes in PBS (pH = 7.4), with 19 – 23 repetitions per condition. All agents were used at the concentration of 0.022 µM. Values represent Spearman's rank correlation coefficient (rs), p-value, and number of technical replicates (n).

Spearman's correlation between penetration rate and depth					
	rt-PA	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
Spearman's rs	0.3	-1.0	-1.0	-1.0	-1.0
n	23	19	19	23	20
p-value	0.683	0.017	0.017	0.017	0.017

Table S8 Penetration assay: descriptive statistics and p-values for thrombolytic agent penetration capability expressed as penetration rates. Experiments were conducted at 37 °C for 180 minutes in PBS (pH = 7.4), with 19 – 23 repetitions per condition. All agents were used at a concentration of 0.022 µM. **A)** Descriptive statistics for each group (mean ± SD, n = number of technical replicates). **B)** Pairwise p-values from the Kruskal-Wallis test with post-hoc Dunn's correction. Statistical significance was set at $p<0.05$.

A) Penetration rates at individual distances: Descriptive statistics					
	rt-PA	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
0.25 mm					
Mean [RFU/min]	2864	4287	4481	3251	6444
SD [RFU/min]	741	974	1194	1079	1834
Median [RFU/min]	2663	4487	4722	3241	6208
n	23	19	19	23	20
0.50 mm					
Mean [RFU/min]	3120	3877	4069	2960	5439
SD [RFU/min]	860	857	1125	1018	1651
Median [RFU/min]	2833	4169	4381	3122	5374
n	23	19	19	23	20
0.75 mm					
Mean [RFU/min]	3247	3410	3562	2599	4620
SD [RFU/min]	826	809	1004	953	1492
Median [RFU/min]	3018	3772	3798	2820	4481
n	23	19	19	23	20
1.00 mm					
Mean [RFU/min]	3178	2942	3118	2245	3973
SD [RFU/min]	759	747	910	879	1332
Median [RFU/min]	3284	3181	3471	2511	3907
n	23	19	19	23	20
1.25 mm					
Mean [RFU/min]	2899	2429	2625	1856	3362
SD [RFU/min]	645	638	785	742	1191
Median [RFU/min]	2965	2587	2830	2037	3241
n	23	19	19	23	20
B) Penetration rates at individual distances: p-values					
	rt-PA	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
0.25 mm					
rt-PA	-	0.002	<0.001	0.814	<0.001
SAK STAR WT	-	-	0.988	0.051	<0.001
SAK STAR FRIDA	-	-	-	0.012	<0.001
SAK THR174	-	-	-	-	<0.001
0.50 mm					
rt-PA	-	0.305	0.050	>0.999	<0.001
SAK STAR WT	-	-	>0.999	0.170	0.063
SAK STAR FRIDA	-	-	-	0.025	0.347
SAK THR174	-	-	-	-	<0.001
0.75 mm					
rt-PA	-	>0.999	>0.999	0.785	0.010
SAK STAR WT	-	-	>0.999	0.220	0.107
SAK STAR FRIDA	-	-	-	0.028	0.604
SAK THR174	-	-	-	-	<0.001
1.00 mm					
rt-PA	-	>0.999	>0.999	0.027	0.431
SAK STAR WT	-	-	>0.999	0.299	0.094
SAK STAR FRIDA	-	-	-	0.024	0.789
SAK THR174	-	-	-	-	<0.001
1.25 mm					
rt-PA	-	0.802	>0.999	0.001	>0.999
SAK STAR WT	-	-	>0.999	0.450	0.060
SAK STAR FRIDA	-	-	-	0.025	0.783
SAK THR174	-	-	-	-	<0.001

Table S9 Penetration assay: descriptive statistics and p-values for thrombolytic agent penetration capability expressed as the time taken for successful penetration. Experiments were conducted at 37 °C for 180 minutes in PBS (pH = 7.4), with 19 – 23 repetitions per condition. All agents were used at the concentration of 0.022 µM. **A)** Descriptive statistics for each group (mean ± SD, n = number of technical replicates). **B)** Pairwise p-values from Kruskal-Wallis test with post-hoc Dunn's correction. Statistical significance was set at $p<0.05$.

A) Penetration time at individual distances: Descriptive statistics					
	rt-PA	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
0.25 mm					
Mean [min]	134	118	104	132	75
SD [min]	41	32	38	40	36
Median [min]	135	105	105	115	70
n	23	19	19	23	20
0.50 mm					
Mean [min]	132	124	120	147	93
SD [min]	43	29	34	35	39
Median [min]	145	115	115	160	85
n	23	19	19	23	20
0.75 mm					
Mean [min]	135	146	138	166	111
SD [min]	38	29	32	28	36
Median [min]	145	140	140	180	100
n	23	19	19	23	20
1.00 mm					
Mean [min]	147	165	156	170	127
SD [min]	34	17	27	24	33
Median [min]	150	170	160	180	115
n	23	19	19	23	20
1.25 mm					
Mean [min]	162	177	171	174	143
SD [min]	26	9	14	19	30
Median [min]	180	180	180	180	150
n	23	19	19	23	20
B) Penetration time at individual distances: p-values					
	rt-PA	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
0.25 mm					
rt-PA	-	>0.999	0.186	>0.999	<0.001
SAK STAR WT	-	-	>0.999	>0.999	0.048
SAK STAR FRIDA	-	-	-	0.263	0.584
SAK THR174	-	-	-	-	<0.001
0.50 mm					
rt-PA	-	>0.999	>0.999	>0.999	0.015
SAK STAR WT	-	-	>0.999	0.539	0.131
SAK STAR FRIDA	-	-	-	0.310	0.238
SAK THR174	-	-	-	-	<0.001
0.75 mm					
rt-PA	-	>0.999	>0.999	0.041	0.355
SAK STAR WT	-	-	>0.999	0.585	0.048
SAK STAR FRIDA	-	-	-	0.097	0.320
SAK THR174	-	-	-	-	<0.001
1.00 mm					
rt-PA	-	>0.999	>0.999	0.045	0.565
SAK STAR WT	-	-	>0.999	>0.999	0.012
SAK STAR FRIDA	-	-	-	0.710	0.072
SAK THR174	-	-	-	-	<0.001
1.25 mm					
rt-PA	-	0.607	>0.999	0.583	0.078
SAK STAR WT	-	-	>0.999	>0.999	<0.001
SAK STAR FRIDA	-	-	-	>0.999	0.007
SAK THR174	-	-	-	-	<0.001

Table S10 Penetration assay: descriptive statistics and p-values for thrombolytic agent penetration capability expressed as penetration success frequency. Experiments were conducted at 37 °C for 180 minutes in PBS (pH 7.4), with 19 – 23 repetitions (technical replicates, n) per condition. All agents were used at the concentration of 0.022 μM. **A)** Descriptive statistics for each thrombolytic agent. **B)** Pairwise p-values from Fisher's exact test with post-hoc Holm-Bonferroni correction. Statistical significance was set at $p<0.05$.

A) Penetration success frequency: Descriptive statistics					
	rt-PA	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
0.25 mm					
Penetration	16 (70%)	16 (84%)	18 (95%)	17 (74%)	20 (100%)
No penetration	7 (30%)	3 (16%)	1 (5%)	6 (26%)	0 (0%)
n (100%)	23	19	19	23	20
0.50 mm					
Penetration	18 (78%)	16 (84%)	16 (84%)	15 (65%)	19 (95%)
No penetration	5 (22%)	3 (16%)	3 (16%)	8 (35%)	1 (5%)
n (100%)	23	19	19	23	20
0.75 mm					
Penetration	18 (78%)	13 (68%)	14 (74%)	7 (30%)	17 (85%)
No penetration	5 (22%)	6 (32%)	5 (26%)	16 (70%)	3 (15%)
n (100%)	23	19	19	23	20
1.00 mm					
Penetration	15 (65%)	10 (53%)	10 (53%)	4 (17%)	17 (85%)
No penetration	8 (35%)	9 (47%)	9 (47%)	19 (83%)	3 (15%)
n (100%)	23	19	19	23	20
1.25 mm					
Penetration	9 (39%)	3 (16%)	6 (32%)	3 (13%)	16 (80%)
No penetration	14 (61%)	16 (84%)	13 (68%)	20 (87%)	4 (20%)
n (100%)	23	19	19	23	20

B) Penetration frequency in individual distances: p-values					
	rt-PA	**SAK STAR WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
0.25 mm					
rt-PA	-	>0.999	0.433	>0.999	0.100
SAK STAR WT	-	-	>0.999	>0.999	0.736
SAK STAR FRIDA	-	-	-	0.736	>0.999
SAK THR174	-	-	-	-	0.206
0.50 mm					
rt-PA	-	>0.999	>0.999	>0.999	>0.999
SAK STAR WT	-	-	>0.999	>0.999	>0.999
SAK STAR FRIDA	-	-	-	>0.999	>0.999
SAK THR174	-	-	-	-	0.243
0.75 mm					
rt-PA	-	>0.999	>0.999	0.024	>0.999
SAK STAR WT	-	-	>0.999	0.201	>0.999
SAK STAR FRIDA	-	-	-	0.097	>0.999
SAK THR174	-	-	-	-	0.006
1.00 mm					
rt-PA	-	>0.999	>0.999	0.021	0.701
SAK STAR WT	-	-	>0.999	0.184	0.244
SAK STAR FRIDA	-	-	-	0.184	0.244
SAK THR174	-	-	-	-	<0.001
1.25 mm					
rt-PA	-	0.847	>0.999	0.546	0.087
SAK STAR WT	-	-	>0.999	>0.999	<0.001
SAK STAR FRIDA	-	-	-	>0.999	0.029
SAK THR174	-	-	-	-	<0.001

Table S11 SAK Variants Overview - Wild-type staphylokinases and low-immunogenic staphylokinases that were compared in this study. Production yields are expressed in milligrams per litre of culture [mg/L] of all five studied proteins after cultivation and a full purification procedure. Proteins were produced in *E.coli* BL21(DE3) cells for 4 hours at 37 °C and purified by two rounds of immobilised metal affinity chromatography and one run of size-exclusion chromatography with a cleavage step during dialysis.

Protein overview part 1				
Protein	**Mutations**	**Source**	**Yield**	**Reference**
Staphylokinase SAK STAR WT		*Staphylococcus aureus* genome	60 mg/L	[31]
Staphylokinase SAK 42D WT	S34G, G36R, H43R	Bacteriophage genome	10 mg/L	[31]
Staphylokinase SAK STAR FRIDA	K74A, E75A, R77A	Russian "FRIDA" Clinical Trial	40 mg/L	[14]
Staphylokinase SAK THR174	E65D, K74Q, R77E, E80S, D82S, V112T, K130Y, E134R	Collen group / ThromboGenics	20 mg/L	[30]
Staphylokinase SAK SY155	K35A, E65Q, K74R, D82A, S84A, T90A, E99D, T101S, E108A, K109A, K130T, K135R	Collen group	25 mg/L	[29]

Protein overview part 2		
Protein	**Molecular Weight**	**Sequence**
Staphylokinase SAK STAR WT	15.49 kDa	SSSFDKGKYKKGDDASYFEPTGPYLMVNVTGVDSKGNELLSPHYVEFPI KPGTTLTKEKIEYYVEWALDATAYKEFRVVELDPSAKIEVTYYDKNKKKEE TKSFPITEKGFVVPDLSEHIKNPGFNLITKVVIEKK
Staphylokinase SAK 42D WT	15.58 kDa	SSSFDKGKYKKGDDASYFEPTGPYLMVNVTGVDGKRNELLSPRYVEFPI KPGTTLTKEKIEYYVEWALDATAYKEFRVVELDPSAKIEVTYYDKNKKKEE TKSFPITEKGFVVPDLSEHIKNPGFNLITKVVIEKK
Staphylokinase SAK STAR FRIDA	15.29 kDa	SSSFDKGKYKKGDDASYFEPTGPYLMVNVTGVDSKGNELLSPHYVEFPI KPGTTLTKEKIEYYVEWALDATAYAAFAVVELDPSAKIEVTYYDKNKKKEET KSFPITEKGFVVPDLSEHIKNPGFNLITKVVIEKK
Staphylokinase SAK THR174	15.45 kDa	SSSFDKGKYKKGDDASYFEPTGPYLMVNVTGVDSKGNELLSPHYVEFPI KPGTTLTKEKIEYYVDWALDATAYQEFEVVSLSPSAKIEVTYYDKNKKKEE TKSFPITEKGFTVPDLSEHIKNPGFNLITYVVIRKK
Staphylokinase SAK SY155	15.23 kDa	SSSFDKGKYKKGDDASYFEPTGPYLMVNVTGVDSAGNELLSPHYVEFPI KPGTTLTKEKIEYYVQWALDATAYREFRVVELAPAAKIEVAYYDKNKKKDE SKSFPITAAGFVVPDLSEHIKNPGFNLITTVVIERK

Expressed sequence (SAK STAR WT):

MHHHHHHENLYFQSSSFDKGKYKKGDDASYFEPTGPYLMVNVTGVDSKGNELLSPHYVE FPIKPGTTLTKEKIEYYVEWALDATAYKEFRVVELDPSAKIEVTYYDKNKKKEETKSFPITEKG FVVPDLSEHIKNPGFNLITKVVIEKK

Final sequence (SAK STAR WT) after TEV cleavage:

SSSFDKGKYKKGDDASYFEPTGPYLMVNVTGVDSKGNELLSPHYVEFPIKPGTTLTKEKIEY YVEWALDATAYKEFRVVELDPSAKIEVTYYDKNKKKEETKSFPITEKGFVVPDLSEHIKNPGF NLITKVVIEKK

To prevent confusion when the mutations are introduced to the sequence, a uniform numbering is suggested. The amino acids are counted from the first triplet of serines (underlined S1, S2, and S3).

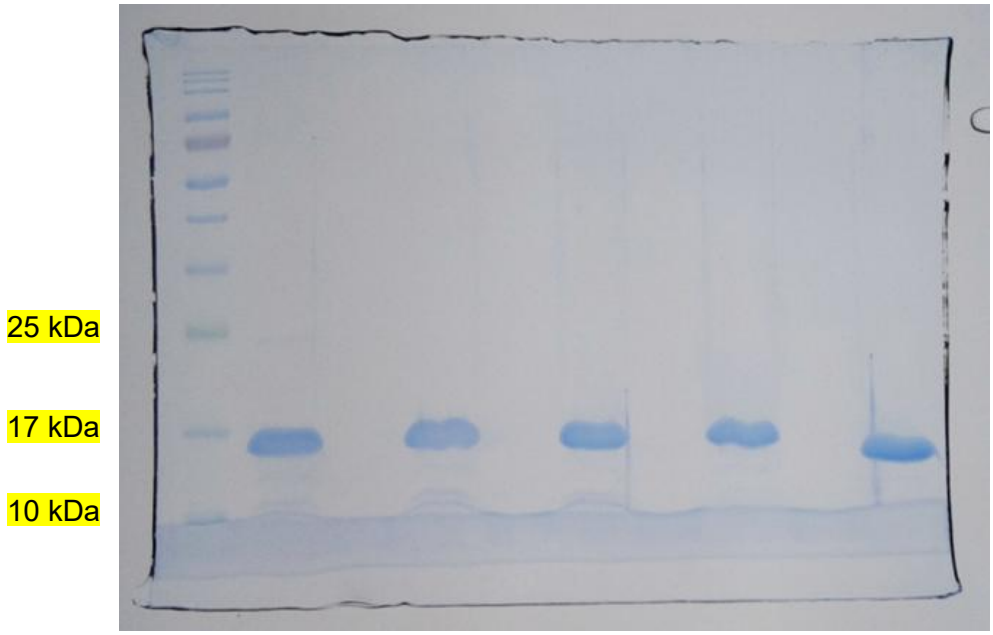


Figure S3 SDS-PAGE gel showing the purity of SAK 42D WT, SAK STAR WT, SAK STAR FRIDA, SAK THR174, and SAK SY155, verifying their purity after the full purification cycle. 10 µL of each sample was loaded onto the gel. The concentration of the sample was around 1 mg/mL. The sample was prepared by mixing 20 µL of the original sample and 20 µL of Laemmli Sample buffer, incubated for 10 minutes at 95 °C, and then run at 150 V, 400 mA for 60 minutes in 15% polyacrylamide gel.

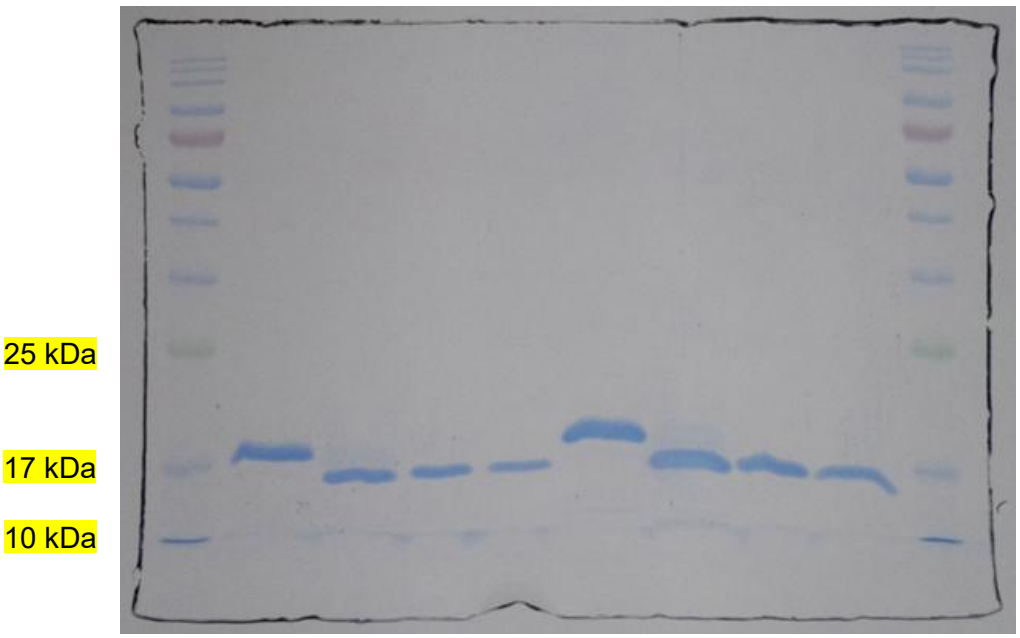


Figure S4 SDS-PAGE gel performed on samples collected during the purification of SAK 42D WT, showing the cleavage of the His tag with TEV sequence (samples after the first metal-affinity purification step, after cleavage by TEV protease and dialysis, after the second metal-affinity purification step, and after gel permeation chromatography (SEC)). The loading was repeated twice with 8 µL and 16 µL of the sample, with a concentration of the sample around 1 mg/mL. The sample was prepared by mixing 20 µL of the original sample and 20 µL of Laemmli Sample buffer, incubated for 10 minutes at 95 °C, and then run at 150 V, 400 mA for 60 minutes in 15% polyacrylamide gel.

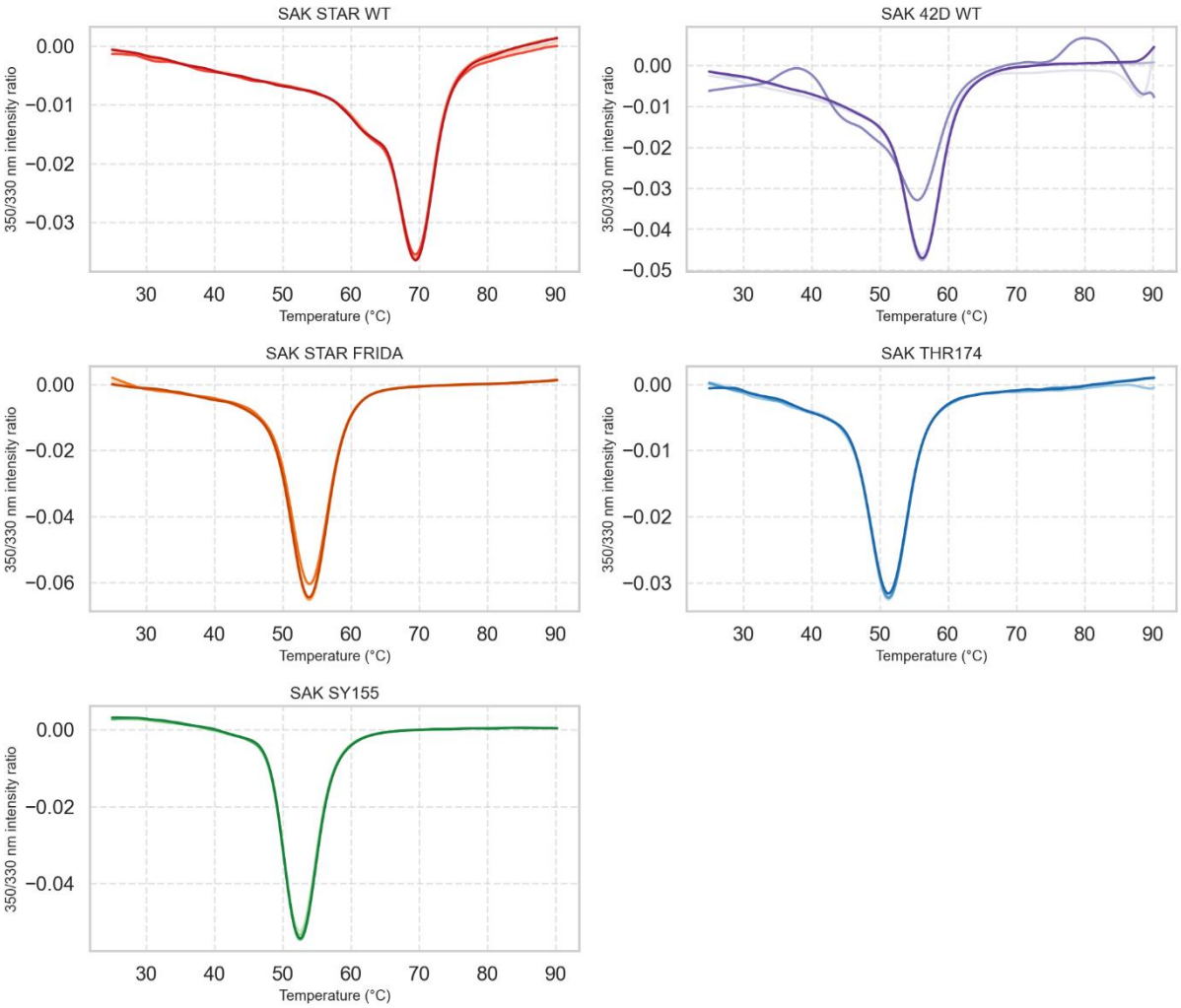


Figure S5 Differential scanning fluorimetry (DSF) curve of thermal stability showing the first derivative of the 350/330 nm fluorescence intensity ratio for all five studied variants in quadruplicate (number of technical replicates, n = 4), replicates are visualised as shades of a certain colour associated with the specific variant: SAK STAR WT in shades of red, SAK 42D WT in shades of violet, SAK STAR FRIDA in shades of orange, SAK THR174 in shades of blue, and SAK SY155 in shades of green. The minimum of the curve represents the melting temperature, while the initial decrease towards the minimum is the onset of melting. Melting was measured from 25 to 90 °C at a rate of increase of 1 °C per minute in PBS (pH 7.4).

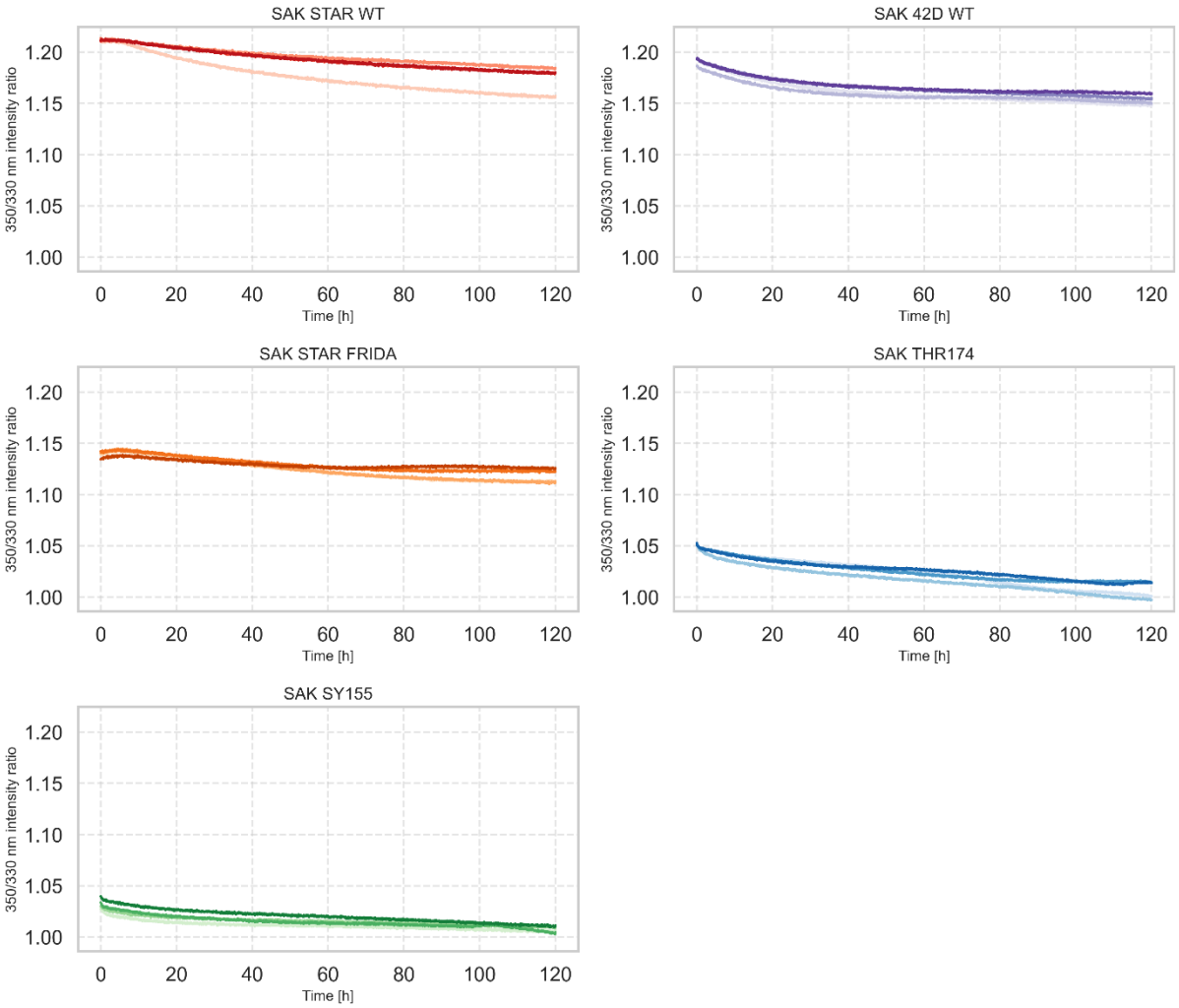


Figure S6 Isothermal DSF measurement of protein aggregation performed at 37 °C for 120 hours in quadruplicate (number of technical replicates, n = 4) in PBS (pH 7.4); replicates are visualised as shades of a certain colour associated with the specific variant: SAK STAR WT in shades of red, SAK 42D WT in shades of violet, SAK STAR FRIDA in shades of orange, SAK THR174 in shades of blue, and SAK SY155 in shades of green. None of the variants showed any significant changes, so we conclude that no aggregation is occurring in this time window.

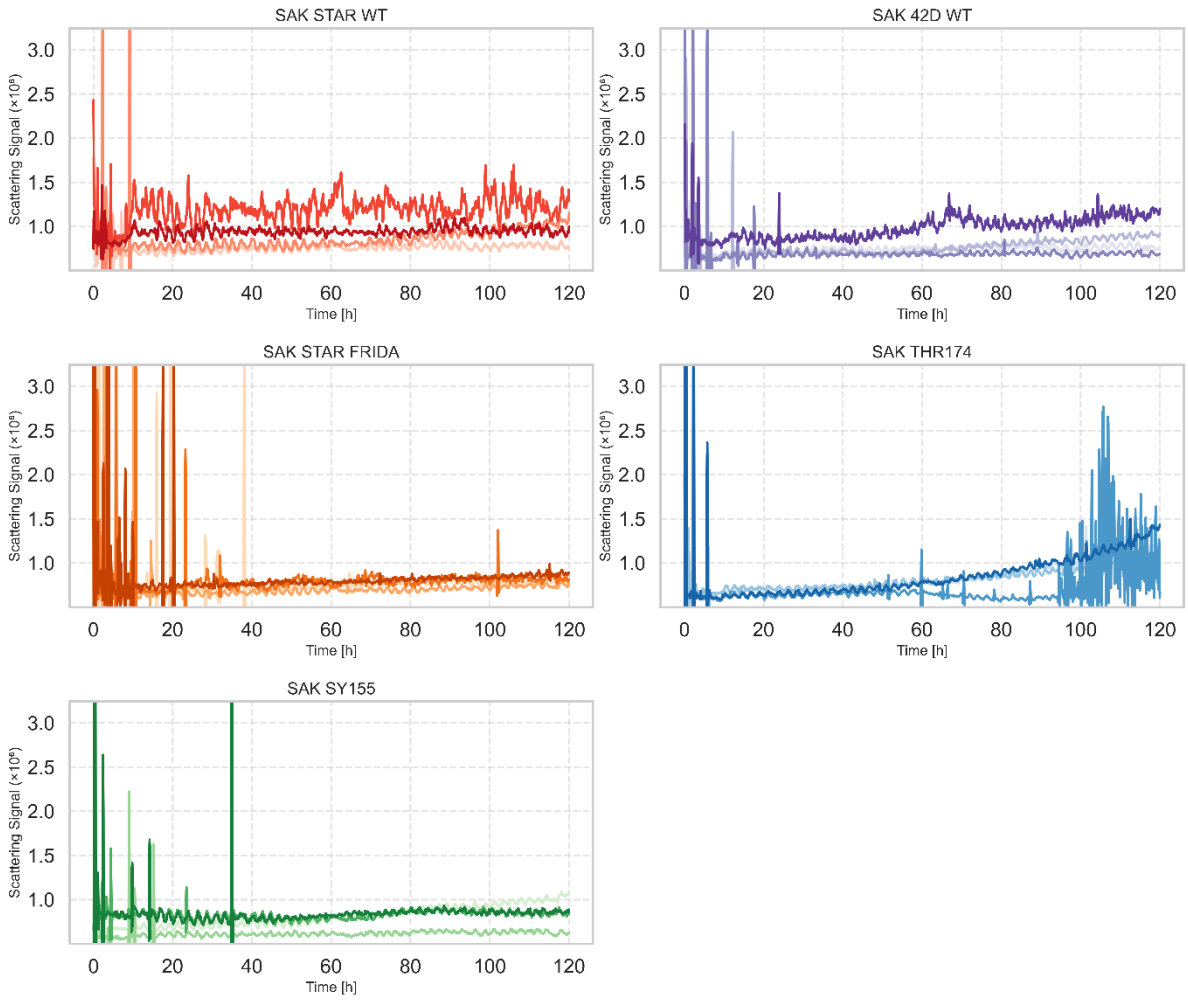


Figure S7 Isothermal DLS measurement of protein aggregation performed at 37 °C for 120 hours in quadruplicate (number of technical replicates, n = 4) in PBS (pH 7.4); replicates are visualised as shades of a certain colour associated with the specific variant: SAK STAR WT in shades of red, SAK 42D WT in shades of violet, SAK STAR FRIDA in shades of orange, SAK THR174 in shades of blue, and SAK SY155 in shades of green. Some noise can be seen in the curves, especially for SAK STAR FRIDA, but the response is limited, which suggests no significant changes are happening to any of the studied variants.

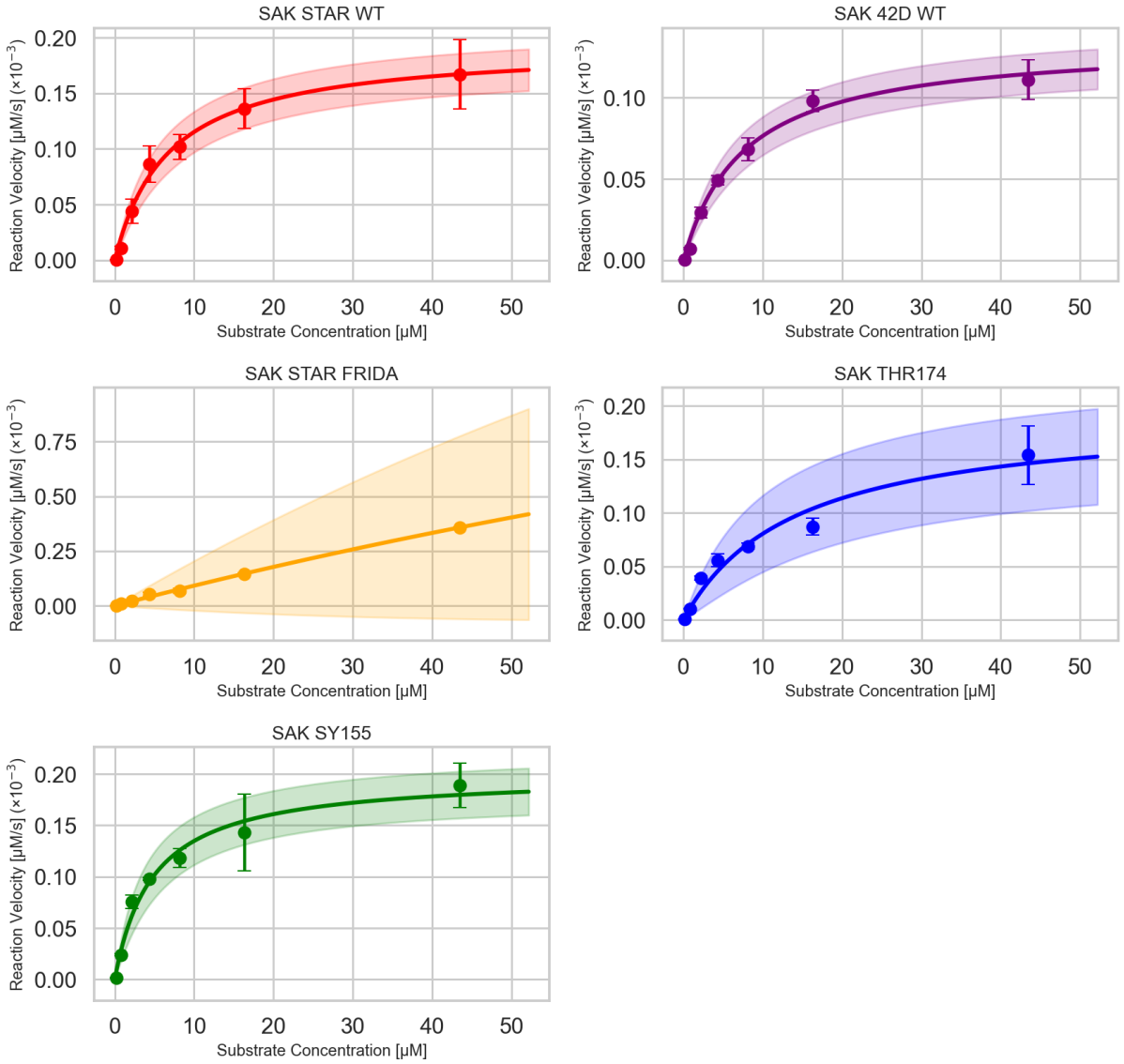


Figure S8 The maximum reaction velocities extracted from the initial parabolic phase at different substrate concentrations. The data were fitted by the Michaelis-Menten equation to obtain the apparent maximum reaction velocity and apparent Michaelis constant for each variant. The experiment was run at 37 °C in PBS buffer (pH = 7.4) with plasminogen concentrations of 0, 0.16, 0.81, 2.17, 4.35, 8.15, 16.3, 43.5 μM and staphylokinase concentration of 0.5 nM. D-VLK-AMC was present at the final concentration of 200 μM. For all variants, except for SAK STAR FRIDA, the fitting was satisfactory. For SAK STAR FRIDA, the kinetic parameters would have to be extrapolated, which significantly decreases their reliability, so an alternative evaluation, a linear fit, was used instead.

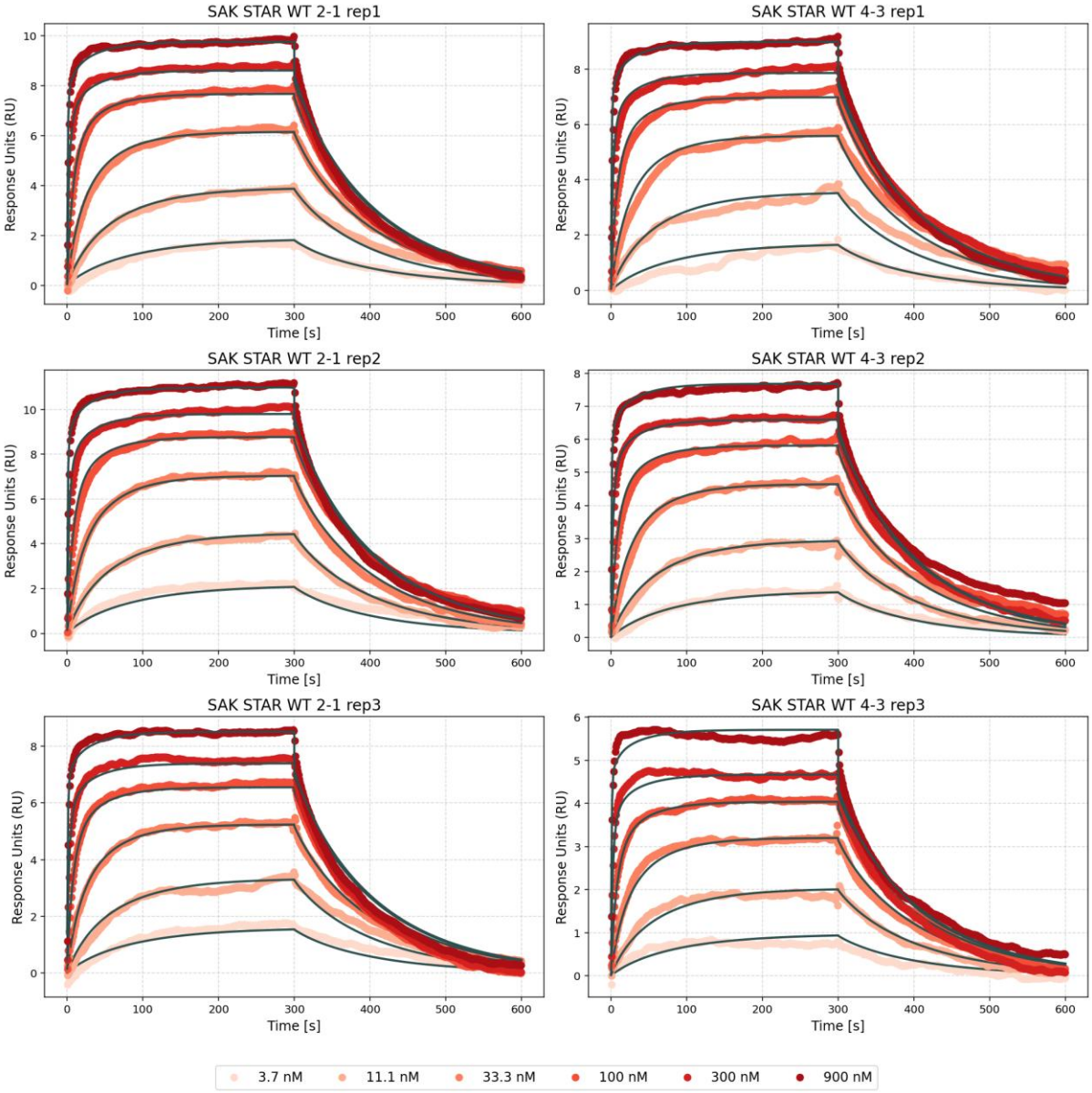


Figure S9 The binding kinetics of SAK STAR WT to human plasmin were analysed by surface-based assays using a CM5 sensor chip. Human plasmin (~850 RU on channel 2, ~550 RU on channel 4) was immobilised by amine coupling, and SAK was injected at concentrations of 3.7, 11, 33, 100, 300, and 900 nM in PBS/Tween80 (pH 7.4) at 25 °C. Binding curves were fitted numerically to a model shown in Scheme 1 in the Methods section. Curves labelled 2-1 refer to data obtained from channels 1 and 2, while curves labelled 4-3 refer to data obtained from channels 3 and 4.

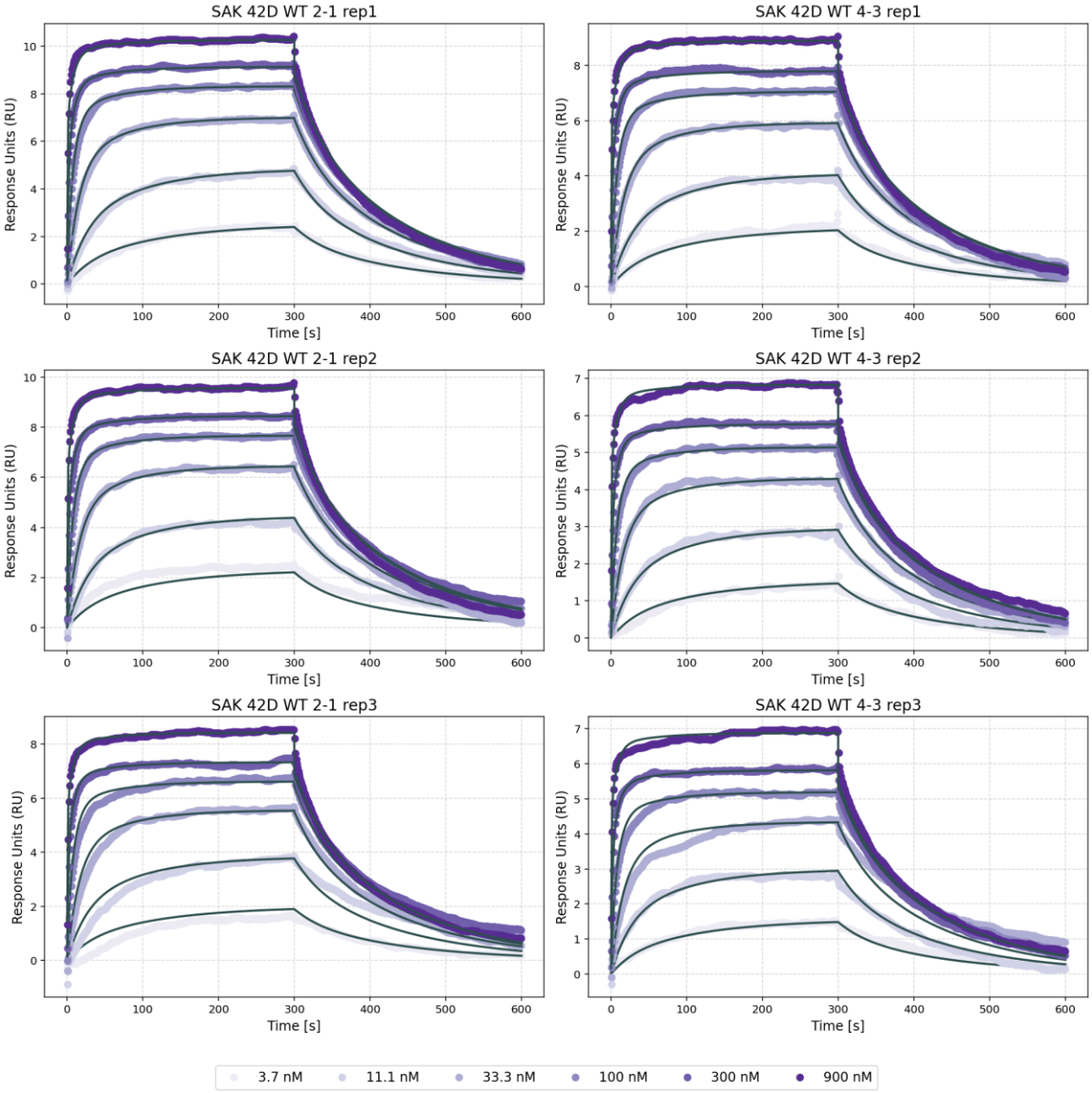


Figure S10 The binding kinetics of SAK 42D WT to human plasmin were analysed by surface-based assays using a CM5 sensor chip. Human plasmin (~850 RU on channel 2, ~550 RU on channel 4) was immobilised by amine coupling, and SAK was injected at concentrations of 3.7, 11, 33, 100, 300, and 900 nM in PBS/Tween80 (pH 7.4) at 25 °C. Binding curves were fitted numerically to a model shown in Scheme 1 in the Methods section. Curves labelled 2-1 refer to data obtained from channels 1 and 2, while curves labelled 4-3 refer to data obtained from channels 3 and 4.

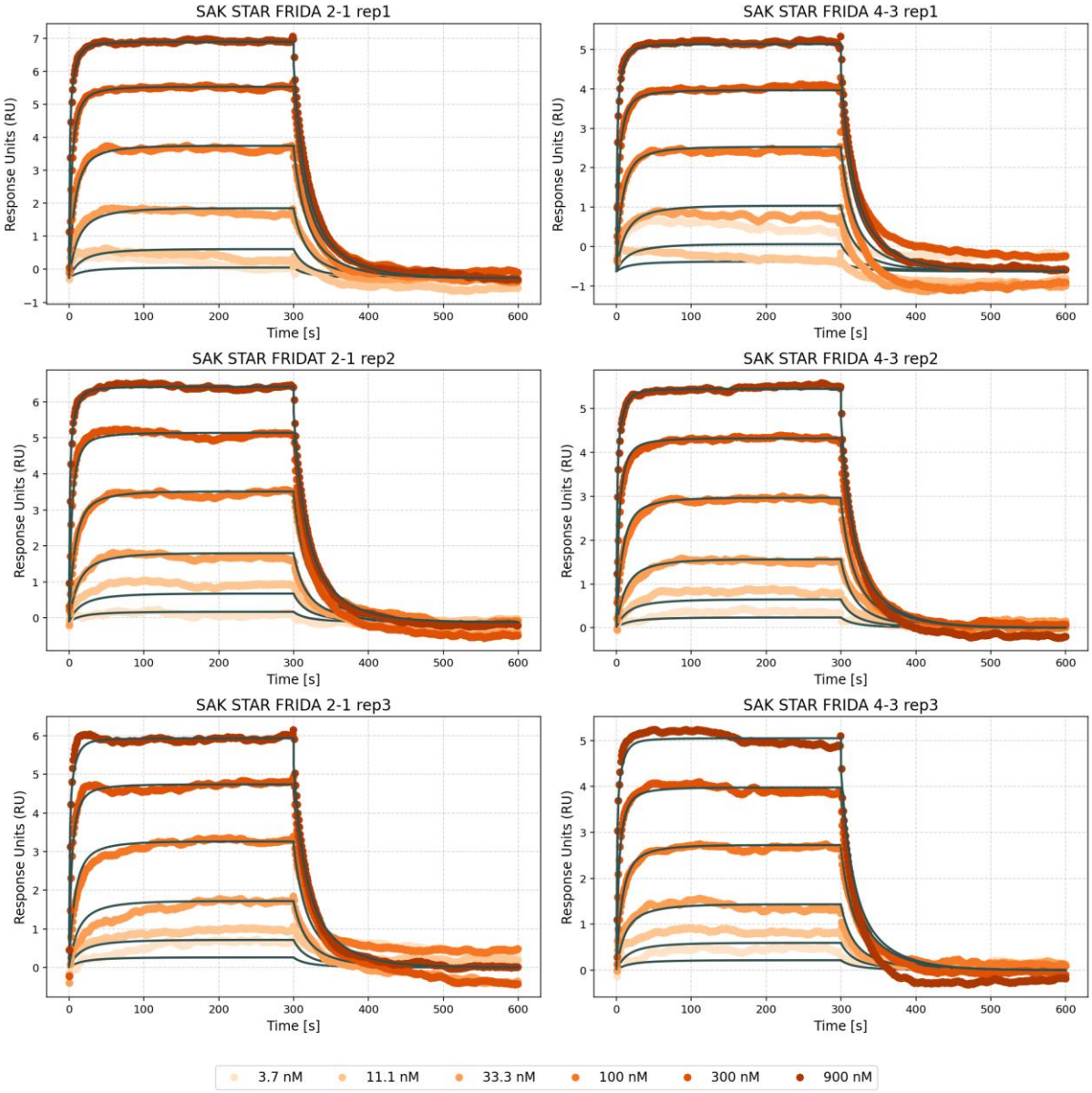


Figure S11 The binding kinetics of SAK STAR FRIDA to human plasmin were analysed by surface-based assays using a CM5 sensor chip. Human plasmin (~850 RU on channel 2, ~550 RU on channel 4) was immobilised by amine coupling, and SAK was injected at concentrations of 3.7, 11, 33, 100, 300, and 900 nM in PBS/Tween80 (pH 7.4) at 25 °C. Binding curves were fitted numerically to a model shown in Scheme 1 in the Methods section. Curves labelled 2-1 refer to data obtained from channels 1 and 2, while curves labelled 4-3 refer to data obtained from channels 3 and 4.

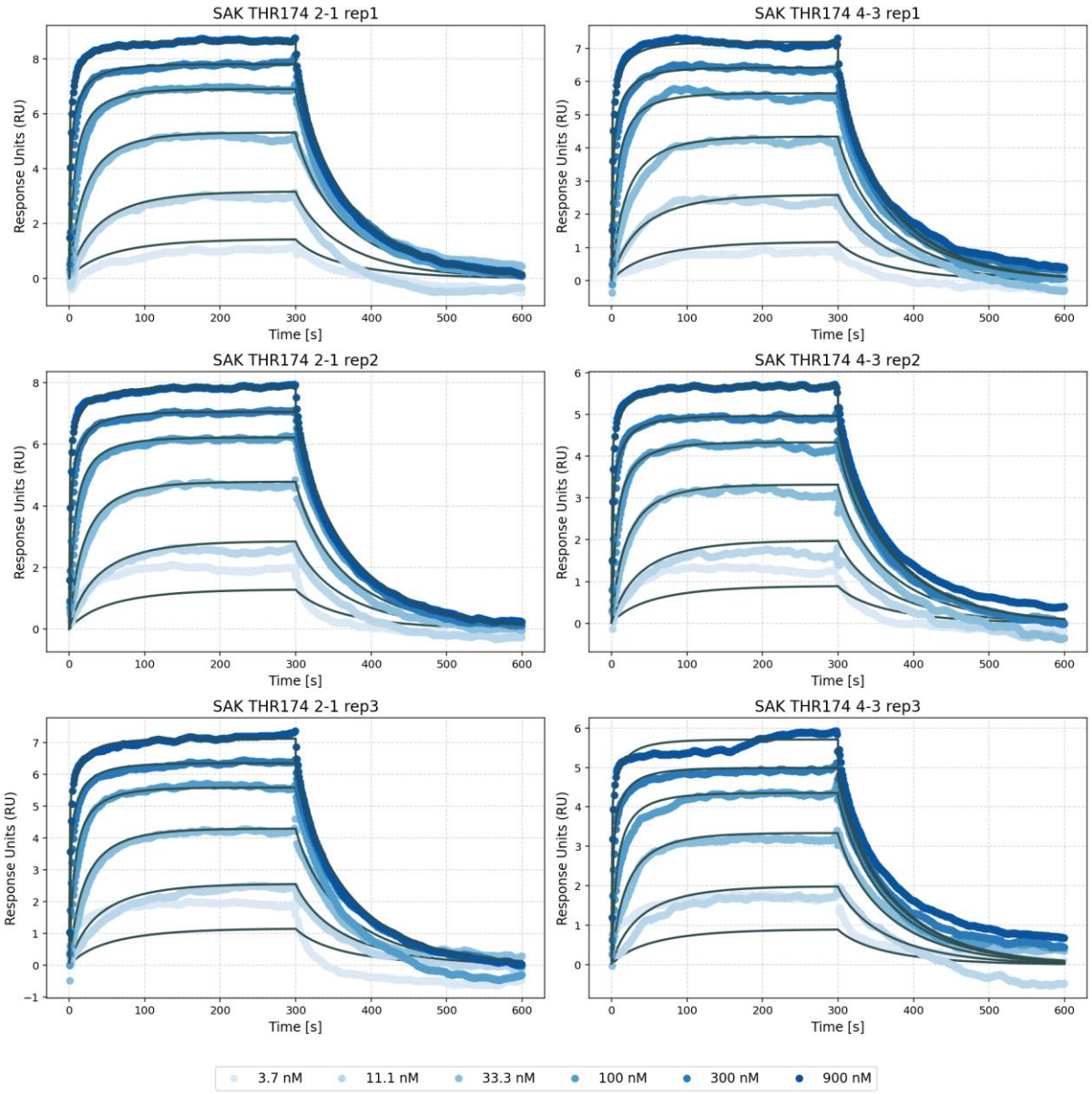


Figure S12 The binding kinetics of SAK THR174 to human plasmin were analysed by surface-based assays using a CM5 sensor chip. Human plasmin (~850 RU on channel 2, ~550 RU on channel 4) was immobilised by amine coupling, and SAK was injected at concentrations of 3.7, 11, 33, 100, 300, and 900 nM in PBS/Tween80 (pH 7.4) at 25 °C. Binding curves were fitted numerically to a model shown in Scheme 1 in the Methods section. Curves labelled 2-1 refer to data obtained from channels 1 and 2, while curves labelled 4-3 refer to data obtained from channels 3 and 4.

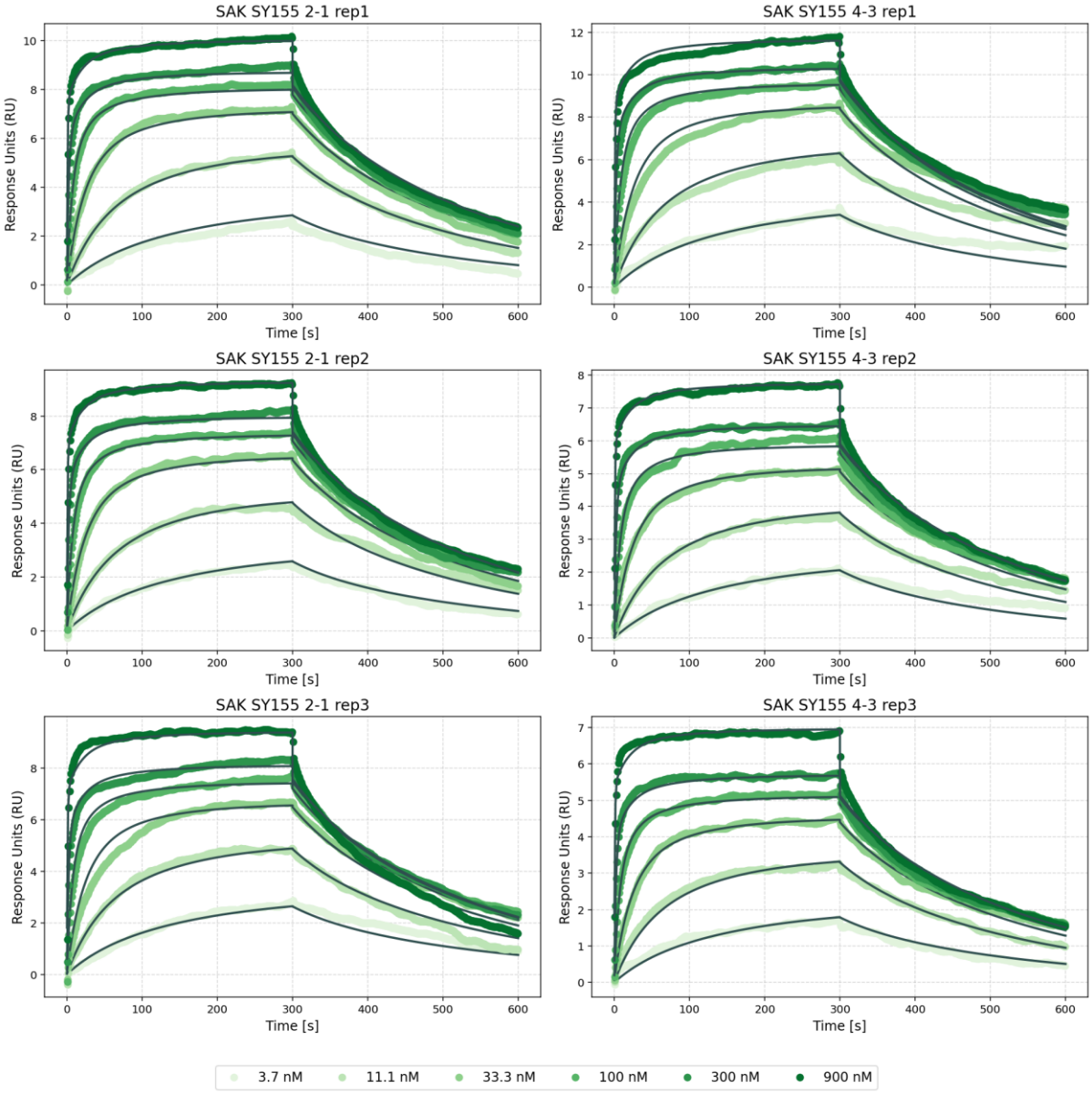
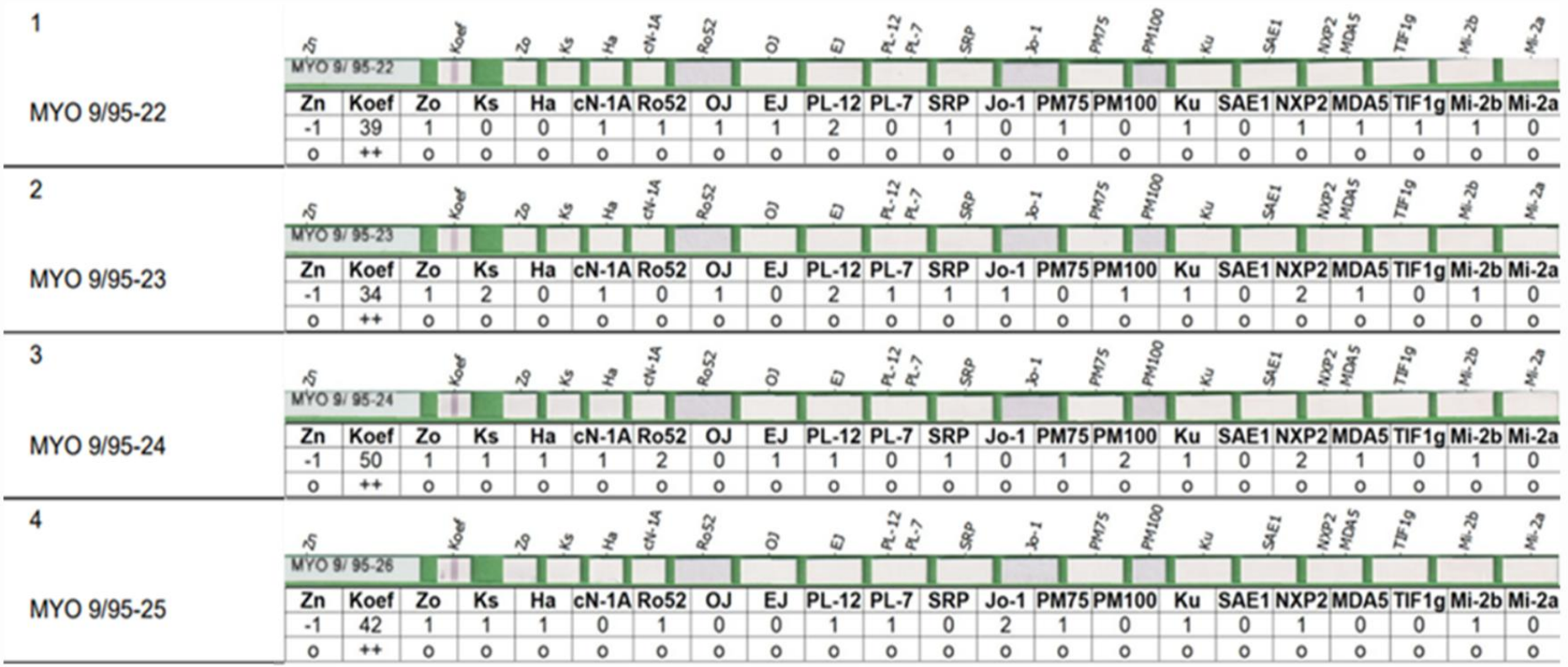


Figure S13 The binding kinetics of SAK SY155 to human plasmin were analysed by surface-based assays using a CM5 sensor chip. Human plasmin (~850 RU on channel 2, ~550 RU on channel 4) was immobilised by amine coupling, and SAK was injected at concentrations of 3.7, 11, 33, 100, 300, and 900 nM in PBS/Tween80 (pH 7.4) at 25 °C. Binding curves were fitted numerically to a model shown in Scheme 1 in the Methods section. Curves labelled 2-1 refer to data obtained from channels 1 and 2, while curves labelled 4-3 refer to data obtained from channels 3 and 4.



Zn	Koef	Zo	Ks	Ha	cN-1A	Ro52	OJ	EJ	PL-12	PL-7	SRP	Jo-1	PM75	PM100	Ku	SAE1	NXP2	MDA5	TIF1g	Mi-2b	Mi-2a
-1	39	1	0	0	1	1	1	1	2	0	1	0	1	0	1	0	1	1	1	1	0
o	++	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o

Zn	Koef	Zo	Ks	Ha	cN-1A	Ro52	OJ	EJ	PL-12	PL-7	SRP	Jo-1	PM75	PM100	Ku	SAE1	NXP2	MDA5	TIF1g	Mi-2b	Mi-2a
-1	34	1	2	0	1	0	1	0	2	1	1	1	0	1	1	0	2	1	0	1	0
o	++	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o

Zn	Koef	Zo	Ks	Ha	cN-1A	Ro52	OJ	EJ	PL-12	PL-7	SRP	Jo-1	PM75	PM100	Ku	SAE1	NXP2	MDA5	TIF1g	Mi-2b	Mi-2a
-1	50	1	1	1	1	2	0	1	1	0	1	0	1	2	1	0	2	1	0	1	0
o	++	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o

Zn	Koef	Zo	Ks	Ha	cN-1A	Ro52	OJ	EJ	PL-12	PL-7	SRP	Jo-1	PM75	PM100	Ku	SAE1	NXP2	MDA5	TIF1g	Mi-2b	Mi-2a
-1	42	1	1	1	0	1	0	0	1	1	0	2	1	0	1	0	1	0	0	1	0
o	++	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o

Figure S14 Reactivity of murine sera with myositis-associated/specific human autoantigens. 1 – 4 pooled sera of mice vaccinated with individual SAK versions with adjuvant. 1 - SAK THR174, 2 - SAK SY155, 3 - SAK STAR FRIDA, 4 - SAK STAR WT. Autoantibodies against the following autoantigens were tested: Mi-2α, Mi-2β, TIF1γ (transcriptional intermediary factor 1), MDA5 (melanoma differentiation-associated protein 5), NXP2 (nuclear matrix protein 2), SAE1 (small ubiquitin-like modifier 1-activating enzyme), Ku, PM-Scl100 (polymyositis/scleroderma), PM-Scl75, Jo-1 (histidyl-tRNA synthetase), SRP (signal recognition particle), PL-7 (threonyl-tRNA synthetase), PL-12 (alanyl-tRNA-synthetase), EJ (glycyl-tRNA synthetase), OJ (isoleucyl-tRNA synthetase), Ro-52 (TRIM21).

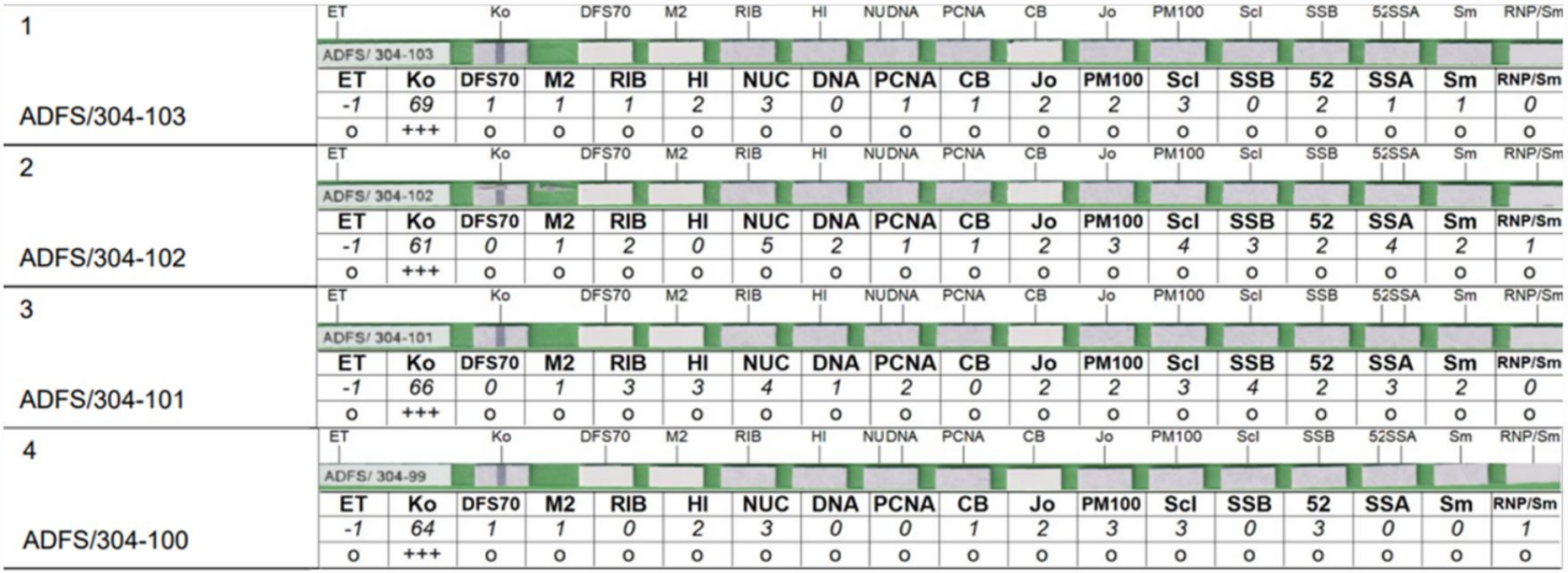


Figure S15 Reactivity of murine sera with nuclear human autoantigens. 1 – 4 pooled sera of mice vaccinated with individual SAK versions with adjuvant. 1 - SAK THR174, 2 - SAK SY155, 3 - SAK STAR FRIDA, 4 - SAK STAR WT. Autoantibodies against the following autoantigens were tested: nRNP/Sm, Sm (Smith), SS-A/Ro, Ro-52 (TRIM21), SS-B/La, Scl-70 (topoisomerase I), PM-Scl (polymyositis/scleroderma), Jo-1 (histidyl-tRNA synthetase), CENP B (centromere protein B), PCNA (proliferating cell nuclear antigen), dsDNA, nucleosomes, histones, ribosomal proteins, AMA M2, DFS70 (dense fine speckled, 70kDa molecular weight protein).

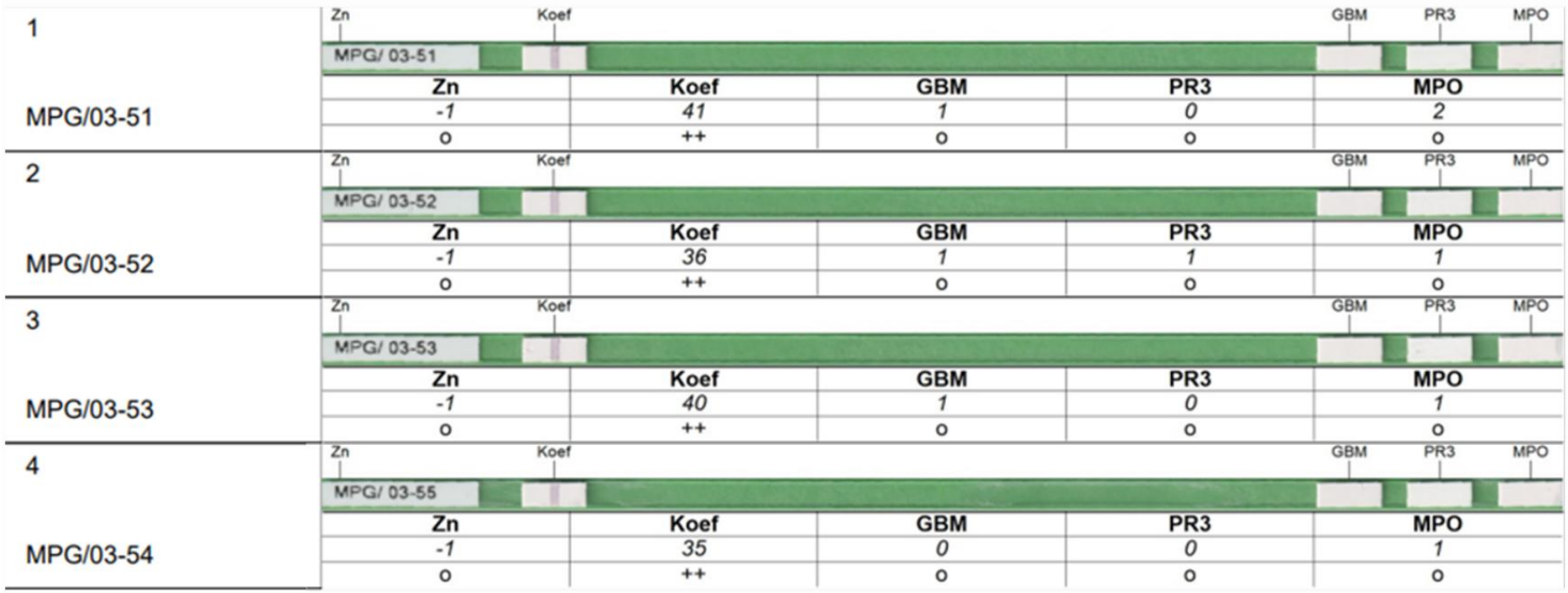


Figure S16 Reactivity of murine sera with MPO, PR3, and GBM human autoantigens. 1 – 4 pooled sera of mice vaccinated with individual SAK versions with adjuvant. 1 - SAK THR174, 2 - SAK SY155, 3 - SAK STAR FRIDA, 4 - SAK STAR WT. Autoantibodies against the following autoantigens were tested: GBM (glomerular basement membrane antigen - collagen α3(IV)), PR3 (neutrophil protease 3), and MPO (neutrophil myeloperoxidase).

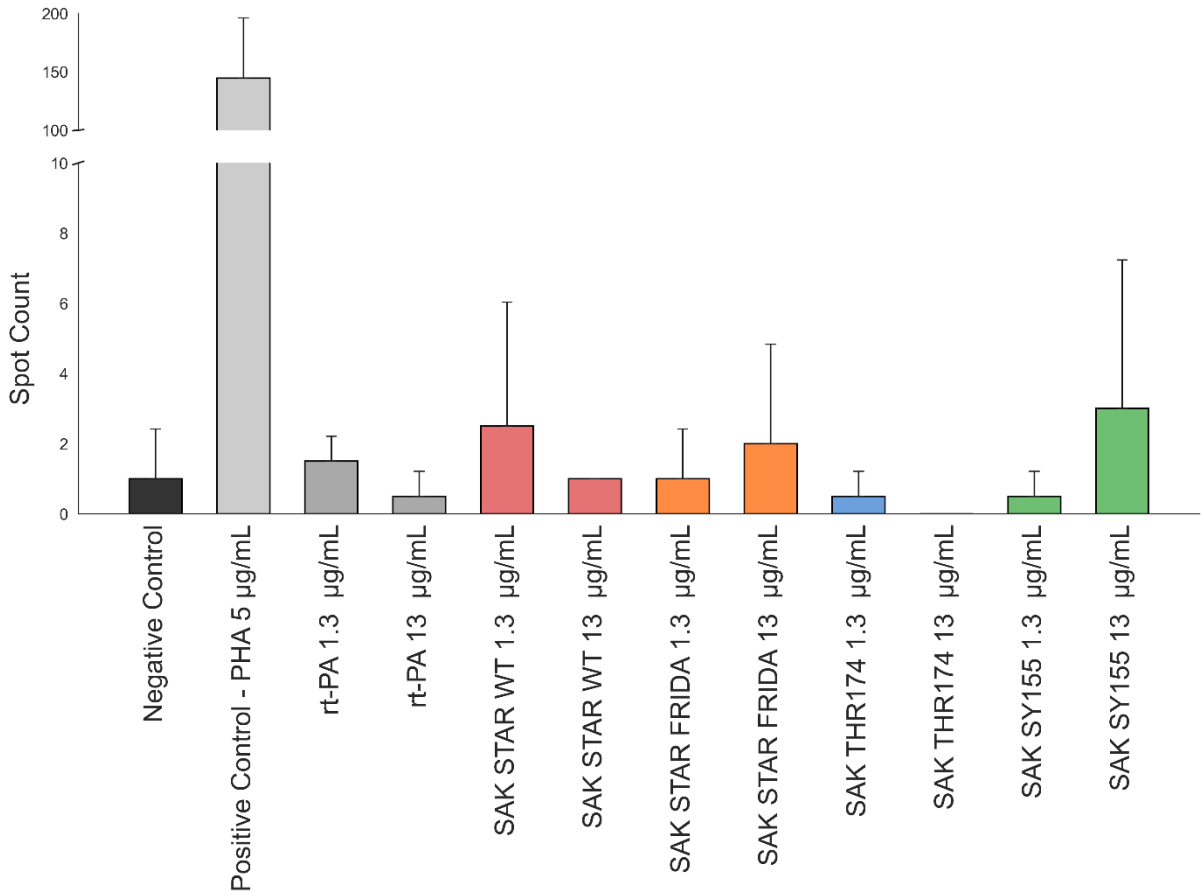


Figure S17 FluoroSpot analysis of IFN-γ. CD8-depleted PBMCs were stimulated with medium alone (negative control), positive control (PHA, 5 µg/mL), or with rt-PA and SAK variants (1.3 and 13 µg/mL) for 3 days. IFN-γ secretion was quantified using fluorophore-labelled antibodies and analysed with an ImmunoSpot® reader (520 nm). Data are shown as mean spot counts ± standard deviation (number of biological replicates, n = 2).

Table S12 Monocyte activation test (MAT) statistics - Descriptive statistics and p-values for endotoxin removal confirmation, presented as IL-6 release. **(A)** Descriptive statistics for each condition (mean, SD = standard deviation, median, n = number of donors). **(B)** Pairwise p-values from Kruskal-Wallis test with post-hoc Dunn's correction, with all conditions compared to the negative control and 0.007 EU/mL LPS (clinically acceptable limit). Statistical significance was set at $p<0.05$.

(A) MAT: Descriptive statistics													
	Negative control	**LPS 0.007 EU/mL**	**LPS 0.07 EU/mL**	**rt-PA 1.3 µg/mL**	**rt-PA 13 µg/mL**	**SAK STAR WT 1.3 µg/mL**	**SAK STAR WT 13 µg/mL**	**SAK STAR FRIDA 1.3 µg/mL**	**SAK STAR FRIDA 13 µg/mL**	**SAK THR174 1.3 µg/mL**	**SAK THR174 13 µg/mL**	**SAK SY155 1.3 µg/mL**	**SAK SY155 13 µg/mL**
Mean [pg/mL]	0.20	1.84	94.28	0.22	0.16	0.43	0.83	0.92	2.77	0.72	1.16	0.56	0.29
SD [pg/mL]	0.34	2.43	55.72	0.28	0.21	0.46	0.82	0.60	2.87	0.58	1.35	0.59	0.40
Median [pg/mL]	0.02	0.51	76.35	0.09	0.03	0.22	0.53	1.06	1.54	0.51	0.62	0.32	0.08
n	10	11	11	10	9	9	11	6	5	4	6	8	6
(B) MAT: p-values													
	Negative control	**LPS 0.007 EU/mL**	**LPS 0.07 EU/mL**	**rt-PA 1.3 µg/mL**	**rt-PA 13 µg/mL**	**SAK STAR WT 1.3 µg/mL**	**SAK STAR WT 13 µg/mL**	**SAK STAR FRIDA 1.3 µg/mL**	**SAK STAR FRIDA 13 µg/mL**	**SAK THR174 1.3 µg/mL**	**SAK THR174 13 µg/mL**	**SAK SY155 1.3 µg/mL**	**SAK SY155 13 µg/mL**
Negative control	-	0.319	<0.001	>0.999	>0.999	>0.999	0.673	0.286	0.079	>0.999	0.732	>0.999	>0.999
LPS 0.007 EU/mL	0.319	-	0.019	0.518	0.421	>0.999	>0.999	>0.999	>0.999	>0.999	>0.999	>0.999	>0.999

Table S13 T lymphocyte proliferation statistics - Descriptive statistics and p-values for T lymphocyte proliferation, presented as percentage of proliferation. **(A)** Descriptive statistics for each condition (mean, SD = standard deviation, median, n = number of donors). **(B)** Pairwise p-values from ordinary one-way ANOVA with Tukey's post hoc correction, with all conditions compared against each other. Statistical significance was set at $p<0.05$.

(A) T lymphocyte proliferation: Descriptive statistics												
	Negative control	**PHA 5 µg/mL**	**rt-PA 1.3 µg/mL**	**rt-PA 13 µg/mL**	**SAK STAR WT 1.3 µg/mL**	**SAK STAR WT 13 µg/mL**	**SAK STAR FRIDA 1.3 µg/mL**	**SAK STAR FRIDA 13 µg/mL**	**SAK THR174 1.3 µg/mL**	**SAK THR174 13 µg/mL**	**SAK SY155 1.3 µg/mL**	**SAK SY155 13 µg/mL**
Mean [%]	1.19	95.20	0.96	0.61	1.23	2.83	1.12	1.18	0.99	1.53	0.80	1.95
SD [%]	1.46	4.98	1.01	0.89	1.59	5.19	1.57	1.34	1.21	2.64	0.97	3.64
Median [%]	0.71	97.40	0.89	0.19	0.65	0.34	0.52	1.09	0.67	0.28	0.52	0.20
n	4	4	4	4	4	4	4	4	4	4	4	4
(B) T lymphocyte proliferation: p-values												
	Negative control	**PHA 5 µg/mL**	**rt-PA 1.3 µg/mL**	**rt-PA 13 µg/mL**	**SAK STAR WT 1.3 µg/mL**	**SAK STAR WT 13 µg/mL**	**SAK STAR FRIDA 1.3 µg/mL**	**SAK STAR FRIDA 13 µg/mL**	**SAK THR174 1.3 µg/mL**	**SAK THR174 13 µg/mL**	**SAK SY155 1.3 µg/mL**	**SAK SY155 13 µg/mL**
Negative control	-	<0.001	>0.999	>0.999	>0.999	0.999	>0.999	>0.999	>0.999	>0.999	>0.999	>0.999
PHA 5 µg/mL	-	-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
rt-PA 1.3 µg/mL	-	-	-	>0.999	>0.999	0.997	>0.999	>0.999	>0.999	>0.999	>0.999	>0.999
rt-PA 13 µg/mL	-	-	-	-	>0.999	0.987	>0.999	>0.999	>0.999	>0.999	>0.999	>0.999
SAK STAR WT 1.3 µg/mL	-	-	-	-	-	0.999	>0.999	>0.999	>0.999	>0.999	>0.999	>0.999
SAK STAR WT 13 µg/mL	-	-	-	-	-	-	0.999	0.999	0.997	>0.999	0.994	>0.999
SAK STAR FRIDA 1.3 µg/mL	-	-	-	-	-	-	-	>0.999	>0.999	>0.999	>0.999	>0.999
SAK STAR FRIDA 13 µg/mL	-	-	-	-	-	-	-	-	>0.999	>0.999	>0.999	>0.999
SAK THR174 1.3 µg/mL	-	-	-	-	-	-	-	-	-	>0.999	>0.999	>0.999
SAK THR174 13 µg/mL	-	-	-	-	-	-	-	-	-	-	>0.999	>0.999
SAK SY155 1.3 µg/mL	-	-	-	-	-	-	-	-	-	-	-	>0.999

Table S14 Descriptive statistics for clot formation and lysis assay expressed as lysis time. The concentration of SAK variants was 2.4 nM. The experiment was performed at 37 °C in PBS/Ca^{2+}/Tween80 buffer (pH 7.4). **A)** Descriptive statistics for each group (mean, SD = standard deviation, median, n = number of technical replicates). **B)** Pairwise p-values from the Kruskal-Wallis test with post-hoc Dunn's correction. Statistical significance was set at $p<0.05$.

A) Clot formation and lysis assay: Descriptive statistics					
	SAK STAR WT	**SAK 42D WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
Mean [min]	19.3	20.7	50.8	24.4	16.8
SD [min]	2.4	2.4	4.6	1.4	1.8
Median [min]	19.1	20.7	51.3	24.3	16.4
n	11	11	11	11	11
B) Clot formation and lysis assay: p-values					
	SAK STAR WT	**SAK 42D WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
SAK STAR WT	-	0.380	<0.001	0.010	0.194
SAK 42D WT	-	-	<0.001	0.079	0.042
SAK STAR FRIDA	-	-	-	0.088	<0.001
SAK THR174	-	-	-	-	<0.001

Table S15 Determination of antibodies against individual SAK variants together with negative control after vaccination of laboratory BALB/c mice. ELISA panels were coated with SAK STAR WT with a protein concentration of 5 µg/mL in carbonate buffer (100 mM bicarbonate/carbonate, pH 9.6) at 4 °C overnight. Sera of mice (dilution 1:500) immunised with individual SAK variants or with none (negative control) were applied to the wells of ELISA plates. The values are absorbance data measured at 450 nm. * this sample was not measured

Antibody determination using ELISA panels					
	SAK STAR WT	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**	**Negative control**
Mouse 1	0.222	0.164	0.248	0.172	0.216
Mouse 2	0.153	0.208	0.478	0.162	0.199
Mouse 3	0.140	0.306	0.187	0.169	*

Table S16 Best-fit estimates of kinetic and thermodynamic parameters determined via numerical integration of rate equations based on the model illustrated in Scheme 1. Standard errors (S.E.) were calculated from the covariance matrix. Parameter confidence intervals (lower/upper limits) were rigorously defined by confidence contour analysis using a χ^2 threshold of 0.98 (scanning resolution grid of 25 points). Since the scaling parameters are tightly constrained, the extended χ^2 threshold of 0.9 and the scanning resolution grid of 35 points were applied.

SPR affinity kinetic rates – standard error					
	SAK STAR WT	**SAK 42D WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
k_1 [$nM^{-1}*s^{-1}$]($\times10^{-3}$)	0.58 ± 0.07	1.3 ± 0.2	0.55 ± 0.1	1.06 ± 0.62	0.90 ± 0.04
k_{-1} [s^{-1}] ($\times10^{-3}$)	82 ± 7	88 ± 3	162 ± 18	93 ± 7	26 ± 2
k_2 [$nM^{-1}*s^{-1}$]($\times10^{-3}$)	36 ± 10	51 ± 2	34 ± 3	20 ± 7	37 ± 1
k_{-2} [s^{-1}] ($\times10^{-3}$)	73 ± 25	65 ± 7	28 ± 8	62 ± 33	10 ± 1
k_3 [$nM^{-1*}s^{-1}$]($\times10^{-3}$)	0.58 ± 0.02	0.57 ± 0.02	0.39 ± 0.06	0.55 ± 0.05	0.42 ± 0.05
k_{-3} [s^{-1}] ($\times10^{-3}$)	8.8 ± 0.6	7.1 ± 0.4	75 ± 13	12 ± 1	20 ± 3
SPR affinity kinetic rates – confidence intervals					
	SAK STAR WT	**SAK 42D WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
k_1 [$nM^{-1}*s^{-1}$]($\times10^{-3}$)	0.54 – 0.81	1.0 – 1.4	0.51 – 0.91	0.85 – 1.66	0.68 – 0.95
k_{-1} [s^{-1}] ($\times10^{-3}$)	56 – 97	56 – 97	130 – 253	75 – 117	13 – 46
k_2 [$nM^{-1}*s^{-1}$]($\times10^{-3}$)	15 – 45	41 – 60	28 – 54	5 – 39	33 – 96
k_{-2} [s^{-1}] ($\times10^{-3}$)	69 – 177	62 – 67	25 – 34	13 – 238	8 – 16
k_3 [$nM^{-1*}s^{-1}$]($\times10^{-3}$)	0.53 – 0.59	0.51 – 0.62	0.35 – 0.45	0.52 – 0.56	0.34 – 0.63
k_{-3} [s^{-1}] ($\times10^{-3}$)	8.5 – 9.0	6.8 – 7.4	66 – 83	12 – 13	16 – 32
SPR affinity scaling parameters – standard error					
	SAK STAR WT	**SAK 42D WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
a_1	4.25 ± 0.09	3.96 ± 0.07	2.34 ± 0.70	4.20 ± 0.13	5.38 ± 0.07
a_2	4.87 ± 0.10	3.65 ± 0.07	2.11 ± 0.63	3.77 ± 0.12	4.88 ± 0.07
a_3	3.62 ± 0.08	3.13 ± 0.06	1.98 ± 0.57	3.39 ± 0.11	4.98 ± 0.07
a_4	3.86 ± 0.08	3.35 ± 0.06	1.83 ± 0.55	3.42 ± 0.11	6.44 ± 0.09
a_5	3.20 ± 0.07	2.42 ± 0.05	1.72 ± 0.52	2.61 ± 0.08	3.89 ± 0.05
a_6	2.20 ± 0.05	2.45 ± 0.05	1.57 ± 0.47	2.63 ± 0.08	3.38 ± 0.05
b	1.26 ± 0.11	2.98 ± 0.12	3.53 ± 1.07	2.23 ± 0.32	1.71 ± 0.02
c	2.15 ± 0.05	2.38 ± 0.04	3.87 ± 1.56	2.10 ± 0.07	1.31 ± 0.03
d ($*10^{-3}$)	1.5 ± 0.1	1.6 ± 0.1	0.79 ± 0.01	0.91 ± 0.01	2.0 ± 0.1
SPR affinity scaling parameters – confidence intervals					
	SAK STAR WT	**SAK 42D WT**	**SAK STAR FRIDA**	**SAK THR174**	**SAK SY155**
a_1	4.25 – 4.28	3.94 – 3.97	2.22 – 2.41	4.11 – 4.20	5.27 – 5.45
a_2	4.87 – 4.91	3.63 – 3.66	2.02 – 2.17	3.70 – 3.78	4.78 – 4.94
a_3	3.62 – 3.64	3.12 – 3.14	1.79 – 1.93	3.33 – 3.40	4.87 – 5.04
a_4	3.86 – 3.88	3.33 – 3.35	1.70 – 1.85	3.35 – 3.43	6.29 – 6.52
a_5	3.20 – 3.23	2.41 – 2.43	1.62 – 1.75	2.56 – 2.61	3.81 – 3.94
a_6	2.20 – 2.21	2.43 – 2.45	1.48 – 1.59	2.58 – 2.63	3.31 – 3.42
b	1.26 – 1.36	2.95 – 2.99	3.00 – 3.63	2.19 – 2.26	1.65 – 2.01
c	2.14 – 2.23	2.34 – 2.41	3.71 – 4.01	1.94 – 2.16	0.89 – 1.38
d ($*10^{-3}$)	1.32 – 1.73	1.43 – 1.76	0.43 – 1.44	0.50 – 1.36	1.85 – 2.12

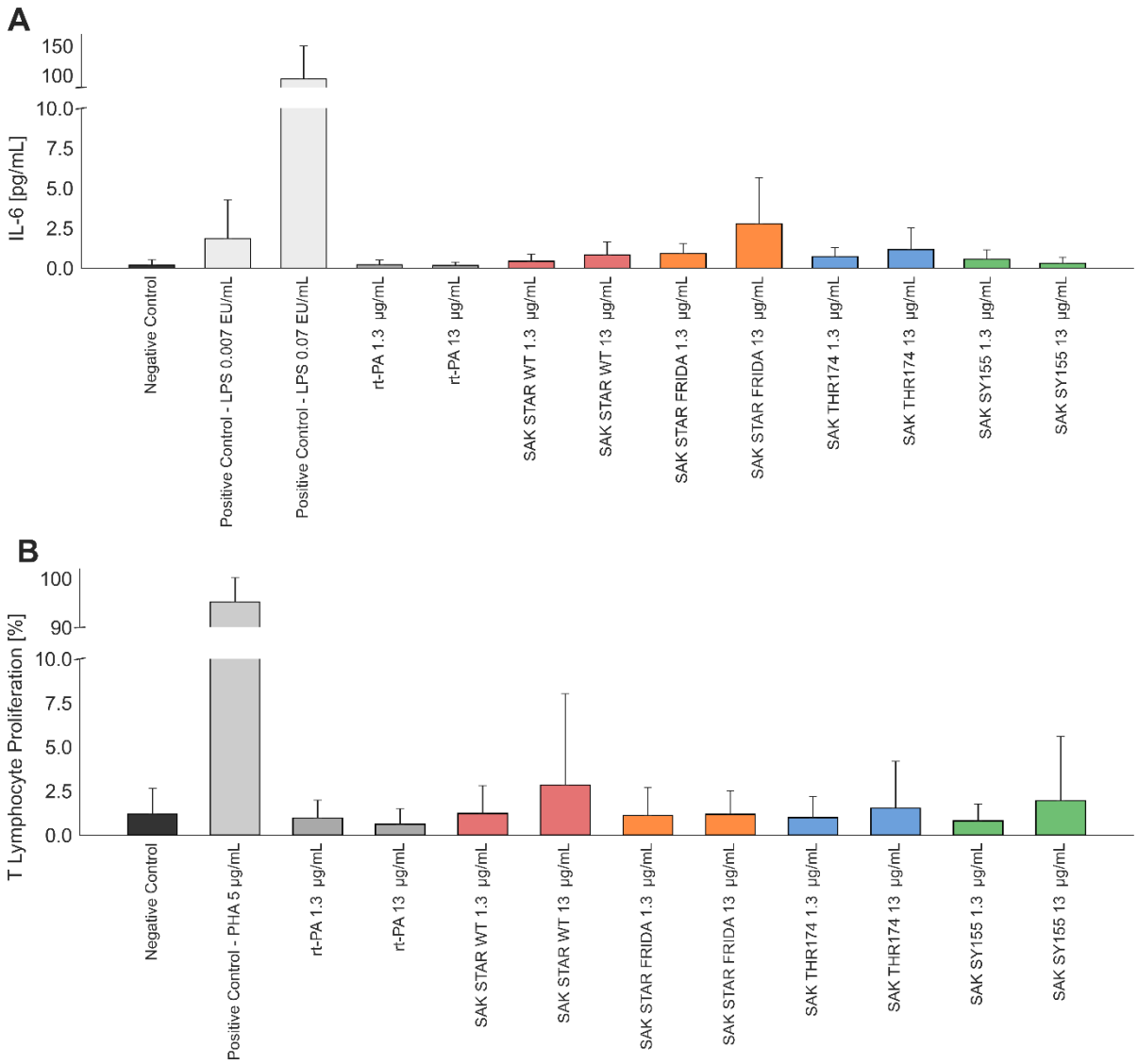


Figure S18 Immunological characterisation of SAK variants using Monocyte activation test (MAT) and T lymphocyte proliferation test. A) Monocyte activation test (MAT). IL-6 release was measured in 10-fold diluted human blood stimulated with negative control, LPS at 0.007 EU/mL (clinically acceptable limit), and LPS at 0.07 EU/mL (10-fold higher, assay functionality control), as well as with rt-PA and SAK variants at 1.3 and 13 µg/mL. Data are shown as mean ± standard deviation from 4 – 11 independent donors. Kruskal-Wallis test with Dunn's post hoc correction was performed against negative control and 0.007 EU/mL LPS. B) T lymphocyte proliferation test. Proliferation of CD8-depleted PBMCs from four healthy donors was assessed following stimulation with negative control, positive control (PHA 5 µg/mL), rt-PA (1.3 and 13 µg/mL), or SAK variants (1.3 and 13 µg/mL). Data are shown as mean ± standard deviation. One-way ANOVA with Tukey's post hoc correction was performed between all conditions.

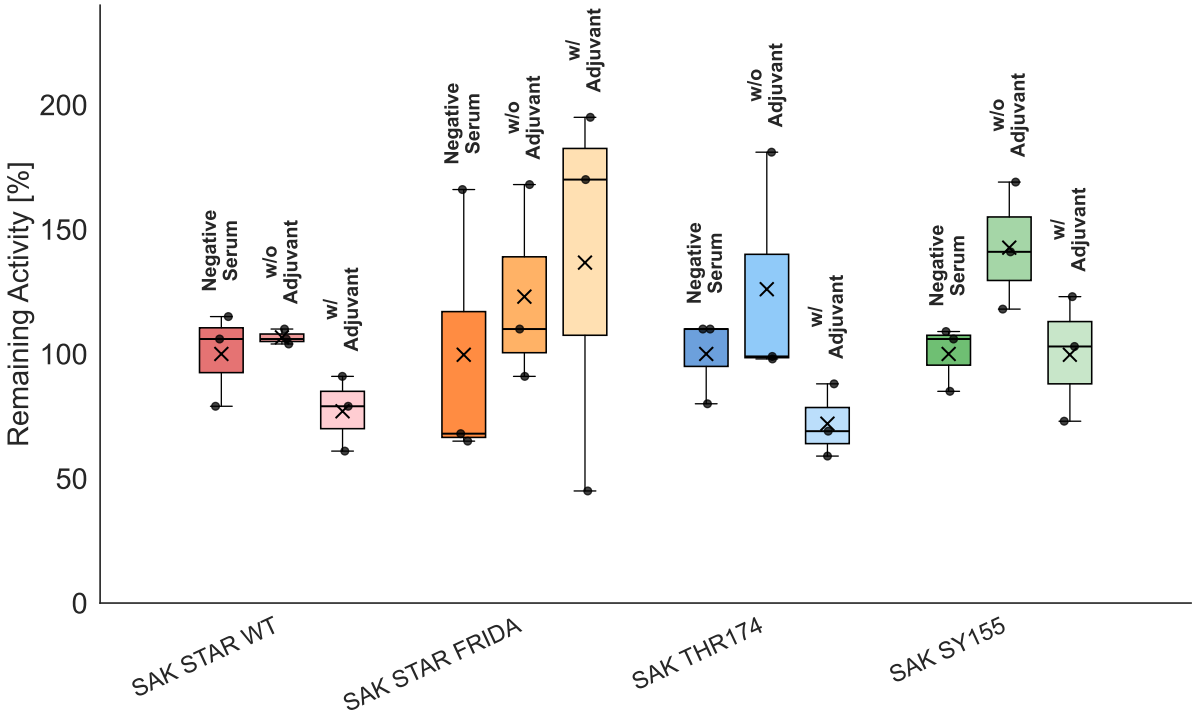


Figure S19 Comparison of serum inhibitory potency after administration of four SAK variants under different conditions: negative control, administration without adjuvant, and administration with adjuvant. Boxplots represent the distribution of three biological replicates for each condition, with whiskers indicating the full data range (min–max). Individual replicate values are shown as black dots, while black crosses indicate mean values. For each protein variant, condition-specific measurements are displayed using different tonal shades of the same base colour: dark shades represent negative serum controls, medium shades represent samples without adjuvant, and light shades represent samples with adjuvant.

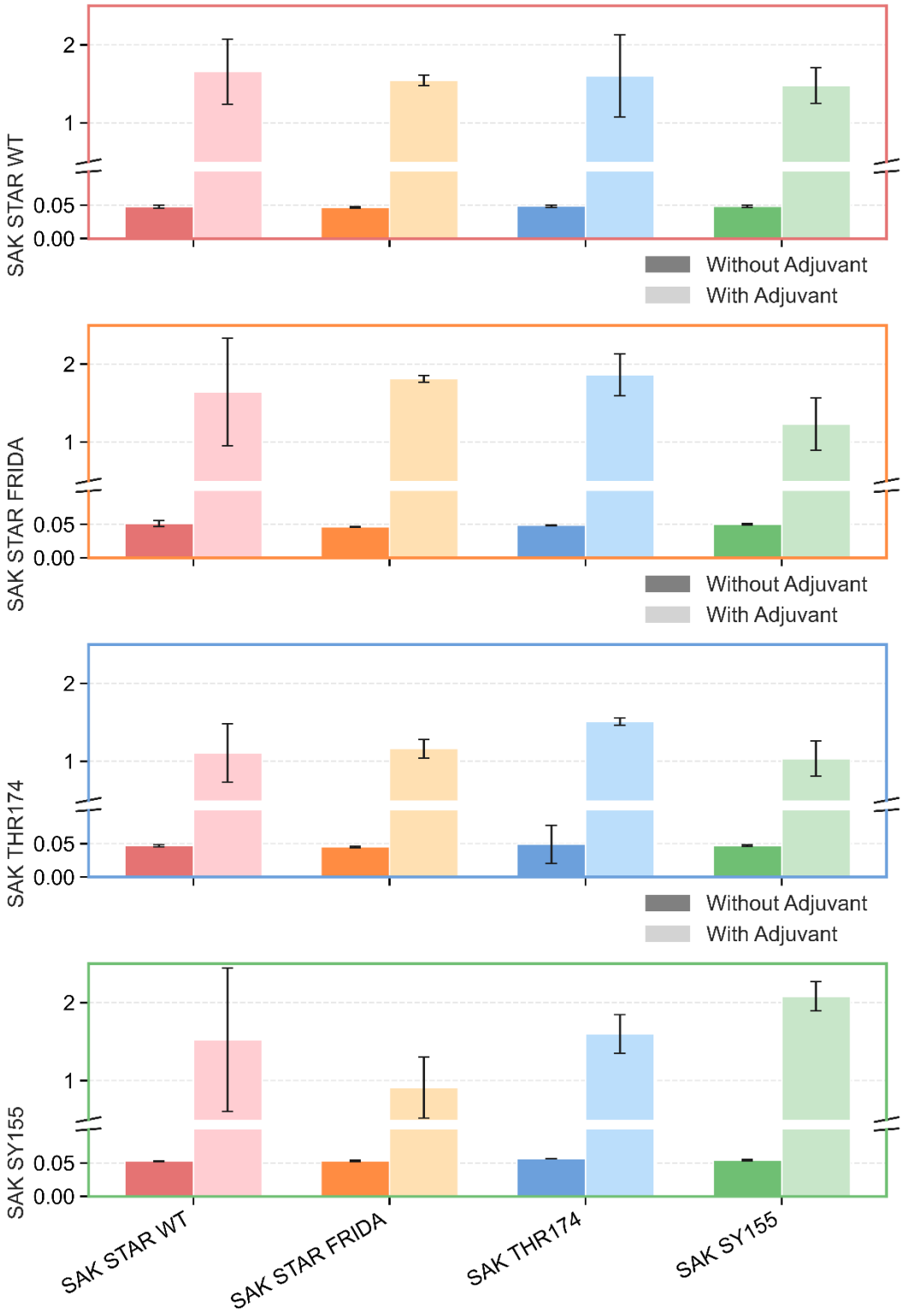


Figure S20 Determination of antibodies against individual SAK variants after vaccination of laboratory BALB/c mice. ELISA panels were coated with respective variants of SAK and sera of mice (dilution 1:500) immunised with individual SAK variants without (darker colour bars) and with (lighter colour bars) aluminium adjuvant were applied to respective wells of ELISA plates. The SAK variant used for coating is indicated on the left side of the individual subplots. The values are absorbance data measured at 450 nm. Data are shown as mean ± standard deviation from two independent ELISA replicates.

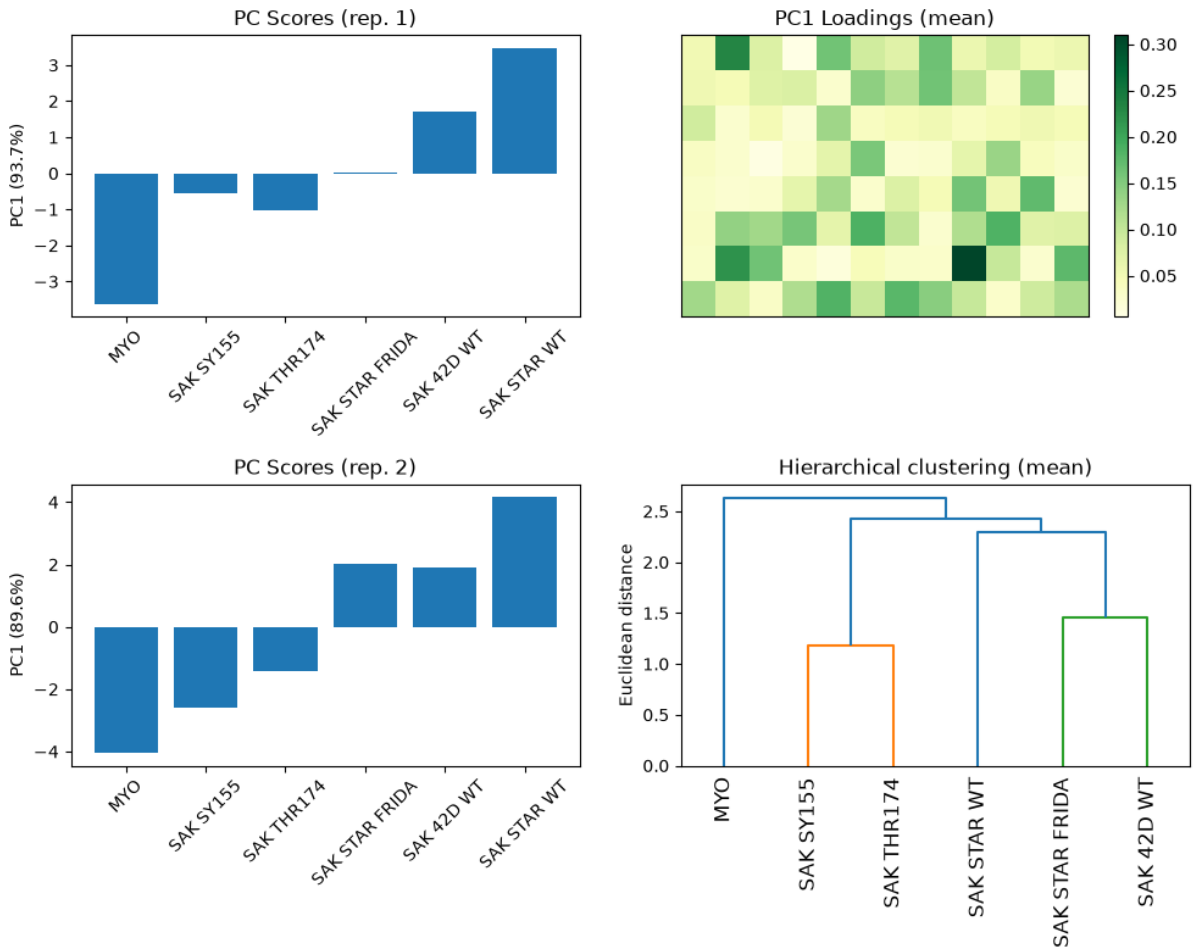


Figure S21 Data analysis of reactivity of human sera for individual SAK variants. Left: the first principal component of the centred non-normalised absorbance values of human sera reactivity for each independent replicate; Top right: the loadings graph for the first principal component of the mean values of absorbances (ordering is consistent with Figure 4); Bottom right: hierarchical clustering of the SAK variants and the mock protein (Myo) for mean values of absorbances.