The contact system proteases play disparate roles in streptococcal sepsis

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Methods:

Bacterial strains and culture conditions

The *S. pyogenes* strains AP1 (40/58) has been described previously [1]. Bacteria were grown overnight in Todd-Hewitt broth (THB; Becton Dickinson) at 37°C and 5% CO_2.

Human plasma

Pooled plasma obtained from healthy donors was purchased from Affinity Biologicals Inc. PK- and FXII-deficient plasmas were purchased from George King Bio-Medical.

Infection of HepG2 cells

HepG2 cells were cultured in RPMI 1640 Medium with L-glutamin (Gibco), phenol red and 10% FCS at 37 °C with 5% CO_2. $1x10^6$ cells were plated and after 24 h the medium was replaced with fresh medium without FCS. Cells were infected with *S. pyogenes* (MOI 1:10) for 6 h. After 6h of incubation cells were harvested for RNA analysis or washed with PBS and further incubated with fresh medium containing 1% PenStrep for 24 h.

mRNA analysis

Total RNA was isolated from HepG2 cells or homogenized mouse liver with RNeasy Plus Mini Kit (Qiagen). RNA quality was checked with Agilent RNA 6000 Nano Kit (Agilent Technologies) and RNA concentration determined with Qubit™ RNA HS Assay Kit (Invitrogen). All analyses were performed according to the manufacturer's instructions. 800 ng total RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and the complementary DNA obtained used for real-time quantitative PCR. Reaction mixture (20 µl) containing gene specific nuclease assay, Taqman Universal PCR Master Mix (Applied Biosystems) and cDNA was amplified as follows: denaturation at 95 °C for 10 min and 45 cycles at 95 °C for 15 sec and 60 °C for 1 min. GAPDH (human or rodent) was used as housekeeping gene. Relative expression was calculated employing the $2^{-\Delta\Delta ct}$ method.

Clotting assays

All clotting times were measured using an Amelung coagulometer. Activated partial thromboplastin time (aPTT) was measured by incubating mouse plasma for 1 minute followed by the addition of equal amounts of Dapptin, containing silica, sulfatide and phospholipids

(Technoclone) for 60 seconds at 37°C. Clotting was initiated by the addition of 25 mM $CaCl_2$. For the prothrombin time assay (PT), clotting was initiated by the addition of Technoplastin HIS (PT reagent, Technoclone).

Clot lysis time

A clot was generated in 10 µl human normal, FXII- or PK-deficient plasma by addition of 10 µl PT-Reagent. In some experiment's CTI (75 µg/ml), PKSI (10 µM), FXIIa (50 µg/ml) or kallikrein (KK; 50 µg/ml) was added before clot was induced. The clot was incubated for 5 min at 37°C before Streptokinase (100 Units), uPA (10 µg) or tPa (10 µg) was added. Time until clot lysis was determined in a coagulometer.

Measurement of FXII and PK in plasma

Total PK or FXII amount in plasma was quantified by a sandwich ELISA (Affinity Biologicals Inc.). Briefly, affinity-purified antibodies detecting PPK, PK or FXII and FXIIa were coated onto the wells of a microtiter plate overnight at 4 °C. Any remaining binding sites on the plastic wells were blocked for 1.5 h at room temperature with bovine serum albumin. The plates were washed and plasma samples, in appropriate dilution, were applied over night at 4 °C. After washing, a peroxidase conjugated second antibody to PK or FXII was added. Peroxidase activity was expressed by incubation with o-phenylenediamine (OPD). After development for 10 min the reaction was quenched with addition of H_2SO_4 and the color produced was quantified using a microplate reader. The color was proportional to the concentration of contact factors present in the samples.

Alternatively, proteins in plasma were separated and blotted as described [1], followed by incubation with anti-human FXII (Nordic-MUbio) or anti human PK (Affinity Biologicals). Blots were incubated with secondary fluorophore-labeled antibodies (LI-COR) and imaged on Odyssey Imager (LI-COR). PPK and FXII relative plasma protein levels were determined by densitometry analysis (ImageStudioLite 5.2.5).

Plasmin activity in human plasma

Plasmin activity in human normal, FXII- or PK-deficient plasma (diluted 1:10 in HEPES-saline, 10 mM HEPES, 137 mM NaCl) was determined after addition of streptokinase (20 Units), uPA

(20 µg) or tPa (20 µg) and the chromogenic substrate S-2403 (0.36 mM, Chromogenix) followed by incubation for 3 h at 37 °C, during measurement at 405 nm in an ELISA reader.

Measurement of plasminogen in plasma or preparations of purified proteins

Total plasminogen amount in plasma or purified proteins was quantified by ELISA (Technoclone GmbH). In purified FXII, FXIIa, PPK, KK or fibrinogen no plasminogen was detected.

Plasmin-antiplasmin (PAP) complexes in infected mice plasma

PAP complexes were detected by ELISA in EDTA plasma of infected mice according to the instruction of the manufacture (Abbexa Ltd.).

Proteolytic potential for PK/FXIIa activtiy in mouse plasma

Citrated Plasma from 4 mice/group was pooled and 10 µl pooled plasma was activated with 10 µl Dapptin (Technoclone). Activity was determined over 60 min in a microplate reader by addition of 1 mM chromogenic substrate S-2302 (Chromogenix) for PK and FXIIa. Plasma incubated with Dapptin and 100 µl HEPES-saline was used as blank.

Fibrinogen degradation

Fibrinogen (30 µg/ml) was mixed with either plasmin (40 nM), plasminogen (38 nM) and streptokinase (10 U), FXII (40 nM), PK (38 nM), or a combination of FXII and PK or PBS as negative control. The mix were incubated at 37 °C and at indicated time points samples were mixed with SDS-sample buffer and analyzed by SDS-Page and Western Blot using 12% gradient SDS-polyacrylamide gels followed by immunoblotting with a fibrinogen antibody (Santa Cruz). Blots were incubated with secondary fluorophore-labeled antibody (LI-COR), imaged on Odyssey Imager (LI-COR), and relative fibrinogen levels were determined by densitometry analysis (ImageStudioLite 5.2.5).

Mouse plasma clot escape experiments

S. pyogenes AP1 was grown to mid-logarithmic phase (OD_{620nm}= 0.4) in TH broth. Citrated plasma of 4 mice/group was pooled and, if indicated, PPK-ASO plasma was pre-incubated with human PK (50 µg/ml). Plasma was mixed with *S. pyogenes* (final concentration $1x10^7$ CFU) and clotting was induced by addition of thrombin (1 U) and $CaCl_2$ (25 mM). The clot was

incubated for 5 min at 37°C for stabilization and overlayed with PBS containing 1% of the original plasma. After 2 or 4 h of incubation at 37 °C the supernatant and the ribolysed clot were plated to determine the CFU/ml [2].

Scanning electron microscopy

150 µl human plasma was mixed with 150 µl PT-reagent and incubated for 30 min at 37°C and fixed. Mouse plasma clots were derived from pooled plasma of 4mice/group. 50 µl mouse plasma was mixed with *S. pyogenes* (10^9 CFU/ml) and a clot was induced with thrombin and $CaCl_2$ as described above. The clots were incubated for 4 hours at 37°C, fixed and processed for critical point drying and mounting as described before. Specimens were analyzed with a field emission scanning electron microscope (Merlin compact, Carl Zeiss Microscopy GmbH).

Histology

Mouse organs were fixed in 4% paraformaldehyde (in PBS, pH7.4) and processed for routine histopathologic evaluation as described before [3]. Tissues were stained with Mayers hematoxylin and eosin (HE, MORPHISTO, Germany) or with Martius scarlet blue (MSB Lendrum, Fibrin-Red; Erythrocytes-Yellow, Collagen, Elastic Fibers, Basement Membranes – Blue, Epithelia - Red). Fibrin areas > 5 µm were counted in each organ and graded on a scale of 0 to 3 (0= absent; 1 = up to 20; 2 = 20 - 50 and 3 = more than 50 fibrin areas).

D-dimer ELISA

S. pyogenes AP1 was grown to mid-logarithmic phase (OD_{620nm}= 0.4). 100 µl bacteria ($2x10^8$ CFU/ml) were mixed with 100 µl human normal, FXII- or PK-def. plasma and, if indicated, PPK- or FXII def. plasma was pre-incubated with human KK or FXIIa (50 µg/ml). A clot was generated by addition of thrombin (1U) and $CaCl_2$ (25 mM). The clot was incubated for 5 min at 37 °C, and overlayed with PBS containing 1 % of the original plasma. 50 µl supernatant was taken at indicated time points and stored at -20°C. The D-dimer ELISA (Technoclone) was performed according to the manufacturer's instructions**.**

Proteome analysis

Proteome analysis was performed with EDTA plasma samples from five healthy and five *S. pyogenes*-infected mice, respectively. Samples corresponding to 100 µg of protein were mixed

with solubilization buffer (1.5% sodium deoxycholate (SDC), 10 mM dithiothreitol, and 50 mM ammonium bicarbonate (ABC)), incubated at 95 °C for 5 min, and alkylated with 15 mM iodoacetamide for 20 min at room temperature. After dilution with two volumes of 50 mM ABC, sequencing grade trypsin (Promega GmbH, Mannheim, Germany) was added in an enzyme/protein ratio of approximately 1:100 to a final volume of 320 µl. Digestion was performed at 37 °C for 16 h. SDC was removed from the solutions of digested proteins using the phase transfer surfactant method as previously described [4]. Finally, the peptide solutions were desalted with OASIS HLB 1cc Vac Cartridges (Waters, Manchester, UK).

Peptide samples corresponding to approximately 100 ng of digested protein supplemented with 40 fmol of Hi3 E. coli standard for protein absolute quantification (Waters) were separated using a nanoAcquity UPLC system (Waters). The UPLC system was coupled to a Synapt G2-S mass spectrometer (Waters) operated in data-independent mode ($HDMS^E$) as previously described. Samples were measured once without technical replication.

Protein identification and label free quantification was performed using Progenesis QI for Proteomics version 4.0 (Nonlinear Dynamics, Newcastle upon Tyne, UK) as described [5]. For the database search, a database containing 16970 reviewed protein sequences from *Mus musculus* (UniProt release 2018_04) appended with the sequences of ClpB from *E. coli* (P63284) and porcine trypsin was used. Proteins which showed at least 1.5-fold increase or decrease, respectively, and ANOVA p-values < 0.05 for the comparison between healthy and infected animals were regarded as significant.

Animal experiments

Eight-week-old female BALB/c mice with a weight of 16 – 18 g (Charles River Laboratories) were treated with ASOs through intraperitoneal injections, with a dose of 800 µg/mouse, twice per week for 3 weeks (total 7 injections, each with 800 µg ASO/mouse).

The subcutaneous infection model with *S. pyogenes* AP1 strain and determination of bacterial dissemination were performed as described previously [2]. Briefly, mice were anesthetized with isoflurane and injected with 1.5 - 2 x 10^7 CFU growing *S. pyogenes* AP1 bacteria in an air

pouch on the neck. 6 or 24 h after infection organs and blood were plated, respectively citrated plasma was prepared for clotting and substrate assays.

Multiplex cytokine quantification assay

A panel of plasma cytokines (n=20) was determined according to the manufacturer's instructions of the Procartaplex™ Multiplex Immunoassay (ThermoFisher Scientific, Berlin, Germany). Briefly, plates were coated with magnetic beads, followed by addition of standards and plasma samples from infected and healthy control mice (n=5/group; 25 µl/well). Upon washing, detection antibody was added. Data acquisition was done after incubation with streptavidin-PE. Samples were run on a Bioplex 2000 (Bio-Rad Laboratories GmbH, Munich, Germany). In combination with the Bio-Plex Manager Software, sample concentration (pg/ml) was calculated by plotting against the corresponding standard curve.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (v5.o and 6.0). The P value was determined by using the unpaired t test (comparison of two groups), if not otherwise indicated. All samples were analysed in triplicates, and all experiments were performed at least three times, if not otherwise declared. The bars in the figures indicate the standard deviations (SD).

Supplemental Table 1. Plasma proteins from infected mice quantified by mass spectrometry, compared to healthy mice

		Protein abundance (fmol on column)							
		Control animals (n = 5)			24 h infected animals (n = 5)			Comparative analysis	
Protein name	Unique peptides	Mean	SD	CV	Mean	SD	CV	Fold Change infected/control	Anova (p)
Actin_ cytoplasmic 1	4	0.93	0.12	12.96	2.14	0.56	26.32	2.29	2.73E-04
Adiponectin	3	1.37	0.39	28.07	0.68	0.31	45.61	0.50	1.33E-02
Afamin	19	9.18	0.92	10.03	4.50	0.32	7.15	0.49	1.28E-06
Alpha-1-acid glycoprotein 1	8	1.76	0.50	28.34	14.54	1.85	12.75	8.26	2.71E-07
Alpha-1-acid glycoprotein 2	3	0.54	0.02	4.46	9.55	1.72	17.99	17.80	1.85E-09
Alpha-1-antitrypsin 1-1	7	30.69	1.88	6.12	21.40	6.58	30.74	0.70	4.71E-02
Alpha-1-antitrypsin 1-2	7	30.18	2.92	9.66	21.40	3.96	18.48	0.71	7.01E-03
Alpha-2-antiplasmin	16	7.05	0.55	7.75	6.33	0.77	12.17	0.90	1.18E-01
Alpha-2-HS-glycoprotein	11	59.46	8.08	13.59	32.84	4.97	15.14	0.55	2.09E-04
Alpha-2-macroglobulin-P	7	0.10	0.04	42.57	0.37	0.20	54.96	3.65	1.93E-02
Alpha-amylase 1	2	0.37	0.14	39.46	0.37	0.31	84.50	1.00	9.43E-01
Angiotensinogen	4	1.37	0.42	30.94	0.90	0.37	41.30	0.66	9.60E-02
Antithrombin-III	23	38.05	4.06	10.67	25.15	3.78	15.02	0.66	1.07E-03
Apolipoprotein A-I	35	36.50	3.33	9.13	37.91	5.54	14.62	1.04	6.80E-01
Apolipoprotein A-IV	30	18.91	2.07	10.93	30.95	5.73	18.52	1.64	1.30E-03
Apolipoprotein C-I	3	5.56	1.04	18.77	4.53	1.57	34.70	0.82	1.99E-01
Apolipoprotein C-III	4	17.21	0.93	5.38	8.47	2.50	29.46	0.49	3.90E-04
Apolipoprotein C-IV	2	2.88	0.42	14.73	0.96	0.51	53.83	0.33	5.79E-04
Apolipoprotein D	5	1.55	0.26	17.02	1.91	0.45	23.47	1.24	1.46E-01
Apolipoprotein E	21	6.40	1.17	18.31	16.57	4.05	24.43	2.59	1.57E-04
Apolipoprotein M	3	1.70	0.36	21.05	0.75	0.25	33.20	0.44	8.43E-04
Beta-2-glycoprotein 1	21	9.21	0.77	8.37	6.67	0.61	9.18	0.72	3.94E-04
Beta-2-microglobulin	3	4.62	0.83	18.07	2.32	0.71	30.41	0.50	1.98E-03
BPI fold-containing family A member 2	5	0.44	0.46	105.43	0.95	1.44	150.52	2.17	5.44E-01
C4b-binding protein	3	1.62	0.08	4.74	1.37	0.40	29.02	0.85	1.65E-01
Carbonic anhydrase 2	3	0.63	0.58	91.78	0.63	0.49	78.78	0.99	9.78E-01
Carboxylesterase 1C	16	49.97	6.59	13.20	32.77	7.39	22.55	0.66	5.23E-03
Carboxypeptidase B2	8	0.37	0.03	7.27	1.29	0.13	9.98	3.44	4.95E-08
Carboxypeptidase N catalytic chain	6	1.57	0.22	14.02	0.67	0.23	34.47	0.42	2.95E-04
Carboxypeptidase N subunit 2	12	3.64	0.15	4.00	1.45	0.23	15.89	0.40	8.67E-07
Cathepsin B	2	0.09	0.04	41.21	0.17	0.10	56.92	1.98	1.04E-01
CD5 antigen-like	11	2.08	0.37	17.56	1.12	0.25	22.55	0.54	1.19E-03
Ceruloplasmin	53	10.59	2.19	20.67	29.56	5.69	19.26	2.79	7.16E-05
Chaperone protein ClpB	5	39.17	4.94	12.60	39.71	16.27	40.98	1.01	8.29E-01
Clusterin	12	5.89	0.67	11.38	3.20	0.41	12.73	0.54	6.55E-05
Coagulation factor X	6	13.89	1.51	10.90	11.50	2.49	21.70	0.83	9.70E-02
Coagulation factor XII	5	0.77	0.13	17.01	0.58	0.16	28.27	0.75	6.92E-02
Coagulation factor XIII A chain	3	0.45	0.13	28.34	0.20	0.11	56.43	0.45	1.18E-02
Coagulation factor XIII B chain	6	0.75	0.05	6.69	0.33	0.04	12.20	0.44	3.61E-07
Complement C1q subcomponent subunit B	2	0.78	0.11	14.32	0.50	0.16	31.96	0.64	1.23E-02
Complement C1s-A subcomponent	3	0.55	0.16	28.24	0.45	0.16	35.81	0.82	3.40E-01
Complement C3	110	22.38	5.28	23.61	29.78	6.23	20.91	1.33	7.22E-02
Complement C4-B	28	1.66	0.35	20.85	1.83	0.47	25.92	1.10	5.61E-01
Complement C5	14	1.63	0.41	24.86	1.14	0.36	31.29	0.70	7.01E-02
Complement component C8 beta chain	2	0.37	0.06	16.02	0.33	0.11	33.52	0.89	4.63E-01
Complement component C8 gamma chain	2	0.40	0.14	34.15	0.25	0.07	27.09	0.63	6.03E-02
Complement factor B	28	5.61	0.62	11.06	10.45	2.05	19.61	1.86	2.39E-04
Complement factor D	5	4.47	1.59	35.67	2.36	0.71	30.17	0.53	2.81E-02
Complement factor H	51	7.52	0.78	10.37	11.46	1.49	13.01	1.52	5.62E-04
Complement factor I	17	4.21	0.38	9.14	2.76	0.34	12.47	0.66	3.33E-04
Corticosteroid-binding globulin	7	11.00	2.09	18.98	5.12	1.04	20.39	0.47	2.25E-04
C-reactive protein	5	0.35	0.09	25.15	2.35	0.39	16.57	6.74	1.83E-07
Extracellular matrix protein 1	2	0.56	0.11	20.55	0.51	0.09	18.52	0.92	5.08E-01
Fetuin-B	8	5.06	1.05	20.76	4.90	1.22	24.86	0.97	7.93E-01
Fibrinogen alpha chain	39	44.98	4.60	10.22	123.72	14.66	11.85	2.75	6.06E-07
Fibrinogen beta chain	51	33.79	3.43	10.15	96.06	16.84	17.53	2.84	2.52E-06
Fibrinogen gamma chain	37	46.61	4.75	10.18	118.84	5.76	4.85	2.55	6.78E-08
Fructose-bisphosphate aldolase A	2	0.04	0.03	84.25	0.58	0.40	68.93	14.84	1.09E-02
Gelsolin	25	13.16	2.09	15.92	7.00	1.11	15.90	0.53	1.87E-04
Glutathione peroxidase 3	6	3.58	0.48	13.42	3.84	0.69	17.98	1.07	5.44E-01
Granulins	2	0.12	0.02	20.62	0.23	0.06	23.83	1.97	2.81E-03

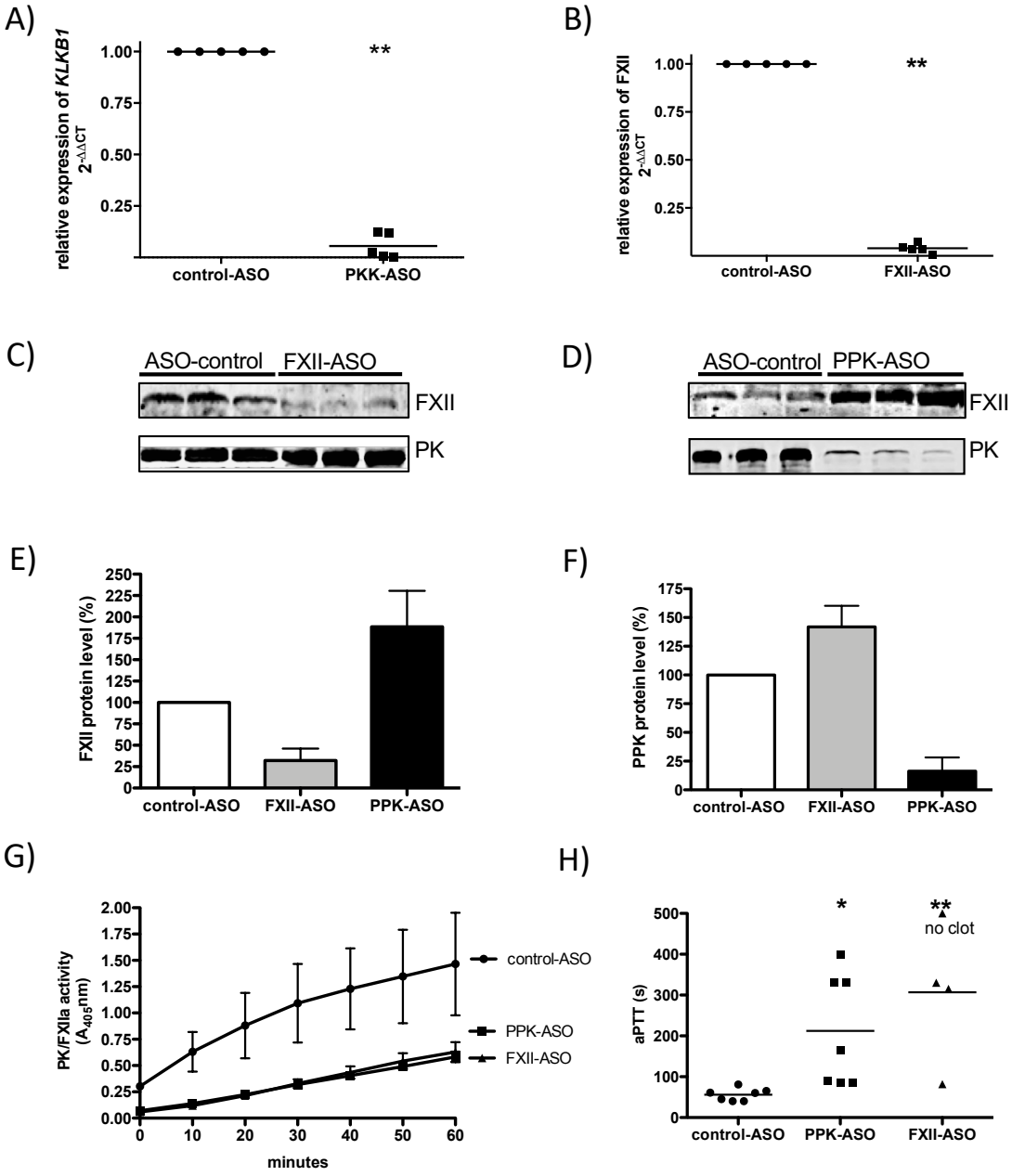
H-2 class I histocompatibility antigen_ Q10 alpha chain	12	4.78	0.67	14.06	2.03	0.55	27.32	0.42	2.00E-04
Haptoglobin	27	4.93	0.19	3.78	66.40	13.43	20.22	13.46	1.55E-09
Hemoglobin subunit alpha	8	25.42	23.64	93.00	24.87	24.27	97.60	0.98	9.93E-01
Hemoglobin subunit beta-1	6	53.00	51.86	97.83	43.89	35.41	80.69	0.83	9.53E-01
Hemoglobin subunit beta-2	6	18.73	20.42	109.00	16.41	14.16	86.29	0.88	9.33E-01
Hemopexin	36	32.21	4.68	14.55	132.05	19.99	15.14	4.10	4.64E-07
Heparin cofactor 2	4	1.46	0.35	23.71	0.28	0.09	32.32	0.19	1.50E-05
Hepatocyte growth factor activator	2	0.93	0.15	16.68	0.42	0.12	28.18	0.46	3.31E-04
Histidine-rich glycoprotein	11	5.07	0.58	11.36	3.29	1.00	30.46	0.65	1.06E-02
Ig alpha chain C region	7	2.30	0.25	10.85	5.31	2.15	40.56	2.31	3.08E-03
Ig gamma-1 chain C region secreted form	8	14.07	2.10	14.92	9.45	1.76	18.58	0.67	4.62E-03
Ig gamma-2A chain C region_ A allele	4	3.55	1.25	35.21	1.79	0.80	44.78	0.50	2.38E-02
Ig gamma-2B chain C region	8	6.35	0.97	15.29	4.16	1.91	46.00	0.66	3.93E-02
Ig gamma-3 chain C region	4	3.51	0.66	18.84	1.14	0.13	11.02	0.32	4.16E-06
Ig heavy chain V region 441	7	1.61	0.52	32.10	0.91	0.27	30.15	0.56	2.29E-02
Ig heavy chain V region AC38 205.12	2	3.09	0.40	12.87	2.63	0.71	26.98	0.85	2.03E-01
Ig heavy chain V regions TEPC 15/S107/HPCM1/HPCM2/HPCM3	2	1.68	0.52	31.11	0.92	0.30	32.41	0.55	1.75E-02
Ig heavy chain V-III region A4	2	0.36	0.12	33.83	0.35	0.11	32.32	0.96	8.68E-01
Ig kappa chain V19-17	2	1.26	0.33	25.97	0.93	0.31	32.77	0.74	1.42E-01
Ig kappa chain V-II region 26-10	3	2.72	0.36	13.21	1.77	0.56	31.52	0.65	1.55E-02
Ig kappa chain V-V region K2 (Fragment)	2	0.49	0.13	26.19	0.36	0.10	27.83	0.73	9.88E-02
Ig kappa chain V-V region L7 (Fragment)	2	0.75	0.12	16.27	0.38	0.12	32.71	0.50	1.26E-03
Ig kappa chain V-VI region XRPC 44	3	1.65	0.47	28.30	0.53	0.20	38.34	0.32	5.87E-04
Ig lambda-1 chain C region	3	2.21	0.53	24.07	1.47	0.31	21.27	0.67	2.40E-02
Immunoglobulin heavy constant mu	22	20.55	3.33	16.20	13.61	2.70	19.86	0.66	6.51E-03
Immunoglobulin J chain	3	1.87	0.41	22.18	1.75	0.45	25.79	0.93	6.32E-01
Immunoglobulin kappa constant	5	4.25	0.81	19.05	3.38	0.82	24.27	0.79	1.09E-01
Inhibitor of carbonic anhydrase	23	3.95	0.42	10.66	3.13	0.65	20.66	0.79	3.96E-02
Inter alpha-trypsin inhibitor_ heavy chain 4	39	5.04	1.27	25.08	31.22	7.88	25.25	6.19	3.42E-06
Inter-alpha-trypsin inhibitor heavy chain H1	12	2.28	0.23	10.10	1.75	0.25	14.42	0.77	7.83E-03
Inter-alpha-trypsin inhibitor heavy chain H2	12	4.44	1.38	31.13	3.92	1.27	32.41	0.88	5.68E-01
Inter-alpha-trypsin inhibitor heavy chain H3	12	1.69	0.25	15.05	5.17	0.62	11.97	3.07	8.08E-07
Interleukin-1 receptor accessory protein	6	1.37	0.11	7.79	0.62	0.17	27.99	0.45	6.01E-05
Kininogen-1	23	24.88	1.00	4.03	21.90	3.26	14.87	0.88	1.02E-01
Leukemia inhibitory factor receptor	5	1.01	0.18	17.80	0.39	0.12	30.24	0.38	1.30E-04
Lipopolysaccharide-binding protein	4	0.02	0.01	31.65	0.88	0.38	43.45	40.77	2.45E-04
Lumican	4	2.25	0.38	16.96	1.15	0.36	31.33	0.51	1.81E-03
Mannose-binding protein A	2	0.65	0.14	21.21	0.65	0.26	39.13	1.01	9.85E-01
Mannose-binding protein C	5	2.24	0.56	24.88	0.71	0.27	37.52	0.32	2.66E-04
Monocyte differentiation antigen CD14	11	0.06	0.07	117.05	4.48	1.02	22.80	79.69	6.53E-08
Murinoglobulin-1	33	27.53	3.54	12.86	17.29	1.65	9.56	0.63	1.80E-04
Murinoglobulin-2	2	20.39	1.64	8.05	12.98	1.85	14.21	0.64	2.88E-04
N-acetylmuramoyl-L-alanine amidase	2	0.90	0.17	18.37	0.44	0.16	35.67	0.48	1.55E-03
Neutrophil gelatinase-associated lipocalin	6	0.18	0.05	25.60	2.73	0.35	12.91	15.38	3.81E-09
Peroxiredoxin-2	2	0.23	0.30	126.91	0.22	0.23	104.80	0.94	9.51E-01
Phosphatidylcholine-sterol acyltransferase	5	0.89	0.20	22.32	1.92	0.73	38.24	2.15	6.66E-03
Phosphatidylinositol-glycan-specific phospholipase D	8	0.94	0.17	17.80	0.20	0.07	35.09	0.21	7.41E-06
Plasma kallikrein	12	2.37	0.46	19.50	0.88	0.30	33.87	0.37	2.42E-04
Plasma protease C1 inhibitor	14	3.09	0.28	8.94	5.65	0.33	5.79	1.83	1.61E-06
Plasminogen	48	14.09	1.68	11.89	11.16	1.81	16.19	0.79	2.76E-02
Pregnancy zone protein	74	70.53	6.76	9.59	58.21	6.41	11.01	0.83	1.75E-02
Properdin	5	15.57	2.10	13.50	11.53	1.06	9.20	0.74	3.88E-03
Prosaposin	5	0.06	0.01	9.10	0.27	0.13	47.26	4.19	6.26E-03
Protein AMBP	8	6.28	1.06	16.92	8.86	2.10	23.68	1.41	3.86E-02
Protein Z-dependent protease inhibitor	7	0.89	0.14	16.21	2.82	0.51	18.22	3.18	5.73E-06
Proteoglycan 4	3	0.00	0.00	83.59	0.52	0.11	20.83	1320.59	2.48E-06
Prothrombin	35	6.56	0.46	6.95	6.56	1.00	15.17	1.00	9.29E-01
Retinol-binding protein 4	5	2.40	0.20	8.18	0.20	0.08	40.14	0.08	2.17E-09
Serine protease inhibitor A3K	16	81.33	14.99	18.43	102.42	16.80	16.40	1.26	6.98E-02

Serine protease inhibitor A3M	6	0.47	0.14	29.24	2.09	0.54	25.68	4.41	3.22E-05
Serine protease inhibitor A3N	18	2.20	0.20	9.05	28.22	7.23	25.63	12.85	3.04E-08
Serotransferrin	69	165.38	18.69	11.30	127.82	20.61	16.12	0.77	1.41E-02
Serum albumin	75	641.07	86.07	13.43	493.57	77.28	15.66	0.77	2.16E-02
Serum amyloid A-1 protein	6	0.26	0.27	105.06	44.73	13.42	29.99	172.06	1.06E-08
Serum amyloid A-2 protein	7	0.35	0.16	46.48	33.82	11.61	34.34	95.72	2.66E-08
Serum amyloid A-3 protein	5	0.39	0.21	53.68	11.81	4.52	38.31	30.22	2.92E-07
Serum amyloid A-4 protein	7	1.00	0.19	19.35	4.09	0.45	11.05	4.09	2.39E-07
Serum amyloid P-component	9	6.90	1.75	25.38	32.22	5.70	17.68	4.67	4.44E-06
Serum paraoxonase/arylesterase 1	7	3.61	0.43	11.89	3.16	0.88	27.78	0.87	2.80E-01
Sulfhydryl oxidase 1	12	0.96	0.08	8.18	2.26	0.39	17.01	2.35	1.12E-05
Transthyretin	11	19.11	4.16	21.75	9.78	1.52	15.55	0.51	5.89E-04
Trypsin	4	30.05	6.16	20.51	26.24	4.27	16.28	0.87	2.95E-01
Vascular non-inflammatory molecule 3	10	0.55	0.06	11.37	2.85	1.01	35.26	5.22	3.53E-05
Vitamin D-binding protein	34	26.20	3.44	13.14	23.70	5.25	22.13	0.90	3.46E-01
Vitronectin	11	5.80	0.37	6.34	7.54	1.46	19.33	1.30	2.66E-02
Zinc-alpha-2-glycoprotein	10	2.37	0.16	6.57	1.42	0.18	12.76	0.60	3.34E-05

Supplemental Table 2: Plasma proteins from infected mice detected by mass spectrometry analysis that were significant upregulated, compared to healthy mice

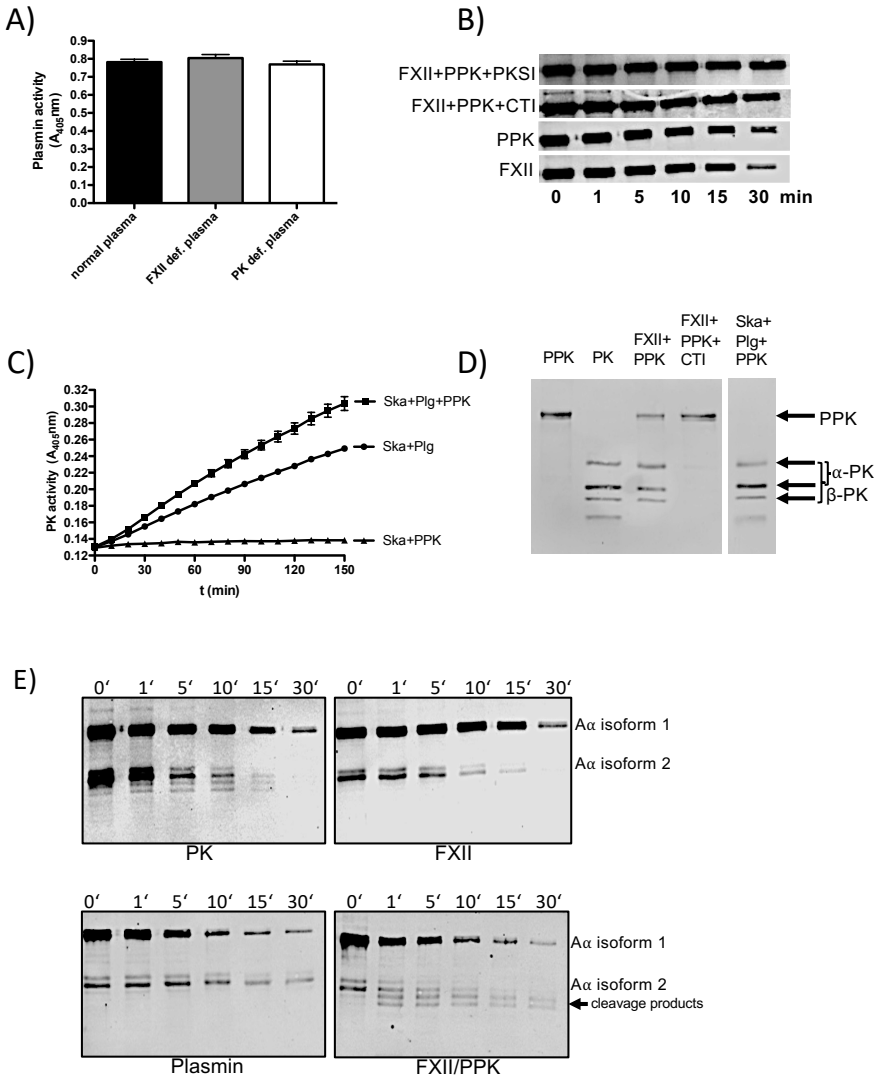
Accession	Protein	Fold Change	Anova (p)
Q9JM99	Proteoglycan 4	1320,59	2,48E-06
P05366	Serum amyloid A-1 protein	172,06	1,06E-08
P05367	Serum amyloid A-2 protein	95,72	2,66E-08
P10810	Monocyte differentiation antigen CD14	79,69	6,53E-08
Q61805	Lipopolysaccharide-binding protein	40,77	2,45E-04
P04918	Serum amyloid A-3 protein	30,22	2,92E-07
P07361	Alpha-1-acid glycoprotein 2	17,80	1,85E-09
P11672	Neutrophil gelatinase-associated lipocalin	15,38	3,81E-09
P05064	Fructose-bisphosphate aldolase A	14,84	1,09E-02
Q61646	Haptoglobin	13,46	1,55E-09
Q91WP6	Serine protease inhibitor A3N	12,85	3,04E-08
Q60590	Alpha-1-acid glycoprotein 1	8,26	2,71E-07
P14847	C-reactive protein	6,74	1,83E-07
A6X935	Inter alpha-trypsin inhibitor_ heavy chain 4	6,19	3,42E-06
Q9QZ25	Vascular non-inflammatory molecule 3	5,22	3,53E-05
P12246	Serum amyloid P-component	4,67	4,44E-06
Q03734	Serine protease inhibitor A3M	4,41	3,22E-05
Q61207	Prosaposin	4,19	6,26E-03
Q91X72	Hemopexin	4,10	4,64E-07
P31532	Serum amyloid A-4 protein	4,09	2,39E-07
Q6GQT1	Alpha-2-macroglobulin-P	3,65	1,93E-02
Q9JHH6	Carboxypeptidase B2	3,44	4,95E-08
Q8R121	Protein Z-dependent protease inhibitor	3,18	5,73E-06
Q61704	Inter-alpha-trypsin inhibitor heavy chain H3	3,07	8,08E-07
Q8K0E8	Fibrinogen beta chain	2,84	2,52E-06
Q61147	Ceruloplasmin	2,79	7,16E-05
E9PV24	Fibrinogen alpha chain	2,75	6,06E-07
P08226	Apolipoprotein E	2,59	1,57E-04
Q8VCM7	Fibrinogen gamma chain	2,55	6,78E-08
Q8BND5	Sulfhydryl oxidase 1	2,35	1,12E-05
P01878	Ig alpha chain C region	2,31	3,08E-03
P60710	Actin_ cytoplasmic 1	2,29	2,73E-04
P16301	Phosphatidylcholine-sterol acyltransferase	2,15	6,66E-03
P28798	Granulins	1,97	2,81E-03
P04186	Complement factor B	1,86	2,39E-04
P97290	Plasma protease C1 inhibitor	1,83	1,61E-06
P06728	Apolipoprotein A-IV	1,64	1,30E-03
P06909	Complement factor H	1,52	5,62E-04

Supplemental Figures:



suppl. Figure 1: Treatment with PPK- or FXII-ASO s reduced target mRNA expression in the liver and inhibited contact system activation.

Groups (n=4-7) of mice were intraperitoneal treated with PPK- or FXII-ASO (800 µg/mouse) two times a week over a period of 3 weeks. A,B) Liver tissue was taken for quantification of Klkb1 and F12 mRNA levels. Man Whitney test, **p<0.01 C, D) PPK and FXII immunoblot analysis on 3 individual samples E,F) Quantification of PPK and FXII relative plasma protein levels by densitometry. G) Plasma from 4 mice/group were pooled and proteolytic potential of PK/FXIIa was measured using chromogenic substrate S-2302. H) aPTT in plasma of treated mice were measured in a coagulometer (*p<0.05, **p<0.01)



suppl. Figure 2 : Plasmin activity, fibrinogen degradation and PPK activation induced by streptokinase

A) Plasmin activity in plasma was determined 60 minutes after addition of streptokinase. B) Western Blot analysis of fibrinogen incubated for up to 30 minutes with PPK, FXII, FXII/PK (+PKSI or CTI). C) Streptokinase (10 U) was incubated with 100 nM PPK (Ska+PPK), or 100 nM plasminogen (Ska+Plg) or a combination of Plg and PPK (Ska+Plg+PPK) in the presence of 3 µg fibrinogen. PK activity was measured using the Subtrat S-2302 for PK. D) Western Blot analysis of PPK and PK. PPK was incubated for 10 minutes with FXII (FXII+PPK), and CTI (FXII+PPK+CTI), or with plasminogen and streptokinase (Ska+Plg+PPK). All samples were incubated in the presence of fibrinogen. E) Western Blot analysis of fibrinogen incubated for up to 30 minutes with PK, FXII, FXII/PPK, or plasmin. The antibody detects the alpha chain of fibrinogen.

References:

1. Oehmcke S, Shannon O, Köckritz-Blickwede von M, et al. Treatment of invasive streptococcal infection with a peptide derived from human high-molecular weight kininogen. *Blood*. 2009;114(2):444–451.
2. Oehmcke S, Westman J, Malmström J, et al. A novel role for pro-coagulant microvesicles in the early host defense against streptococcus pyogenes. *PLoS Pathog.* 2013;9(8):e1003529.
3. Loof TG, Mörgelin M, Johansson L, et al. Coagulation, an ancestral serine protease cascade, exerts a novel function in early immune defense. *Blood*. 2011;118(9):2589–2598.
4. Pappesch R, Warnke P, Mikkat S, et al. The Regulatory Small RNA MarS Supports Virulence of Streptococcus pyogenes. *Sci Rep*. 2017;7(1):12241.
5. Oehmcke-Hecht S, Nass LE, Wichura JB, et al. Deletion of the L-Lactate Dehydrogenase Gene ldh in Streptococcus pyogenes Leads to a Loss of SpeB Activity and a Hypovirulent Phenotype. *Front Microbiol*. 2017;8:1841.