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(71) Applicant (for all designated States except US): **NOVO NORDISK A/S** [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **HILDEN, Ida** [DK/DK]; Novo Nordisk A/S, Corporate Patents, Novo Alle, DK-2880 Bagsvaerd (DK). **PESCHKE, Bernd** [DE/DK]; Novo Nordisk A/S, Corporate Patents, Novo Alle, DK-2880 Bagsvaerd (DK). **BREINHOLT, Jens** [DK/DK]; Novo Nordisk A/S, Corporate Patents, Novo Alle, DK-2880 Bagsvaerd (DK). **KOFOD-HANSEN, Mikael** [DK/DK]; Novo Nordisk A/S, Corporate Patents, Novo Alle, DK-2880 Bagsvaerd (DK).

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(54) Title: COAGULATION FACTOR-TARGETING TO TLT-1 ON ACTIVATED PLATELETS

(57) Abstract: The current invention relates to: procoagulant proteins which may, for example, be fusion proteins or chemical conjugates; methods of producing said procoagulant proteins; polynucleotides that encode said fusion proteins and cells that expresses them. Furthermore, the current invention relates to procoagulant proteins for use as a medicament. Individuals that have a coagulopathy, such as haemophilia A and B with or without inhibitors, may be treated with the procoagulant proteins of the current invention.



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TITLE

Coagulation factor-targeting to TLT-1 on activated platelets

FIELD OF THE INVENTION

5 The current invention relates to procoagulant proteins, polynucleotides that encode procoagulant fusion proteins, cells that express procoagulant fusion proteins, a process for preparing procoagulant proteins and uses of said procoagulant proteins.

BACKGROUND OF THE INVENTION

10 Normal resting platelets freely flow throughout the blood circulation when the endothelium is intact. When the single-layered endothelial barrier is damaged, resting platelets adhere to subendothelial structures by means of glycoprotein (GP) receptors. For example, GPIaIIa and GPVI bind collagen; GPIcIIa binds fibronectin; GPIcIIa binds laminin and GPIb-V-IX binds von Willebrand Factor (vWF) polymers. Adhesion to the ex-

15 travascular tissue components exposed following a vessel injury in conjunction with the influence of factors produced locally at the site of injury, e.g. the serine protease thrombin, lead to activation of the platelets. In the complex process of activation, platelets change shape and expose certain phospholipids on their surface. Also, receptors already present on the platelet surface in the resting state become activated upon platelet activation. Additionally, platelet activation leads to the release and surface exposure of molecules which in the resting state are stored intracellularly in alpha and dense granules and

20 thus not present on the surface of platelets in the resting state. GPIIbIIIa is an example of a platelet receptor present on the surface of both resting and activated platelets. GPIIbIIIa exists on resting platelets in a closed and inactive conformation and during platelet activation assumes an open and active conformation capable of binding its ligands, including fibrinogen and fibrin. An example of a receptor stored intracellularly in

25 alpha granules in resting platelets, but released and exposed on the surface of the activated platelet is TREM-like transcript 1 (TLT-1) (Washington et al., Blood, 104, 1042–1047 (2004), Gattis et al., Journal of Biological Chemistry, 281, 13396–13403 (2006)) to which the present application relates.

30 The blood coagulation cascade is initiated when tissue factor (TF) bearing cells in the subendothelium are exposed to components circulating in the blood. Exposure of TF to circulating coagulation factor VIIa (FVIIa) triggers the formation of small amounts of thrombin, which serves as a procoagulant signal leading to further recruitment and activation of platelets adhered to the site of injury. The coagulation is further propagated and amplified on the surface of the activated platelets, eventually leading to a burst of

thrombin generation, which in turn lead to activation and polymerization of fibrinogen to fibrin fibers, cross-linking and stabilizing the haemostatic clot. A feature attributed to several of the components of the coagulation cascade is their ability to specifically associate with the phospholipid membrane of activated platelets. To this end, FVIIa as well as e.g. coagulation factors IX and X (FIX and FX, respectively) and their corresponding activated forms (FVIIa, FIXa and FXa, respectively), possess a γ -carboxyglutamic acid rich region (Gla domain) which enables them to be directed and bind to the surface of activated platelets. Coagulation factor VIII (FVIII) is associated with activated platelets by binding via its light chain. The mechanism of coagulation factor XI (FXI) binding to platelets is more controversial but growing evidence suggests that platelets affect FXI and FXIa and that binding of FXI to platelets requires residues in the FXI A3 domain (Emsley et al., 2010, Blood, Vol. 115, p. 2569).

Membrane binding strongly enhances the activity of coagulation factors such as FVIIa. However, their interactions with platelet membranes are of varying affinity. For example, the binding constant (K_D) for FVIIa to the platelet surface is in the low micromolar range. Improved platelet binding and localisation of the coagulation factors to the activated platelet surface may enhance their activity. A means of doing so is thus desirable.

In subjects with a coagulopathy, such as human beings with haemophilia A, B or C, various steps of the coagulation cascade are rendered dysfunctional due to, for example, the absence or insufficient presence of a functional coagulation factor. Such dysfunction of one part of coagulation results in insufficient blood coagulation leading to spontaneous bleeds e.g. in joints and potentially life-threatening bleeding.

An object of the current invention is to provide a compound that is suitable for use as a procoagulant drug in such subjects. A second object of the current invention is to provide procoagulant molecules that have increased activity, compared to the coagulation factors from which they derive. A third object of the current invention is to provide molecules that up-regulate blood coagulation in a physiologically suitable microenvironment. A fourth object of the current invention is to provide procoagulant molecules that have longer half lives than the coagulation factor from which they derive. A fifth object of the current invention is to provide procoagulant molecules that do not give rise to a drop in platelet count. A further object of the current invention is to direct a coagulation factor to the surface of activated platelets. One particular object of the invention is to enhance the generation of FIXa and/or FXa on the surface of the activated platelet. Thus, the object is to enable the initiation of blood coagulation on the surface of activated platelets that are located intravascularly or extravascularly.

WO06/096828 discloses chimeric proteins that comprise soluble tissue factor (sTF) and a phosphatidyl serine (PS) binding domain, such as Annexin V. PS is exposed on the surface of activated cells, such as monocytes, endothelial cells and cells undergoing apoptosis, as well as on activated and resting platelets. The chimeric proteins are both pro-coagulant and anti-coagulant; the latter due to the fact that, in higher doses, constructs compete with coagulation factors in binding to PS on activated platelets. Thus, the chimeric proteins of WO06/096828 have a different set of properties than the procoagulant proteins described herein.

SUMMARY OF THE INVENTION

The procoagulant proteins of the present invention are specifically targeted to activated platelets, present at sites of injury. Proteins of the present invention are based upon the identification of particular receptors and component epitopes that appear on platelet membranes when platelets are no longer resting but are activated or in the process of being activated. Thus, the invention relates to a method of enhancing coagulation on the surface of activated platelets.

Procoagulant proteins of the invention comprise (i) at least one coagulation factor, covalently attached to (ii) an antibody or a fragment thereof that is capable of binding (iii) a receptor, and/or a fragment or variant thereof, which is exposed on the surface of activated platelets and is exposed to a lesser degree (and in some assays not detectably exposed) on the surface of resting platelets. TLT-1 is an example of such a receptor and procoagulant proteins may, for example, bind, to TLT-1 (16-162), TLT-1 (20-125) or TLT-1 (126-162). The coagulation factor may be a serine protease in the zymogen form, e.g., FVII, FIX or FX or the corresponding activated form FVIIa, FIXa, and FXa, or a derivative of a serine protease; FV or a derivative thereof, FVIII or a derivative thereof or FXI or a derivative thereof. Coagulation factor and antibody or antibody fragment are optionally joined by means of a linker. Procoagulant proteins of the invention may be fusion proteins or chemical conjugates. Hence, the invention also relates to their manufacture. One process for preparing a composition that comprises at least a procoagulant protein of the invention involves chemically conjugating (i), a TLT-1 antibody or fragment thereof, with one reactive group (RS1) of a linker and reacting (ii), the coagulation factor, with another reactive group (RS2) of said linker.

In this case, said linker may be a polymer, such as polyethylene glycol (PEG).

The current invention also provides the following: an isolated nucleotide sequence that encodes any one of the procoagulant proteins according to the current invention; a vector that comprises an isolated nucleotide sequence that, in turn, encodes

any one of the procoagulant proteins according to the current invention; an isolated cell that comprises a nucleotide sequence that encodes any one of the procoagulant proteins of the current invention. Said nucleotide sequence may, in turn, be expressed by an intracellular vector. Said isolated cell may be a eukaryotic cell, such as a mammalian cell,
 5 such as a BHK or a CHO or a HEK cell.

Similarly, the invention relates to a procoagulant protein for use as a medicament and for the treatment of a coagulopathy. In one embodiment, a therapeutically effective amount of said protein is parenterally administered, such as intravenously or subcutaneously administered, to an individual in need thereof. Such
 10 individual in need may have any congenital, acquired and/or iatrogenic coagulopathy.

DESCRIPTION OF THE DRAWINGS

Figure 1: Human TLT-1 nucleotide and amino acid sequences. In Figure 1, the nucleotide- and amino acid sequences representing hTLT-1 are shown. Here, nucleotide sequence position 1-45 encode the predicted signal peptide, the nucleotide sequence at
 15 position 46-486 encode the extracellular domain of hTLT-1, the nucleotide sequence position 487-555 encode the transmembrane region and the nucleotide sequence at position 556-933 encode the intracellular domain of hTLT-1.

Figure 2: Nucleotide and amino acid sequences representing the extracellular domain of human TLT-1 containing a C-terminal His-6 tag. In Figure 2, the nucleotide- and amino acid sequences representing the extracellular domain of hTLT-1 with a C-terminal His tag are shown. Here, the underlined sequence (7-15) indicates the positions of a kozak sequence, the nucleotide sequence at position 16-60 encodes the predicted signal peptide, the nucleotide sequence at position 61-501 encode the extracellular domain of hTLT-1 and the nucleotide sequence at position 502-519 encodes the 6xHis tag (in bold). The restriction enzyme sites HindIII (nucleotide sequence at position 1-6) and EcoRI (nucleotide sequence at position 523-528) are also shown and the stop codon is marked with an asterisk (520-522).
 20

Figure 3A-D: The variable domain of 0012LC and 0012HC including Kabat numbering: 3A) The variable domain of 0012LC; 3B) The variable domain of 0012LC – Kabat numbering; 3C) The variable domain of 0012HC; 3D) The variable domain of 0012HC – Kabat numbering. In Figure 3A, the nucleotide sequence at position 1-57 encodes the LC signal peptide sequence; the nucleotide sequences at position 58-396 encodes the variable domain of 0012LC. In Figure 3B, the sequences in bold and grey represent positions of the 0012LC CDRs according to Kabat numbering. In Figure 3C, the
 30 nucleotide sequence at position 1-54 encodes the 0012HC signal peptide, the nucleotide
 35

sequence at position 55-396 encodes the variable domain of 0012HC. In Figure 3D, the sequences in bold and grey represent positions of the 0012HC CDRs according to Kabat numbering.

Figure 4: The variable domain of 0012LC together with constant region of human LC,kappa and a HPC4 tag (encoded by pTT-0012LC.HPC4). In Figure 4, the nucleotide sequences at position 1-57 encodes the LC signal peptide, the nucleotide sequence 58-396 encodes the variable domain of 0012LC, the nucleotide sequence 397-714 encodes the constant region of human 0012LC, kappa and the nucleotide sequence 715-750 encodes a HPC4 tag.

Figure 5: The variable domain of 0012HC together with the constant region of human IgG4 (pTT-0012HC). In Figure 5, the nucleotide sequence 1-54 encodes the HC signal peptide, the nucleotide sequence 55-396 encodes the variable domain of 0012HC and the nucleotide sequence 397-1377 encodes the constant region of human IgG4 (in bold).

Figure 6: Sequence coverage of HX analyzed peptides of TLT-1 in the presence and absence of mAb0023. The primary sequence of hTLT-1 is displayed above the HX analyzed peptides (shown as horizontal bars). Peptides showing similar exchange patterns both in the presence and absence of 0023 are displayed in white whereas peptides showing reduced deuterium incorporation upon 0023 binding are coloured black.

Figure 7: Sequence coverage of HX analyzed peptides of TLT-1 in the presence and absence of mAb0051. The primary sequence of hTLT-1 is displayed above the HX analyzed peptides (shown as horizontal bars). Peptides showing similar exchange patterns both in the presence and absence of 0051 are displayed in white whereas peptides showing reduced deuterium incorporation upon 0051 binding are coloured black.

Figure 8: Sequence coverage of HX analyzed peptides of TLT-1 in the presence and absence of mAb0062. The primary sequence of hTLT-1 is displayed above the HX analyzed peptides (shown as horizontal bars). Peptides showing similar exchange patterns both in the presence and absence of 0062 are displayed in white whereas peptides showing reduced deuterium incorporation upon 0062 binding are coloured black.

Figure 9: Sequence coverage of HX analyzed peptides of TLT-1 in the presence and absence of mAb0061 (mAb0012). The primary sequence of hTLT-1 is displayed above the HX analyzed peptides (shown as horizontal bars). Peptides showing similar exchange patterns both in the presence and absence of 0061 are displayed in white whereas peptides showing reduced deuterium incorporation upon 0061 binding are coloured black.

Figure 10: Sequence coverage of HX analyzed peptides of TLT-1 region 126-162. The primary sequence of hTLT-1 is displayed above the HX analyzed peptides (shown as horizontal bars). All peptides showed reduced deuterium incorporation upon mAb0061 (mAb0012) binding.

Figure 11A: Effect of FVIIa-Fab1029 on TF-induced fibrin clot formation in human whole blood (HWB) from a normal donor measured by thromb-elastography (TEG). Clot formation in re-calcified HWB (curve HWB) is induced by 0.03 pM TF (Innovin®). The curve "Hemophilia" shows the delayed and inadequate clot formation when HWB is supplemented with 10 µg/ml anti-FVIII antibody. Other curves show the curves obtained when the hemophilia-like condition induced by the FVIII antibody is reverted by various concentrations (0; 0.25; 0.5; 1.0 nM) of either FVIIa-Fab1029 or rFVIIa as indicated. The FVIII bypassing activity of FVIIa-Fab1029 is shown to be more potent than that of rFVIIa.

Figure 11B: Effect of FVIIa-Fab1029 on TF-induced fibrin clot formation in human whole blood (HWB) from a normal donor measured by thromb-elastography (TEG). R-time determined from the TEG traces of the experiment shown in Figure 11A.

Citrated-stabilized human whole blood (HWB) is drawn from normal donors. Hemophilia-like conditions are obtained by incubation of HWB with 10 µg/ml anti-FVIII antibody (Sheep anti-Human Factor VIII; Hematologic Technologies Inc) for 30 min at room temp. Clot formation is measured by thrombelastography (5000 series TEG analyzer, Haemoscope Corporation, Niles, IL, USA). Various concentrations (0; 0.25; 0.5; 1.0 nM) of FVIIa-Fab1029 or rFVIIa are added to hemophilia-like citrated HWB. Clotting is initiated when 340 µl of normal or hemophilia-like HWB is transferred to a thrombelastograph cup containing 20 µl 0.2 M CaCl₂ with 0.03 pM lipidated TF (Innovin®, Dade Behring GmbH (Marburg, Germany)). The TEG trace is followed continuously for up to 120 min. The following TEG variables are recorded: R time (clotting time i.e. the time from initiation of coagulation until an amplitude of 2 mm was obtained), α-angle (clot development measured as the angle between the R value and the inflection point of the TEG trace), K (speed of clot kinetics to reach a certain level of clot strength, amplitude = 20 mm), and MA (maximal amplitude of the TEG trace reflecting the maximal mechanical strength of the clot).

Figure 12: Effect of FIX-Fab0135 on TF-induced fibrin clot formation in human whole blood (HWB) from a normal donor measured by thromb-elastography (TEG). Clot formation in re-calcified HWB (curve HWB) is induced by 0.03 pM TF (Innovin®). The curve "Hemophilia" shows the delayed and inadequate clot formation when HWB is supplemented with 10 µg/ml anti-FVIII antibody. Other curves show TEG traces obtained

when the hemophilia A-like condition induced by the FVIII antibody is reverted by various concentrations (0.1; 0.2; 1.0; 5.0; 10 nM) of FIX-Fab0135. TEG curves obtained when FIX fusion protein is replaced by 1 nM rFVIIa or 10 nM rFIX are shown for comparison.

Figure 13: FIX-Fab0135 targeted to TLT-1 enriched phospholipid vesicles

markedly promotes FX activation induced by FVIIa in absence of FVIII. Various concentrations (0.05 – 100 nM) of rFVIIa are incubated for 15 min in Hepes buffer (○) or in Hepes buffer containing 10 nM FIX (□); 10 nM FIXa (■) or 10 nM FIX-Fab0135 (▲). This is then mixed and incubated for 3 min with 100 nM FX and TLT-1 enriched phospholipid vesicles at 1:4,000 dilution in absence or presence of 5 nM FVIII as indicated. The reaction is stopped with EDTA, and FXa generation is measured in a chromogenic assay.

Figure 14: TLT-1 expression on human platelets. The figure shows the TLT-1 number on platelets in whole blood gated on forward and side scatter in flow cytometry analysis. The mean fluorescence within the platelet gate was used to calculate the TLT-1 number using a standard curve obtained from beads with a definite number of binding sites for the detecting antibody. The platelets were activated with protease activated receptor-1 activating peptide SFLLRN (0.3-30 μ M). To ensure maximum TLT-1 expression a combination of SFLLRN (30 μ M) and Convulxin (Cvxln) (100 ng/ml) was used as control. TLT-1 expression was measured by two different anti-TLT-1 antibodies mAb0136; left bar graph and mAb0123; right bar graph. Data are presented as mean $\pm$ sem with the number of blood donors ranging from 2 to 4 in the different groups.

Figure 15: FVIIa-Fab9015 dose-dependently reduced bleeding time (left) and blood loss (right) in TLT-1 KOKI mice with antibody induced haemophilia compared to haemophilic mice receiving vehicle. A dose of 20 nmol/kg FVIIa-Fab9015 was significantly more effective compared to 20 nmol/kg rFVIIa.

Figure 16: Platelet counts in transient haemophilic TLT-1 KOKI mice dosed with FVIIa-Fab9015 (20 or 4 nmol/kg), rFVIIa (20 nmol/kg) or vehicle. The mice were dosed at -5 min, bleeding was observed from 0 to 30 min, and platelet counts were observed for 120 min after induction of bleeding.

Figure 17A: The bar-graph show peak amount thrombin generated by 25 nM of either rFVIIa (unfilled bar) or FVIIa-Fab9015 (filled bar). The thrombin generation was measured under induced haemophilia A conditions (sheep anti-human FVIII polyclonal antibodies) with a fixed platelet concentration of 150000 plts/ μ l. The reaction was started with platelet activation through the addition of PAR-1 activating peptide SFLLRN (30 μ M) and the GPIV agonist Convulxin (100 ng/ml).

Figure 17B: The bar-graph show peak amount thrombin generated by 5 nM of either rFVIIa (unfilled bar) or FVIIa-Fab9015 (filled bar). The thrombin generation was measured under haemophilia A conditions with a fixed platelet concentration of 150000 plts/ μ l. The reaction was started with platelet activation through the addition of PAR-1 activating peptide SFLLRN (30 μ M) and the GPIV agonist Convulxin (100 ng/ml).

Figure 18: The figure shows that the effect of FVIIa-Fab9015 was dependent on platelet activation. 25 nM FVIIa-Fab9015 was added to human platelet rich plasma (150000 plts/ μ l) made haemophilia A like though addition of sheep anti-human FVIII antibodies. Samples with unactivated platelets show poor thrombin generation (dotted line), PAR-1 activating peptide SFLLRN (10 μ M) gives some increase in thrombin generation (broken line) and platelets activated with both SFLLRN (30 μ M) and the GPVI agonist Convulxin (100 ng/ml) gives the largest thrombin generation (solid line).

Figure 19: The figure shows that the increase in thrombin generation with FVIIa-Fab9015 compared to rFVIIa was TLT-1 dependent. The original traces show thrombin generation in human platelet rich plasma (150000 plts/ μ l) made haemophilic A like through the addition of sheep anti-human FVIII polyclonal antibodies. Thrombin generation was started by the addition of PAR-1 activating peptide SFLLRN (30 μ M) and GPVI agonist Convulxin (100 ng/ml). As shown by the traces, FVIIa-Fab9015 (5 nM) (solid line) gives a significant larger thrombin peak than rFVIIa (5 nM) (dotted line). By pre-incubating the samples with soluble TLT-1 (100 nM) the FVIIa-Fab9015 thrombin generating capacity was significantly reduced (broken line). This confirms that the 9015 effect is dependent on TLT-1 binding on the activated platelet.

Figure 20: The figure shows that the increase in thrombin generation with FIX-Fab0155 compared to rFIX was TLT-1 dependent. The original traces show thrombin generation in FIX deficient plasma containing human platelets (150000 plts/ μ l). Thrombin generation was started by the addition of PAR-1 activating peptide SFLLRN (30 μ M) and GPVI agonist Convulxin (100 ng/ml). As shown by the traces, 1 nM FIX-Fab0155 (solid line) gives a significant larger thrombin peak than rFIX (grey line). Pre-incubation of soluble TLT-1 (100 nM) significantly reduced the thrombin generation capacity of FIX-Fab0155 (broken line) confirming TLT-1 dependency.

Figure 21: Autoactivation of FVII-Fab5001 fusion protein in the presence of soluble Tissue Factor (sTF). Proteolytic activity was measured in the presence of phospholipids (PC:PS) and sTF. Results are averages of two independent measurements and given in relative k_{cat}/K_m values compared to wtFVIIa.

Figure 22A: None of the TLT-1 antibodies (mAb0023, mAb0051, mAb0061, mAb0062) affect platelet aggregation. The figure shows original traces from light

transmission measurements in platelet rich plasma where the platelets have been activated with SFLLRN (1 μ M). The samples were pre-incubated with anti-TLT-1 antibody or irrelevant control antibody (10 nM) 3 min before platelet activation.

Figure 22B: None of the TLT-1 antibodies (mAb0023, mAb0051, mAb0061, mAb0062) affects platelet aggregation. The figure shows original traces from light transmission measurements in platelet rich plasma in which the platelets have been activated with SFLLRN (10 μ M). The samples have been pre-incubated with anti-TLT-1 antibody or irrelevant control antibody (10 nM) 3 min before platelet activation

Figure 23: Effect of FIX-mAb0145 on TF-induced fibrin clot formation in human whole blood (HWP) from a normal donor measured by thromb-elastography (TEG). The R time obtained from TEG traces with normal HWP and "hemophilia" blood supplemented with various concentrations of FIX-mAb0145 or rFIX are shown.

Figure 24: The bar-graph show peak amount thrombin generated by 25 nM of the FVIIa-Fab9015, FVIIa-Fab1029 and FVIIa-Fab1001. All three constructs showed increased potency compared to rFVIIa (25 nM). The thrombin generation was measured under induced haemophilia A conditions (sheep anti-human FVIII polyclonal antibodies) with a fixed platelet concentration of 150000 plts/ μ l. The reaction was started with platelet activation through the addition of PAR-1 activating peptide SFLLRN (30 μ M) and the GPIV agonist Convulxin (100 ng/ml). The bars show mean $\pm$ SD of peak thrombin generation (n=2-5).

Figure 25: The figure shows that increased potency of the FVIIa-Fab5001 compared to wild-type rFVIIa. The original traces show thrombin generation in factor VIII deficient plasma containing washed human platelets (150000 plts/ μ l). In the activated samples thrombin generation was started by the addition of the PAR-1 activating peptide SFLLRN (30 μ M) and the GPVI agonist Convulxin (100 ng/ml). As shown by the traces FVIIa-Fab5001 (25 nM) (solid line) gives approximately a four fold larger thrombin peak than wild-type rFVIIa (25 nM) (dotted line). There was no enhancement of the potency with unactivated platelets (broken line).

SEQUENCES

The sequences are as follows:

SEQ ID NO: 1 provides the nucleotide sequence of human (h)TLT-1.

SEQ ID NO: 2 provides the amino acid sequence of hTLT-1.

SEQ ID NO: 3 provides the nucleotide sequence of the extracellular domain of hTLT-1-His6.

SEQ ID NO: 4 provides the amino acid sequence of the extracellular domain of hTLT-1-His6.

SEQ ID NOs: 5 to 8 provide the amino acid sequences of hTLT-1 fragments: hTLT-1.20-125, hTLT-1.16-162, hTLT-1.126-162 and hTLT-1.129-142.

5 SEQ ID NO: 9 provides the nucleotide sequence of the variable domain of mAb 0012 LC.

SEQ ID NO: 10 provides the amino acid sequence of the variable domain of mAb0012 LC.

10 SEQ ID NO: 11 provides the nucleotide sequence of the variable domain of 0012 HC.

SEQ ID NO: 12 provides the amino acid sequence of the variable domain of 0012 HC.

SEQ ID NO: 13 provides the nucleotide sequence of the heavy chain of mAb0012.

15 SEQ ID NO: 14 provides the nucleotide sequence of the light chain of mAb0012 and Fab0012.

SEQ ID NO: 15 provides the nucleotide sequence of the heavy chain of mAb0023.

20 SEQ ID NO: 16 provides the nucleotide sequence of the light chain of mAb0023 and Fab0023.

SEQ ID NO: 17 provides the nucleotide sequence of the heavy chain of mAb0051.

SEQ ID NO: 18 provides the nucleotide sequence of the light chain of mAb0051 and Fab0051.

25 SEQ ID NO: 19 provides the nucleotide sequence of the heavy chain of mAb0052.

SEQ ID NO: 20 provides the nucleotide sequence of the heavy chain of mAb0062.

30 SEQ ID NO: 21 provides the nucleotide sequence of the light chain of mAb0052, Fab0052 and mAb0062.

SEQ ID NO: 22 provides the nucleotide sequence of the heavy chain of mAb0061.

SEQ ID NO: 23 provides the nucleotide sequence of the heavy chain of mAb0082.

35 SEQ ID NO: 24 provides the nucleotide sequence of the light chain of mAb0061, Fab0061, mAb0082 and Fab0082.

SEQ ID NO: 25 provides the nucleotide sequence of Fab0012 VH-CH1.

SEQ ID NO: 26 provides the nucleotide sequence of Fab0023 VH-CH1.

SEQ ID NO: 27 provides the nucleotide sequence of Fab0051 VH-CH1.

SEQ ID NO: 28 provides the nucleotide sequence of Fab0052 VH-CH1.

5 SEQ ID NO: 29 provides the nucleotide sequence of Fab0061 VH-CH1.

SEQ ID NO: 30 provides the nucleotide sequence of Fab0082 VH-CH1.

SEQ ID NO: 31 provides the nucleotide sequence of hIgG4 hinge-CH2-CH3.

SEQ ID NO: 32 provides the amino acid sequence of mAb0012, HC (mouse VH-human IgG4 CH1-CH2-CH3).

10 SEQ ID NO: 33 provides the amino acid sequence of mAb0012, LC (mouse VL - human Kappa CL) and Fab0012, LC (mouse VL - human Kappa CL).

SEQ ID NO: 34 provides the amino acid sequence of mAb0023, HC (mouse VH-human IgG4 CH1-CH2-CH3).

15 SEQ ID NO: 35 provides the amino acid sequence of mAb0023, LC (mouse VL - human Kappa CL) and Fab0023, LC (mouse VL - human Kappa CL).

SEQ ID NO: 36 provides the amino acid sequence of mAb0051, HC (mouse VH-human IgG4 CH1-CH2-CH3).

SEQ ID NO: 37 provides the amino acid sequence of mAb0051, LC (mouse VL - human Kappa CL) and Fab0051, LC (mouse VL - human Kappa CL).

20 SEQ ID NO: 38 provides the amino acid sequence of mAb0052, HC (mouse VH-human IgG4 CH1-CH2-CH3).

SEQ ID NO: 39 provides the amino acid sequence of mAb0052, LC (mouse VL - human Kappa CL); Fab0052, LC (mouse VL - human Kappa CL); mAb0062, LC (mouse VL - human Kappa CL).

25 SEQ ID NO: 40 provides the amino acid sequence of mAb0061, HC (mouse VH-human IgG4 CH1-CH2-CH3).

SEQ ID NO: 41 provides the amino acid sequence of mAb0061, LC (mouse VL - human Kappa CL); Fab0061, LC (mouse VL - human Kappa CL) and mAb0082, LC (mouse VL - human Kappa CL); Fab0082, LC (mouse VL - human Kappa CL).

30 SEQ ID NO: 42 provides the amino acid sequence of mAb0062, HC (mouse VH-human IgG4 CH1-CH2-CH3).

SEQ ID NO: 43 provides the amino acid sequence of mAb0082, HC (mouse VH-human IgG4 CH1-CH2-CH3).

35 SEQ ID NO: 44 provides the amino acid sequence of Fab0012, mouse VH - human IgG4 CH1.

SEQ ID NO: 45 provides the amino acid sequence of Fab0023, mouse VH - human IgG4 CH1.

SEQ ID NO: 46 provides the amino acid sequence of Fab0051, mouse VH - human IgG4 CH1.

5 SEQ ID NO: 47 provides the amino acid sequence of Fab0052, mouse VH - human IgG4 CH1.

SEQ ID NO: 48 provides the amino acid sequence of Fab0082, mouse VH - human IgG4 CH1.

10 SEQ ID NOs: 49-58 provide the amino acid sequences of optional linkers L2-L10. Optional linkers are numbered and listed in Table 3.

SEQ ID NO: 59 provides the amino acid sequence of purification tag HPC4.

SEQ ID NOs: 60-145 provide the nucleic acid sequences of the primers used during the development of the expression constructs described in examples 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19, 20, 24, 25.

15 SEQ ID NO: 146 provides the amino acid sequence of Fab0061 VH-CH1.

SEQ ID NO: 147 provides the amino acid sequence of hIgG4-hinge-CH2-CH3.

SEQ ID NO: 148 provides the amino acid sequence of a His6 tag.

SEQ ID NO: 149 provides the amino acid sequence of hTLT-1.18-188.

SEQ ID NO: 150 provides the nucleic acid sequence of primer no. 1004.

20 SEQ ID NO: 151 provides the nucleic acid sequence of primer no. 1005.

SEQ ID NO: 152 provides the amino acid sequence of Fab0100 HC.

SEQ ID NO: 153 provides the amino acid sequence of Fab0100 LC.

SEQ ID NO: 154 provides the nucleic acid sequence of wild type human Factor FV.

25 SEQ ID NO: 155 provides the amino acid sequence of wild type human Factor FV.

SEQ ID NO: 156 provides the nucleic acid sequence of wild type human Factor FVII.

30 SEQ ID NO: 157 provides the amino acid sequence of wild type human Factor FVII.

SEQ ID NO: 158 provides the nucleic acid sequence of wild type human Factor FVIII.

SEQ ID NO: 159 provides the amino acid sequence of wild type human Factor FVIII.

35 SEQ ID NO: 160 provides the nucleic acid sequence of wild type human Factor FIX.

SEQ ID NO: 161 provides the amino acid sequence of wild type human Factor
FIX.

SEQ ID NO: 162 provides the nucleic acid sequence of wild type human Factor
FX.

5 SEQ ID NO: 163 provides the amino acid sequence of wild type human Factor
FX.

SEQ ID NO: 164 provides the nucleic acid sequence of wild type human Factor
FXI.

10 SEQ ID NO: 165 provides the amino acid sequence of wild type human Factor
FXI.

SEQ ID NO: 166 provides the 0012LC.C36A-HPC4 DNA sequence.

SEQ ID NO: 167 provides the 0012LC.C36A-HPC4 amino acid sequence.

SEQ ID NO: 168 provides the 0012VH-CH1-HPC4 DNA sequence.

SEQ ID NO: 169 provides the 0012VH-CH1-HPC4 amino acid sequence.

15 SEQ ID NO: 170 provides the 0012VH.T60N-CH1-YGPPC DNA sequence.

SEQ ID NO: 171 provides the 0012VH.T60N-CH1-YGPPC.

SEQ ID NO: 172 provides the FIX-L4b-0012LC DNA sequence.

SEQ ID NO: 173 provides the FIX-L4b-0012LC amino acid sequence.

20 SEQ ID NO: 174 provides the amino acid sequence of 0003 Fab-LC: 0062Fab-LC-
HPC4.

SEQ ID NO: 175 provides the amino acid sequence of 0003 Fab-HC: 0062Fab-
VH-CH1-YGPPC.

SEQ ID NO: 176 provides the amino acid sequence of 0197-0000-0074 Fab-HC:
0197-0000-0051Fab-VH-CH1-YGPPC.

25 SEQ ID NO: 177 provides the amino acid sequence of 0074 Fab-LC: 0051Fab-LC-
HPC4.

SEQ ID NO: 178 provides the amino acid sequence of 0004 Fab-HC: 0023Fab-
VH-CH1-YGPPC.

30 SEQ ID NO: 179 provides the amino acid sequence of 0004Fab-LC: 0023Fab-LC-
HPC4.

SEQ ID NO: 180 provides the amino acid sequence of human FVII-L4b-0062VH-
CH1-HPC4.

SEQ ID NO: 181 provides the amino acid sequence of human FVII 407C.

SEQ ID NO: 182 provides the amino acid sequence of human FIX-L4b-0061LC.

DESCRIPTION OF THE INVENTION

Procoagulant proteins of the current invention comprise at least one coagulation factor, or functional variant thereof, and an antibody, or a fragment thereof, that is capable of binding to a receptor that is only present (in the ubiquitous sense of the word) on a platelet undergoing the morphological and functional changes associated with activation or an activated platelet. Such receptors might originate from the alpha- or dense granules of resting platelets, one particular example of such a receptor is TREM-like transcript 1 (TLT-1).

The procoagulant molecules may be fusion proteins. The term "fusion protein" herein refers to proteins that are created through the in-frame joining of two or more DNA sequences, which originally encode separate proteins or peptides, or fragments thereof. Translation of the fusion protein DNA sequence will result in a single protein sequence, which may have functional properties derived from each of the original proteins or peptides. DNA sequences encoding fusion proteins may be created artificially by standard molecular biology methods such as overlapping PCR or DNA ligation and the assembly is performed excluding the stop codon in the first 5'-end DNA sequence while retaining the stop codon in the 3'-end DNA sequence. The resulting fusion protein DNA sequence may be inserted into an appropriate expression vector that supports the heterologous fusion protein expression in standard host organisms such as bacteria, yeast, fungus, insect cells or mammalian cells.

Fusion proteins may contain a linker or spacer peptide sequence that separates the protein or peptide parts which define the fusion protein. The linker or spacer peptide sequence may facilitate the correct folding of the individual protein or peptide parts and may make it more likely for the individual protein or peptide parts to retain their individual functional properties. Linker or spacer peptide sequences may be inserted into fusion protein DNA sequences during the in-frame assembly of the individual DNA fragments that make up the complete fusion protein DNA sequence, i.e., during overlapping PCR or DNA ligation.

Alternatively, the procoagulant proteins of the invention may be conjugates of their constituent coagulation factor and antibody counterparts, such that coagulation factor and antibody are manufactured independently of one another and, thereafter, joined synthetically.

As mentioned above, the procoagulant molecules of the invention may specifically bind TREM-like transcript 1 (TLT-1). Triggering receptors expressed on myeloid cells (TREMs) have a well-established role in the biology of various myeloid lineages, playing important roles in the regulation of innate and adaptive immunity. TLT-1 belongs to the

same family of proteins, though the TLT-1 gene is expressed only in a single lineage, namely megakaryocytes and thrombocytes (platelets) and is exclusively found in the alpha-granules of megakaryocytes and platelets. TLT-1 is a transmembrane protein that is exposed on the surface of activated platelets upon alpha-granule release. To date, TLT-1 has not been found on the surface of resting platelets or on the surface of any other cell types.

The extracellular portion of TLT-1 is known to be composed of a single, immunoglobulin-like (Ig-like) domain connected to the platelet cell membrane by a linker region called the stalk (Gattis et al., *Jour Biol Chem*, 2006, Vol. 281, No. 19, pp. 13396–13403). The Ig-like domain of human TLT-1 (hTLT-1) is composed of 105 residues and is attached to the membrane by the 37-amino acid stalk. Thus, the Ig-like domain of TLT-1 is expected to have considerable freedom of movement.

The putative transmembrane segment of hTLT-1 is 20 amino acids long. TLT-1 also has a cytoplasmic Immune-receptor Tyrosine-based Inhibitory-Motif (ITIM), which may function as an intracellular signal transduction motif.

The role of TLT-1 in platelet biology has not yet been fully elucidated; it has been shown that TLT-1 binds fibrinogen and it is believed that TLT-1 plays a role in regulating coagulation and inflammation at the site of an injury. A soluble form of TLT-1 containing the Ig-like domain has been reported (Gattis et al., *Jour Biol Chem*, 2006, Vol. 281, No. 19, pp. 13396–13403). The specific functions of soluble versus platelet-bound TLT-1 remain to be established.

Giomerarelli et al. (*Thrombosis and Haemostasis* (2007) 97, 955-963) reported the generation of anti-TLT-1 scFv molecules using phage display techniques. Some of the anti-TLT-1 scFv molecules were found to inhibit thrombin-mediated human platelet aggregation. Thus, anti-TLT-1 scFv molecules with such features may have anti-thrombotic properties, similar to anti-GPIIb/IIIa scFv molecules described by Schwartz et al. (*FASEB Journal*, (2004), 18, 1704-1706).

The present invention relates to fusions proteins or conjugates comprising a coagulation factor attached to an anti-TLT-1 antibody, or antigen binding fragments thereof, including scFv. The anti-TLT-1 antibody or fragments thereof serve to target the linked clotting factor to the surface of activated platelets by binding to TLT-1 with the purpose of delivering a procoagulant activity at the activated platelet surface. In this context, inhibition of platelet aggregation is not a desirable property of the anti-TLT-1 antibody. Thus, anti-TLT-1 antibodies of the current invention are preferably not interfering with functions of TLT-1, and in particular do not inhibit platelet aggregation.

A receptor such as TLT-1 comprises epitopes that are useful targets for the procoagulant proteins of the current invention. Procoagulant proteins may bind any part of TLT-1 that would be available for binding *in vivo*, such as surface accessible residues of the Ig-like domain, or part of the stalk. Hence, fusion proteins may bind one or more residues within TLT-1 (20-125), TLT-1 (16-162), TLT-1 (126-162) and/or TLT-1 (129-142) (numbers in parenthesis refer to amino acid residues in SEQ ID NO: 2).

In a preferred embodiment, procoagulant proteins bind the stalk of TLT-1, such as one or more residues of TLT-1 (126-162) or TLT-1 (129-142). Procoagulant proteins that bind to the stalk of TLT-1 are unlikely to interfere with the function of the Ig-like domain.

In another preferred embodiment, fusing the coagulation factor to the C-terminal of an antibody, or fragment thereof, will position the coagulation factor even more favourably on the cell surface of activated platelets, relative to that of FVII and FVIIa.

In another preferred embodiment, procoagulant proteins of the invention bind to TLT-1 without interfering platelet aggregation.

In another embodiment the procoagulant proteins of the invention bind to TLT-1 without competing with fibrinogen binding to TLT-1.

In terms of the current invention, TLT-1 may be from any vertebrate, such as any mammal, such as a rodent (such as a mouse, rat or guinea pig), a lagomorph (such as a rabbit), an artiodactyl (such as a pig, cow, sheep or camel) or a primate (such as a monkey or human being). TLT-1 is, preferably, human TLT-1. TLT-1 may be translated from any naturally occurring genotype or allele that gives rise to a functional TLT-1 protein. A non-limiting example of one human TLT-1 is the polypeptide sequence of SEQ ID NO: 2. SEQ ID NO: 2 includes the signal peptide (residues 1-15 (MGLTLLLLLLLLGLEG) of SEQ ID NO: 2, and the mature TLT-1 polypeptide corresponds to residues 16-311 of SEQ ID NO: 2.

Procoagulant proteins of the invention comprise an antibody, or a fragment thereof, that has been raised against TLT-1. The antibody or fragment thereof may or may not result in a change in the conformational structure of TLT-1. Furthermore, the antibody or fragment thereof may or may not result in intracellular signalling, as a result of binding to TLT-1. In one embodiment, the antibody or fragment thereof is capable of binding to the stalk of TLT-1. Hence, the antibody or fragment thereof utilises a naturally occurring receptor, or portion thereof, in order to achieve the effect that is unique to and provided by the current invention.

The term "antibody" herein refers to a protein, derived from a germline immunoglobulin sequence, that is capable of specifically binding to an antigen which is TLT-1

or a portion thereof. The term includes full length antibodies of any isotype (that is, IgA, IgE, IgG, IgM and/or IgY) and any fragment or single chain thereof.

Full-length antibodies usually comprise at least four polypeptide chains: that is, two heavy (H) chains and two light (L) chains that are interconnected by disulfide bonds.

5 One immunoglobulin sub-class of particular pharmaceutical interest is the IgG family, which may be sub-divided into isotypes IgG1, IgG2, IgG3 and IgG4. IgG molecules are composed of two heavy chains, interlinked by two or more disulfide bonds, and two light chains, each attached to a heavy chain by a disulfide bond. A heavy chain may comprise a heavy chain variable region (VH) and up to three heavy chain constant (CH) regions:
10 CH1, CH2 and CH3. A light chain may comprise a light chain variable region (VL) and a light chain constant region (CL). VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FR). VH and VL regions are typically composed of three CDRs and four FRs, arranged from amino-
15 terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The hypervariable regions of the heavy and light chains form a domain that is capable of interacting with an antigen (TLT-1), whilst the constant region of an antibody may mediate binding of the immunoglobulin to host tissues or factors, including but not limited to various cells of the immune system (effector cells), Fc receptors and the first
20 component (Clq) of the classical complement system.

The antibody component of the procoagulant proteins may be a monoclonal antibody. Such an antibody may be a chimeric antibody, a CDR-grafted antibody, a human antibody, a humanised antibody or an antigen binding portion of any thereof. For the production of antibodies, the experimental animal is a suitable mammal such as a
25 goat, rabbit, rat or mouse.

In structural terms, a monoclonal antibody is represented by a single molecular species having a single binding specificity and affinity for a particular epitope. Monoclonal antibodies (mAbs) for the procoagulant proteins of the invention can be produced by a variety of well-known techniques, including conventional monoclonal antibody
30 methodology *e.g.*, the standard somatic cell hybridization technique of Kohler and Milstein (1975) *Nature* 256: 495, or viral or oncogenic transformation of B lymphocytes. The preferred animal system for preparing hybridomas is the murine system. Hybridoma production in the mouse is a very well-established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art.
35 Fusion partners (*e.g.*, murine myeloma cells) and fusion procedures are also known.

To generate hybridomas producing suitable monoclonal antibodies, splenocytes and/or lymph node cells from immunized mice can be isolated and fused to an appropriate immortalized cell line, such as a mouse myeloma cell line. The resulting hybridomas can be screened for the production of antigen-specific antibodies. The antibody secreting hybridomas can be replated, screened again, and if still positive for suitable IgG, the monoclonal antibodies can be subcloned at least twice by limiting dilution. The stable subclones can then be cultured *in vitro* to generate small amounts of antibody in tissue culture medium for characterization.

Antibodies for the invention may be prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (e.g., a mouse) that is transgenic or transchromosomal for the immunoglobulin genes of interest or a hybridoma prepared therefrom, (b) antibodies isolated from a host cell transformed to express the antibody of interest, e.g., from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial antibody library, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of immunoglobulin gene sequences to other DNA sequences.

Several suitable monoclonal antibodies, shown in Table 1, are herein identified by means of the prefix "mAb" together with a 4-digit number. Hence, the monoclonal antibody may be mAb0012 or a variant thereof. The monoclonal antibody may be mAb0023 or a variant thereof. The monoclonal antibody may be mAb0051 or a variant thereof. The monoclonal antibody may be mAb0061 or a variant thereof. The monoclonal antibody may be mAb0062 or a variant thereof. The monoclonal antibody may be mAb0082 or a variant thereof.

Table 1: Non-limiting examples of suitable monoclonal antibodies

mAb ID	HC	LC
0012	SEQ ID NO: 32	SEQ ID NO: 33
0023	SEQ ID NO: 34	SEQ ID NO: 35
0051	SEQ ID NO: 36	SEQ ID NO: 37
0052	SEQ ID NO: 38	SEQ ID NO: 39
0061	SEQ ID NO: 40	SEQ ID NO: 41
0062	SEQ ID NO: 42	SEQ ID NO: 39
0082	SEQ ID NO: 43	SEQ ID NO: 41

The term "antigen-binding portion" of an antibody refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen, such as

TLT-1 or another target receptor, as described herein. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. The antibody component of the procoagulant proteins may therefore be a fragment of an antibody, such as a fragment of a monoclonal antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include a Fab fragment, a $F(ab')_2$ fragment, a Fab' fragment, a Fd fragment, a Fv fragment, a ScFv fragment, a dAb fragment and an isolated complementarity determining region (CDR). Single chain antibodies such as scFv and heavy chain antibodies such as VHH and camel antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. These antibody fragments may be obtained using conventional techniques known to those of skill in the art, and the fragments may be screened for utility in the same manner as intact antibodies.

A "Fab" fragment includes a variable domain and a constant domain of the light chain and a variable domain and the first constant domain (C_{H1}) of the heavy chain. A Fab' fragment includes one or more carboxy terminal disulphide linkages to the heavy or light chains. $F(ab')_2$ antibody fragments comprise a pair of Fab fragments that are generally covalently linked near their carboxy termini by hinge cysteines. Other chemical couplings of antibody fragments are also known in the art.

An "Fv" fragment is an antibody fragment that contains a complete antigen recognition and binding site, and generally comprises a dimer of one heavy and one light chain variable domain in tight association that can be covalent in nature, for example in a single chain variable domain fragment (scFv). It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H - V_L dimer. Collectively, the six hypervariable regions or a subset thereof confer antigen binding specificity to the antibody. However, even a single variable domain comprising only three hypervariable regions specific for an antigen has the ability to recognize and bind antigen, although usually at a lower affinity than the entire binding site (Cai & Garen, *Proc. Natl. Acad. Sci. USA*, 93: 6280-6285, 1996). For example, naturally occurring camelid antibodies that only have a heavy chain variable domain (VHH) can bind antigen (Desmyter et al., *J. Biol. Chem.*, 277: 23645-23650, 2002; Bond et al., *J. Mol. Biol.* 2003; 332: 643-655).

"Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of an antibody, where these domains are present in a single polypeptide chain.

Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains that enables the scFv to form the desired structure for antigen binding. For a

review of scFv, see Pluckthun, 1994, In: *The Pharmacology of Monoclonal Antibodies*, Vol. 113, Rosenberg and Moore eds. Springer-Verlag, New York, pp. 269-315.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, in which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H and V_L). By using a linker that is too short to allow pairing between the two variable domains on the same chain, the variable domains are forced to pair with complementary domains of another chain, creating two antigen-binding sites. Diabodies are described more fully, for example, in EP 404,097; WO 93/11161; and Hollinger et al., 1993, *Proc. Natl. Acad. Sci. USA*, 90:6444-6448.

The expression "linear antibodies" refers to antibodies as described in Zapata et al., 1995, *Protein Eng.*, 8(10):1057-1062. Briefly, these antibodies contain a pair of tandem Fd segments (V_H - C_H1 - V_H - C_H1) that, together with complementary light chain polypeptides, form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

The term "monobody" as used herein, refers to an antigen binding molecule with a heavy chain variable domain and no light chain variable domain. A monobody can bind to an antigen in the absence of light chains and typically has three hypervariable regions, for example CDRs designated CDRH1, CDRH2, and CDRH3. A heavy chain IgG monobody has two heavy chain antigen binding molecules connected by a disulfide bond. The heavy chain variable domain comprises one or more hypervariable regions, preferably a CDRH3 or HVL-H3 region.

The term "hypervariable region", when used herein, refers to the amino acid residues of an antibody that are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity-determining region" or "CDR" (defined by sequence as residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (defined by structure and differing for each antibody; see, for example: Chothia and Lesk, 1987, *J. Mol. Biol.* 196:901-917). In one example, HVL residues can include, 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain.

In one embodiment of the invention, the TLT-1 binding portion of the procoagulant protein is a Fab fragment. Several suitable Fab fragments, shown in Table

2, are herein identified by means of the prefix "Fab" together with a 4-digit number. The Fab fragment may be Fab0003 or a variant thereof. The Fab fragment may be Fab0004 or a variant thereof. The Fab fragment may be Fab0012 or a variant thereof. The Fab fragment may be Fab0023 or a variant thereof. The Fab fragment may be Fab0051 or a variant thereof. The Fab fragment may be Fab0052 or a variant thereof. The Fab fragment may be Fab0061 or a variant thereof. The Fab fragment may be Fab0062 or a variant thereof. The Fab fragment may be Fab0074 or a variant thereof. The Fab fragment may be Fab0082 or a variant thereof. The Fab fragment may be Fab0084 or a variant thereof.

Table 2: Non-limiting examples of suitable Fab fragments

Fab ID	VH-CH1	LC
Fab0003	SEQ ID NO: 175	SEQ ID NO: 174
Fab0004	SEQ ID NO: 178	SEQ ID NO: 179
Fab0012	SEQ ID NO: 44	SEQ ID NO: 33
Fab0023	SEQ ID NO: 45	SEQ ID NO: 35
Fab0051	SEQ ID NO: 46	SEQ ID NO: 37
Fab0052	SEQ ID NO: 47	SEQ ID NO: 39
Fab0061	SEQ ID NO: 146	SEQ ID NO: 41
Fab0074	SEQ ID NO: 176	SEQ ID NO: 177
Fab0082	SEQ ID NO: 48	SEQ ID NO: 41
Fab0084	SEQ ID NO: 171	SEQ ID NO: 167

As mentioned above, an antibody for the invention may be a human antibody or a humanised antibody. The term "human antibody", as used herein, is intended to include antibodies that have variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. Furthermore, if the antibody contains a constant region, the constant region is also derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

Such a human antibody may be a human monoclonal antibody. Such a human monoclonal antibody may be produced by a hybridoma which includes a B cell obtained from a transgenic nonhuman animal, *e.g.*, a transgenic mouse whose genome comprises a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

Human antibodies may be prepared by *in vitro* immunisation of human lymphocytes followed by transformation of the lymphocytes with Epstein-Barr virus.

The term "human antibody derivatives" refers to any modified form of the human antibody, *e.g.*, a conjugate of the antibody and another agent or antibody.

The term "humanized antibody" herein refers to antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences. Additional framework region modifications may be made within the human framework sequences.

Antibodies of the invention can be tested for binding to the target protein by, for example, standard ELISA or Western blotting. An ELISA assay can also be used to screen for hybridomas that show positive reactivity with the target protein. The binding specificity of an antibody may also be determined by monitoring binding of the antibody to cells expressing the target protein, for example, by flow cytometry.

The specificity of an antibody of the invention for the target protein may be further studied by determining whether or not the antibody binds to other proteins. For example, where it is desired to produce an antibody that specifically binds TLT-1 or a particular part, *e.g.* epitope, of TLT-1, the specificity of the antibody may be assessed by determining whether or not the antibody also binds to other molecules or modified forms of TLT-1 that lack the part of interest.

Polypeptide or antibody "fragments" according to the invention may be made by truncation of the corresponding monoclonal antibodies, *e.g.* by removal of one or more amino acids from the N and/or C-terminal ends of a polypeptide. Up to 10, up to 20, up to 30, up to 40 or more amino acids may be removed from the N and/or C terminal in this way. Fragments may also be generated by one or more internal deletions.

In terms of the current invention, "epitope" refers to the area or region on an antigen (Ag), which is a molecular structure on the surface of an activated platelet, to which the antibody (Ab) portion of the procoagulant protein is capable of specifically binding, *i.e.* the area or region that is in physical contact with the Ab. An antigen's epitope may comprise amino acid residues in the Ag that are directly involved in binding to a Ab (the immunodominant component of the epitope) and other amino acid residues, which are not directly involved in the binding, such as amino acid residues of the Ag

which are effectively blocked by the Ab (in other words, the amino acid residue is within the "solvent-excluded surface" and/or the "footprint" of the Ab). The term epitope herein includes both types of binding sites within any particular region of a receptor, such as TLT-1, that specifically binds to an anti-TLT-1 antibody, or fragment thereof, unless
5 otherwise stated (e.g., in some contexts the invention relates to antibodies that bind directly to particular amino acid residues). Receptors such as TLT-1 may comprise a number of different epitopes, which may include, without limitation, (1) linear peptide epitopes, (2) conformational epitopes which include of one or more non-contiguous amino acids located near each other in the mature receptor conformation; and (3) post-
10 translational epitopes, which include, either in whole or part, of molecular structures covalently attached to TLT-1, such as carbohydrate groups.

The epitope for a given antibody (Ab)/antigen (Ag) pair can be defined and characterized at different levels of detail using a variety of experimental and computational epitope mapping methods. The experimental methods include
15 mutagenesis, X-ray crystallography, Nuclear Magnetic Resonance (NMR) spectroscopy, Hydrogen deuterium eXchange Mass Spectrometry (HX-MS) and various competition binding methods. As each method relies on a unique principle, the description of an epitope is intimately linked to the method by which it has been determined. Thus, the epitope for a given Ab/Ag pair will be defined differently depending on the epitope
20 mapping method employed.

At its most detailed level, the epitope for the interaction between the Ag and the Ab can be defined by the spatial coordinates defining the atomic contacts present in the Ag-Ab interaction, as well as information about their relative contributions to the binding thermodynamics. At a less detailed level the epitope can be characterized by the spatial
25 coordinates defining the atomic contacts between the Ag and Ab. At a further less detailed level the epitope can be characterized by the amino acid residues that it comprises as defined by a specific criterium, e.g. distance between atoms in the Ab and the Ag. At a further less detailed level the epitope can be characterized through function, e.g. by competition binding with other Abs. The epitope can also be defined more
30 generically as comprising amino acid residues for which substitution by another amino acid will alter the characteristics of the interaction between the Ab and Ag.

In the context of an X-ray derived crystal structure defined by spatial coordinates of a complex between an Ab, e.g. a Fab fragment, and its Ag, the term epitope is herein, unless otherwise specified or contradicted by context, specifically defined as platelet
35 receptor residues characterized by having a heavy atom (i.e. a non-hydrogen atom) within a distance of 4 Å from a heavy atom in the Ab.

From the fact that descriptions and definitions of epitopes, dependent on the epitope mapping method used, are obtained at different levels of detail, it follows that comparison of epitopes for different Abs on the same Ag can similarly be conducted at different levels of detail.

5 Epitopes described on the amino acid level, e.g. determined from an X-ray structure, are said to be identical if they contain the same set of amino acid residues. Epitopes are said to overlap if at least one amino acid is shared by the epitopes. Epitopes are said to be separate (unique) if no amino acid residue is shared by the epitopes.

 Epitopes characterized by competition binding are said to be overlapping if the
10 binding of the corresponding Ab's are mutually exclusive, i.e. if binding of one Ab excludes simultaneous binding of the other Ab. The epitopes are said to be separate (unique) if the Ag is able to accommodate binding of both corresponding Ab's simultaneously. Furthermore, there are instances when one or more antibodies do not have overlapping epitopes but can not bind simultaneously. Due to tertiary and
15 quaternary structure of an antigen, one antibody may not be able to access its epitope due to previous binding of another antibody.

 Procoagulant proteins of the invention may be capable of binding to the same epitope as mAb0012. Procoagulant proteins may be capable of binding to the same epitope as mAb0023. Procoagulant proteins may be capable of binding to the same
20 epitope as mAb0051. Procoagulant proteins may be capable of binding to the same epitope as mAb0061. Procoagulant proteins may be capable of binding to the same epitope as mAb0062. Procoagulant proteins may be capable of binding to the same epitope as mAb0082.

 The epitope may comprise one or more residues selected from the group
25 consisting of K133, I134, G135, S136, L137, A138, N140, A141, F142, S143, D144, P145 and A146 of SEQ ID NO: 4 (corresponding to K133, I134, G135, S136, L137, A138, N140, A141, F142, S143, D144, P145 and A146 of SEQ ID NO: 2).

 The epitope may comprise one or more residues selected from the group consisting of V17, Q18, C19, H20, Y21, R22, L23, Q24, D25, V26, K27, A28, L63, G64,
30 G65, G66, L67, L68, G89, A90, R91, G92, P93, Q94, I95 and L96 of SEQ ID NO: 5 (corresponding to V36, Q37, C38, H39, Y40, R41, L42, Q43, D44, V45, K46, A47, L82, G83, G84, G85, L86, L87, G108, A109, R110, G111, P112, Q113, I114 and L115 of SEQ ID NO: 2).

 The epitope may comprise one or more residues selected from the group
35 consisting of L36, P37, E38, G39, C40, Q41, P42, L43, V44, S45, S46, A47, V73, T74, L75, Q76, E77, E78, D79, A80, G81, E82, Y83, G84, C85, M86, R91, G92, P93, Q94, I95,

L96, H97, R98, V99, S100 and L101 of SEQ ID NO: 5 (corresponding to L55, P56, E57, G58, C59, Q60, P61, L62, V63, S64, S65, A66, V92, T93, L94, Q95, E96, E97, D98, A99, G100, E101, Y102, G103, C104, M105, R110, G111, P112, Q113, I114, L115, H116, R117, V118, S119 and L120 of SEQ ID NO: 2).

5 The epitope may comprise one or more residues selected from the group consisting of V17, Q18, C19, H20, Y21, R22, L23, Q24, D25, V26, K27, A28, R91, G92, P93, Q94, I95, L96, H97, R98, V99, S100 and L101 of SEQ ID NO: 5 (corresponding to V36, Q37, C38, H39, Y40, R41, L42, Q43, D44, V45, K46, A47, R110, G111, P112, Q113, I114, L115, H116, R117, V118, S119 and L120 of SEQ ID NO: 2).

10 The epitope may comprise one or more residues selected from the group consisting of E5, T6, H7, K8, I9, G10, S11, L12, A13, E14, N15, A16, F17, S18, D19 and P20 of SEQ ID NO: 7 (corresponding to E130, T131, H132, K133, I134, G135, S136, L137, A138, E139, N140, A141, F142, S143, D144 and P145 of SEQ ID NO: 2).

15 The epitope may comprise one or more residues selected from the group consisting of K8, I9, G10, S11, L12, A13, N15, A16, F17, S18, D19, P20 and A21 of SEQ ID NO: 7 (corresponding to K133, I134, G135, S136, L137, A138, N140, A141, F142, S143, D144, P145 and A146 of SEQ ID NO: 2).

20 The definition of the term "paratope" is derived from the above definition of "epitope" by reversing the perspective. Thus, the term "paratope" refers to the area or region on the Ab to which an Ag specifically binds, i.e. to which it makes physical contact to the Ag.

25 The paratope may comprise one or more residues selected from the group consisting of H50, N52, Y56, H58, Y73, F79, S115, T116, V118 and Y120 of the anti-TLT-1 light (L) chain (SEQ ID NO: 33), and residues V20, F45, R49, Y50, W51, E68, T75, N77, S116, G117, V118 and T120 of the anti-TLT-1 heavy (H) chain (SEQ ID NO: 32).

30 In the context of an X-ray derived crystal structure defined by spatial coordinates of a complex between an Ab, e.g. a Fab fragment, and its Ag, the term paratope is herein, unless otherwise specified or contradicted by context, specifically defined as Ag residues characterized by having a heavy atom (i.e. a non-hydrogen atom) within a distance of 4 Å from a heavy atom in the platelet receptor.

35 The epitope and paratope for a given antibody (Ab)/antigen (Ag) pair may be identified by routine methods. For example, the general location of an epitope may be determined by assessing the ability of an antibody to bind to different fragments or variants of TLT-1. The specific amino acids within TLT-1 that make contact with an antibody (epitope) and the specific amino acids in an antibody that make contact with TLT-1 (paratope) may also be determined using routine methods, such as those

described in the examples. For example, the antibody and target molecule may be combined and the Ab/Ag complex may be crystallised. The crystal structure of the complex may be determined and used to identify specific sites of interaction between the antibody and its target.

5 Fusion proteins comprising an antibody or fragment thereof may also be defined in terms of their complementarity-determining regions (CDRs). The term "complementarity-determining region" or "hypervariable region" herein refers to the amino acid residues of an antibody that are responsible for antigen binding. The complementarity-determining regions or "CDRs" are generally comprised of amino acid
10 residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light-chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy-chain variable domain; (Kabat et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and/or those residues from a "hypervariable loop" (residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in
15 the light-chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy-chain variable domain; Chothia and Lesk, J. Mol. Biol 1987;196:901-917). Typically, the numbering of amino acid residues in this region is performed by the method described in Kabat et al., *supra*. Phrases such as "Kabat position", "Kabat residue", and "according to Kabat" herein refer to this numbering system for heavy chain
20 variable domains or light chain variable domains. Using the Kabat numbering system, the actual linear amino acid sequence of a peptide may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or CDR of the variable domain. For example, a heavy chain variable domain may include amino acid insertions (residue 52a, 52b and 52c according to Kabat) after residue 52 of CDR H2 and inserted
25 residues (e.g. residues 82a, 82b, and 82c, etc. according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

30 The term "framework region" or "FR" residues refer to those VH or VL amino acid residues that are not within the CDRs, as defined herein.

In one embodiment, the heavy chain of an antibody, or fragment thereof, for the procoagulant proteins of the invention comprises:

- a CDR1 sequence of amino acids 50 to 54 (DYFMY) of SEQ ID NO: 34, wherein one of these amino acids may be substituted by a different amino acid; and/or

- a CDR2 sequence of amino acids 69 to 85 (YISNGGDSSSYPTDKG) of SEQ ID NO: 34, wherein one, two, three or four of these amino acids may be substituted by a different amino acid; and/or
- a CDR3 sequence of amino acids 118 to 129 (NKNWDDYYDMY) of SEQ ID NO: 34, wherein one, two or three of these amino acids may be substituted by a different amino acid.

In another embodiment, the light chain of an antibody, or fragment thereof, for the procoagulant proteins of the invention comprises:

- a CDR1 sequence of amino acids 44 to 60 (KSSQSLLNSRTRKNYLA) of SEQ ID NO: 35, wherein one, two, three or four of these amino acids may be substituted with a different amino acid; and/or
- a CDR2 sequence of amino acids 76 to 82 (WASTRES) of SEQ ID NO: 35, wherein one or two of these amino acids may be substituted with a different amino acid; and/or
- a CDR3 sequence of amino acids 115 to 122 (KQSYNLLT) of SEQ ID NO: 35, wherein one or two of these amino acids may be substituted with a different amino acid.

In another embodiment, the heavy chain of an antibody, or fragment thereof, for the procoagulant proteins of the invention comprises:

- a CDR1 sequence of amino acids 50 to 54 (DYSMH) of SEQ ID NO: 36, wherein one of these amino acids may be substituted by a different amino acid; and/or
- a CDR2 sequence of amino acids 69 to 85 (VISTYYGDVRYNQKFKG) of SEQ ID NO: 36, wherein one, two, three or four of these amino acids may be substituted by a different amino acid; and/or
- a CDR3 sequence of amino acids 118 to 129 (APMITTGAWFAY) of SEQ ID NO: 36, wherein one, two or three of these amino acids may be substituted by a different amino acid.

In another embodiment, the light chain of an antibody, or fragment thereof, for the procoagulant proteins of the invention comprises:

- a CDR1 sequence of amino acids 44 to 54 (KASQSVSNDVA) of SEQ ID NO: 37, wherein one, two or three of these amino acids may be substituted with a different amino acid; and/or
- a CDR2 sequence of amino acids 70 to 76 (YASSRYT) of SEQ ID NO: 37, wherein one or two of these amino acids may be substituted with a different amino acid; and/or
- a CDR3 sequence of amino acids 109 to 117 (QQDYSSPYT) of SEQ ID NO: 37, wherein one or two of these amino acids may be substituted with a different amino acid.

In another embodiment, the heavy chain of an antibody, or fragment thereof, for the procoagulant proteins of the invention comprises:

- a CDR1 sequence of amino acids 50 to 54 (SHWIE) of SEQ ID NO: 42, wherein one of these amino acids may be substituted by a different amino acid; and/or
- 5 • a CDR2 sequence of amino acids 69 to 85 (EILPGSGNTNYNEKFKG) of SEQ ID NO: 42, wherein one, two, three or four of these amino acids may be substituted by a different amino acid; and/or
- a CDR3 sequence of amino acids 118 to 130 (GYGLNYDWYFDV) of SEQ ID NO: 42, wherein one, two or three of these amino acids may be substituted by a different
- 10 amino acid.

In another embodiment, the light chain of an antibody, or fragment thereof, for the procoagulant proteins of the invention comprises:

- a CDR1 sequence of amino acids 44 to 54 (RASQDISNYLN) of SEQ ID NO: 39, wherein one, two or three of these amino acids may be substituted with a different
- 15 amino acid; and/or
- a CDR2 sequence of amino acids 70 to 76 (YTSRLHS) of SEQ ID NO: 39, wherein one or two of these amino acids may be substituted with a different amino acid; and/or
- a CDR3 sequence of amino acids 109 to 117 (QQDTKLPLYT) of SEQ ID NO: 39, wherein one or two of these amino acids may be substituted with a different amino
- 20 acid.

In another embodiment, the heavy chain of an antibody, or fragment thereof, for the procoagulant proteins of the invention comprises:

- a CDR1 sequence of amino acids 49 to 53 (RYWMT) of SEQ ID NO: 40, wherein one of these amino acids may be substituted by a different amino acid; and/or
- 25 • a CDR2 sequence of amino acids 68 to 84 (EINPDSSTINYNPSLKD) of SEQ ID NO: 40, wherein one, two, three or four of these amino acids may be substituted by a different amino acid; and/or
- a CDR3 sequence of amino acids 117 to 121 (GVFTS) of SEQ ID NO: 40, wherein one, two or three of these amino acids may be substituted by a different amino acid.

30 In another embodiment, the light chain of an antibody, or fragment thereof, for the procoagulant proteins of the invention comprises:

- a CDR1 sequence of amino acids 43 to 58 (RSSQSLVHRNGNTYFH) of SEQ ID NO: 41, wherein one, two, three or four of these amino acids may be substituted with a different amino acid; and/or
- 35 • a CDR2 sequence of amino acids 74 to 80 (KVSNRFS) of SEQ ID NO: 41, wherein one or two of these amino acids may be substituted with a different amino acid; and/or

- a CDR3 sequence of amino acids 113 to 121 (SQSTHVPYT) of SEQ ID NO: 41, wherein one or two of these amino acids may be substituted with a different amino acid.

In another embodiment, the heavy chain of an antibody, or fragment thereof, for the procoagulant proteins of the invention comprises:

- a CDR1 sequence of amino acids 49 to 53 (RYWMT) of SEQ ID NO: 32, wherein one of these amino acids may be substituted by a different amino acid; and/or
- a CDR2 sequence of amino acids 68 to 84 (EINPDSSTINYTPSLKD) of SEQ ID NO: 32, wherein one, two, three or four of these amino acids may be substituted by a different amino acid; and/or
- a CDR3 sequence of amino acids 117 to 121 (GVFTS) of SEQ ID NO: 32, wherein one, two or three of these amino acids may be substituted by a different amino acid.

In another embodiment, the light chain of an antibody, or fragment thereof, for the procoagulant proteins of the invention comprises:

- a CDR1 sequence of amino acids 43 to 58 (RSSQSLVHRNGNTYFH) of SEQ ID NO: 33, wherein one, two, three or four of these amino acids may be substituted with a different amino acid; and/or
- a CDR2 sequence of amino acids 74 to 80 (KVSNRFS) of SEQ ID NO: 33, wherein one or two of these amino acids may be substituted with a different amino acid; and/or
- a CDR3 sequence of amino acids 113 to 121 (SQSTHVPYT) of SEQ ID NO: 33, wherein one or two of these amino acids may be substituted with a different amino acid.

Monoclonal antibodies, or fragments thereof, for the procoagulant proteins of the current invention may be glycosylation variants. Glycosylation variants of antibodies are variants in which the glycosylation pattern of an antibody is altered. By altering is meant deleting one or more carbohydrate moieties found in the antibody, adding one or more carbohydrate moieties to the antibody, changing the composition of glycosylation (glycosylation pattern), the extent of glycosylation.

Antibodies are glycosylated at conserved positions in their constant regions (Jefferis and Lund, *Chem. Immunol.* 1997; 65:111-128; Wright and Morrison, *Trends Biotechnol.* 1997; 15:26-32). The oligosaccharide side chains of the immunoglobulins can affect a protein's function (Boyd et al., *Mol. Immunol.* 1996; 32:1311-1318), and the intramolecular interaction between portions of the glycoprotein can affect the conformation and presented three-dimensional surface of the glycoprotein. Oligosaccharides may also serve to target a given glycoprotein to certain molecules based upon specific recognition structures. For example, it has been reported that in

agalactosylated IgG, the oligosaccharide moiety "flips" out of the inter-CH₂ space and terminal N-acetylglucosamine residues become available to bind mannose binding protein (Malhotra et al., *Nature Med.* 1995; 1:237-243). Removal by glycopeptidase of the oligosaccharides from CAMPATH-1H (a recombinant humanized murine monoclonal IgG1 antibody which recognizes the CDw52 antigen of human lymphocytes) produced in Chinese Hamster Ovary (CHO) cells resulted in a complete reduction in complement mediated lysis (CMCL) (Boyd et al., *Mol. Immunol.* 1996; 32:1311-1318), while selective removal of sialic acid residues using neuraminidase resulted in no loss of DMCL. Glycosylation of antibodies has also been reported to affect antibody-dependent cellular cytotoxicity (ADCC). In particular, CHO cells with tetracycline-regulated expression of $\beta(1,4)$ -N-acetylglucosaminyltransferase III (GnTIII), a glycosyltransferase catalyzing formation of bisecting GlcNAc, was reported to have improved ADCC activity (Umana et al. *Nature Biotech.* 1999; 17:176-180).

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites). Similarly, removal of glycosylation sites can be accomplished by amino acid alteration within the native glycosylation sites of the antibody.

The amino acid sequence is usually altered by altering the underlying nucleic acid sequence. Nucleic acid molecules encoding amino acid sequence variants of a TLT-1 antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the TLT-1 antibody.

The glycosylation (including glycosylation pattern) of antibodies may also be altered without altering the amino acid sequence or the underlying nucleotide sequence. Glycosylation largely depends on the host cell used to express the antibody. Since the cell type used for expression of recombinant glycoproteins, e.g. antibodies, as potential
5 therapeutics is rarely the native cell, significant variations in the glycosylation pattern of the antibodies can be expected (see, e.g. Hse et al., *J. Biol. Chem.* 1997; 272:9062-9070). In addition to the choice of host cells, factors which affect glycosylation during recombinant production of antibodies include growth mode, media formulation, culture density, oxygenation, pH, purification schemes and the like. Various methods have been
10 proposed to alter the glycosylation pattern achieved in a particular host organism including introducing or overexpressing certain enzymes involved in oligosaccharide production (U.S. Pat. Nos. 5,047,335; 5,510,261 and 5,278,299). Glycosylation, or certain types of glycosylation, can be enzymatically removed from the glycoprotein, for example using endoglycosidase H (Endo H). In addition, the recombinant host cell can be
15 genetically engineered, e.g. make defective in processing certain types of polysaccharides. These and similar techniques are well known in the art.

The glycosylation structure of antibodies can be readily analyzed by conventional techniques of carbohydrate analysis, including lectin chromatography, NMR, Mass spectrometry, HPLC, GPC, monosaccharide compositional analysis, sequential enzymatic
20 digestion, and HPAEC-PAD, which uses high pH anion exchange chromatography to separate oligosaccharides based on charge. Methods for releasing oligosaccharides for analytical purposes are also known, and include, without limitation, enzymatic treatment, elimination using harsh alkaline environment to release mainly O-linked structures, and chemical methods using anhydrous hydrazine to release both N- and O-linked
25 oligosaccharides.

In addition to their TLT-1-binding portion, which involves a monoclonal antibody or fragment thereof, procoagulant proteins of the invention also comprise a coagulation factor component, whose function is to upregulate blood coagulation in the vicinity of the activated platelet.

30 Coagulation factors of procoagulant fusion proteins or conjugates of the present invention may be in their inactive form or in their activated form. The coagulation factor may be a serine protease, in which case the inactive form of the coagulation factor corresponds to the zymogen form and the activated form corresponds to the catalytically active form. The coagulation factor may be a FVII polypeptide (FVII or FVIIa), a FVIII polypeptide (i.e. FVIII or FVIIIa), a FIX polypeptide (FIX or FIXa), a FX polypeptide (FX or
35 FXa) or a FXI polypeptide (FXI or FXIa). FVII, FVIII, FIX, FX and FXI polypeptides of the

present invention also includes variants, such as truncated variants, and derivatives of said coagulation factors. FVII, FIX, FX and FXI variants will include truncated des-Gla variants, i.e. variants of said coagulation factors lacking the Gla-domain responsible for interaction with phospholipid membranes of said coagulation factors.

5 If the coagulation factor is a FVII polypeptide, the FVII component may be able to bind to tissue factor and it is, preferably, able to cleave FIX or FX. If the coagulation factor is a FVIII polypeptide, the FVIII component is, preferably, able to bind to FIXa and support cleavage of FX. If the coagulation factor is a FIXa polypeptide, it is preferably able to cleave FX. If the coagulation factor is FXa, then it is preferably able to cleave
10 prothrombin (FII). If the coagulation factor is a FXIa polypeptide then it is preferably able to cleave FIX.

In one particular embodiment, the coagulation factor is a FV polypeptide. Factor V is synthesized by the liver and secreted Factor V circulates in plasma as a 330-kDa single-chain polypeptide that is the inactive procoagulant (Huang et al. (2008)

15 Haemophilia 14: 1164-9). FV is composed of 2196 amino acids, including a 28 amino acids signal peptide. It is composed of six domains A1 (Aa 30-329), A2 (Aa 348-684), B (Aa 692-1573), A3 (Aa 1578-1907), C1 (Aa 1907-2061), and C2 (Aa 2066-2221). The A and C domains of the two proteins are approximately 40% homologous with the equivalent domains of FVIII, but the B domains are not conserved. As is the case with
20 FVIII, FV activity is tightly regulated via site-specific proteolysis. Thrombin, and to a lesser extent Factor Xa (FXa), are primarily responsible for FV activation via proteolytic cleavages at positions Arg⁷⁰⁹-Ser⁷¹⁰, Arg¹⁰¹⁸-Thr¹⁰¹⁹ and Arg¹⁵⁴⁵-Ser¹⁵⁴⁶. These cleavages release the B domain and create a dimeric molecule composed of a 105-kDa heavy chain that contains the A1 and A2 domains and a 71- to 74-kDa light chain that contains the
25 A3, C1, and C2 domains. These two chains are held together by calcium at residues Asp¹³⁹ and Asp¹⁴⁰ and hydrophobic interactions. The heavy chain provides the contacts for both FXa and prothrombin, whereas the two C domains in the light chain are needed for the interaction of FVa with the phospholipid surface. Thus, Factor V is active as a cofactor for FXa of the thrombinase complex and the activated FXa enzyme requires
30 calcium and FVa to convert prothrombin to thrombin on the cell surface membrane. The A3 domain in the light chain is involved in both FXa and phospholipid interactions. Taken together, the two FVa chains link FXa to the phospholipid surface formed by the platelet plug at the site of injury and enable FXa to efficiently bind and cleave prothrombin to generate thrombin. Factor V is able to bind to activated platelets. Although FV is
35 predominately found as a soluble component in blood plasma, a fraction of FV is also present in the α -granula of platelets, which is important for normal hemostasis as

evidenced by platelet specific Factor V deficiency (Janeway et al. (1996) Blood 87: 3571-8).

One wild type human Factor V sequence is provided in SEQ ID NO: 155. The term "Factor V polypeptide" herein refers to wild type Factor V molecules as well as FV variants, FV derivatives and FV conjugates. Such variants, derivatives and conjugates may exhibit substantially the same, or improved, biological activity relative to wild-type human Factor V.

For the purpose of the current invention, Factor V may be plasma-derived or recombinantly produced, using well known methods of production and purification. The degree and location of glycosylation, gamma-carboxylation and other post-translational modifications may vary depending on the chosen host cell and its growth conditions.

Factor V polypeptides may be tested using commercially available clotting assays, such as the *in vitro* Hemoclot Factor V Reagent assay (Aniara, Ohio, USA: Cat. No. ACK071K).

In one particular embodiment, the coagulation factor is a FVIIa polypeptide. Factor VII (FVII) is a glycoprotein primarily produced in the liver. The mature protein is composed of 406 amino acid residues and is composed of four domains as defined by homology. There is an N-terminal Gla domain followed by two epidermal growth factor (EGF)-like domains and a C-terminal serine protease domain. FVII circulates in plasma as a single-chain molecule. Upon activation to activated FVII (FVIIa), the molecule is cloven between residues Arg152 and Ile153, resulting in a two-chain protein held together by a disulphide bond. The light chain contains the Gla and EGF-like domains, whereas the heavy chain is the protease domain. FVIIa requires binding to its cell-surface cofactor tissue factor to become fully biologically active.

The term "Factor VII(a)" herein encompasses the uncloven zymogen, Factor VII, as well as the cloven and thus activated protease, Factor VIIa. "Factor VII(a)" includes natural allelic variants of FVII(a) that may exist and occur from one individual to another. One wild type human Factor VIIa sequence is provided in SEQ ID NO: 157, as well as in Proc Natl Acad Sci USA 1986; 83:2412-2416.

The term "Factor VII(a) polypeptide" herein refers to wild type Factor VIIa molecules as well as FVII(a) variants, FVII(a) derivatives and FVII(a) conjugates. Such variants, derivatives and conjugates may exhibit substantially the same, or improved, biological activity relative to wild-type human Factor VIIa.

The term "FVII(a) variant", as used herein, is intended to designate Factor FVII having the sequence of SEQ ID NO: 157, wherein one or more amino acids of the parent protein have been substituted by another amino acid and/or wherein one or more amino

acids of the parent protein have been deleted and/or wherein one or more amino acids have been inserted in protein and/or wherein one or more amino acids have been added to the parent protein. Such addition can take place either at the N-terminal end or at the C-terminal end of the parent protein or both. The "analogue" or "analogues" within this definition still have FVII activity in its activated form. In one embodiment a variant is at least 90% identical with the sequence of SEQ ID NO: 157. In another embodiment a variant is at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, , such as at least 99% identical with the sequence of SEQ ID NO: 157. As used herein, any reference to a specific position refers to the corresponding position in SEQ ID NO: 157.

Non-limiting examples of FVII(a) variants that have substantially the same or increased proteolytic activity compared to recombinant wild type human Factor VII(a) include those disclosed in WO 01/83725, WO 02/22776, WO 02/077218, WO 03/027147, WO 03/037932, WO 04/029090, WO 05/024006, WO 07/031559 and EP 05108713.8, US 7173000 B2 ; and JP4451514 B2.

The term "improved biological activity" refers to FVII(a) polypeptides that exhibit i) substantially the same or increased proteolytic activity compared to recombinant wild type human Factor VIIa in the presence and/or absence of tissue factor or ii) to FVII(a) polypeptides with substantially the same or increased TF affinity compared to recombinant wild type human Factor VIIa or iii) to FVII(a) polypeptides with substantially the same or increased half life in plasma compared to recombinant wild type human Factor VIIa, or iv) to FVII(a) polypeptides with substantially the same or increased affinity for the activated platelet. The biological activity of a FVIIa polypeptide may be measured using a variety of assays known to the person skilled in the art, such as the *in vitro* hydrolysis and *in vitro* proteolysis assays described in examples 26 and 27.

For the purpose of the current invention, Factor VII(a) may be plasma-derived or recombinantly produced, using well known methods of production and purification. The degree and location of glycosylation, gamma-carboxylation and other post-translational modifications may vary depending on the chosen host cell and its growth conditions.

Gamma-Carboxylated residues in the FVII sequence below are represented by " γ ".

SEQ ID NO: 157: Wild type human coagulation Factor VII

ANAF γ L γ LRPGSL γ R γ CK γ γ QCSF γ γ AR γ IFKDA γ RTKLFWISYSDGDQCASSPCQNGGSCDQLQSYICF
CLPAFEGRNCE γ THKDDQLICVNENGGCEQYCS γ DHTG γ TKRSCRCHEGYSL γ LADGV γ SCTPTVEYPCKIP
ILEKNASKPQGRIVGGKVC γ PKGECPWQV γ LLLVNGAQLCGGTLINTI γ WVVSAAHCFKIKNWRNLI γ AVL
GEHDLSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALLR γ LHQPVVLT γ DHVVPLCLPERTFSERTLAFV

RFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCLQQSRKVGDSPNITEYMFCAGYSDGSKDSCK
GDSGGPHATHYRGTWYLTGIVSWGQGCAVGHFGVYTRV SQYIEWLQKLMRSEPRPGVLLRAPFP

In another particular embodiment, the coagulation factor is a FVIII polypeptide.

- 5 Factor VIII (FVIII) is a large, complex glycoprotein that is primarily produced by hepatocytes. FVIII is composed of 2351 amino acids, including a signal peptide, and contains several distinct domains as defined by homology. There are three A-domains, a unique B-domain, and two C-domains. The domain order can be listed as NH₂-A1-A2-B-A3-C1-C2-COOH. FVIII circulates in plasma as two chains, separated at the B-A3 border.
- 10 The chains are connected by bivalent metal ion-bindings. The A1-A2-B chain is termed the heavy chain (HC) while the A3-C1-C2 is termed the light chain (LC). Small acidic regions C-terminal of the A1 (the a₁ region) and A2 (the a₂ region) and N-terminal of the A3 domain (the a₃ region) play important roles in its interaction with other coagulation proteins, including thrombin and von Willebrand factor (vWF or VWF), the carrier protein
- 15 for FVIII, *in vivo*.

Endogenous FVIII molecules circulate *in vivo* as a pool of molecules with B domains of various sizes, the shortest having C-terminal at position 740, i.e. at the C-terminal of A2-a₂. These FVIII molecules with B-domains of different length all have full procoagulant activity. Upon activation with thrombin, FVIII is cloven C-terminal of A1-a₁

20 at position 372, C-terminal of A2-a₂ at position 740, and between a₃ and A3 at position 1689, the latter cleavage releases the a₃ region, with concomitant loss of affinity for vWF. The activated FVIII molecule is termed FVIIIa. Activation allows interaction of FVIIIa with phospholipid surfaces like activated platelets and activated factor IX (FIXa): the tenase complex is formed, allowing efficient activation of factor X (FX).

25 The terms "Factor VIII(a)" and "FVIII(a)" include both FVIII and FVIIIa. "Factor VIII" or "FVIII" herein refers to a human plasma glycoprotein that is a member of the intrinsic coagulation pathway and is essential to blood coagulation. "Native FVIII" is the human FVIII molecule derived from the full length sequence as shown in SEQ ID NO: 159 (amino acid 1-2332). "FVIII(a)" includes natural allelic variants of FVIII(a) that may exist

30 and occur from one individual to another.

FVIII molecules/variants may be B domain-truncated FVIII molecules wherein the remaining domains correspond closely to the sequences as set forth in amino acid numbers 1-740 and 1649-2332 of SEQ ID NO: 159. In such variants, as well as in FVIII derived from the full-length sequence, mutations may be introduced in order to, for

35 example, reduce vWF binding capacity. Amino acid modifications, such as substitutions and deletions, may be introduced into the molecule in order to modify the binding

capacity of FVIII with various other components such as LRP, various receptors, other coagulation factors, cell surfaces, introduction and/or abolishment of glycosylation sites. Other mutations that do not abolish FVIII activity may also be accommodated in a FVIII molecule/variant that may be used for the purposes of the present invention.

5 The B domain of FVIII spans amino acids 741-1648 of SEQ ID NO: 159. The B domain is cloven at several different sites, generating large heterogeneity in circulating plasma FVIII molecules. The exact function of the heavily glycosylated B domain is unknown. What is known is that the B domain is indispensable for FVIII activity in the coagulation cascade. Recombinant FVIII is thus frequently produced in the form of B
10 domain-deleted/truncated variants.

Endogenous full length FVIII is synthesized as a single-chain precursor molecule. Prior to secretion, the precursor is cloven into the heavy chain and the light chain. Recombinant B domain-deleted FVIII can be produced by means of two different strategies. Either the heavy chain without the B-domain and the light chain are
15 synthesized individually as two different polypeptide chains (two-chain strategy) or the B domain-deleted FVIII is synthesized as a single precursor polypeptide chain (single-chain strategy) that is cloven into the heavy and light chains in the same way as the full-length FVIII precursor.

In a B domain-deleted FVIII precursor polypeptide, produced by the single-chain
20 strategy, the heavy and light chain moieties are often separated by a linker. To minimize the risk of introducing immunogenic epitopes in the B domain-deleted FVIII, the sequence of the linker is preferably derived from the FVIII B-domain. As a minimum, the linker must comprise a recognition site for the protease that cleaves the B domain-deleted FVIII precursor polypeptide into the heavy and light chain. In the B domain of full
25 length FVIII, amino acid 1644-1648 constitutes this recognition site. The thrombin cleavage site leading to removal of the linker on activation of B domain-deleted FVIII is located in the heavy chain. Thus, the size and amino acid sequence of the linker is unlikely to influence its removal from the remaining FVIII molecule by thrombin activation. Deletion/truncation of the B domain is an advantage for production of FVIII.
30 Nevertheless, parts of the B domain can be included in the linker without reducing the productivity. The negative effect of the B domain on productivity has not been attributed to any specific size or sequence of the B domain.

FVIII molecules for the present invention are capable of functioning in the coagulation cascade in a manner that is functionally similar, or equivalent, to FVIII,
35 inducing the formation of FXa via interaction with FIXa on an activated platelet and supporting the formation of a blood clot. FVIII activity can be assessed *in vitro* using

techniques well known in the art. Clot analyses, FX activation assays (often termed chromogenic assays), thrombin generation assays and whole blood thromboelastography are examples of such *in vitro* techniques, two of which are described in examples 28 and 29. FVIII molecules according to the present invention have FVIII activity that is at least
5 that of native human FVIII.

The term "FVIII variant", as used herein, is intended to designate Factor FVIII having the sequence of SEQ ID NO: 159, wherein one or more amino acids of the parent protein have been substituted by another amino acid and/or wherein one or more amino acids of the parent protein have been deleted and/or wherein one or more amino acids
10 have been inserted in protein and/or wherein one or more amino acids have been added to the parent protein. Such addition can take place either at the N-terminal end or at the C-terminal end of the parent protein or both. The "analogue" or "analogues" within this definition still have FVIII activity in its activated form. In one embodiment a variant is at least 90% identical with the sequence of of SEQ ID NO: 159. In another embodiment a
15 variant is at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identical with the sequence of of SEQ ID NO: 159. As used herein, any reference to a specific position refers to the corresponding position in SEQ ID NO: 159.

For the purpose of the current invention, FVIII may be plasma-derived or
20 recombinantly produced, using well known methods of production and purification. The degree and location of glycosylation, gamma-carboxylation and other post-translational modifications may vary depending on the chosen host cell and its growth conditions.

In another particular embodiment, the coagulation factor is a Factor IX polypeptide. Factor IX (FIX) is, in its active form FIXa, a trypsin-like serine protease that
25 serves a key role in haemostasis by generating, as part of the tenase complex, most of the factor Xa required to support proper thrombin formation during coagulation (reviewed in (Hoffman and Monroe, III 2001)).

Factor IX (FIX) is a vitamin K-dependent coagulation factor with structural similarities to factor VII, prothrombin, factor X, and protein C. The circulating zymogen
30 form is composed of of 415 amino acids divided into four distinct domains comprising an N-terminal γ -carboxyglutamic acid-rich (Gla) domain, two EGF domains and a C-terminal trypsin-like serine protease domain. Activation of FIX occurs by limited proteolysis at Arg¹⁴⁵-Ala¹⁴⁶ and Arg¹⁸⁰-Val¹⁸¹ releasing a 35-aa fragment, the so-called activation peptide (Schmidt and Bajaj 2003). The activation peptide is heavily glycosylated,
35 containing two N-linked and up to four O-linked glycans.

"Factor IX" or "FIX", as used herein, refers to a human plasma Factor IX glycoprotein that is a member of the intrinsic coagulation pathway and is essential to blood coagulation. "Factor IX(a)" includes natural allelic variants of FIX(a) that may exist and occur from one individual to another. Factor IX(a) may be plasma-derived or recombinantly produced using well known methods of production and purification. The degree and location of glycosylation, gamma-carboxylation and other post-translation modifications may vary depending on the chosen host cell and its growth conditions. Unless otherwise specified or indicated, Factor IX means any functional human Factor IX protein molecule in its normal role in coagulation, including any fragment, analogue and derivative thereof.

One example of a "wild type FIX" is the full length human FIX molecule, as shown in SEQ ID NO: 161.

The terms "FIX analogue", as used herein, is intended to designate Factor FIX having the sequence of SEQ ID NO: 161, wherein one or more amino acids of the parent protein have been substituted by another amino acid and/or wherein one or more amino acids of the parent protein have been deleted and/or wherein one or more amino acids have been inserted in protein and/or wherein one or more amino acids have been added to the parent protein. Such addition can take place either at the N-terminal end or at the C-terminal end of the parent protein or both. The "analogue" or "analogues" within this definition still have FIX activity in its activated form. In one embodiment a variant is at least 90% identical with the sequence of SEQ ID NO: 161. In a further embodiment a variant is at least 95% identical with the sequence of of SEQ ID NO: 161. As used herein any reference to specific positions refers to the corresponding position in SEQ ID NO: 161. Non-limiting examples of FIX(a) variants that have substantially the same activity, FVIII bypassing activity or increased proteolytic activity compared to recombinant wild type human Factor IX(a) include those disclosed in Milanov P, Ivanciu L, Abriss D, Quade-Lyssy P, Miesbach W, Alesci S, Tonn T, Grez M, Seifried E, Schüttrumpf (2012) J.Blood 119: 602-11 and US 2011/0217284 A1. Unless otherwise specified, Factor IX domains include the following amino acid residues: Gla domain being the region from residue Tyr1 to residue Lys43; EGF1 being the region from residue Gln44 to residue Leu84; EGF2 being the region from residue Asp85 to residue Arg145; the Activation Peptide being the region from residue Ala146 to residue Arg180; and the Protease Domain being the region from residue Val181 to Thr414. The light chain refers to the region encompassing the Gla domain, EGF1 and EGF2, while the heavy chain refers to the Protease Domain.

Factor IX may be plasma-derived or recombinantly produced, using well known methods of production and purification. The degree and location of glycosylation, gamma-carboxylation and other post-translational modifications may vary depending on the chosen host cell and its growth conditions.

A commercially available assay kit known as 'Hyphen BioMed Chromogenic Factor IX kit (Aniara)' may be used to assess the activity level of the FIX polypeptide. In this assay, Factor XIa activates Factor IX into Factor IXa, which together with activated Factor VIII:C, phospholipids and Ca^{2+} , activates Factor X into Factor Xa. The amount of generated Factor Xa was measured at 405 nm by the amount of pNA released from the Factor Xa specific chromogenic substrate Sxa-11.

Gamma-Carboxylated residues in the FVII sequence below are represented by " γ ".

SEQ ID NO: 161: Wild type human coagulation Factor IX

YNSGKL $\gamma\gamma$ FVQGNL γ R γ CM $\gamma\gamma$ KCSF $\gamma\gamma$ AR γ VF γ NT γ RTT γ FWKQYVDGDQCESNPCLNGGSCCKDDINSY
 ECWCPCFGFEGKNCELDVTCTNIKNGRCEQFCKNSADNKVVCSTEGYRLAENQKSCEPAVPFPCGRV
 SVSQTSLKTRAEAVFPDVDYVNSTEAEITLDNITQSTQSFNDFTRVVGGEDAKPGQFPWQVVLNGK
 VDAFCGGSIVNEKWIVTAAHCVETGVKITVVAGEHNIEETEHEQKRNIVIRIIPHHNYN AAINKYNH
 DIALLELDEPLVLNSYVTPICIAADKEYNIFLKFGSGYVSGWGRVFHKGRSALVLQYLRVPLVDRATCLR
 STKFTYNNMFCAGFHEGGRDSCQGDSGGPHVTEVEGTSFLTGIISWGEECAMKGKYGIYTKVSRVY
 NWIKEKTKLT

In another particular embodiment, the coagulation factor is a FX polypeptide. Coagulation factor X (FX) is a vitamin K-dependent coagulation factor with structural similarities to factor VII, prothrombin, factor IX (FIX), and protein C. It is synthesised with a 40-residue pre-pro-sequence containing a hydrophobic signal sequence (Aa 1-31) that targets the protein for secretion. The pro-peptide is important for directing γ -carboxylation to the light chain of Factor X. The circulating human FX zymogen is composed of 445 amino acids divided into four distinct domains comprising an N-terminal gamma-carboxyglutamic acid rich (Gla) domain, two EGF domains, and a C-terminal trypsin-like serine protease domain. The mature two-chain form of FX is composed of a light chain (Aa41-179) and a heavy chain (Aa183-488) held together by a disulfide bridge (Cys¹⁷² - Cys³⁴²). The light chain contains 11 Gla residues, which are important for Ca^{2+} -dependent binding of FX to negatively charged phospholipid membranes. Wild-type human coagulation factor X has two N-glycosylation sites (Asn²²¹ and Asn²³¹) and two O-glycosylation sites (Thr¹⁹⁹ and Thr²¹¹) in the activation peptide. β -hydroxylation occurs at Asp¹⁰³ in the first EGF domain, resulting in β -hydroxyaspartic acid (Hya). Activation of FX

occurs by limited proteolysis at Arg²³⁴-Ile²³⁵ releasing a 52 amino acid activation peptide (Aa 183-234). In the extrinsic pathway, this occurs upon exposure of Tissue factor (TF) on the membrane of subendothelial cells to plasma and subsequent activation of FVIIa. Activation via the intrinsic pathway occurs with the interaction of factor IXa, factor VIIIa, calcium and acidic phospholipid surfaces. Prothrombin is the most important substrate of Factor Xa, but the activation requires FXa's co-factor factor Va, calcium and acidic phospholipid surface. FX deficiency is a rare autosomal recessive bleeding disorder with an incidence of 1:1,000,000 in the general population (Dewerchin et al. (2000) Thromb Haemost 83: 185-190). Although it produces a variable bleeding tendency, patients with a severe FX deficiency tend to be the most seriously affected among patients with rare coagulation defects. The prevalence of heterozygous FX deficiency is about 1:500, but is usually clinically asymptomatic.

One example of a "wild type FX" is the full length human FX molecule, as shown in SEQ ID NO: 163.

"Factor X polypeptide" herein refers to any functional Factor X protein molecule capable of activating thrombin, including fragments, analogues and derivatives of SEQ ID NO: 163.

The term "FX analogue", as used herein, is intended to designate Factor FX having the sequence of SEQ ID NO: 163, wherein one or more amino acids of the parent protein have been substituted by another amino acid and/or wherein one or more amino acids of the parent protein have been deleted and/or wherein one or more amino acids have been inserted in protein and/or wherein one or more amino acids have been added to the parent protein. Such addition can take place either at the N-terminal end or at the C-terminal end of the parent protein or both. The "analogue" or "analogues" within this definition still have FX activity in its activated form. In one embodiment a variant is at least 90 % identical with the sequence of SEQ ID NO: 163. In a further embodiment a variant is at least 95% identical with the sequence of SEQ ID NO: 163. As used herein any reference to specific positions refers to the corresponding position in SEQ ID NO: 163.

FX may be plasma-derived or recombinantly produced, using well known methods of production and purification. The degree and location of glycosylation, gamma-carboxylation and other post-translational modifications may vary depending on the chosen host cell and its growth conditions.

In another particular embodiment, the coagulation factor is a Factor XI polypeptide. Factor XI (FXI) is the zymogen of a blood coagulation protease, factor XIa (FXIa), which contributes to hemostasis through activation of factor IX. The factor is

produced by the liver (Emsley et al. (2010) Blood 115:2569-77). The protein is a 160-kDa disulfide-linked dimer of identical 607 amino acid subunits, each containing 4 90- or 91-amino acid repeats called apple domains (from the N-terminus: A1: Aa 20-103, A2: Aa 110-193, A3: Aa 200-283, A4: Aa 291-374) and a C-terminal trypsin-like catalytic domain. The protein is expressed with a signal peptide Aa1-18. The structure is different from those of the well-characterized vitamin K-dependent coagulation proteases. FXI circulates in blood as a complex with high molecular weight kininogen (HK). Prekallikrein (PK), the zymogen of the protease -kallikrein, is a monomeric homolog of FXI with the same domain structure that also circulates in complex with HK. The zymogen factor is activated into factor XIa by Factor XIIa (FXIIa), thrombin, and is also autocatalytic. Cleavage activation occurs at the activation loop containing the Arg369-Ile370 cleavage site. Since FXI deficiency causes relatively mild bleeding, FXI has a speculative role in early fibrin generation. FXIa is postulated to be part of a feedback loop that sustains thrombin generation through FIX activation to consolidate coagulation. Certain tissues with robust fibrinolytic activity seem important for FXIa activity, including oropharynx and urinary tract, as these are common sites of bleeding in FXI-deficient patients. Congenital FXI deficiency is associated with a mild to moderate bleeding disorder. More than 180 other FXI gene mutations associated with FXI deficiency have been reported (<http://www.factorxi.org>, <http://www.isth.org>), including more than 100 single amino acid (missense) substitutions. Severe deficiency is prevalent in people of Jewish ancestry (Seligsohn et al. (2007) Thromb Haemost. 98:84-89). The carrier rate is approximately 5% in Ashkenazi Jews, with severe (homozygous) deficiency found in 1 in 450 persons. As an example, a severe mutation at Glu117Stop results in a truncated protein and homozygotes lacks completely plasma FXI antigen.

Factor XIa activates factor IX by selectively cleaving Arg¹⁴⁵-Ala¹⁴⁶ and Arg¹⁸⁰-Val¹⁸¹.

The term "FXI analogue", as used herein, is intended to designate Factor XI having the sequence of SEQ ID NO: 165, wherein one or more amino acids of the parent protein have been substituted by another amino acid and/or wherein one or more amino acids of the parent protein have been deleted and/or wherein one or more amino acids have been inserted in protein and/or wherein one or more amino acids have been added to the parent protein. Such addition can take place either at the N-terminal end or at the C-terminal end of the parent protein or both. The "analogue" or "analogues" within this definition still have FX activity in its activated form. In one embodiment a variant is at least 90% identical with the sequence of SEQ ID NO: 165. In a further embodiment a variant is at least 95% identical with the sequence of SEQ ID NO: 165. As used herein

any reference to a specific positions refers to the corresponding position in SEQ ID NO: 165.

FXI may be plasma-derived or recombinantly produced, using well known methods of production and purification. The degree and location of glycosylation, and other post-translational modifications may vary depending on the chosen host cell and its growth conditions.

The term "identity", as known in the art, refers to a relationship between the sequences of two or more polypeptides, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptides, as determined by the number of matches between strings of two or more amino acid residues. "Identity" measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (i.e., "algorithms"). Identity of related polypeptides can be readily calculated by known methods. Such methods include, but are not limited to, those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; and Carillo et al., SIAM J. Applied Math. 48, 1073 (1988).

Preferred methods for determining identity are designed to give the largest match between the sequences tested. Methods of determining identity are described in publicly available computer programs. Preferred computer program methods for determining identity between two sequences include the GCG program package, including GAP (Devereux et al., Nucl. Acid. Res. 12, 387 (1984); Genetics Computer Group, University of Wisconsin, Madison, Wis.), BLASTP, BLASTN, and FASTA (Altschul et al., J. Mol. Biol. 215, 403-410 (1990)). The BLASTX program is publicly available from the National Center for Biotechnology Information (NCBI) and other sources (BLAST Manual, Altschul et al. NCB/NLM/NIH Bethesda, Md. 20894; Altschul et al., supra). The well known Smith Waterman algorithm may also be used to determine identity.

For example, using the computer algorithm GAP (Genetics Computer Group, University of Wisconsin, Madison, Wis.), two polypeptides for which the percent sequence identity is to be determined are aligned for optimal matching of their respective amino acids (the "matched span", as determined by the algorithm). A gap opening penalty (which is calculated as 3 times the average diagonal; the "average diagonal" is the

average of the diagonal of the comparison matrix being used; the "diagonal" is the score or number assigned to each perfect amino acid match by the particular comparison matrix) and a gap extension penalty (which is usually {fraction (1/10)} times the gap opening penalty), as well as a comparison matrix such as PAM 250 or BLOSUM 62 are used in conjunction with the algorithm. A standard comparison matrix (see Dayhoff et al., Atlas of Protein Sequence and Structure, vol. 5, supp.3 (1978) for the PAM 250 comparison matrix; Henikoff et al., Proc. Natl. Acad. Sci USA 89, 10915-10919 (1992) for the BLOSUM 62 comparison matrix) is also used by the algorithm.

Preferred parameters for a peptide sequence comparison include the following:

Algorithm: Needleman et al., J. Mol. Biol. 48, 443-453 (1970); Comparison matrix: BLOSUM 62 from Henikoff et al., PNAS USA 89, 10915-10919 (1992); Gap Penalty: 12, Gap Length Penalty: 4, Threshold of Similarity: 0.

The GAP program is useful with the above parameters. The aforementioned parameters are the default parameters for peptide comparisons (along with no penalty for end gaps) using the GAP algorithm.

Hence, the procoagulant proteins of the current invention comprise (i) at least one coagulation factor component and (ii) an antibody or fragment thereof that is capable of binding a receptor, and/or a fragment thereof, wherein the receptor is present only (in the non-ubiquitous sense of the word) on the surface of activated platelets. In one preferred embodiment, said receptor is TLT-1. The procoagulant proteins of the current invention are preferably engineered such that their constituent parts may function independently of one another. For example, said coagulation factor component of the current invention is capable of upregulating blood coagulation. Likewise, said "antibody" component of the invention is preferably able to bind a receptor such as TLT-1, unhindered by the presence of said coagulation factor component. The carboxy terminus of the coagulation factor component may be covalently attached to the amino terminus of the antibody component of the construct, or vice versa. Said antibody component of the construct will preferably not bind to or demonstrate little affinity for any other triggering receptor expressed on myeloid cells (TREM). The construct of the current invention may or may not comprise a linker between said coagulation factor and said antibody constituents. Said optional linker may be any one of the linkers described in Table 3, or may be any other linker that binds both coagulation factor and antibody constituent parts of the construct, such that both are functional. In one embodiment, the coagulation factor and anti-TLT-1 components are expressed as fusion proteins. In one embodiment, the coagulation factor and anti-TLT-1 components are chemically conjugated.

Procoagulant proteins wherein part (ii) is a mAb may comprise two coagulation factor polypeptides (part (i)). The coagulation factor may be fused to a HC of the mAb; The coagulation factor may be fused to a LC of the mAb. The coagulation factor may be fused to a antibody, or fragment thereof, which, in turn, is fused to a HC of the mAb or a LC of the mAb.

Thus, a procoagulant protein of the present invention may comprise (i) at least one FV polypeptide and (ii) an antibody, or fragment thereof, that is capable of binding TLT-1.

A procoagulant protein of the present invention may comprise (i) at least one FVII polypeptide and (ii) an antibody, or fragment thereof, that is capable of binding TLT-1.

A procoagulant protein of the present invention may comprise (i) at least one FVIII polypeptide and (ii) an antibody, or fragment thereof, that is capable of binding TLT-1.

A procoagulant protein of the present invention may comprise (i) at least one FIX polypeptide and (ii) an antibody, or fragment thereof, that is capable of binding TLT-1.

A procoagulant protein of the present invention may comprise (i) at least one FX polypeptide and (ii) an antibody, or fragment thereof, that is capable of binding TLT-1.

A procoagulant protein of the present invention may comprise (i) at least one FXI polypeptide and (ii) an antibody, or fragment thereof, that is capable of binding TLT-1.

Procoagulant proteins may further comprise a linker. Non-limiting examples of linker amino acid sequences, which may be utilised when the procoagulant proteins are manufactured as fusion proteins, are shown in Table 3. Hence, said linker may be L1. The linker may be L2. The linker may be L3. The linker may be L4. The linker may be L5. The linker may be L6. The linker may be L7. The linker may be L8. The linker may be L9. The linker may be L10.

Table 3: Non-limiting examples of optional linkers

Linker ID	Length (AA)	Linker sequence
L0	0	no linker
L1	2	GS
L2	7	GSGGGGS
L3	12	GSGGGGSGGGGS
L4a	17	GSGGGGSGGGGSGGGGS
L4b	17	GGGGSGGGGSGGGGSGS
L5	22	GGGGSGSGGGGSGGGGSGGGGS
L6	27	GGGGSGGGGSGSGGGGSGGGGSGGGGS

L7	32	GGGGS GGGGS GGGGS GSGGGGS GGGGS GGGGS
L8	37	GGGGS GGGGS GGGGS GGGGS GSGGGGS GGGGS GGGGS
L9	42	GGGGS GGGGS GGGGS GGGGS GSGGGGS GGGGS GGGGS
L10	16	YGPPSPSSPAPEFLGG

As mentioned above, the extracellular part of TLT-1 is composed of an immunoglobulin-like domain and a stalk. Procoagulant proteins of the invention may be capable of binding to either of these. When part (ii) of the procoagulant protein is capable of binding the immunoglobulin-like domain, a longer linker may allow part (i) of said fusion protein to adapt a functionally relevant position and orientation on the surface of the activated platelet, thereby facilitating its function.

A procoagulant protein that is capable of binding the stalk of TLT-1 is adjacent to the platelet membrane. A procoagulant protein that is capable of binding the stalk may comprise a linker but the inclusion of a linker does not necessarily affect the function of the coagulation factor part of the fusion protein.

As described above, procoagulant proteins of the invention are capable of binding a receptor that is present on platelets that are undergoing activation or that are fully activated, such as TLT-1. The term "binding affinity" is intended to refer to the property of procoagulant proteins, or the antibody component of procoagulant proteins, to bind or not to bind to their target. Binding affinity may be quantified by determining the binding constant (K_D) for an antibody component and its target. Similarly, the specificity of binding of an antibody component to its target may be defined in terms of the comparative binding constants (K_D) of the antibody for its target as compared to the binding constant with respect to the antibody and another, non-target molecule.

Typically, the K_D for the antibody with respect to the target will be 2-fold, preferably 5-fold, more preferably 10-fold less than K_D with respect to the other, non-target molecule such as unrelated material or accompanying material in the environment. More preferably, the K_D will be 50-fold less, even more preferably 100-fold less, and yet more preferably 200-fold less.

The value of this binding constant can be determined directly by well-known methods, and can be computed even for complex mixtures by methods such as those, for example, set forth in Caceci et al. (Byte 9:340-362, 1984). For example, the K_D may be established using a double-filter nitrocellulose filter binding assay such as that disclosed by Wong & Lohman (Proc. Natl. Acad. Sci. USA 90, 5428-5432, 1993). Other standard assays to evaluate the binding ability of antibodies towards targets are known in the art, including for example, ELISAs, Western blots, RIAs, and flow cytometry analysis. The

binding kinetics (e.g., association rate and dissociation rate constants) and binding affinity of the antibody can also be assessed by standard assays known in the art, such as by surface plasmon resonance (SPR) analysis.

A competitive binding assay can be conducted in which the binding of the antibody to the target is compared to the binding of the target by another, known ligand of that target, or another antibody.

K_D values for the antibody, or fragment thereof, of the invention may also be at least $1 \times 10^{-15}M$, such as at least $1 \times 10^{-14}M$, such as at least $1 \times 10^{-13}M$, such as at least $1 \times 10^{-12}M$, such as at least $1 \times 10^{-11}M$, such as at least $1 \times 10^{-10}M$, such as approximately $3 \times 10^{-9}M$, such as at least $1 \times 10^{-9}M$, or at least $1 \times 10^{-8}M$. An antibody of the invention may have a K_d (or K_i) for its target of $1 \times 10^{-7}M$ or less, $1 \times 10^{-8}M$ or less or $1 \times 10^{-9}M$ or less.

Preferred K_D values for the antibody, or fragment thereof, may be $1 \times 10^{-15}M$ to $1 \times 10^{-14}M$, such as $1 \times 10^{-14}M$ to $1 \times 10^{-13}M$, $1 \times 10^{-13}M$ to $1 \times 10^{-12}M$, such as $1 \times 10^{-12}M$ to $1 \times 10^{-11}M$, such as $1 \times 10^{-11}M$ to $1 \times 10^{-10}M$, such as $1 \times 10^{-10}M$ to $1 \times 10^{-9}M$ such as approximately $3 \times 10^{-9}M$, such as $1 \times 10^{-9}M$ to $2 \times 10^{-8}M$.

An antibody or fragment thereof that specifically binds its target may bind its target with a high affinity, such as a K_D as discussed above, and may bind to other, non-target molecules with a lower affinity. For example, the antibody may bind to a non-target molecules with a K_D of $1 \times 10^{-6}M$ or more, more preferably $1 \times 10^{-5}M$ or more, more preferably $1 \times 10^{-4}M$ or more, more preferably $1 \times 10^{-3}M$ or more, even more preferably $1 \times 10^{-2}M$ or more. A procoagulant protein of the invention is preferably capable of binding to its target with an affinity that is at least two-fold, 10-fold, 50-fold, 100-fold, 200-fold, 500-fold, 1,000-fold or 10,000-fold or greater than its affinity for binding to another non-target molecule, such as other TREMs than TLT-1.

The functional effects of the invented procoagulant proteins may be assessed by means of various *in vitro* and *in vivo* experiments. *In vitro* experiments may be designed to assess the function of the fusion proteins as a whole, as well as their component (i) coagulation factor and (ii) antibody parts. *In vivo*, fusion proteins may be tested in a tail-bleeding model in haemophilic mice that are transfused with human platelets. Furthermore *in vivo*, fusion proteins may be tested in a tail-bleeding model in haemophilic mice with the human TLT-1 gene inserted ("humanized" with respect to TLT-1).

As mentioned above, the procoagulant proteins may be provided in the form of fusion proteins or chemical conjugates. In the former case, the invention also relates to polynucleotides that encode the procoagulant proteins of the invention. Thus, a

polynucleotide of the invention may encode any procoagulant protein as described herein. The terms "nucleic acid molecule" and "polynucleotide" are used interchangeably herein and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Non-limiting examples of polynucleotides include a gene, a gene fragment, messenger RNA (mRNA), cDNA, recombinant polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide of the invention may be provided in isolated or purified form.

A nucleic acid sequence which "encodes" a selected polypeptide is a nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vivo*, when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. For the purposes of the invention, such nucleic acid sequences can include, but are not limited to, cDNA from viral, prokaryotic or eukaryotic mRNA, genomic sequences from viral or prokaryotic DNA or RNA, and even synthetic DNA sequences. A transcription termination sequence may be located 3' to the coding sequence.

Polynucleotides of the invention can be synthesised according to methods well known in the art, as described by way of example in Sambrook et al. (1989, Molecular Cloning - a laboratory manual; Cold Spring Harbor Press).

The nucleic acid molecules of the present invention may be provided in the form of an expression cassette which includes control sequences operably linked to the inserted sequence, thus allowing for expression of the antibody of the invention *in vivo*. These expression cassettes, in turn, are typically provided within vectors (e.g., plasmids or recombinant viral vectors). Such an expression cassette may be administered directly to a host subject. Alternatively, a vector comprising a polynucleotide of the invention may be administered to a host subject. Preferably the polynucleotide is prepared and/or administered using a genetic vector. A suitable vector may be any vector which is capable of carrying a sufficient amount of genetic information, and allowing expression of a polypeptide of the invention.

The present invention thus includes expression vectors that comprise such polynucleotide sequences. Such expression vectors are routinely constructed in the art of molecular biology and may for example involve the use of plasmid DNA and appropriate initiators, promoters, enhancers and other elements, such as for example polyadenylation signals which may be necessary, and which are positioned in the correct orientation, in order to allow for expression of a peptide of the invention. Other suitable

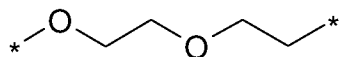
vectors would be apparent to persons skilled in the art. By way of further example in this regard we refer to Sambrook et al.

The invention also includes isolated cells that have been modified to express fusion proteins according to the invention. Such cells include transient, or – preferably – stable higher eukaryotic cell lines, such as mammalian cells or insect cells; lower eukaryotic cells, such as yeast; or prokaryotic cells such as bacterial cells. Particular examples of cells which may be modified by insertion of vectors or expression cassettes encoding for a construct of the invention include mammalian HEK293T, CHO, HeLa and COS cells. Preferably the cell line selected will be one which is not only stable, but also allows for mature glycosylation and cell surface expression of a polypeptide.

Such cell lines of the invention may be cultured using routine methods to produce a fusion protein or construct according to the invention. Alternatively, polynucleotides, expression cassettes or vectors of the invention may be administered to a cell from a subject *ex vivo* and the cell then returned to the body of the subject.

Alternatively, procoagulant proteins may be obtained by chemical conjugation of the antibody (such as a mAb), or fragment thereof, and the coagulation factor. In this case, a linker between the two proteins may contain one or more chemical moieties which are not present in those amino acids that are encoded by DNA.

In one embodiment, a chemical moiety used in the linker comprises the biradical with the structure



wherein * shows the positions of connection of this biradical.

The term "biradical" refers to an even-electron chemical compound with two free radical centres which act independently of one another.

In another embodiment, a chemical moiety used in the linker comprises a polymer: a macromolecule composed of repeating structural units that are typically connected by covalent chemical bonds. Such a polymer may be hydrophilic.

The term hydrophilic or "water-soluble" refers to moieties that have some detectable degree of solubility in water. Methods to detect and/or quantify water solubility are well known in the art.

Exemplary water-soluble polymers according to the invention include peptides, saccharides, (poly)ethers, (poly)amines, (poly)carboxylic acids and the like. Peptides can have mixed sequences and be composed of a single amino acid, e.g., (poly)lysine. An exemplary polysaccharide is (poly)sialic acid. An exemplary (poly)ether is (poly)ethylene

glycol. (Poly)ethylene imine is an exemplary polyamine, and (poly)acrylic acid is a representative (poly)carboxylic acid.

The hydrophilic polymer according to the present invention is, preferably, non-naturally occurring. In one example, the non-naturally occurring modifying group is a polymeric modifying group, in which at least one polymeric moiety is non-naturally occurring. In another example, the non-naturally occurring modifying group is a modified carbohydrate. The locus of functionalization with the modifying group is selected such that it does not prevent the "modified sugar" from being added enzymatically to a polypeptide. "Modified sugar" also refers to any glycosyl mimetic moiety that is functionalized with a modifying group and which is a substrate for a natural or modified enzyme, such as a glycosyltransferase.

Many other polymers are also suitable for the invention. Polymer backbones that are non-peptidic and water-soluble, are particularly useful in the invention. Examples of suitable polymers include, but are not limited to, other poly(alkylene glycols), such as poly(propylene glycol) ("PPG"), copolymers of ethylene glycol and propylene glycol and the like, poly(oxyethylated polyol), poly(olefinic alcohol), poly(vinylpyrrolidone), poly(hydroxypropylmethacrylamide), poly(α -hydroxy acid), poly(vinyl alcohol), polyphosphazene, polyoxazoline, poly(N-acryloylmorpholine), such as described in U.S. Patent No. 5,629,384, which is incorporated by reference herein in its entirety, as well as copolymers, terpolymers, and mixtures thereof.

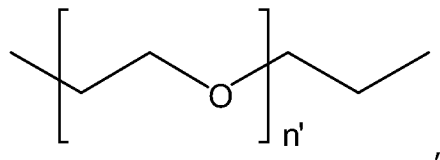
The polymeric linker may alter a property of the procoagulant protein, such as its bioavailability, biological activity or its half-life in the body.

The polymeric linker is, preferably, linear.

Although the molecular weight of each individual polymer chain may vary, it is typically in the range of from about 1,000 Da (1 kDa) to about 40,000 Da (40 kDa), such as about 1,000 Da to about 12,000 Da such as about 2,000 Da to about 11,000 Da, such as about 2,000 Da to about 3,000 Da; about 3,000 Da to about 4,000 Da; about 4,000 to about 5,000 Da; about 5,000 to about 6,000 Da; about 6,000 to about 7,000 Da; about 7,000 to about 8,000 Da; about 8,000 to about 9,000 Da; about 9,000 to about 10,000 Da; or about 10,000 to about 11,000 Da. It should be understood that these sizes represent estimates rather than exact measures. According to a preferred embodiment, the molecules according to the invention are conjugated with a heterogeneous population of hydrophilic polymers.

In a particular embodiment, a chemical moiety used in the linker comprises polyethylene glycol (PEG).

The term "PEG" herein refers to a biradical comprising the structure



wherein n' is an integer larger than 1.

PEG is prepared by polymerization of ethylene oxide and is commercially available over a wide range of molecular weights. The PEG for use according to the present invention is, preferably, linear.

Furthermore, "PEG" may refer to a polyethylene glycol compound, or derivative thereof, with or without coupling agents, coupling or activating moieties (e.g., with carboxylic acid/active ester, keto, alkoxyamine, thiol, triflate, tresylate, aziridine, oxirane, alkyne, azide or a maleimide moiety).

In one particular embodiment the PEG for use according to the invention is monodisperse. In another particular embodiment, the PEG for use according to the invention is polydisperse.

Polydisperse PEG is composed of PEG molecules that have various molecular weights. The size distribution can be characterized statistically by its weight average molecular weight (M_w) and its number average molecular weight (M_n), the ratio of which is called the polydispersity index (M_w/M_n) (see e.g. "Polymer Synthesis and Characterization", J. A. Nairn, University of Utah, 2003). M_w and M_n can be measured by mass spectroscopy.

The polydispersity index may be a number that is greater than or equal to one and it may be estimated from Gel Permeation Chromatographic data. When the polydispersity index is 1, the product is monodisperse and is thus made up of compounds with a single molecular weight. When the polydispersity index is greater than 1 the polymer is polydisperse, and the polydispersity index tells how broad the distribution of polymers with different molecular weights is. The polydispersity index typically increases with the molecular weight of the PEG. In particular embodiments, the polydispersity index of the PEG for use according to the invention is i) below 1.06, ii) below 1.05, iii) below 1.04, iv) below 1.03 or v) between 1.02 and 1.03.

Different forms of PEG are available, depending on the initiator used for the polymerization process.

Numerous methods for conjugation of PEG substituents are described in *Advanced Drug Delivery Reviews*, 2002, 54, 459–476, *Nature Reviews Drug Discovery*, 2003, 2, 214–221 DOI:10.1038/nrd1033, *Adv Polym Sci*, 2006, 192, 95–134, DOI 10.1007/12_022, Springer-Verlag, Berlin Heidelberg, 2005, and references therein. Alternatively, conjugation of the hydrophilic polymer substituent could take place by use

of enzymatic methods. Such methods are for instance use of glycosyltransferases as described in WO2003/031464 or use of transglutaminases as described in WO2006134148.

To effect covalent attachment of the polymer molecule(s) to the polypeptide, the hydroxyl end groups of the polymer molecule are provided in activated form, i.e. with reactive functional groups. Suitable activated polymer molecules are commercially available, e.g. from Sigma-Aldrich Corporation, St. Louis, MO, USA, Rapp Polymere GmbH, Tübingen, Germany, or from PolyMASC Pharmaceuticals plc, UK. Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g. as disclosed in WO 90/13540. Specific examples of activated PEG polymers are disclosed in U.S. Pat. No. 5,932,462 and U.S. Pat. No. 5,643,575. Furthermore, the following publications disclose useful polymer molecules and/or PEGylation chemistries: WO2003/031464, WO2004/099231.

The conjugation of the monoclonal antibody, fragment thereof or coagulation factor with the activated polymer molecules may be conducted by use of any conventional method, e.g. as described in the following references (which also describe suitable methods for activation of polymer molecules): R. F. Taylor, (1991), "Protein immobilisation. Fundamental and applications", Marcel Dekker, N.Y.; S. S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking", CRC Press, Boca Raton; G. T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques", Academic Press, N.Y., 'Bioconjugate Techniques, Second Edition, Greg T. Hermanson, 2008, Amsterdam, Elsevier). The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the attachment group(s) of the polypeptide (examples of which are given further above), as well as the functional groups of the polymer (e.g. being amine, hydroxyl, carboxyl, aldehyde, sulfhydryl, succinimidyl, maleimide, vinylsulfone or haloacetate). The PEGylation may be directed towards conjugation to all available attachment groups on the polypeptide (i.e. such attachment groups that are exposed at the surface of the polypeptide) or may be directed towards one or more specific attachment groups, e.g. the N-terminal amino group. Furthermore, the conjugation may be achieved in one step or in a stepwise manner.

In another embodiment, a chemical moiety used as the linker is hydroxyethyl starch. The term "hydroxyethyl starch" (HES/HAES), as used herein, refers to a nonionic starch derivative. Different types of hydroxyethyl starches are typically described by their average molecular weight, typically around 130 to 200 kDa.

In another embodiment, a chemical moiety used in the linker comprises polysialic acid.

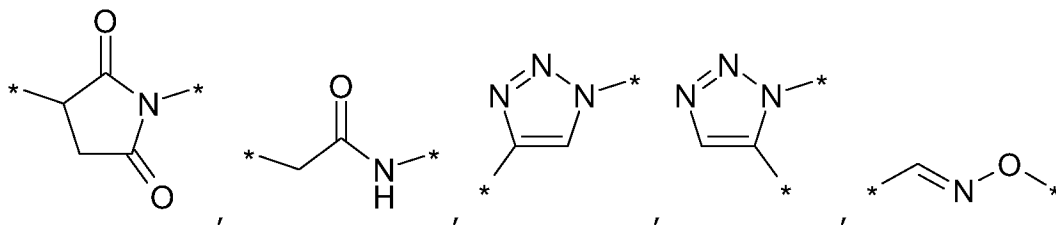
In another embodiment, a chemical moiety used in the linker is attached to at least one of the proteins to a glycan: a polysaccharide or an oligosaccharide that is attached to a protein.

In another embodiment, a chemical moiety used in the linker is attached to at least one of the proteins to an *O*-linked glycan.

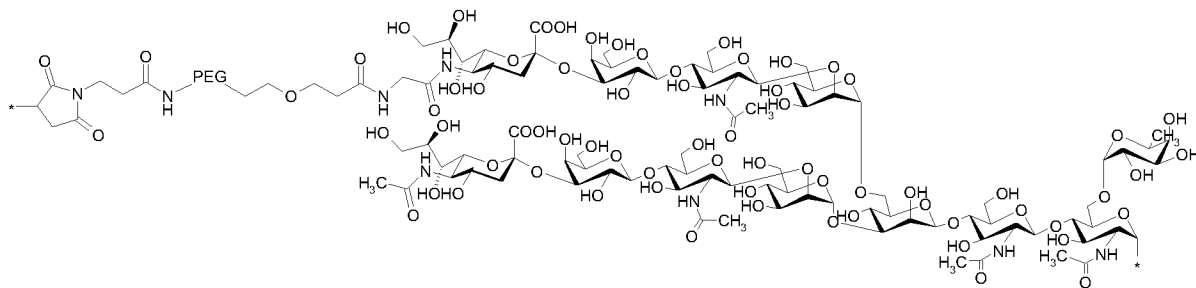
In another embodiment, a chemical moiety used in the linker is attached to at least one of the proteins to an *N*-linked glycan.

Both N-glycans and O-glycans are attached to proteins such as mAbs and coagulation factors by the cells producing these proteins. The cellular N-glycosylation machinery recognizes and glycosylates N-glycosylation signals (N-X-S/T motifs) in the amino acid chain, as the nascent protein is translocated from the ribosome to the endoplasmic reticulum (Kiely et al. 1976; Glabe et al. 1980). Likewise, O-glycans are attached to specific O-glycosylation sites in the amino acid chain, but the motifs triggering O-glycosylation are much more heterogeneous than the N-glycosylation signals, and our ability to predict O-glycosylation sites in amino acid sequences is still inadequate (Julenius et al. 2004). Methods of conjugating polypeptides with various polymeric side groups are described e.g. in WO0331464.

In another embodiment, a chemical moiety used in the linker comprises a chemical moiety, which is used to attach said linker to at least one of the proteins with a structure selected of the biradicals:

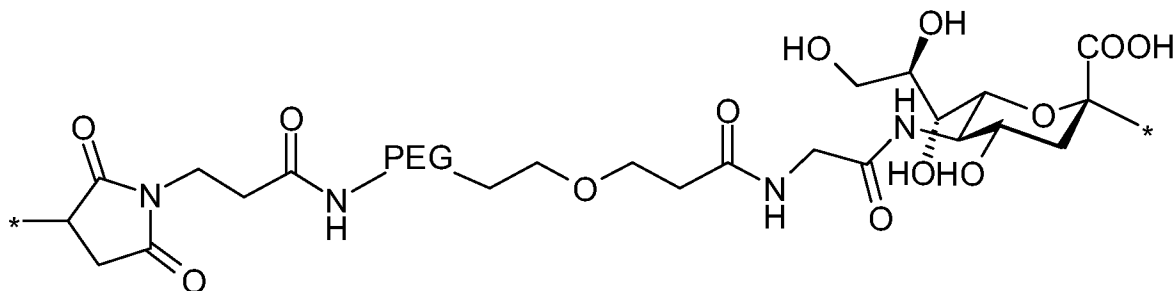


In one embodiment, the linker comprises the biradical structure of



wherein * shows the positions of connection of this biradical.

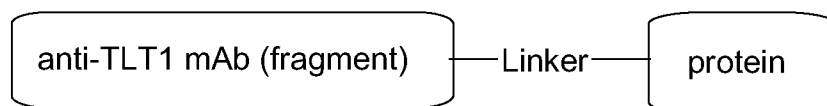
In another embodiment, the linker comprises the structure



wherein * shows the positions of connection of this biradical.

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A compound of the general formula



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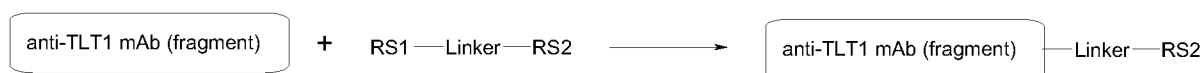
wherein "anti-TLT-1 mAb (fragment)" may be a full size mAb against TLT-1 or a fragment or an analogue intellectually derived thereof such as but not limited to, a FAB-fragment or a sc-FAB with none, one or more point mutations, the linker may be a water soluble polymer such as but not limited to e.g. PEG, polysialic acid, or hydroxyethyl starch, and protein is any protein which is thought to has one or more improved properties when attached to anti-TLT-1 mAb (fragment) may be for example prepared in a two step procedure.

15

During the first step, a linker, with two different reactive groups RS1 and RS2 may be attached to the anti-TLT-1 mAb (fragment). The reaction may be run with low site selectivity or in a selective way, such that RS1 only reacts at one or few position of the anti TLT-1 mAb (fragment). As a non-exclusive example, RS1 could be an aldehyde and react by reductive amination only with N-termini of the anti TLT-1 mAb (fragment) by reductive amination, known to a person trained in the art. Another non-exclusive example RS1 could be a maleimide group, which may react with a free thiol on the anti TLT-1 mAb (fragment).

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25



During the second step, the reactive group RS2 may be reacted with low site selectivity or site selectivity with a FVIIa molecule. As a non-exclusive example, a site selective reaction at FVIIa may be obtained when RS2 is a sialic acid derivative, which can react in the presence of a suitable enzyme such as but not limited to ST3Gal-III with N-linked glycans, which do not end exclusively with sialic acids.



The order of attachment of the linker to the two proteins, namely the anti-TLT-1 mAb (fragment) and the protein may be switched, thereby attaching the RS1-Linker-RS2 molecule first to the protein molecule and then to the anti TLT-1 mAb (fragment).

In another aspect, the present invention provides compositions and formulations comprising molecules of the invention, such as the fusion proteins, polynucleotides, vectors and cells described herein. For example, the invention provides a pharmaceutical composition that comprises one or more fusion proteins of the invention, formulated together with a pharmaceutically acceptable carrier.

Accordingly, one object of the invention is to provide a pharmaceutical formulation comprising such an antibody which is present in a concentration from 0.25 mg/ml to 250 mg/ml, and wherein said formulation has a pH from 2.0 to 10.0. The formulation may further comprise a buffer system, preservative(s), tonicity agent(s), chelating agent(s), stabilizers and surfactants. The use of preservatives, isotonic agents, chelating agents, stabilizers and surfactants in pharmaceutical compositions is well-known to the skilled person. Reference may be made to Remington: *The Science and Practice of Pharmacy*, 19th edition, 1995.

In one embodiment, the pharmaceutical formulation is an aqueous formulation. Such a formulation is typically a solution or a suspension. The terms "aqueous formulation" is defined as a formulation comprising at least 50% w/w water. Likewise, the term "aqueous solution" is defined as a solution comprising at least 50%w/w water, and the term "aqueous suspension" is defined as a suspension comprising at least 50%w/w water.

In another embodiment, the pharmaceutical formulation is a freeze-dried formulation, to which the physician or the patient adds solvents and/or diluents prior to use.

In a further aspect, the pharmaceutical formulation comprises an aqueous solution of such an antibody, and a buffer, wherein the antibody is present in a

concentration from 1 mg/ml or above, and wherein said formulation has a pH from about 2.0 to about 10.0.

The term "treatment", as used herein, refers to the medical therapy of any human or other animal subject in need thereof. Said subject is expected to have undergone physical examination by a medical practitioner, who has given a tentative or definitive diagnosis which would indicate that the use of said specific treatment is beneficial to the health of said human or other animal subject. The timing and purpose of said treatment may vary from one individual to another, according to the status quo of the subject's health. Preventative or prophylactic administration of antibodies of the invention is also contemplated, with prevention being defined as delaying or averting manifestation aggravation of one or more symptoms of a disease or disorder. Thus, said treatment may be prophylactic, palliative, symptomatic and/or curative.

In terms of the present invention, prophylactic, palliative, symptomatic and/or curative treatments may represent separate aspects of the invention.

A coagulopathy that results in an increased haemorrhagic tendency may be caused by any qualitative or quantitative deficiency of any pro-coagulative component of the normal coagulation cascade or any upregulation of fibrinolysis. Such coagulopathies may be congenital and/or acquired and/or iatrogenic and are identified by a person skilled in the art.

Non-limiting examples of congenital hypocoagulopathies are haemophilia A, haemophilia B, Factor VII deficiency, Factor X deficiency, Factor XI deficiency, von Willebrand's disease and thrombocytopenias such as Glanzmann's thrombasthenia and Bernard-Soulier syndrome.

A non-limiting example of an acquired coagulopathy is serine protease deficiency caused by vitamin K deficiency; such vitamin K-deficiency may be caused by administration of a vitamin K antagonist, such as warfarin. Acquired coagulopathy may also occur following extensive trauma. In this case otherwise known as the "bloody vicious cycle", it is characterised by haemodilution (dilutional thrombocytopaenia and dilution of clotting factors), hypothermia, consumption of clotting factors and metabolic derangements (acidosis). Fluid therapy and increased fibrinolysis may exasperate this situation. Said haemorrhage may be from any part of the body.

Haemophilia A with "inhibitors" (that is, allo-antibodies against factor VIII) and haemophilia B with "inhibitors" (that is, allo-antibodies against factor IX) are non-limiting examples of coagulopathies that are partly congenital and partly acquired.

A non-limiting example of an iatrogenic coagulopathy is an overdosage of anticoagulant medication – such as heparin, aspirin, warfarin and other platelet aggregation

inhibitors – that may be prescribed to treat thromboembolic disease. A second, non-limiting example of iatrogenic coagulopathy is that which is induced by excessive and/or inappropriate fluid therapy, such as that which may be induced by a blood transfusion.

In one embodiment of the current invention, haemorrhage is associated with
5 haemophilia A or B. In another embodiment, haemorrhage is associated with haemophilia A or B with acquired inhibitors. In another embodiment, haemorrhage is associated with von Willebrand's disease. In another embodiment, haemorrhage is associated with severe tissue damage. In another embodiment, haemorrhage is associated with severe trauma. In another embodiment, haemorrhage is associated with surgery. In another embodiment,
10 haemorrhage is associated with haemorrhagic gastritis and/or enteritis. In another embodiment, the haemorrhage is profuse uterine bleeding, such as in placental abruption. In another embodiment, haemorrhage occurs in organs with a limited possibility for mechanical haemostasis, such as intracranially, intraaurally or intraocularly. In another embodiment, haemorrhage is associated with anticoagulant therapy.

15 In a further embodiment, haemorrhage may be associated with thrombocytopenia. In individuals with thrombocytopenia, constructs of the current invention may be co-administered with platelets.

The following is a non-limiting list of embodiments of the present invention:

20 *Embodiment 1:* A procoagulant protein comprising (i) at least one coagulation factor, covalently attached to (ii) an antibody, or fragment thereof, that is capable of binding (iii) TLT-1, and/or a fragment or variant thereof.

25 *Embodiment 2:* The procoagulant protein according to embodiment 1, wherein (iii) is TLT-1, or a fragment or variant thereof.

Embodiment 3: The procoagulant protein according to embodiment 2, wherein
30 (iii) is TLT-1 (16-162).

Embodiment 4: The procoagulant protein according to embodiment 2, wherein
(iii) is TLT-1 (20-125).

Embodiment 5: The procoagulant protein according to embodiment 2, wherein
35 (iii) is TLT-1 (126-162).

Embodiment 6: The procoagulant protein according to any one of embodiments 1-2, wherein (i) is a serine protease or a derivative thereof.

5 *Embodiment 7:* The procoagulant protein according to embodiment 3, wherein (i) is a Factor VII polypeptide.

Embodiment 8: The procoagulant protein according to embodiment 3, wherein (i) is a Factor IX polypeptide.

10 *Embodiment 9:* The procoagulant protein according to embodiment 3, wherein (i) is a Factor X polypeptide.

15 *Embodiment 10:* The procoagulant protein according to any one of embodiments 1-2, wherein (i) is a Factor V polypeptide.

Embodiment 11: The procoagulant protein according to any one of embodiments 1-2, wherein (i) is a Factor VIII polypeptide.

20 *Embodiment 12:* The procoagulant protein according to any one of embodiments 1-2, wherein (i) is a Factor XI polypeptide.

Embodiment 13: The procoagulant protein according to any one of embodiments 1-6, wherein (ii) is a monoclonal antibody or a fragment thereof.

25 *Embodiment 14:* The procoagulant protein according to embodiment 10, wherein (ii) is a Fab fragment, a F(ab')₂ fragment, a Fab' fragment, a Fd fragment, a Fv fragment, a ScFv fragment, a dAb fragment or an isolated complementarity determining region (CDR).

30 *Embodiment 15:* The procoagulant protein according embodiment 11, wherein (ii) is a Fab fragment.

35 *Embodiment 16:* The procoagulant protein according to any one of embodiments 13-15, wherein the epitope of (ii) comprises one or more residues selected from the group consisting of V17, Q18, C19, H20, Y21, R22, L23, Q24, D25, V26, K27, A28, L63, G64, G65, G66, L67, L68, G89, A90, R91, G92, P93, Q94, I95 and L96 of SEQ ID NO: 5.

Embodiment 17: The procoagulant protein according to any one of embodiments 13-15, wherein (ii) is an antibody, or a fragment thereof, which is capable of binding to the same epitope as mAb0023.

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Embodiment 18: A procoagulant protein according to any of embodiments 16-17, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 50 to 54 (DYFMY) of SEQ ID NO: 34 , wherein one of these amino acids may be substituted by a different amino acid; and/or
- 10 • a CDR2 sequence of amino acids 69 to 85 (YISNGGDSSSYPTVKG) of SEQ ID NO: 34 , wherein one, two, three or four of these amino acids may be substituted by a different amino acid; and/or
- a CDR3 sequence of amino acids 118 to 129 (NKNWDDYYDMDY) of SEQ ID NO: 34 , wherein one, two or three of these amino acids may be substituted by a different
- 15 amino acid.

Embodiment 19: A procoagulant protein according to any of embodiments 16-18, wherein the light chain of (ii) comprises:

- a CDR1 sequence of amino acids 44 to 60 (KSSQSLLNSRTRKNYLA) of SEQ ID NO: 35, wherein one, two, three or four of these amino acids may be substituted with a
- 20 different amino acid; and/or
- a CDR2 sequence of amino acids 76 to 82 (WASTRES) of SEQ ID NO: 35, wherein one or two of these amino acids may be substituted with a different amino acid; and/or
- a CDR3 sequence of amino acids 115 to 122 (KQSYNLLT) of SEQ ID NO: 35, wherein
- 25 one or two of these amino acids may be substituted with a different amino acid.

Embodiment 20: A procoagulant protein according to any of embodiments 16-17, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 50 to 54 (DYFMY) of SEQ ID NO: 34 , wherein one of these amino acids may be substituted by a different amino acid; and/or
- 30 • a CDR2 sequence of amino acids 69 to 85 (YISNGGDSSSYPTVKG) of SEQ ID NO: 34, wherein one, two, three or four of these amino acids may be substituted by a different amino acid; and/or
- a CDR3 sequence of amino acids 118 to 129 (NKNWDDYYDMDY) of SEQ ID NO: 34 , wherein one, two or three of these amino acids may be substituted by a different
- 35 amino acid.

and wherein the light chain of (ii) comprises:

- a CDR1 sequence of amino acids 44 to 60 (KSSQSLLNSRTRKNYLA) of SEQ ID NO: 35, wherein one, two, three or four of these amino acids may be substituted with a different amino acid; and/or
- a CDR2 sequence of amino acids 76 to 82 (WASTRES) of SEQ ID NO: 35, wherein one or two of these amino acids may be substituted with a different amino acid; and/or
- a CDR3 sequence of amino acids 115 to 122 (KQSYNLLT) of SEQ ID NO: 35, wherein one or two of these amino acids may be substituted with a different amino acid.

Embodiment 21: A procoagulant protein according to embodiment 20, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 50 to 54 (DYFMY) of SEQ ID NO: 34 ; and
- a CDR2 sequence of amino acids 69 to 85 (YISNGGDSSSYPTVKG) of SEQ ID NO: 34; and
- a CDR3 sequence of amino acids 118 to 129 (NKNWDDYYDMDY) of SEQ ID NO: 34 , and wherein the light chain of (ii) comprises:
 - a CDR1 sequence of amino acids 44 to 60 (KSSQSLLNSRTRKNYLA) of SEQ ID NO: 35; and
 - a CDR2 sequence of amino acids 76 to 82 (WASTRES) of SEQ ID NO: 35; and
 - a CDR3 sequence of amino acids 115 to 122 (KQSYNLLT) of SEQ ID NO: 35.

Embodiment 22: The procoagulant protein according to any one of embodiments 13-15, wherein the epitope of (ii) comprises one or more residues selected from the group consisting of L36, P37, E38, G39, C40, Q41, P42, L43, V44, S45, S46, A47, V73, T74, L75, Q76, E77, E78, D79, A80, G81, E82, Y83, G84, C85, M86, R91, G92, P93, Q94, I95, L96, H97, R98, V99, S100 and L101 of SEQ ID NO: 5.

Embodiment 23: The procoagulant protein according to any one of embodiments 13-15, wherein (ii) is an antibody, or a fragment thereof, which is capable of binding to the same epitope as mAb0051.

Embodiment 24: A procoagulant protein according to any one of embodiments 22-23, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 50 to 54 (DYSMH) of SEQ ID NO: 36, wherein one of these amino acids may be substituted by a different amino acid; and/or

- a CDR2 sequence of amino acids 69 to 85 (VISTYYGDVRYNQKFKG) of SEQ ID NO: 36, wherein one, two, three or four of these amino acids may be substituted by a different amino acid; and/or
- a CDR3 sequence of amino acids 118 to 129 (APMITTGAWFAY) of SEQ ID NO: 36, wherein one, two or three of these amino acids may be substituted by a different amino acid.

Embodiment 25: A procoagulant protein according to any of embodiments 22-24, wherein the light chain of (ii) comprises:

- a CDR1 sequence of amino acids 44 to 54 (KASQSVSNDVA) of SEQ ID NO: 37, wherein one, two or three of these amino acids may be substituted with a different amino acid; and/or
- a CDR2 sequence of amino acids 70 to 76 (YASSRYT) of SEQ ID NO: 37, wherein one or two of these amino acids may be substituted with a different amino acid; and/or
- a CDR3 sequence of amino acids 109 to 117 (QQDYSSPYT) of SEQ ID NO: 37, wherein one or two of these amino acids may be substituted with a different amino acid.

Embodiment 26: A procoagulant protein according to any one of embodiments 22-23, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 50 to 54 (DYSMH) of SEQ ID NO: 36, wherein one of these amino acids may be substituted by a different amino acid; and/or
- a CDR2 sequence of amino acids 69 to 85 (VISTYYGDVRYNQKFKG) of SEQ ID NO: 36, wherein one, two, three or four of these amino acids may be substituted by a different amino acid; and/or
- a CDR3 sequence of amino acids 118 to 129 (APMITTGAWFAY) of SEQ ID NO: 36, wherein one, two or three of these amino acids may be substituted by a different amino acid.

and wherein the light chain of (ii) comprises:

- a CDR1 sequence of amino acids 44 to 54 (KASQSVSNDVA) of SEQ ID NO: 37, wherein one, two or three of these amino acids may be substituted with a different amino acid; and/or
- a CDR2 sequence of amino acids 70 to 76 (YASSRYT) of SEQ ID NO: 37, wherein one or two of these amino acids may be substituted with a different amino acid; and/or

- a CDR3 sequence of amino acids 109 to 117 (QQDYSSPYT) of SEQ ID NO: 37, wherein one or two of these amino acids may be substituted with a different amino acid.

5 *Embodiment 27:* A procoagulant protein according to embodiment 26, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 50 to 54 (DYSMH) of SEQ ID NO: 36; and
- a CDR2 sequence of amino acids 69 to 85 (VISTYYGDVRYNQKFKG) of SEQ ID NO: 36; and
- 10 • a CDR3 sequence of amino acids 118 to 129 (APMITTGAWFAY) of SEQ ID NO: 36; and wherein the light chain of (ii) comprises:
 - a CDR1 sequence of amino acids 44 to 54 (KASQSVSNDVA) of SEQ ID NO: 37; and
 - a CDR2 sequence of amino acids 70 to 76 (YASSRYT) of SEQ ID NO: 37; and
 - a CDR3 sequence of amino acids 109 to 117 (QQDYSSPYT) of SEQ ID NO: 37.

15

Embodiment 28: The procoagulant protein according to any one of embodiments 13-15, wherein the epitope of (ii) comprises one or more residues selected from the group consisting of V17, Q18, C19, H20, Y21, R22, L23, Q24, D25, V26, K27, A28, R91, G92, P93, Q94, I95, L96, H97, R98, V99, S100 and L101 of SEQ ID NO: 5.

20

Embodiment 29: The procoagulant protein according to any one of embodiments 13-15, wherein (ii) is an antibody, or a fragment thereof, which is capable of binding to the same epitope as mAb0062.

25 *Embodiment 30:* A procoagulant protein according to any one of embodiments 28-29, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 50 to 54 (SHWIE) of SEQ ID NO: 42, wherein one of these amino acids may be substituted by a different amino acid; and/or
- a CDR2 sequence of amino acids 69 to 85 (EILPGSGNTNYNEKFKG) of SEQ ID NO: 42, wherein one, two, three or four of these amino acids may be substituted by a different amino acid; and/or
- 30 • a CDR3 sequence of amino acids 118 to 130 (GYGLNYDWYFDV) of SEQ ID NO: 42, wherein one, two or three of these amino acids may be substituted by a different amino acid.

35

Embodiment 31: A procoagulant protein according to any of embodiments 28-30, wherein the light chain of (ii) comprises:

- a CDR1 sequence of amino acids 44 to 54 (RASQDISNYLN) of SEQ ID NO: 39, wherein one, two or three of these amino acids may be substituted with a different amino acid; and/or
- a CDR2 sequence of amino acids 70 to 76 (YTSRLHS) of SEQ ID NO: 39, wherein one or two of these amino acids may be substituted with a different amino acid; and/or
- a CDR3 sequence of amino acids 109 to 117 (QQDTKLPYT) of SEQ ID NO: 39, wherein one or two of these amino acids may be substituted with a different amino acid.

Embodiment 32: A procoagulant protein according to any of embodiments 28-31, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 50 to 54 (SHWIE) of SEQ ID NO: 42, wherein one of these amino acids may be substituted by a different amino acid; and/or
- a CDR2 sequence of amino acids 69 to 85 (EILPGSGNTNYNEKFKG) of SEQ ID NO: 42, wherein one, two, three or four of these amino acids may be substituted by a different amino acid; and/or
- a CDR3 sequence of amino acids 118 to 130 (GYGLNYDWYFDV) of SEQ ID NO: 42, wherein one, two or three of these amino acids may be substituted by a different amino acid.

and wherein the light chain of (ii) comprises:

- a CDR1 sequence of amino acids 44 to 54 (RASQDISNYLN) of SEQ ID NO: 39, wherein one, two or three of these amino acids may be substituted with a different amino acid; and/or
- a CDR2 sequence of amino acids 70 to 76 (YTSRLHS) of SEQ ID NO: 39, wherein one or two of these amino acids may be substituted with a different amino acid; and/or
- a CDR3 sequence of amino acids 109 to 117 (QQDTKLPYT) of SEQ ID NO: 39, wherein one or two of these amino acids may be substituted with a different amino acid.

Embodiment 33: A procoagulant protein according to embodiment 32, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 50 to 54 (SHWIE) of SEQ ID NO: 42; and
- a CDR2 sequence of amino acids 69 to 85 (EILPGSGNTNYNEKFKG) of SEQ ID NO: 42; and

- a CDR3 sequence of amino acids 118 to 130 (GYGLNYDWYFDV) of SEQ ID NO: 42; and wherein the light chain of (ii) comprises:
- a CDR1 sequence of amino acids 44 to 54 (RASQDISNYLN) of SEQ ID NO: 39; and
- a CDR2 sequence of amino acids 70 to 76 (YTSRLHS) of SEQ ID NO: 39; and
- 5 • a CDR3 sequence of amino acids 109 to 117 (QQDTKLPTYT) of SEQ ID NO: 39.

Embodiment 34: The procoagulant protein according to any one of embodiments 13-15, wherein the epitope of (ii) comprises one or more residues selected from the group consisting of E5, T6, H7, K8, I9, G10, S11, L12, A13, E14, N15, A16, F17, S18,
10 D19, P20 and A21 of SEQ ID NO: 7.

Embodiment 35: The procoagulant protein according to embodiment 34, wherein said residues are K8, I9, G10, S11, L12, A13, N15, A16, F17, S18, D19, P20 and A21.

15 *Embodiment 36:* The procoagulant protein according to any one of embodiments 13-15, wherein the epitope of (ii) comprises one or more residues selected from the group consisting of K118, I119, G120, S121, L122, A123, E124, N125, A126, F127 of SEQ ID NO: 6.

20 *Embodiment 37:* The procoagulant protein according to any one of embodiments 13-15, wherein (ii) is an antibody, or a fragment thereof, which is capable of binding to the same epitope as mAb0061 or mAb0082.

Embodiment 38: A procoagulant protein according to any one of embodiments
25 34-37, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 49 to 53 (RYWMT) of SEQ ID NO: 40, wherein one of these amino acids may be substituted by a different amino acid; and/or
- a CDR2 sequence of amino acids 68 to 84 (EINPDSSTINYNP SLKD) of SEQ ID NO: 40, wherein one, two, three or four of these amino acids may be substituted by a
30 different amino acid; and/or
- a CDR3 sequence of amino acids 117 to 121 (GVFTS) of SEQ ID NO: 40, wherein one, two or three of these amino acids may be substituted by a different amino acid.

Embodiment 39: A procoagulant protein according to any of embodiments 34-
35 38, wherein the light chain of (ii) comprises:

- a CDR1 sequence of amino acids 43 to 58 (RSSQSLVHRNGNTYFH) of SEQ ID NO: 41, wherein one, two, three or four of these amino acids may be substituted with a different amino acid; and/or
- a CDR2 sequence of amino acids 74 to 80 (KVSNRFS) of SEQ ID NO: 41, wherein one or two of these amino acids may be substituted with a different amino acid; and/or
- a CDR3 sequence of amino acids 113 to 121 (SQSTHVPYT) of SEQ ID NO: 41, wherein one or two of these amino acids may be substituted with a different amino acid.

Embodiment 40: A procoagulant protein according to any of embodiments 34-39, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 49 to 53 (RYWMT) of SEQ ID NO: 40, wherein one of these amino acids may be substituted by a different amino acid; and/or
- a CDR2 sequence of amino acids 68 to 84 (EINPDSSTINYNP SLKD) of SEQ ID NO: 40, wherein one, two, three or four of these amino acids may be substituted by a different amino acid; and/or
- a CDR3 sequence of amino acids 117 to 121 (GVFTS) of SEQ ID NO: 40, wherein one, two or three of these amino acids may be substituted by a different amino acid.

and wherein the light chain of (ii) comprises:

- a CDR1 sequence of amino acids 43 to 58 (RSSQSLVHRNGNTYFH) of SEQ ID NO: 41, wherein one, two, three or four of these amino acids may be substituted with a different amino acid; and/or
- a CDR2 sequence of amino acids 74 to 80 (KVSNRFS) of SEQ ID NO: 41, wherein one or two of these amino acids may be substituted with a different amino acid; and/or
- a CDR3 sequence of amino acids 113 to 121 (SQSTHVPYT) of SEQ ID NO: 41, wherein one or two of these amino acids may be substituted with a different amino acid.

Embodiment 41: A procoagulant protein according to embodiment 40, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 49 to 53 (RYWMT) of SEQ ID NO: 40; and
- a CDR2 sequence of amino acids 68 to 84 (EINPDSSTINYNP SLKD) of SEQ ID NO: 40; and
- a CDR3 sequence of amino acids 117 to 121 (GVFTS) of SEQ ID NO: 40;

and wherein the light chain of (ii) comprises:

- a CDR1 sequence of amino acids 43 to 58 (RSSQSLVHRNGNTYFH) of SEQ ID NO: 41; and
- a CDR2 sequence of amino acids 74 to 80 (KVSNRFS) of SEQ ID NO: 41; and

- a CDR3 sequence of amino acids 113 to 121 (SQSTHVPYT) of SEQ ID NO: 41.

Embodiment 42: A procoagulant protein according to any of embodiments 34-37, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 49 to 53 (RYWMT) of SEQ ID NO: 50, wherein one of these amino acids may be substituted by a different amino acid; and/or
- a CDR2 sequence of amino acids 68 to 84 (EINPDSSTINYAPSLKD) of SEQ ID NO: 50, wherein one, two, three or four of these amino acids may be substituted by a different amino acid; and/or
- a CDR3 sequence of amino acids 117 to 121 (GVFTS) of SEQ ID NO: 50, wherein one of these amino acids may be substituted by a different amino acid;
and wherein the light chain of (ii) comprises:
 - a CDR1 sequence of amino acids 43 to 58 (RSSQSLVHRNGNTYFH) of SEQ ID NO: 41, wherein one, two, three or four of these amino acids may be substituted with a different amino acid; and/or
 - a CDR2 sequence of amino acids 74 to 80 (KVSNRFS) of SEQ ID NO: 41, wherein one or two of these amino acids may be substituted with a different amino acid; and/or
 - a CDR3 sequence of amino acids 113 to 121 (SQSTHVPYT) of SEQ ID NO: 41, wherein one or two of these amino acids may be substituted with a different amino acid.

Embodiment 43: A procoagulant protein according to embodiment 42, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 49 to 53 (RYWMT) of SEQ ID NO: 50; and
- a CDR2 sequence of amino acids 68 to 84 (EINPDSSTINYAPSLKD) of SEQ ID NO: 50; and
- a CDR3 sequence of amino acids 117 to 121 (GVFTS) of SEQ ID NO: 50;
and wherein the light chain of (ii) comprises:
 - a CDR1 sequence of amino acids 43 to 58 (RSSQSLVHRNGNTYFH) of SEQ ID NO: 41; and
 - a CDR2 sequence of amino acids 74 to 80 (KVSNRFS) of SEQ ID NO: 41; and
 - a CDR3 sequence of amino acids 113 to 121 (SQSTHVPYT) of SEQ ID NO: 41.

Embodiment 44: The procoagulant protein according to any one of embodiments 13-15, wherein the paratope of (ii) comprises one or more residues selected from the group consisting of H50, N52, Y56, H58, Y73, F79, S115, T116, V118 and Y120 of the anti-TLT-1 light (L) chain (SEQ ID NO: 33), and residues V20, F45, R49, Y50, W51, E68, T75, N77, S116, G117, V118 and T120 of the anti-TLT-1 heavy (H) chain (SEQ ID NO: 32)

Embodiment 45: The procoagulant protein according to any one of embodiments 13-15 and 44, wherein the epitope of (ii) comprises one or more residues selected from the group consisting of K133, I134, G135, S136, L137, A138, N140, A141, F142, S143, D144, P145 and A146 of SEQ ID NO: 4.

5

Embodiment 46: The procoagulant protein according to any one of embodiments 13-15, wherein (ii) is an antibody, or a fragment thereof, which is capable of binding to the same epitope as mAb0012.

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Embodiment 47: A procoagulant protein according to any of embodiments 44-46, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 49 to 53 (RYWMT) of SEQ ID NO: 32, wherein one of these amino acids may be substituted by a different amino acid; and/or
- a CDR2 sequence of amino acids 68 to 84 (EINPDSSTINYTPSLKD) of SEQ ID NO: 32, wherein one, two, three or four of these amino acids may be substituted by a different amino acid; and/or
- a CDR3 sequence of amino acids 117 to 121 (GVFTS) of SEQ ID NO: 32, wherein one, two or three of these amino acids may be substituted by a different amino acid.

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Embodiment 48: A procoagulant protein according to any of embodiments 44-47, wherein the light chain of (ii) comprises:

- a CDR1 sequence of amino acids 43 to 58 (RSSQSLVHRNGNTYFTH) of SEQ ID NO: 33, wherein one, two, three or four of these amino acids may be substituted with a different amino acid; and/or
- a CDR2 sequence of amino acids 74 to 80 (KVSNRFS) of SEQ ID NO: 33, wherein one or two of these amino acids may be substituted with a different amino acid; and/or
- a CDR3 sequence of amino acids 113 to 121 (SQSTHVPYT) of SEQ ID NO: 33, wherein one or two of these amino acids may be substituted with a different amino acid.

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Embodiment 49: A procoagulant protein according to any of embodiments 44-48, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 49 to 53 (RYWMT) of SEQ ID NO: 32, wherein one of these amino acids may be substituted by a different amino acid; and/or

- a CDR2 sequence of amino acids 68 to 84 (EINPDSSTINYTPSLKD) of SEQ ID NO: 32, wherein one, two, three or four of these amino acids may be substituted by a different amino acid; and/or
- a CDR3 sequence of amino acids 117 to 121 (GVFTS) of SEQ ID NO: 32, wherein one,
5 two or three of these amino acids may be substituted by a different amino acid
and wherein the light chain of (ii) comprises:
- a CDR1 sequence of amino acids 43 to 58 (RSSQSLVHRNGNTYFTH) of SEQ ID NO: 33, wherein one, two, three or four of these amino acids may be substituted with a different amino acid; and/or
- 10 • a CDR2 sequence of amino acids 74 to 80 (KVSNRFS) of SEQ ID NO: 33, wherein one or two of these amino acids may be substituted with a different amino acid; and/or
- a CDR3 sequence of amino acids 113 to 121 (SQSTHVPYT) of SEQ ID NO: 33, wherein one or two of these amino acids may be substituted with a different amino acid.

15 *Embodiment 50:* A procoagulant protein according to embodiment 49, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 49 to 53 (RYWMT) of SEQ ID NO: 32; and
- a CDR2 sequence of amino acids 68 to 84 (EINPDSSTINYTPSLKD) of SEQ ID NO: 32;
20 and
- a CDR3 sequence of amino acids 117 to 121 (GVFTS) of SEQ ID NO: 32;
and wherein the light chain of (ii) comprises:
- a CDR1 sequence of amino acids 43 to 58 (RSSQSLVHRNGNTYFTH) of SEQ ID NO: 33; and
- 25 • a CDR2 sequence of amino acids 74 to 80 (KVSNRFS) of SEQ ID NO: 33; and
- a CDR3 sequence of amino acids 113 to 121 (SQSTHVPYT) of SEQ ID NO: 33.

30 *Embodiment 51:* The procoagulant protein according to any one of embodiments 1-50, wherein (ii) is a human monoclonal antibody or a fragment thereof.

Embodiment 52: The procoagulant protein according to any one of embodiments 1-50, wherein (ii) is a chimeric antibody or a fragment thereof.

35 *Embodiment 53:* The procoagulant protein according to any one of embodiments 1-50, wherein (ii) is a humanised antibody or a fragment thereof.

Embodiment 54: The procoagulant protein according to any one of embodiments 51-53, wherein the isotype of (ii) is IgG.

5 *Embodiment 55:* The procoagulant protein according to embodiment 54, wherein the isotype is IgG1, IgG2 or IgG4.

Embodiment 56: The procoagulant protein according to embodiment 55, wherein the isotype is IgG4.

10 *Embodiment 57:* The procoagulant protein according to any one of embodiments 1-56, further comprising a linker between (i) and (ii).

15 *Embodiment 58:* The procoagulant protein according to any one of embodiments 1-57, which is a fusion protein.

Embodiment 59: The procoagulant protein according to any one of embodiments 1-57, which is a conjugate of (i) and (ii).

20 *Embodiment 60:* The conjugate according to embodiment 59, wherein (i) and (ii) are covalently connected by a linker comprising polyethyleneglycol (PEG).

Embodiment 61: The conjugate according to any one of embodiments 59-60, wherein (i) and (ii) are covalently conjugated via a glycan of at least one of said proteins.

25 *Embodiment 62:* A process for preparing a composition comprising at least one conjugate according to any one of embodiments 59-61, comprising chemically conjugating (i) the -TLT-1 antibody or fragment thereof with one reactive group (RS1) of a linker and reacting (ii) the coagulation factor with another reactive group (RS2) of said linker.

30 *Embodiment 63:* The process as defined in embodiment 62, wherein (i) is a monoclonal antibody.

35 *Embodiment 64:* The process as defined in embodiment 62, wherein (i) is a fragment of a monoclonal antibody.

Embodiment 65: The process as defined in embodiment 64, wherein (i) is a Fab fragment.

5 *Embodiment 66:* The process as defined in embodiment 65, wherein said Fab fragment contains one Cys mutation in the constant region.

Embodiment 67: The process as defined in embodiment 62, wherein (i) is a sc-Fab fragment.

10 *Embodiment 68:* The process as defined in any one of embodiments 62-67, wherein (ii) is a FV polypeptide.

Embodiment 69: The process as defined in any one of embodiments 62-67, wherein (ii) is a FVIIa polypeptide.
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Embodiment 70: The process as defined in any one of embodiments 62-67, wherein (ii) is a FVIII polypeptide.

Embodiment 71: The process as defined in any one of embodiments 62-67, wherein (ii) is a FIX polypeptide.
20

Embodiment 72: The process as defined in any one of embodiments 62-67, wherein (ii) is a FX polypeptide.

25 *Embodiment 73:* The process as defined in any one of embodiments 62-67, wherein (ii) is a FXI polypeptide.

Embodiment 74: The process as defined in any one of embodiments 62-73, wherein said linker is a water soluble.
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Embodiment 75: The process as defined in any one of embodiments 62-74, wherein said linker is polymer.

Embodiment 76: The process as defined in any one of embodiments 74-75, wherein (ii) is polyethylene glycol (PEG).
35

Embodiment 77: The process as defined in any one of embodiments 74-75, wherein (ii) is polysialic acid.

5 *Embodiment 78:* The process as defined in any one of embodiments 74-75, wherein (ii) is hydroxyethyl starch.

Embodiment 79: The process as defined in any one of embodiments 62-78, wherein RS1 is an aldehyde.

10 *Embodiment 80:* The process as defined in any one of embodiments 62-78, wherein RS1 is a maleimide group.

Embodiment 81: The process as defined in any one of embodiments 62-78, wherein RS1 is activated carbohydrate derivative capable of reacting in an enzyme-catalysed reaction.

15

Embodiment 82: The process as defined in any one of embodiments 81, wherein RS1 is an activated sialic acid derivative capable of reacting in an enzyme-catalysed reaction.

20

Embodiment 83: The process as defined in embodiment 82, wherein RS1 is O²-[5']cytidyl- ξ -neuraminic acid.

Embodiment 84: The process as defined in any one of embodiments 62-83, wherein RS2 is an aldehyde.

25

Embodiment 85: The process as defined in any one of embodiments 62-83, wherein RS2 is a maleimide group.

30 *Embodiment 86:* The process as defined in any one of embodiments 62-83, wherein RS2 is activated carbohydrate derivative capable of reacting in an enzyme-catalysed reaction.

Embodiment 87: The process as defined in any one of embodiments 62-83, wherein RS2 is a sialic acid derivative.

35

Embodiment 88: The procoagulant protein according to any one of embodiments 1-61, in which (ii) has a K_D of less than 100 nM, such as less than 10 nM.

5 *Embodiment 89:* A method of targeting a coagulation factor, or a functional fragment thereof, to the surface of activated platelets, said method comprising the contacting of activated platelets with a procoagulant protein according to any one of embodiments 1-61.

10 *Embodiment 90:* A procoagulant protein according to any one of embodiments 1-61 for use as a medicament.

Embodiment 91: The procoagulant protein of embodiment 87 for use as a procoagulant.

15 *Embodiment 92:* A pharmaceutical formulation comprising the procoagulant protein according to any one of embodiments 1-61.

20 *Embodiment 93:* The procoagulant protein according to any one of embodiments 1-61 or the pharmaceutical formulation according to embodiment 92 for use in the treatment of a coagulopathy.

Embodiment 94: Use of the procoagulant protein according to any one of embodiments 1-61 for the manufacture of a medicament for the treatment of a coagulopathy.

25

Embodiment 95: Use according to any one of embodiments 93 or 94, wherein said coagulopathy is haemophilia A, with or without inhibitors, or haemophilia B, with or without inhibitors.

30 *Embodiment 96:* A method of treating coagulopathy, comprising administering an effective amount of the procoagulant protein according to any one of embodiments 1-61 or the formulation of embodiment 93 to an individual in need thereof.

35 *Embodiment 97:* The method according to embodiment 95, wherein said coagulopathy is haemophilia A, with or without inhibitors, and haemophilia B, with or without inhibitors.

Embodiment 98: A polynucleotide that encodes the procoagulant protein according to any one of embodiments 1-61.

5 *Embodiment 99:* An isolated cell that comprises the fusion protein according to any one of embodiments 1-61 and/or the polynucleotide according to embodiment 97.

Embodiment 100: A procoagulant protein to any one of embodiments 1-61, wherein (ii) bind to TLT-1 without competing with fibrinogen binding to TLT-1.

10 *Embodiment 101:* A procoagulant protein to any one of embodiments 1-61, wherein (ii) does not inhibit platelet aggregation.

The present invention is further illustrated by the following examples which should not be construed as further limiting. The contents of all figures and all references cited
15 throughout this application are expressly incorporated herein by reference.

EXAMPLES

In the examples, anti-TLT-1 antibodies and fragments thereof, e.g. Fab fragments, were used to target coagulation factors to activated platelets. To ease interpretation of the data presented in the examples, Table 3a summarizes information regarding
20 some of the anti-TLT-1 antibodies and fragments thereof further described below. In Table 3A, parent antibodies, variants, fragments, fusions and conjugates thereof are listed with reference to the parent mAb and type of protein. **Name** refers to the name of the protein, **Parent** refers to the antibody from which the anti-TLT-1 mAb, Fab or fusion/conjugate is derived and **Type** defines if the protein is a mAb, a Fab, a DNA fusion
25 (fusion) with a coagulation factor or a chemical conjugate with a coagulation factor (conjugate).

Table 3A: Overview of antibodies (mAbs), antibody fragments (Fabs) and Fusion-proteins

Name	Parent	Type
mAb0012	mAb0012	mAb
mAb0023	mAb0023	mAb
mAb0051	mAb0051	mAb
mAb0052	mAb0052	mAb
mAb0061	mAb0012	mAb
mAb0062	mAb0052	mAb
mAb0082	mAb0012	mAb
Fab0003	mAb0052	Fab
Fab0004	mAb0023	Fab
Fab0012	mAb0012	Fab
Fab0023	mAb0023	Fab
Fab0051	mAb0051	Fab
Fab0052	mAb0052	Fab
Fab0061	mAb0012	Fab
Fab0074	mAb0051	Fab
Fab0082	mAb0012	Fab
Fab0084	mAb0012	Fab
FVIIa-Fab1001	mAb0012	Conjugate (FVIIa)
FVIIa-Fab1029	mAb0012	Conjugate (FVIIa)
FVII-Fab5001	mAb0052	Fusion (FVII)
FVIIa-Fab9015	mAb0012	Conjugate (FVIIa)
FIX-Fab0135	mAb0012	Fusion (FIX)
FIX-mAb0145	mAb0012	Conjugate (FIX)
FIX-Fab0155	mAb0012	Conjugate (FIX)

Example 1: Cloning and expression of hTLT-1 ECD-His antigen.

Nucleotide sequences encoding the extracellular domain of human TLT-1 (hTLT-1) (Figure 1) together with a C-terminal His-6 tag were PCR amplified with a forward primer containing a HindIII recognition site together with a kozak sequence, and a reverse primer containing a stop codon and an EcoRI recognition site (Figure 2). The HindIII- and EcoRI digested PCR fragment was inserted into the HindIII- and EcoRI sites

of a pTT-based expression vector. The pTT vector is essentially described in Durocher, Y. et al., (2002) Nucleic Acid Res, 30: E9. The resulting expression plasmid was designated pTT-hTLT-1 ECD-His. The nucleotide and amino acid sequences for hTLT-1 ECD-His is shown in SEQ ID NO: 3 and 4. pTT-hTLT-1 ECD-His was transfected into HEK293-6E suspension cells in order to transiently express hTLT-1 ECD-His. HEK293-6E cells were grown in Freestyle HEK293 medium (GIBCO, cat. no. 12338-018) supplemented with 1% P/S (GIBCO cat. no. 15140-122), 0.1% pluronic (GIBCO, cat. no. 24040-032) and 25 ug/mL Geneticin (GIBCO, cat. no. 10131-019) and cells were transfected at a cell density of 1 mill/mL using 293fectin (Invitrogen, cat. no. 12347-019). For each liter of HEK293-6E cells, the transfection was performed by diluting 1 mg of pTT-hTLT-1 ECD-His DNA into 30 mL Optimem (dilution A) and by diluting 1 mL 293fectin into 30 mL Optimem (GIBCO, cat. no. 51985-026, dilution B). Dilution A and B were mixed and incubated at room temperature for 30 minutes. The transfection mix was hereafter added to the HEK293-6E cells and cells were incubated at 37°C in a humidified incubator with orbital rotation (125 rpm). Five to seven days post-transfection, cells were removed by centrifugation and the resulting hTLT-1 ECD-His containing supernatants were sterile-filtrated prior to purification.

Example 2: Purification and characterisation of hTLT-1 ECD-His protein.

Purification of the hTLT-1 ECD-His protein was conducted as a 2-step process composed of 1) His-affinity chromatography using the Cobalt-loaded resin TALON (Clontech, cat. no. 635506) and 2) anion-exchange chromatography using the fine-particle resin Source 15Q (GE Healthcare, cat. no. 17-0947). The purifications were conducted using an ÄktaExplorer chromatography system (GE Healthcare, cat. no. 18-1112-41). The buffer systems used for the first purification step was an equilibration buffer composed of 20 mM Hepes, pH 7.0, 150 mM NaCl, a wash buffer composed of 20 mM Hepes, pH 7.0, 0.5 M NaCl and an elution buffer composed of 20 mM Hepes, pH 7.0, 150 mM Imidazole. The cell supernatant was applied directly without any adjustments onto a pre-equilibrated TALON column. The column was washed with 20 column volumes of equilibration buffer, 20 column volumes of wash buffer and last with 20 column volumes of equilibration buffer. The protein was eluted isocratically in approximately 5 column volumes of elution buffer. The molecular mass of the eluted protein was analysed using SDS-PAGE/Coomassie NuPage 4–12% Bis-Tris gels (Invitrogen, cat. no. NP0321BOX) and Matrix Assisted Laser Desorption Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) setup on a Micro-flex system (Bruker Daltonics). Here, two distinct protein masses were observed of approximately 16.7 and 33.4 kDa of almost

equal amounts. The observed masses corresponded to monomer and dimer forms of hTLT-1 ECD-His. Reducing the protein resulted in complete abolishment of the 33.4 kDa protein, while intensifying the 16.7 kDa protein as judged from a SDS-PAGE/Coomassie analysis. Thus, the hTLT-1 ECD-His protein contained an interlinked C-C dimer. To segregate monomer from dimer, a second purification step was employed. The buffer systems used for this purification step was an equilibration buffer composed of 50 mM Hepes, pH 8.0 and an elution buffer composed of 50 mM Hepes, pH 8.0, 1 M NaCl. The sample was adjusted to a pH of 8.0 using 1 M NaOH and then diluted to a conductivity of approximately 10 mS/cm. The protein was applied to a pre-equilibrated Source 15Q column, washed with 5 column volumes of equilibration and eluted using 0 – 100% elution buffer over 20 column volumes. Based on UV280 monitoring, two peaks were apparent with almost base-line separation. Analyzing fractions over the two peaks using SDS-PAGE/Coomassie, MALDI-TOF MS and Dynamic Light-Scattering (DLS) using a Dynapro instrument (Wyatt Technology) analyses showed the presence of monomer hTLT-1 ECD-His protein in the peak eluting first and Cys-Cys dimer in the peak eluting second. A pool was prepared containing the monomer hTLT-1 ECD-His protein only. The final protein integrity was analyzed based on a Size-Exclusion High-Performance Liquid Chromatographic (SEC-HPLC) method setup on an Agilent LC 1100/1200 system and using a BIOsep-SEC-S3000 300x7.8mm column (Phenomenex, cat. no. 00H-2146-K0) and a running buffer composed of 200 mM NaPhosphate pH 6.9, 300 mM NaCl and 10% isopropanol. The protein eluted as a single symmetric peak at a retention time of approximately 9.9 min at a flow rate of 1 ml/min.

A batch of hTLT-1 ECD-His was prepared for an immunization study for production of monoclonal anti-TLT-1 antibodies. Thus, the protein was dialyzed into an isotonic PBS buffer using a Slide-A-Lyzer Dialysis Cassette 10kDa MWCO (Pierce, cat. no. 66453). To measure the final protein concentration, a NanoDrop spectrophotometer (Thermo Scientific) was used together with an extinction coefficient of 0.55.

Example 3: Preparation of monoclonal TLT-1 antibodies.

RBF mice were immunized by injecting 50 µg of hTLT-1 ECD-His. FCA subcutaneously followed by two injections with 20µg of hTLT-1 ECD-His in FIA. High responder mice were boosted intravenously with 25µg of hTLT-1 ECD-His and the spleens were harvested after 3 days. Spleen cells were fused with the myeloma Fox cell line. Supernatants were screened for hTLT-1 specific antibody production in a specific ELISA and in a FACS assay utilizing hTLT-1- or Mock-transfected CHO cells as positive and negative target cells, respectively. A secondary screen was done on resting versus dual

agonistic activated platelets of human, cynomolgous monkey, dog, rabbit or mouse origin.

Example 4: Cloning and Sequencing of anti-TLT-1 mAb LC and HC cDNAs from hybridoma.

Total RNA was extracted from four different anti-TLT-1 mAb expressing hybridoma designated: 0012Hyb, 0023Hyb, 0051Hyb and 0052Hyb. The RNA was extracted from hybridoma cells using RNeasy mini kit (Qiagen, cat. no. 74106) and an aliquot of the resulting RNA was used as template for first-stranded cDNA synthesis using SMART RACE cDNA Amplification kit (Clontech, cat. no. 634914) following the instruction of the manufacturer for 5' RACE and using 5' RACE CDS primer A together with SMART II A RNA oligonucleotide. The light chain (LC) and heavy chain (HC) coding region cDNAs from each of the four anti-TLT-1 hybridomas were hereafter PCR amplified using UPM forward primer mix together with either a mouse LC, kappa specific reverse primer (reverse primer number 339, 348, or 610) or together with a reverse primer recognizing mouse IgG1, IgG2a, IgG2b or IgG3 sequences (reverse primer number 341, 347, 613, 614, 615, or 616, primer sequences are shown in Table 4 and SEQ ID NOs 60-145). The PCR reactions were performed using phusion PCR mix (FinnZymes, cat no.: F-531L). The resulting PCR fragments were cloned using Zero blunt Topo PCR cloning kit for sequencing (Invitrogen, cat. no. K287540) and sequenced. The variable domain sequences for 0012LC and HC are shown in Figure 3.

Example 5: Development of pTT-0012HC, pTT-0023HC, pTT-0051HC and pTT-0052HC expression constructs.

The HC variable domain (V_H) encoding DNA sequences isolated from each of the four different anti-TLT-1 hybridomas were PCR amplified with forward primers containing a *HinDIII* restriction enzyme site and reverse primers containing a *NheI* restriction enzyme site for cloning purposes. The 0012 V_H , 0023 V_H , 0051 V_H and 0052 V_H DNA sequences were PCR amplified using phusion PCR mix (FinnZymes, cat No. F-531L) with the following primer number pairs: 490 (forward) + 491 (reverse), 546 (forward) + 547 (reverse), 627 (forward) + 628 (reverse), and 617 (forward) + 618 (reverse, primer sequences are shown in Table 4 and SEQ ID NOs 60-145), respectively, and inserted into the *HinDIII* and *NheI* restriction enzyme sites of a pTT based vector designated pTT-hIgG4, containing the constant region encoding sequences for human IgG4 HC (ie CH1-hinge-CH2-CH3). The pTT vector is essentially described in Durocher, Y. et al., (2002) Nucleic Acid Res, 30: E9 (Figure 22). The resulting vectors were designated pTT-0012HC

(Figure 5), pTT-0023HC, pTT-0051HC, and pTT-0052HC. The anti-TLT-1 HC amino acid sequences encoded by the expression vectors are shown (SEQ. ID NO: 0012HC: 32, 0023HC: 34, 0051HC: 36, 0052HC: 38).

5 Example 6: Development of pTT-0012LC, pTT-0023LC, pTT-0051LC and pTT-0052LC expression constructs.

The LC variable domain (V_L) encoding DNA sequences isolated from each of the four different anti-TLT-1 hybridomas were PCR amplified with forward primers containing a HindIII restriction enzyme site and reverse primers containing a BsiWI restriction enzyme site for cloning purposes. The 0012 V_L , 0023 V_L , 0051 V_L and 0052 V_L DNA sequences were PCR amplified with the following primers number pairs: 493 (forward) + 495 (reverse), 548 (forward) + 549 (reverse), 492 (forward) + 494 (reverse), and 619 (forward) + 620 (reverse primer sequences are shown in Table 4 and SEQ ID NOs 60-145), respectively, and inserted into the HindIII and BsiWI restriction enzyme sites of a pTT-based vector designated, pTT-hLC,Kappa, containing the constant region encoding sequences for human LC, kappa. The resulting vectors were designated pTT-0012LC, pTT-0023LC, pTT-0051LC, and pTT-0052LC. The anti-TLT-1 LC amino acid sequence encoded by the expression vectors are shown in (SEQ ID NO: 0012LC: 33, 0023LC: 35, 0051LC: 37, 0052LC: 39).

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Example 7: Development of pTT-0012HC.T60N, pTT-0012HC.T60A, pTT-0012LC.C36A and pTT-0052HC.C91Y.

The 0012 V_H amino acid sequence contains a potential N-linked glycosylation site (T60, kabat numbering) and the 0012 V_L and the 0052 V_H amino acid sequences each contain an unpaired Cys (C36 and C91, respectively, kabat numbering). Expression vectors encoding 0012HC.T60N or 0012HC.T60A or 0012LC.C36A or 0052HC.C91Y were developed using site directed mutagenesis (Quickchange II, Stratagene, Catalog number 20523-5) following the instructions of the manufacturer. The site-directed mutagenesis reactions were performed using a) pTT-0012HC DNA as template and primer numbers 682 (forward) + 683 (reverse) for pTT-0012HC.T60N, b) pTT-0012HC DNA as template and primer numbers 688 (forward) + 689 (reverse) for pTT-0012HC.T60A, c) pTT-0012LC DNA as template and primer numbers 598 (forward) + 599 (reverse) for pTT-0012LC.C36A, d) pTT-0052HC DNA as template and the following primer numbers 684 (forward) + 685 (reverse, primer sequences are shown in Table 4 and SEQ ID NOs 60-145) for pTT-0052HC.C91Y. The resulting expression vectors were sequenced in order to verify DNA sequences. The anti-TLT-1 HC and LC amino acid sequence encoded by the

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pTT-0012HC.T60N, pTT-0012HC.T60A and pTT-0012LC.C36A expression vectors are shown in (SEQ ID NO: 0012HC.T60N (also called 0061HC): 40, 0012HC.T60A (also called 0082HC): 43, 0012LC.C36A (also called 0061LC): 41). The 0012LC.C36A amino acid sequence is also shown without the N-terminal signal peptide sequence in SEQ ID NO: 153.

Example 8: Development of pTT-0012LC-HPC4, pTT-0012LC.C36A-HPC4, pTT-0023LC-HPC4, pTT-0051LC-HPC4 and pTT-0052LC-HPC4 expression constructs.

V_L encoding DNA sequences isolated from each of the four different anti-TLT-1 hybridomas were PCR amplified with forward primers containing a HindIII restriction enzyme site and reverse primers containing a BsiWI restriction enzyme site for cloning purposes. 0012V_L, 0012V_L.C36A, 0023V_L, 0051V_L and 0052V_L DNA sequences were PCR amplified with the following primer numbers: 493 (forward) + 495 (reverse), 493 (forward) + 495 (reverse), 548 (forward) + 549 (reverse), 492 (forward) + 494 (reverse), and 619 (forward) + 620 (reverse, primer sequences are shown in Table 4 and SEQ ID NOs: 60-145) respectively, using phusion PCR mix (FinnZymes, cat No. F-531L). The human C_Lκ encoding sequence was PCR amplified with forward primer number 486 and reverse primer number 485. Forward primer number 486 contains a BsiWI restriction enzyme site and reverse primer 485 encodes a HPC4 tag followed by a stop codon and contains a 3' flanking EcoRI site for cloning purposes. The PCR reaction was performed using phusion PCR mix (FinnZymes, cat No. F-531L). HindIII+BsiWI digested 0012V_L PCR fragment was mixed with BsiWI+EcoRI digested human C_Lκ-HPC4 PCR fragment and inserted into the HindIII + EcoRI sites of a pTT-based expression vector resulting in pTT-0012LC-HPC4 (Figure 4). In order to develop corresponding expression vectors encoding the LC-HPC4 version of the remaining four anti-TLT-1 LC sequences, the 0012V_L sequence in pTT-0012LC-HPC4 was excised with HindIII+ BsiWI and replaced with HindIII+BsiWI digested 0023V_L, 0051V_L, 0052V_L and 0012V_L.C36A PCR fragments. The resulting four expression vectors were designated: pTT-0023LC-HPC4, pTT-0051LC-HPC4, pTT-0052LC-HPC4 and pTT-0012LC.C36A.HPC4 (Figure 4B). The amino acid sequences encoded by pTT-0012LC.C36A-HPC4, pTT-0023LC-HPC4, pTT-0051LC-HPC4 and pTT-0052LC-HPC4 are shown in SEQ ID NO: 0012LC.C36A-HPC4 (also called 0061LC-HPC4): 167, 0023LC-HPC4: 179, 0051LC-HPC4: 177, 0052LC-HPC4 (also called 0062LC-HPC4): 174.

Example 9: Development of pTT-0012V_H.T60N-CH1-YGPPC, pTT-0023V_H-CH1-YGPPC, pTT-0051V_H-CH1-YGPPC, and pTT-0052V_H.C91Y-CH1-YGPPC expression constructs.

The 0012V_H.T60N-CH1-YGPPC, 0023V_H-CH1-YGPPC, 0051V_H-CH1-YGPPC and 0052V_H.C91Y-CH1-YGPPC sequence (YGPPC is a partial human IgG4 hinge amino acid sequence) was PCR amplified from pTT-0012HC.T60N, pTT-0023HC, pTT-0051HC and pTT-0052HC.C91Y respectively using forward and reverse primer pairs: 572 (forward) + 698 (reverse), 576 (forward) + 698 (reverse), 627 (forward)+698 (reverse) and 617 (forward)+698 (reverse), respectively. The forward primers contain a HindIII restriction enzyme site and the reverse primer 698 contains a stop codon and an EcoRI site for cloning purposes. The resulting PCR fragment was digested with HindIII+EcoRI and inserted into the HindIII+EcoRI sites of a pTT based vector. The resulting expression vectors were designated pTT-0012V_H.T60N-CH1-YGPPC (Figure 4A), pTT-0023V_H-CH1-YGPPC, pTT-0051V_H-CH1-YGPPC and pTT-0052V_H.C91Y-CH1-YGPPC. The amino acid sequences encoded by pTT-0012V_H.T60N-CH1-YGPPC (Figure 4A), pTT-0023V_H-CH1-YGPPC, pTT-0051V_H-CH1-YGPPC and pTT-0052V_H.C91Y-CH1-YGPPC is shown in SEQ ID NO: 0012V_H.T60N-CH1-YGPPC (also called 0061V_H-CH1-YGPPC): 171, 0023V_H-CH1-YGPPC: 178, 0051V_H-CH1-YGPPC 176, 0052V_H.C91Y-CH1-YGPPC (also called 0062V_H-CH1-YGPPC): 175.

Example 10: Development of pTT-0012V_H-CH1, pTT-0012V_H-CH1-HPC4, pTT-0023V_H-CH1, pTT-0023V_H-CH1-HPC4, pTT-0051V_H-CH1, pTT-0051V_H-CH1-HPC4, pTT-0052V_H-CH1 and pTT-0052V_H-CH1-HPC4 expression constructs.

The 0012V_H, 0023V_H, 0051V_H, and 0052V_H sequences isolated from 0012Hyb, 0023Hyb, 0051Hyb, 0052Hyb were PCR amplified with primer numbers: 490 (forward) + 491 (reverse), 546 (forward) + 547 (reverse), 627 (forward) + 628 (reverse), 617 (forward) + 618 (reverse, primer sequences are shown in Table 4 and SEQ ID NOs 60-145), respectively, using phusion PCR mix (FinnZymes, cat No. F-531L). All forward primers (490, 546, 627, and 617) contained a HindIII site and all reverse primers (491, 547, 628, and 618) contained a NheI site for cloning purposes. The human IgG₄ CH1 region was PCR amplified either with primer numbers: 489 (forward) + 488 (reverse), or primer numbers 489 (forward) + 487 (reverse). Forward primer number 489 contained a NheI site, the 488 reverse primer number contained a stop codon and an EcoRI site, and the 487 reverse primer number contained an HPC4 tag encoding sequence, a stop codon followed by an EcoRI site for cloning purposes. HindIII+NheI digested 0012V_H PCR fragment was combined with either NheI+EcoRI digested human IgG₄ CH1 PCR fragment

or with NheI+EcoRI digested human IgG₄ CH1-HPC4 PCR fragment and cloned into the HindIII+EcoRI sites for a pTT based vector. The resulting vectors were designated pTT-0012V_H-CH1 and pTT-0012V_H-CH1-HPC4, respectively. The pTT-0012V_H-CH1-HPC4 vector is shown in Figure 5B. The 0012V_H-CH1-HPC4 amino acid and DNA sequences are shown in SEQ ID NOs 168-169. The V_H domains of pTT-0012V_H-CH1 and of pTT-0012V_H-CH1-HPC4 were excised by HindIII+NheI digestion and HindIII+NheI digested 0197-0000-0023V_H, 0197-0000-0051V_H and 0197-0000-0052V_H PCR fragments were inserted. The resulting expression vectors were designated: pTT-0023V_H-CH1, pTT-0023V_H-CH1-HPC4, pTT-0051V_H-CH1, pTT-0051V_H-CH1-HPC4, pTT-0052V_H-CH1, and pTT-0052V_H-CH1-HPC4.

Example 11: Development of pTT-0012V_H.T60N-CH1, pTT-0012V_H.T60N-CH1-HPC4, pTT-0052V_H.C91Y-CH1 and pTT-0052V_H.C91Y-CH1-HPC4 expression constructs.

The 0012V_H.T60N-CH1 and 0052V_H.C91Y-CH1 sequence (including the signal peptide encoding sequence) was PCR amplified from pTT-0012HC.T60N and pTT-0052HC.C91Y, respectively using phusion PCR mix (FinnZymes, cat No. F-531L). For the 0012V_H.T60N-CH1 PCR fragment the forward primer number 572 containing a HindIII restriction enzyme site and reverse primer number 488 containing a EcoRI site for cloning purposes or reverse primer 487 containing a HPC4 tag encoding sequence together with a EcoRI site for cloning purposes were employed. For the 0052V_H.C91Y-CH1 PCR fragment the forward primer number 617 together with either reverse primer number 488 or 487 were employed (primer sequences are shown in Table 4 and SEQ ID NOs 60-145). The resulting PCR fragments were digested with HindIII+ EcoRI and inserted into the HindIII + EcoRI sites of a pTT based vector. The resulting expression vectors were designated pTT-0012V_H.T60N-CH1, pTT-0012V_H.T60N-CH1-HPC4, pTT-0052V_H.C91Y-CH1 and pTT-0052V_H.C91Y-CH1-HPC4. The amino acid sequence encoded by pTT-0012V_H.T60N-CH1 is shown in SEQ ID NO: 0012V_H.T60N-CH1 (also called 0061VH-CH1): 146 while the corresponding amino acid sequence without the N-terminal signal peptide sequence is shown in SEQ ID NO: 152.

Example 12: Development of pTT-FIX-L4b-0012LC and pTT-FIX-L4b-0012LC.C36A expression construct.

The FIX DNA sequence (including the signalpeptide encoding sequence) was PCR amplified from human FIX DNA sequences using forward primer 753 and reverse primer 754. Forward primer 753 inserts a 5'end HindIII restriction enzyme site and reverse primer 754 inserts a 17 amino acid long glycine-serine linker (L4b:

GGGGSGGGSGGGSGGS) containing a 3'end BamHI restriction enzyme site for cloning purposes. The FIX-L4b PCR fragment was inserted into the HindIII + BamHI sites of pTT-hTF.1-219-L4b-0012LC i.e. replacing hTF.1-219 DNA sequences and resulting in the FIX-L4b-0012LC expression construct designated pTT-FIX-L4b-0012LC (Figure 5A). The FIX-L4b-0012LC amino acid and DNA sequences are shown in SEQ ID NO: 172-173.

In order to develop an expression plasmid encoding pTT-FIX-L4b-0012LC.C36A the 0012LC.C36A coding region excluding the signal peptide sequence was PCR amplified using pTT-0012LC.C36A as template and using forward primer 1055 containing a 5'end BamHI site and reverse primer 1056 containing a stop codon and an EcoRI site for cloning purposes (Table 4). The resulting PCR fragment was inserted into the BamHI and EcoRI sites of pTT-FIX-L4b-0012LC i.e. replacing the 0012LC DNA sequence with 0012LC.C36A DNA sequence. The resulting expression vector was called pTT-FIX-L4b-0012LC.C36A and encode the amino acid sequences shown in SEQ ID NO: FIX-L4b-0012LC.C36A (also called FIX-L4b-0061LC): 182.

Example 13: Development of pTT-FVII-L4b-0052V_H.C91Y-CH1-HPC4 expression construct.

The human FVII DNA sequence (including the signalpeptide encoding sequence) was PCR amplified from human FVII DNA sequences using forward primer 751 and reverse primer 752. Forward primer 751 inserts a 5'end HindIII restriction enzyme site and reverse primer 752 inserts a 17 amino acid long glycine-serine linker (L4b: GGGGSGGGSGGGSGGS) containing a 3'end BamHI restriction enzyme site for cloning purposes. The FVII-L4b PCR fragment was inserted into the HindIII + BamHI sites of pTT-hTF.1-219-L4b-0012LC i.e. replacing hTF.1-219 DNA sequences and resulting in the FVII-L4b-0012LC expression construct designated pTT-FVII-L4b-0012LC. The 0052V_H.C91Y-CH1-HPC4 encoding DNA sequence was PCR amplified using pTT-0052V_H.C91Y-CH1-HPC4 vector as template and using forward primer 1236 containing a 5'end BamHI restriction enzyme site and using reverse primer 1095 containing a stop codon and an EcoRI site for cloning purposes. The resulting PCR fragment was inserted into the BamHI + EcoRI site of pTT-FVII-L4b-0012LC ie replacing the 0012LC sequence and resulting in the expression vector designated pTT-FVII-L4b-0052V_H.C91Y-CH1-HPC4. The FVII-L4b-0052V_H.C91Y-CH1-HPC4 amino acid is shown in SEQ ID NO: FVII-L4b-0052VH.C91Y-CH1-HPC4 (also called FVII-L4b-0062VH-CH1-HPC4): 180.

Example 14: Transient transfection of HEK293-6E cells.

All mAb, Fab, and fusion proteins were expressed in HEK293-6E suspension cells by transient transfecting expression plasmids into cells. Individual plasmids combinations underlying the resulting specific protein compounds are shown in Table 5. HEK293-6E cells were grown in Freestyle HEK293 medium (GIBCO, cat. no. 12338-018) supplemented with 1% P/S (GIBCO cat. no. 15140-122), 0.1% pluronic (GIBCO, cat. no. 24040-032) and 25 ug/mL Geneticin (GIBCO, cat. no. 10131-019) and cells were transfected at a cell density of approximately 1 mill/mL using 293fectin (Invitrogen, cat. no. 12347-019) according to the instructions of the manufacturer. In brief, for each liter of HEK293-6E cells, the transfection was performed by diluting a total of 1 mg of DNA into 30 mL Optimem (dilution A) and by diluting 1 mL 293fectin into 30 mL Optimem (GIBCO, cat. no. 51985-026, dilution B). Dilution A and B were mixed and incubated at room temperature for 30 minutes. The transfection mix was hereafter added to the HEK293-6E cells and cells were incubated at 37°C in a humified incubator with orbital rotation (125 rpm). Five to seven days post-transfection, cells were removed by centrifugation and the resulting cell culture supernatants were sterile-filtrated prior to purification. For all transient transfection experiments using co-transfection of 2 expression plasmids, the plasmids were cotransfected in a 1:1 (ug:ug) plasmid ratio using a total DNA amount of 1mg for each liter of HEK293-6E cells to be transfected.

Table 4: Primer numbers and sequences.

Primer No.	Primer sequence (5' to 3')
339	act gga tgg tgg gaa gat gga tac agt
341	aga tcc agg ggc tag cgg ata gac aga
347	cct gta gga cca gag ggc tcc aag gac act
348	gga gct ggt ggt ggc atc tca gga cct ttg
448	ttt aaa aag ctt gcc gcc acc atg gag acc cct gcc tgg ccc cgg gtc
467	gga acc tcc ccc gcc tga tcc ccc gcc acc aga ccc gcc acc tcc ttc tct aaa ttc ccc ttt ctc ctg gcc cat
484	gaa ttt agc ggc cgc gaa ttc gga tcc gga acc tcc ccc gcc tga tcc
485	aaa ttt gaa ttc tta ctt gcc gtc gat cag tct ggg gtc cac ctg gtc ctc aca ctc tcc cct gtt gaa gct ctt tgt gac
486	a cgg atc tct agc aag ctt cgt acg gtg gc
487	aaa ttt gaa ttc tta ctt gcc gtc gat cag tct ggg gtc cac ctg gtc ctc ttt gga ctc aac tct ctt gtc cac ctt ggt
488	aaa ttt gaa ttc tta ttt gga ctc aac tct ctt gtc cac ctt ggt
489	acg gat ctc tag c aag ctt gct agc ac caa

490	aaa ttt aag ctt gcc gcc acc atg gat ttt ggg ctg att ttt ttt att gtt gct
491	aaa ttt gct agc tgc aga gac agt gac cag agt ccc ttg gcc cca
492	aaa ttt aag ctt gcc gcc acc atg aag tca cag acc cag gtc ttc gta ttt
493	aaa ttt aag ctt gcc gcc acc atg aag ttg cct gtt ggg ctg ttg gtg ctg
494	aaa ttt <u>cgt acg</u> ttc tat ttc cag ctt ggt ccc ccc tc
495	aaa ttt cgt acg ttt tat ttc cag ctt ggt ccc ccc tcc gaa
546	aaa ttt <u>aag ctt</u> gcc gcc acc atg aac ttg ggg ctc agc ttg att ttc ctt gtc
547	aaa ttt <u>gct agc</u> tga gga gac ggt gac tga ggt tcc ttg acc
548	aaa ttt <u>aag ctt</u> gcc gcc acc atg gat tca cag gcc cag gtt ctt ata ttg ctg
549	aaa ttt <u>cgt acg</u> ttt cag ctc cag ctt ggt ccc agc acc gaa
551	aaa ttt aaa ttt gga tcc gat gtt gtg atg acc caa act cca ctc tcc
572	aaa ttt aag ctt gcc gcc acc atg gat ttt ggg ctg att ttt ttt att gtt gct
576	aaa ttt aag ctt gcc gcc acc atg aac ttg ggg ctc agc ttg att ttc ctt
598	gga aac acc tat ttt cat tgg gcc ctg cag aaa cca ggc cag tct
599	aga ctg gcc tgg ttt ctg cag ggc cca atg aaa ata ggt gtt tcc
610	gctctaga cta aca ctc att cct gtt gaa gct ctt g
613	aaaaa tctagaata gac aga tgg ggg tgt cgt ttt ggc
614	aaaaa tctaga ctt gac cag gca tcc tag agt ca
615	aaaaa tctaga agg ggc cag tgg ata gac tga tgg
616	aaaaa tctaga agg gac caa ggg ata gac aga tgg
617	aaa ttt <u>aag ctt</u> gcc gcc acc atg gaa tgg acc tgg gtc ttt ctc ttc ct
618	aaa ttt <u>gct agc</u> tga gga gac ggt gac cgt ggt ccc tgc
619	aaa ttt <u>aag ctt</u> gcc gcc acc atg atg tcc tct gct cag ttc ctt ggt
620	aaa ttt <u>cgt acg</u> ttt cat ctc cag ttt ggt ccc ccc tcc
627	<u>aaa ttt aag ctt gcc gcc acc atg ggt tgg agc tgt atc atc ttc ttt ct</u>
628	aaa ttt <u>gct agc</u> tgc aga gac agt gac cag agt ccc ttg
682	gat agc agt acg ata aac tat aac cca tct cta aag gat aaa ttc
683	gaa ttt atc ctt tag aga tgg gtt ata gtt tat cgt act gct atc
684	tct gag gac tct gcc gtc tat <u>tac</u> tgt gca aga ggg tac tac ggt
685	acc gta gta ccc tct tgc aca <u>gta</u> ata gac ggc aga gtc ctc aga
688	gat agc agt acg ata aac tat <u>gcg</u> cca tct cta aag gat aaa ttc
689	gaa ttt atc ctt tag aga tgg <u>cgc</u> ata gtt tat cgt act gct atc
698	ttt aaa gaa ttc tca gca tgg ggg acc ata ttt gga ctc aac tct ctt
751	ttt aaa aag ctt gcc gcc acc atg gtc tcc cag gcc ctc <u>agg</u> ctc ctc
752	ttt aaa gga tcc gga acc tcc ccc gcc tga tcc ccc gcc acc aga ccc gcc acc tcc ggg aaa tgg ggc tgc cag gag gac tcc tgg
753	ttt aaa aag ctt gcc gcc acc atg cag cgc gtg aac atg atc atg gca g
754	ttt aaa gga tcc gga acc tcc ccc gcc tga tcc ccc gcc acc aga ccc gcc acc tcc agt gag ctt tgt ttt ttc ctt aat cca gtt gac ata
1055	aaa ttt gga tcc gat gtt gtg atg acc caa act cca ctc tcc

1056	aaa ttt gaa ttc cta aca ctc tcc cct gtt gaa gct ctt tgt
1095	Aaatgatttgcctcccatatgtccttc
1236	aaa ttt ggatcc cag gtc cag ctg cag cag tct gga gct

Table 5: Overview of plasmid combination underlying the expression of protein ID 0061 (SEQ40+41), 0023 (SEQ34+35), 0051 (SEQ36+37), 0062 (SEQ42+39), 0084 (SEQ171+SEQ167), 0074 (SEQ176+177), 0003 (SEQ175+174), 0004 (SEQ178+179), 0135 (SEQ169+SEQ173), 0145 (SEQ32+173) and protein ID 5001 (SEQ180+39).

Protein ID	LC plasmid	HC plasmid	Protein name
0061	pTT-0012LC.C36A	pTT-0012HC.T60N	<u>antiTLT-1 0061mab:</u> (0012HC.T60N) ₂ ;(0012LC.C36A) ₂
0023	pTT-0023LC	pTT-0023HC	<u>antiTLT-1 0023mab:</u> (0023HC) ₂ ;(0023LC) ₂
0051	pTT-0051LC	pTT-0051HC	<u>antiTLT-1 0051mab:</u> (0051HC) ₂ ;(0051LC) ₂
0062	pTT-0052LC	pTT-0052HC.C91Y	<u>antiTLT-1 0062mab:</u> (0052HC.C91Y) ₂ ;(0052LC) ₂
0084	pTT-0012LC.C36A-HPC4	pTT-0012VH.T60N-YGPPC	<u>antiTLT-1 0084Fab-YGPPC:</u> (0012VH.T60N-CH1-YGPPC);(0012LC.C36A-HPC4)
0074	pTT-0051LC-HPC4	pTT-0051VH-CH1-YGPPC	<u>antiTLT-1 0074Fab-YGPPC:</u> (0051LC-HPC4);(0051VH-CH1-YGPPC)
0003	pTT-0052LC-HPC4	pTT-0052VH.C91Y-CH1-YGPPC	<u>antiTLT-1 0003Fab-YGPPC</u> (0052LC-HPC4);(0052VH.C91Y-CH1-YGPPC)
0004	pTT-0023LC-HPC4	pTT-0023VH-CH1-YGPPC	<u>antiTLT-1 0004Fab-YGPPC</u> (0023LC-HPC4);(0023VH-CH1-YGPPC)
0135	pTT-FIX-L4b-0012LC	pTT-0012VH-CH1-HPC4	<u>FIX-antiTLT-1 Fab:</u> (FIX-L4b-0012LC);(0012VH-CH1-HPC4)
0145	pTT-FIX-L4b-0012LC	pTT-0012HC	<u>FIX-antiTLT-1 mAb:</u> (FIX-L4b-0012LC) ₂ ;(0012HC) ₂
5001	pTT-0052LC	pTT-FVII-L4b-0052V _H .C91Y-CH1-HPC4	<u>FVII-antiTLT-1 Fab:</u> (0052LC);(FVII-L4b-0052V _H .C91Y-CH1-HPC4)

Example 15: Purification and characterization of recombinantly expressed anti-TLT-1 Fabs (Fab0084, Fab0074, Fab0003 and Fab0004).

Purification of the anti-TLT-1 Fabs (0084, 0074, 0003 and 0004) was conducted using affinity chromatography based on the resin kappaSelect (GE Healthcare, cat. no. 17-5458-01). The four Fabs contain a single free cysteine residue, each included in the sequence. The purification was conducted using an ÄktaExplorer chromatography system (GE Healthcare, cat. no. 18-1112-41). The buffer systems used for the purification step was an equilibration/wash buffer composed of 10 mM NaPhosphate, pH 7.5, 150 mM NaCl and an elution buffer composed of 20 mM Formic acid, pH 3.0. No adjustments of the cleared cell supernatant were conducted prior to application on a pre-equilibrated column packed with the kappaSelect resin. The column was washed with 5 column volumes of equilibration/wash buffer. The protein was eluted isocratically in approximately 4 column volumes of elution buffer. The eluate was adjusted to pH 7 using 0.5 M NaPhosphate, pH 9.0. The molecular mass of the eluted protein was analysed using SDS-PAGE/Coomassie NuPage 4–12% Bis-Tris gels (Invitrogen, cat. no. NP0321BOX) and Liquid Chromatography Electrospray Ionisation Time-of-Flight Mass Spectrometry method setup on an Agilent 6210 instrument and a desalting column MassPREP (Waters, cat. no. USRM10008656). A pure and homogenous protein with an expected molecular mass was observed. A size-exclusion high-performance liquid chromatographic (SEC-HPLC) analysis setup on an Agilent LC 1100/1200 system and using a BIOSep-SEC-S3000 300x7.8mm column (Phenomenex, cat. no. 00H-2146-K0) and a running buffer composed of 200 mM NaPhosphate pH 6.9, 300 mM NaCl and 10% isopropanol was also conducted. Here, the protein eluted as a single symmetric peak at a retention time of approximately 9.1 min at a flow rate of 1 ml/min. To measure final protein concentration, a NanoDrop spectrophotometer (Thermo Scientific) was used together with an extinction coefficient of 1.29.

Example 16: Purification and characterization of recombinantly expressed FIX-anti-TLT-1 Fab fusion protein (FIX-Fab0135).

Purification of FIX-Fab0135 was conducted using an affinity chromatography method based on an anti-HPC4 resin (Roche, cat. no. 11815024001). The purification was conducted using an ÄktaExplorer chromatography system (GE Healthcare, cat. no. 18-1112-41). The buffer systems used for the purification step was an equilibration buffer composed of 20 mM Hepes, pH 7.5, 1.0 mM CaCl_2 , 100 mM NaCl and 0.005% (v/v) Tween-80, a wash buffer composed of 20 mM Hepes, pH 7.5, 1.0 mM CaCl_2 , 1.0 M NaCl and 0.005% (v/v) Tween-80, and an elution buffer composed of 20 mM Hepes, pH 7.5, 5.0 mM EDTA and 100 mM NaCl. Cell supernatants were adjusted with 1 mM CaCl_2 final concentration and a pH of 7.5 and applied onto a pre-equilibrated anti-HPC4

column. The column was washed with 5 column volumes of equilibration buffer, 5 column volumes of wash buffer and last with 5 column volumes of equilibration buffer. The protein was eluted isocratically in approximately 4 column volumes of elution buffer. The protein was analyzed using SDS-PAGE/Coomassie and Matrix Assisted Laser Desorption
5 Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) setup on a Micro-flex system (Bruker Daltonics), showing that a pure and homogenous protein with a molecular mass of 106 kDa was obtained. Since the theoretical mass of the amino acid sequence for the FIX-Fab0135 construct was 95.9 kDa, the expressed protein contained post-translational modifications. To measure the final protein concentrations, a NanoDrop
10 spectrophotometer (Thermo Scientific) was used together with extinction coefficient of 1.29.

Example 17: pcDNA3.1(+)-hTLT-1 ECD-HPC4 ala mutant plasmids.

Forty hTLT-1 ECD-HPC4 Ala mutant expression constructs were designed
15 according to Table 6. The expression constructs were developed by external contractor GENEART AG (Im Gewerbepark B35, 93059 Regensburg, Germany) and all 40 expression constructs were made based on the expression vector designated pcDNA3.1(+). Aliquots of DNA for each of the 40 hTLT-1 ECD-HPC4 pcDNA3.1(+) expression construct were transfected into HEK293-6E suspension cells in order to transiently express each hTLT-1
20 ECD-HPC4 Ala mutant protein (Table 6). Transient transfection and culturing of HEK293-6E cells were performed as described in example 14.

Table 6

wt	Wild-type
1	L22A
2	V25A
3	Q27A
4	V30A
5	L35A
6	H39A
7	R41A
8	L42A
9	Q43A
10	K46A
11	Q48A
12	F54A

13	L55A
14	P56A
15	E57A
16	Q60A
17	D68A
18	R69A
19	R70A
20	R75A
21	L82A
22	L86A
23	E90A
24	M91A
25	T93A
26	Q95A
27	E96A
28	E97A
29	D107A
30	R110A
31	H116A
32	R117A
33	S119A
34	P125A
35	E126A
36	E128A
37	E130A
38	S136A
39	N140A
40	K159A

Example 18: Purification and characterisation of monoclonal anti-TLT-1 antibodies (mAb0012, mAb0023, mAb0051, mAb0061, mAb0062).

5 Purification of the recombinantly expressed monoclonal anti-TLT-1 antibodies described in Table 5 was conducted by a 2-step process composed of affinity chromatography using a Protein A MabSelect SuRe resin (GE Healthcare, cat. no. 17-5438-01) and gel filtration chromatography using a 26/60 Superdex 200 PrepGrade column (GE Healthcare, cat no. 17-1071-01). Purifications were conducted using an

ÄktaExplorer chromatography system (GE Healthcare, cat. no. 18-1112-41). The buffer systems used for the affinity purification step was an equilibration buffer composed of 20 mM NaPhosphate pH 7.2, 150 mM NaCl, an elution buffer composed of 10 mM Formic acid pH 3.5 and an pH-adjustment buffer composed of 0.5 M NaPhosphate pH 9.0. Cell supernatants were applied directly without any adjustments onto a pre-equilibrated MabSelect SuRe column. The column was washed with 15 column volumes of equilibration buffer and the monoclonal antibodies were eluted isocratically in approximately 2-5 column volume of elution buffer. The pooled fractions were adjusted to neutral pH using the described pH-adjustment buffer immediately after elution. The protein was further purified and buffer exchanged using said gel filtration column. The running buffer used for size exclusion chromatography was a 25 mM His pH 6.5, 135 mM NaCl. The flow rate used was 2.5 ml/min and the monoclonal anti-TLT-1 antibodies eluted as single peaks at approximately 0.4 column volumes. Based on analyses of fractions over the entire peak using the previously described SEC-HPLC method (as described in example 2), pools were prepared which contained pure antibody protein eluting as symmetric peaks at approximately 8.5 min. and with a minimum content of earlier eluting high-molecular weight protein.

The purified antibodies were characterized using the previously described SDS-PAGE/Coomassie (as described in example 2) and SEC-HPLC methods, showing that all antibody protein preparations produced were highly homogenous. All antibodies displayed expected heavy chain components of approximately 50 kDa and light chain components of approximately 25 kDa when using reducing conditions prior to running the SDS-PAGE/Coomassie analyses. Intact molecular mass determinations were performed using a Liquid Chromatography Electrospray Ionisation Time-of-Flight Mass Spectrometry method setup on an Agilent 6210 instrument and a desalting column MassPREP (Waters, cat. no. USRM10008656). The buffer system used was an equilibration buffer composed of 0.1% Formic acid in LC-MS graded-H₂O and an elution buffer composed of 0.1% Formic acid in LC-MS graded-ACN. All antibodies displayed intact molecular masses of 147.2 – 148.6 kDa, which is approximately 2.7 – 3.1 kDa above the theoretical masses of the amino acid sequences for each of the antibodies. Thus, all the recombinantly expressed anti-TLT-1 antibodies displayed post-translational modifications corresponding to expected HC N-glycosylations. Final purities of 95 – 99% were obtained for the six antibodies. To verify the N-terminal sequence of the cloned and purified anti-TLT-1 antibodies, EDMAN degradations were performed using an automated sequenator system (Applied Biosystems 494 Protein Sequencer). 10–20 degradation cycles were conducted for each antibody. Here, expected light and heavy chain sequences were confirmed for

the six cloned anti-TLT-1 antibodies. To measure the final protein concentrations, a NanoDrop spectrophotometer (Thermo Scientific) was used together with specific extinction coefficients for each of the six antibodies ranging from 1.34–1.51.

5 **Example 19: mAb binding and competition of different mABs for binding to TLT-1.**

Materials:

Table 7: Reagents

Reagent	Source
TLT-1-His	Example 1
mAb0061	Example 18
mAb0023	Example 18
mAb0051	Example 18
mAb0062	Example 18
Fab0074	Example 15
Fab0084	Example 15
Fab0003	Example 15
Fab0004	Example 15
All other reagents	Biacore Human Antibody Capture Kit (BR-1008-39) Anti-His mAb (R&D # MAB050)

10 Method: The mAbs of interest were either immobilized directly to a CM5 chip or by capture via a human Fc capture mAb immobilized to a CM5 chip, while TLT-1-His was immobilized by capture via an anti-His mAb CM5 chip for analysis of Fabs. Reagents that were used are shown in Table 7.

15 *mAb direct capture:* The TLT-1 mAbs were immobilised to a level of approx 500-1000 RU on a CM5 chip (50 µg/ml diluted in Na-acetate, pH 4.0) using the standard procedure recommended by the supplier. Two-fold dilutions of TLT-1 from 200 nM to 0.2 nM were tested for binding to the mAbs. Running and dilution buffer: 10 mM HEPES, 150 mM, 0.005% p20, pH 7.4. Regeneration was obtained by 10 mM Glycine, pH 1.7.

20 *mAb capture via human Fc mAb:* Human Fc mAb was immobilised to approx 10.000 RU. The mAb of interest was added (approx 100 nM). Two-fold dilutions of TLT-1 from 200

nM to 0.2 nM were tested. Running and dilution buffer: 10 mM HEPES, 150 mM, 0.005% p20, pH 7.4. Regeneration was obtained in 3 M MgCl_2 .

TLT-1-His capture via anti-His mAb (R&D # MAB050): The anti-His mAb was immobilised to approx 9.000 RU. The TLT-1-His was added to a level of approx 30-40 RU (10 $\mu\text{g/ml}$ diluted in 10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20) using the standard procedure recommended by the supplier. 8-fold dilutions of Fabs from 3 to 0.047 $\mu\text{g/ml}$ were tested for binding to the TLT-1-His. Running and dilution buffer: 10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20. Regeneration was obtained by 3 M MgCl_2 .

Determination of kinetic and binding constants (k_{on} , k_{off} , K_D) was obtained assuming a 1:1 interaction of TLT-1 and fibrinogen using the Biacore T100 evaluation software.

Competition: Competitional binding interaction analysis was obtained by Surface Plasmon Resonance in a Biacore T-100 analysing binding of various TLT-1 mAbs to TLT-1 when bound to immobilised mAb0012 (or an alternative mAb). Direct immobilization to a CM5 chip of the mAbs to a level of 5000-10000 RU was achieved in 10 mM sodium acetate pH 4.5-5.0. This was followed by binding of 50 nM TLT-1 and after 2 min of dissociation followed by binding of the three other mAbs to be tested for competition. Running and dilution buffer: 10 mM HEPES, 150 mM, 0.005% p20, pH 7.4. Regeneration was obtained by 10 mM Glycine, pH 1.7.

Results:

Table 8

TLT-1 binding	k_a (1/M)	k_d (1/s)	K_D (M) TLT-1	Biacore technique
mAb0061	9.32E+05	0.003499	3.75E-09	capture
mAb0023	2.87E+05	0.00125	4.36E-09	direct
mAb0051	2.45E+05	0.00472	19.3E-09	direct
mAb0062	3.26E+05	0.00134	4.12E-09	direct
Fab0074	3.35E+06	0.00798	2.38E-09	capture
Fab0084	5.31E+06	0.00524	0.99E-09	capture
Fab0003	5.36E+05	0.00279	5.20E-09	capture
Fab0004	1.15E+06	0.00195	1.70E-09	capture

Table 9: SPR analysis. Binding constant for binding to TLT-1. Competition with mAb0012

mAb ID	Competition with mAb0012	Competition with mAb0023	Competition with mAb0051	Competition with mAb0062
mAb0061	Yes	No	no	no
mAb0023		Yes	no	yes
mAb0051			yes	no
mAb0062				yes

Conclusion: Binding constants for mAb0061, mAb0023, mAb0051 and mAb0062 and Fab0074, Fab0084, Fab0003 and Fab0004 were estimated by Biacore analysis (see Table 8). mAb0061 and mAb0051 do not compete with any of the other mAbs for binding (see Table 9). mAb0023 and mAb0062 do compete with each other (see Table 9).

Example 20: Preparation of 20:80 PS:PC vesicles and cloning, expression, refolding and relipidation of TLT-1.

Relipidated TLT-1 in 20:80 PS:PC vesicles were prepared using Triton X-100 as detergent as described in Smith and Morrissey (2005) J. Thromb. Haemost., 2, 1155-1162 except that TLT-1 was used instead of TF.

Materials

LB medium Kanamycin (50 mg/ml). Kanamycin Sigma K-0254

1000 mM IPTG (IPTG Sigma I-6758)

Lysis buffer: 1x Bugbuster (Novagen) in 50mM Tris-HCl, 100mM NaCl, 2mM EDTA, pH 8.0. Add 0.5mg/ml lysozyme + DNaseI. Add 1x Complete Inhibitor Cocktail (Roche)

IB-Wash buffer 1: 1:10 bugbuster in IB-buffer. Add 50µg/ml lysozyme + 0.5x Complete Inhibitor Cocktail (Roche)

IB-Wash buffer 2: 1:10 Bugbuster in IB-buffer

IB-Buffer: 50mM Tris-HCl, 100mM NaCl, 2mM EDTA, pH 8.0

GndHCl buffer: 6M Guanidinium HCl, 50mM Tris-HCl, 50 mM NaCl, 0.1% Triton X-100 red., pH 8.0

Refolding buffer: 50mM Tris-HCl, 800mM Arginine, 0.1% Triton X-100, 5mM reduced glutathione, 0.5mM oxidized glutathione pH 8.5

Dialysis buffer: 20mM Tris-HCl, 0.1% Triton X-100, pH 8.0

DTT: Reduced glutathione (Sigma G4251) Oxidized glutathione Sigma G4376

PC: 10mg/ml L- α -phosphatidylcholine (Egg, chicken) in chloroform (Avanti Polar Lipids Inc.) Catalog No. 840051C. Mw 760.09

PS: 10mg/ml L- α -phosphatidylserine sodium salt (Brain, porcine) in chloroform. (Avanti

Polar Lipids Inc.) Catalog No. 840032C. Mw 812.05

Triton X-100: 10% Triton X-100, hydrogenated, protein grade detergent, sterile filtered.

Calbiochem. Catalog No. 648464 Concentration 159 mM (Mw 628)

HBS buffer: 50mM HEPES, 100mM NaCl, pH 7.4

- 5 Bio-Beads: Bio-Beads SM2 Adsorbent, 20-50 mesh BioRad Laboratories, Catalog No. 152-3920.

Method

Expression: TLT-1 (hTLT-1.18-188; SEQ ID NO: 149) including extracellular domain,

- 10 linker and transmembrane domain was cloned into pET24a using primers 1004 (SEQ ID NO: 150) and 1005 (SEQ ID NO: 151) and pTT-hTLT-1 as template. Standard techniques for DNA preparation were employed. Transformation was performed into BL21 (DE3).

Overnight Culture: 1 x 50 ml LB medium in 250 ml flasks (plastic) and 50 µl of 50 mg/ml Kanamycin + 1 coloni (transformation) from BL21 plate were mixed. The culture was

- 15 incubated ON at 37°C, 220 rpm. Starter-culture: 2 x 500 ml LB medium in 2L flasks (plastic) with 300 µl of 50 mg/ml Kanamycin was added. 10 ml ON culture TLT-1 lip/pET24a in BL21 (DE3) was added and OD₆₀₀ followed. Incubated at 37°C, 220 rpm.

Induction: 2 x 500 ml with TLT-1 lip/pET24a ~ BL21 (DE3) in LB. 25°C ~ 0.2 mM IPTG was added (100 µl of 1M) to the cell culture when OD₆₀₀ reached between 0.6 - 0.8.

- 20 This was incubated for 3h at 25°C, 220 rpm. The culture was harvested after 3h and centrifuged for 30 min at 4600 rpm. The supernatant was discarded. The pellet was stored at -20°C. Lysis of Inclusion bodies: The *E. coli* pellet was resuspended in 5ml lysis buffer/g pellet. MgSO₄ was added to 5mM to support DNaseI activity. Cell suspension

- 25 was incubated on shaking platform for 20 min at room temperature. The lysate was cleared by centrifugation 20000g (8500 rpm) for 20 min at 4°C. The pellet was resuspended in 100 ml IB-Wash buffer. Suspension was mixed by gentle vortexing and incubated at RT for 5 min. Suspension was centrifuged at 20000g for 20 min at 4°C to collect inclusion bodies. Inclusion bodies were resuspended in 100 ml IB-Wash buffer 2.

Sample was centrifuged at 20000g for 20 min at 4°C to collect inclusion bodies. The pellet was resuspended in 100 ml water and centrifuged at 20000g for 20 min at 4°C to collect inclusion bodies. Refolding: The pellet was resolubilised in x ml GdnHCl buffer (20 ml). The final concentration of TLT-1 (A280 was measured) was 1-2 mg/ml. DTT (400 µl) was added to final concentration of 20 mM. Complete solubilization was ensured by magnetic stirring for ~1-2.5 hrs (1.5 h) at RT. Insoluble material was removed by

- 30 centrifugation at 20000g for 20 min. A peristaltic pump was used slowly (overnight) to transfer the GdnHCl/protein solution (20 ml) to >20x Refolding buffer (400 ml) at 4°C.

The refolding buffer was stirred quickly to ensure rapid dilution. Pump run was obtained at Flow rate 1x, speed 2.5, 4°C and left overnight at 4°C. Precipitated protein was removed by centrifugation at 20000g (8500 rpm) in 50 ml tubes for 30 min. The TLT-1 lip was concentrated from 400 ml to 120 ml in Amico-filter 76mm dia., 10.000 MWCO at 4.5 bar. The protein was checked on an SDS-Page by EtOH-precipitation because of the GdnHCl in the sample. 2x 500 µl and 2x 25 µl were concentrated in 0.5 ml tubes with 10.000 MWCO. 50 µl sample + 9 vol. ice-cold 99% EtOH (450 µl) was mixed and placed at -20°C for 10 min. The sample was centrifuged at full speed 13.000 rpm for 5 min. The supernatant was discarded. The pellet was washed with 450 µl ice-cold 96% EtOH + 50 µl MQ. Centrifuge again. Let dry (EtOH must be eliminated before SDS-PAGE). 100 µl was resuspended 1x sample buffer

PS:PC preparation and relipidation: The exact protocol described in Smith SA & Morrissey JH (2004) "Rapid and efficient incorporation of tissue factor into liposomes". *J. Thromb. Haemost.* 2:1155-1162 was followed for relipidation of TLT-1.

Example 21: Analysis of fibrinogen binding to TLT-1 and binding competition between TLT-1 mAbs and fibrinogen.

TLT-1 binds fibrinogen as tested by SPR analysis. Furthermore, simultaneous binding of fibrinogen and each of the four mAbs: mAb0012, mAb0023, mAb0051 and mAb0062 was tested by SPR analysis in a Biacore T100 instrument.

Materials used are shown in Table 10.

Table 10

Reagent	Source
TLT-1	Example 2
mAb0012	Example 18
mAb0023	Example 18
mAb0051	Example 18
mAb0062	Example 18
Fibrinogen	HCI-0150R Haematologic technologies
All other reagents	Biacore GE Healthcare

Method:

Human TLT-1 was immobilised to a level of approx 1000 RU on a CM5 chip (50 µg/ml diluted in Na-acetate, pH 4.0) using the standard procedure recommended by the

supplier. Four-fold dilutions of human fibrinogen from 200 nM to 0.2 nM were tested for binding to the immobilized TLT-1. Running and dilution buffer: 10 mM HEPES, 150 mM, 0.005% p20, pH 7.4. Regeneration was obtained by 10 mM Glycine, pH 1.7.

Determination of kinetic and binding constants (k_{on} , k_{off} , K_D) was obtained assuming a

5 1:1 interaction of TLT-1 and fibrinogen using the Biacore T100 evaluation software (Table 11).

Competition of the different mAbs for binding to TLT-1 and fibrinogen simultaneously was tested by immobilisation of each of the mAbs to approximately 10000-15000 RU at a CM5 chip followed by binding of 50 nM TLT-1 followed after 2-3
10 min dissociation by varying concentrations of the mAbs to be tested for competition. Regeneration of the chip was obtained by 10 mM Glycine, pH 1.7 (Table 12).

Results:

Table 11: TLT-1 binding to fibrinogen

	ka (1/M)	kd (1/s)	K_D (M)
TLT-1- fibrinogen Binding	4171	3.92×10^{-4}	9.40E-08

Table 12: Competition with fibrinogen. The mAb of interest was immobilised to a chip. Addition of TLT-1 was followed by fibrinogen (a sandwich).

mAb ID	Competition with fibrinogen
mAb0012	no
mAb0023	yes
mAb0051	no
mAb0062	yes

Conclusion:

20 TLT-1 (HCI-0150R) binds fibrinogen. mAb0023 and mAb0062 compete with this binding site. mAb0012 and mAb0051 do not compete.

Example 22: Epitope mapping by hydrogen exchange mass spectrometry (HX-MS).

25 The HX-MS technique has been employed to identify the TLT-1 binding epitopes covered by the four monoclonal antibodies mAb0023, mAb0051, mAb0062 and mAb0061.

For the mapping experiments hTLT-1.20-125, hTLT-1.16-162 and hTLT-1.126-162 corresponding to SEQ ID NO 5, 6 and 7, respectively, were used. All proteins were buffer exchanged into PBS pH 7.4 before experiments.

5 Method: HX-MS experiments.

Instrumentation and data recording. The HX experiments were automated by a Leap robot (H/D-x PAL; Leap Technologies Inc.) operated by the LeapShell software (Leap Technologies Inc.), which performed initiation of the deuterium exchange reaction, reaction time control, quench reaction, injection onto the UPLC system and digestion time
10 control. The Leap robot was equipped with two temperature controlled stacks maintained at 20°C for buffer storage and HX reactions and maintained at 2°C for storage of protein and quench solution, respectively. The Leap robot furthermore contained a cooled Trio VS unit (Leap Technologies Inc.) holding the pepsin-, pre- and analytical columns, and the LC tubing and switching valves at 1°C. The switching valves have been upgraded from
15 HPLC to Microbore UHPLC switch valves (Cheminert, VICI AG). For the inline pepsin digestion, 100 µL quenched sample containing 200 pmol TLT-1 was loaded and passed over a Poroszyme® Immobilized Pepsin Cartridge (2.1 × 30 mm (Applied Biosystems)) using a isocratic flow rate of 200 µL/min (0.1% formic acid:CH₃CN 95:5). The resulting peptides were trapped and desalted on a VanGuard pre-column BEH C18 1.7 µm (2.1 × 5
20 mm (Waters Inc.)). Subsequently, the valves were switched to place the pre-column inline with the analytical column, UPLC-BEH C18 1.7 µm (2.1 × 100 mm (Waters Inc.)), and the peptides separated using a 9 min gradient of 15–40% B delivered at 150 µL/min from an AQUITY UPLC system (Waters Inc.). The mobile phases consisted of A: 0.1% formic acid and B: 0.1% formic acid in CH₃CN. The ESI MS data, and the separate data
25 dependent MS/MS acquisitions (CID) and elevated energy (MS^E) experiments were acquired in positive ion mode using a Q-ToF Premier MS (Waters Inc.). Leucine-enkephalin was used as the lock mass ([M+H]⁺ ion at *m/z* 556.2771) and data was collected in continuum mode.

Data analysis. Peptic peptides were identified in separate experiments using
30 standard CID MS/MS or MS^E methods (Waters Inc.). MS^E data were processed using BiopharmaLynx 1.2 (version 017). CID data-dependent MS/MS acquisition was analyzed using the MassLynx software and in-house MASCOT database.

HX-MS raw data files were subjected to continuous lockmass-correction. Data analysis, i.e., centroid determination of deuterated peptides and plotting of in-exchange
35 curves, was performed using HX-Express ((Version Beta); Weis et al., J. Am. Soc. Mass Spectrom. 17, 1700 (2006)).

Epitope mapping of mAb0023: Amide hydrogen/deuterium exchange (HX) was initiated by a 30-fold dilution of hTLT-1.20-125 in the presence or absence of mAb0023 into the corresponding deuterated buffer (i.e. PBS prepared in D₂O, 96% D₂O final, pH 7.4 (uncorrected value)). All HX reactions were carried out at 20°C and contained 4 µM hTLT-1.20-125 in the absence or presence of 2.4 µM mAb0023 thus giving a 1.2 fold molar excess of mAb binding sites. At appropriate time intervals ranging from 10 sec to 8 hours, aliquots of the HX reaction were quenched by an equal volume of ice-cold quenching buffer (1.35M TCEP) resulting in a final pH of 2.6 (uncorrected value).

Epitope mapping of mAbs 0051 and 0062: Epitope mapping of mAb0051 and mAb0062 were performed in a separate experiment using hTLT-1.20-125 and carried out similarly to the mapping of mAb0023 as described above.

Epitope mapping of mAb0061: Epitope mapping of mAb0061 was performed in two separate experiments using either the hTLT-1.16-162 protein or the hTLT-1.126-162 peptide.

Experiments were performed similarly as described above for mAb0023. However, the pepsin column was placed at room temperature for experiments using hTLT-1.126-162. This results in an increased pepsin digestion efficacy with minimal additional exchange loss.

Results

Epitope Mapping of mAb0023

The HX time-course of 20 peptides, covering 100% of the primary sequence of TLT-1, were monitored in the presence and absence mAb0023 for 10 sec to 8 hours.

The observed exchange pattern in the presence or absence of mAb0023 can be divided into two different groups: One group of TLT-1 peptides display an exchange pattern that is unaffected by the binding of mAb0023 and another group of TLT-1 peptides that show protection from exchange upon mAb0023 binding. The regions displaying protection upon mAb0023 binding encompass peptides covering TLT-1 residues 36-51, 79-91 and 105-120. By comparing the relative amounts of exchange protection within each peptide the epitope for mAb0023 can be narrowed to residues 36-47, VQCHYRLQDVKA (50%), 82-87, LGGGLL (30%), 108-115, GARGPQIL (20%) with the relative exchange protection for each segment noted in parenthesis. An overview of the peptide map for the 0023 epitope is shown in Figure 6.

Epitope Mapping of mAb0051

The HX time-course of 22 peptides, covering 100% of the primary sequence of TLT-1, were monitored in the presence and absence mAb0051 for 10 sec to 1000 sec.

The observed exchange pattern in the presence or absence of mAb0051 can be divided into two different groups: one group of TLT-1 peptides display an exchange pattern that is unaffected by the binding of mAb0051 and a group that is affected. The regions displaying protection upon mAb0051 binding encompass peptides covering residues 52-66, 92-120. By comparing the relative amounts of exchange protection within each peptide the epitope for mAb0051 can be narrowed to residues 55-66, LPEGCQPLVSSA (75%) and 110-120, RGPQILHRVSL (25%) as well as a weak interaction in the 92-105 stretch. An overview of the peptide map for the 0051 epitope is shown in Figure 7.

Epitope Mapping of mAb0062

The HX time-course of 22 peptides, covering 100% of the primary sequence of TLT-1, were monitored in the presence and absence mAb0062 for 10 sec to 1000 sec.

The observed exchange pattern in the presence or absence of mAb0062 can be divided into two different groups: One group of TLT-1 peptides display an exchange pattern that is unaffected by the binding of mAb0062 and another group of TLT-1 peptides that show protection. The regions displaying protection upon mAb0062 binding encompass peptides covering residues 36-51 and 105-120. By comparing the relative amounts of exchange protection within each peptide the epitope for mAb0062 can be narrowed to 36-47, VQCHYRLQDVKA (60%) and 110-120, RGPQILHRVSL (40%). An overview of the peptide map for the 0062 epitope is shown in Figure 8.

Epitope Mapping of mAb0061

The epitope for mAb0061 was mapped in two separate experiments using either the hTLT-1.16-162 protein or the hTLT-1.126-162.

For hTLT-1.16-162 the HX time-course of 19 peptides, covering 85% of the primary sequence of TLT-1, were monitored in the presence and absence mAb0061 for 10 sec to 8 hours. Due to an O-glycosylation at residue S148, no information could be recorded beyond residue 141.

The observed exchange pattern in the presence or absence of mAb0061 can be divided into two different groups: One group of TLT-1 peptides display an exchange pattern that is unaffected by the binding of mAb0061 and another group of TLT-1 peptides that show protection from exchange upon mAb0061 binding. The regions

displaying protection upon mAb0061 binding encompass peptides covering residues 121-141. However, it is important to note that no information is given in this experiment for residue 142 and beyond. By comparing the relative amounts of exchange protection within each peptide the epitope for mAb0061 can be narrowed to begin at residue 130.

5 In order to gain full information on the mAb0061 epitope, the mapping experiment was repeated using the peptide hTLT-1.126-162. This peptide binds mAb0061 with high affinity and it is not modified by glycosylation. Thus it should be able to give HX-MS information for the entire region.

10 The HX time-course of 12 peptides, covering the entire 126-162 TLT-1 region were monitored in the presence and absence mAb0061 for 10 sec to 3000 sec.

All the peptides in this 126-162 region display protection from exchange upon mAb0061 binding. By comparing the relative amounts of exchange protection within each peptide the epitope for mAb0061 can be narrowed to be within residues 130-145, ETHKIGSLAENAFSDP. An overview of the peptide map for the 0061 epitope is shown in
15 Figure 9 and 10.

Example 23: Production, characterization and binding analyses of hTLT-1 ECD-HPC4 Ala mutants.

hTLT-1 ECD-HPC4 Alanine mutant constructs were designed according to Table
20 6. The expression constructs were developed by external contractor GENEART AG (Im Gewerbepark B35, 93059 Regensburg, Germany) and all expression constructs were made based on the expression vector designated pcDNA3.1(+). Aliquots of DNA for each of the 40 hTLT-1 ECD-HPC4 pcDNA3.1(+) expression construct were transfected into HEK293-6E suspension cells in order to transiently express each hTLT-1 ECD-HPC4 Ala
25 mutant protein (Table 6). Transient transfection and culturing of HEK293 6e cells were performed as described in example A.

Seven days post-transfection, cells were removed by centrifugation and the resulting hTLT-1 ECD-HPC4 Ala mutant protein containing supernatants were sterile-filtrated prior to analyses. The concentration of expressed hTLT-1 ECD-HPC4 Ala mutant
30 protein in the cleared cell supernatant was determined using a combination of RP-HPLC and SDS-PAGE/Coomassie analyses. These ranged from 4 – 40 µg/mL containing variable degree of dimer formation. As described previously for production of hTLT protein used for immunization experiments, monomer/dimer forms of the expressed protein were observed for all hTLT ECD-HPC4 Ala mutant constructs. The relative concentration of
35 monomer/dimer hTLT-1 ECD-HPC4 protein was estimated by SDS-PAGE/Coomassie and an average Mw for each mutant preparation was calculated.

All binding studies were run at 25°C, and the samples were stored at 15°C in the sample compartment on a ProteOn Analyzer (BioRad) that measures molecular interactions in real time through surface plasmon resonance. The signal (RU, response units) reported by the ProteOn is directly correlated to the mass on the individual sensor chip surface spots.

Anti-hFc Polyclonal antibody was immobilized onto separate flow cells of a GLM sensor chip using a 1:1 mixture of 0.4 M EDAC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride] and 0.1 M Sulfo-NHS [N-hydroxysulfosuccinimide]. Each antibody was diluted in 10 mM sodium acetate pH 5.0 to a concentration of 50 µg/ml, and was immobilized to an individual flow cell at 30 µl/min for 240s. The antibodies were immobilized to flow cells A1-A6 (horizontal direction). After immobilization, the active sites on the flow cell were blocked with 1 M ethanolamine. The final immobilization level of capture antibody typically ranged from approximately 9,000 to 10,000 RU in one experiment. Capture of the anti-TLT-1 antibodies mAb0023, mAb0051, mAb0061 and mAb0062 was conducted by diluting to 0.5 µg/ml into HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3mM EDTA, 0.05% surfactant P20, pH 7.4) and injected at 30 µl/min for 60s in vertical direction, creating interspot reference points with only anti-human Fc antibodies. The final capture level of test antibodies typically ranged from approximately 200 to 300 RU in one experiment. Binding of wt or Ala mutant hTLT-1 ECD-HPC4 protein was conducted by injecting over parallel flow cells in horizontal direction to allow for comparative analyses of binding to different captured anti-TLT-1 antibodies relative to binding to the interspot references. Each hTLT-1 ECD-HPC4 protein was diluted to 100 nM, based on the calculated average Mw, into HBS-EP buffer and injected at 30 µl/min for 240s. The GLM chip was regenerated after each injection cycle of analyte via one 18s injection of 1 M Formic acid followed by a 18s injection of 50 mM NaOH at 100 µl/min. This regeneration step removed the anti-TLT-1 antibody and any bound TLT-1 from the immobilized capture antibody surface, and allowed for the subsequent binding of the next test sample pair. The regeneration procedure did not remove the directly immobilized anti-human-Fc capture antibody from the chip surface.

Data analysis was performed using the ProteOn Manager™ Software. No significant non-specific binding to the interspot control surfaces was observed. Binding curves were processed by double referencing (subtraction of interspot control surface signals as well as blank buffer injections over captured anti-TLT-1 antibodies). This allowed correction for instrument noise, bulk shift and drift during sample injections. Binding signal at 10s after stop of analyte injection was normalized to level of captured anti-TLT-1 antibody and presented as binding relative to wt hTLT-1 ECD-HPC4 protein.

The following Ala mutations displayed a significant decrease of binding to respective anti-TLT-1 compared to wt hTLT-1 ECD-HPC4 protein. mAb0051: F54A < 0.4 wt; M91A < 0.2 wt; R117A < 0.2 wt; S119A < 0.6 wt. mAb0062: R41A < 0.2 wt; L42A < 0.6 wt; Q43A < 0.4 wt; F54A < 0.6 wt; M91A < 0.4 wt; R110A < 0.2 wt; H116A < 0.6 wt. mAb0023: L42A < 0.2 wt; Q43A < 0.2 wt; K46A < 0.2 wt; M91A < 0.4 wt; R110A < 0.2 wt. Since decreased binding could be observed for the hTLT-1 ECD-HPC4 mutant M91A for all 4 anti-TLT-1 antibodies, the residue probably has an important influence on protein stability rather than being part of an actual epitope. mAb0061 did not show a decreased binding to any of the mutated TLT-1 variants tested, indicating that the epitope is not covered by the mutants introduced in the binding study.

Example 24: Crystal structure complexes between anti-TLT-1 Fab and TLT-1 stalk peptides.

Expression of anti-TLT-1 Fab, Fab0100 (identical to Fab0061), for crystallization: The anti-TLT-1 Fab fragment, Fab0100, comprising the heavy chain corresponding to SEQ ID NO: 152 and the light chain corresponding SEQ ID NO: 153 was expressed transiently in HEK293 cells according to the generalized procedure.

Purification of anti-TLT-1 Fab, Fab0100, for crystallization: Purification of said Fab was conducted by a two-step process composed of affinity chromatography using the kappaSelect resin (GE Healthcare, cat. no. 17-5458-01) and size-exclusion chromatography. The purification was conducted using an ÄktaExplorer chromatography system (GE Healthcare, cat. no. 18-1112-41). The buffer systems used for the purification step was an equilibration buffer composed of 10 mM NaPhosphate, pH 7.5 and 150 mM NaCl and an elution buffer composed of 20 mM Formic acid, pH 3.0. The supernatant was adjusted with 1 M NaOH to a pH of 7.5 and applied onto a pre-equilibrated kappaSelect column. The column was washed with 5 column volumes of equilibration buffer and the Fab protein was isocratically eluted using approximately 5 column volumes of elution buffer. The Fab protein was analyzed using SDS-PAGE/Coomassie and LC-MS analyses, showing that a pure and homogenous protein with an expected molecular weight of 46.9 kDa was obtained. To measure the protein concentration, a NanoDrop spectrophotometer (Thermo Scientific) was used together with an extinction coefficient of 1.31. The final polish of the Fab protein was conducted using a size-exclusion column (Superdex200).

Preparation of peptides for crystallization: The TLT-1-stalk peptide hTLT-1.126-162 (SEQ ID NO: 7) was prepared by solid phase peptide synthesis. Likewise, a shorter

version hTLT-1.129-142 of the stalk peptide corresponding to SEQ ID NO: 8 was prepared.

Preparation, crystallization and structure determination of the Fab0100:TLT-1 complexes: Preparation of Fab0100:hTLT-1.126-162: The complex between Fab0100 and hTLT-1.126-162 was prepared by adding two times molar excess of hTLT-1.126-162 to a solution of Fab0100 followed by isolation of the complex by separating excess hTLT-1.126-162 using preparative size exclusion chromatography. Thus, the Fab0100: hTLT-1.126-162 complex was prepared by mixing Fab (1100 μ l, 98 μ M) and hTLT-1.126-162 (155 μ l, 1391 μ M), both in PBS buffer (pH 7.4). The complex was subjected to gel filtration using a Superdex 200 HighLoad 26/60 (GE Healthcare) column eluted with PBS-buffer (pH 7.4) at a flow rate of 1 ml/min. Fractions corresponding to a volume of 3 ml were collected. Fractions containing the desired Fab0100: hTLT-1.126-162 complex were pooled and then concentrated using a centrifugal filter device (Amicon, 10 kDa cut-off) to a protein concentration of 8.6 mg/ml. This preparation was used for crystallization of the Fab0100:hTLT-1.126-162 complex.

Preparation of Fab0100:hTLT-1.129-142: The complex between Fab0100 and the shorter stalk peptide (hTLT-1.129-142) was similarly prepared with the exceptions that the molar ratio between hTLT-1.129-142 and Fab was 1.5:1 and that the gel filtration step was omitted due to weaker binding of hTLT-1.129-142 compared to that of the longer stalk peptide (hTLT-1.126-162).

Crystallization and data collection of Fab0100:hTLT-1.129-142 and Fab0100:hTLT-1.126-162 complexes: Fab0100:hTLT-1.129-142 and Fab0100:hTLT-1.126-162 complexes were at room temperature crystallized by the sitting drop method. Fab0100:hTLT-1.129-142 was crystallized by adding to the protein solution, in a 1:2 volume ratio (precipitant:protein), a precipitation solution containing 0.04 M potassium dihydrogen phosphate, 16% w/v PEG 8,000 and 20% glycerol, while the Fab0100:hTLT-1.126-162 complex was crystallized by adding to the protein solution, in a 1:1 volume ratio (precipitant:protein), a precipitation solution containing 20% w/v PEG 10,000 and 0.10 M Hepes pH 7.5. A crystal of the Fab0100:hTLT-1.129-142 complex was flash frozen in liquid N₂ and during data collection kept at 100 K by a cryogenic N₂ gas stream. Crystallographic data were subsequently collected to 2.14 Å resolution using a Rigaku MicroMax-007 HF rotating anode and a marCCD 165 X-ray detector. Space group determination, integration and scaling of the data were made by the XDS software package (Kabsch,W. (1993) *J. Appl. Crystallogr.* 26, 795-800). Cell parameters of the crystal were determined to be 82.10, 64.99, 107.73 Å, 90°, 95.12° and 90°, for a, b, c, α , β and γ respectively, and the space group was determined to be C2. R_{sym} for

intensities of the data set was calculated to be 6.5%. Coordinates from a Fab model of the PDB-deposited (Berman, H.M. et al. (2000) *Nucleic Acids Res.* 28, 235-242) 1NGZ structure (Yin, J. et al. *PNAS* 100, 856-861) was used for structure determination of the anti-TLT-1 Fab molecule. The 1NGZ Fab model was divided into two domains, the variable and the constant domains, which then were used as independent search models in a Molecular replacement run by the PHASER software program (McCoy, A.J. et al. *Acta Crystallographica Section D Biological Crystallography* 61, 458-464; McCoy, A.J. et al. *J. Appl. Crystallogr.* 40, 658-674) of the CCP4 suite (Bailey, S. (1994) *Acta Crystallogr. Sect. D-Biol. Crystallogr.* 50, 760-763). The ARP-wARP software package (Evrard, G.X. et al. *Acta Crystallographica Section D* 63, 108-117) was subsequently used for automated model building and phasing. Additional crystallographic refinements, using the REFMAC5 software program (Murshudov, G.N. et al. *Acta Crystallogr. Sect. D-Biol. Crystallogr.* 53, 240-255), followed by computer graphics inspection of the electron density maps, model corrections and building, using the COOT software program (Emsley, P. et al. *Acta Crystallogr. Sect. D-Biol. Crystallogr.* 60, 2126-2132), were applied. The procedure was cycled until no further significant improvements could be made to the model. Final calculated R- and R-free after 3 cycles of manual intervention and following refinements were 0.185 and 0.245, respectively, and the model showed a root-mean-square deviation (RMSD) from ideal bond lengths of 0.022 Å.

A crystal of the Fab0100:hTLT-1.126-162 complex was transferred to a cryo-solution containing 75% of the precipitant solution and 25% of glycerol. The crystal was allowed to soak for about 15 seconds, then flash frozen in liquid N₂ and during data collection kept at 100 K by a cryogenic N₂ gas stream. Crystallographic data were subsequently collected to 1.85 Å resolution at beam-line BL911-3 (Ursby, T. et al. (2004) AIP Conference Proceedings 705, 1241-1246) at MAX-lab, Lund, Sweden. Space group determination, integration and scaling of the data were made in the xds software package. Cell parameters for the synchrotron data were determined to be 82.54, 65.32, 108.05 Å, 90°, 95.15° and 90°, for a, b, c, α, β and γ, respectively, and space group was determined to be C2. R_{sym} for intensities of the data set was calculated to be 6.7%. The crystal was isomorphous with the Fab0100:hTLT-1.129-142 crystals and therefore rigid body refinement of the Fab0100:hTLT-1.129-142 complex was used for the original phasing of the Fab0100:hTLT-1.126-162 followed by automated model building and phasing using the ARP-wARP software package. Additional crystallographic refinements, using the REFMAC5 software program, followed by computer graphics inspection of the electron density maps, model corrections and building, using the COOT software program, were applied. The procedure was cycled until no further significant

improvements could be made to the model. Final calculated R- and R-free after 13 cycles of manual intervention and following refinements were 0.171 and 0.223, respectively, and the model showed a RMSD from ideal bond lengths of 0.027 Å (Table 11).

5 Results

As shown in Tables 14 and 15, Anti-TLT-1 effectively binds to the stalk of TLT-1. Using the software program AREAIMOL, of the CCP4 program suite, the average areas excluded in pair-wise interaction between Fab0100 and TLT-1 were calculated to be 764 Å². The average areas excluded in pair-wise interactions gave for the Fab0100:hTLT-1.126-162 complex 656 and 871 Å², for anti-TLT-1 and TLT-1 respectively.

Residues in the TLT-1 peptide (hTLT-1.126-162) making direct contacts to the anti-TLT-1 Fab in the Fab0100: hTLT-1.126-162 complex is defined as the epitope and residues in Fab0100 making direct contacts to hTLT-1.126-162 in the Fab0100: hTLT-1.126-162 complex is defined as the paratope. Epitope and paratope residues were identified by running the CONTACTS software of the CCP4 program suite using a cut-off distance of 4.0 Å between the anti-TLT-1 Fab and the TLT-1 molecule. The results of the contact calculations for the Fab0100:hTLT-1.126-162 complex of the crystal structures are shown in Tables 14 and 15. The resulting TLT-1 epitope for Fab0100 was found to comprise the following residues of SEQ ID NO: 7: Lys 8 (133), Ile 9 (134), Gly 10 (135), Ser 11 (136), Leu 12 (137), Ala 13 (138), Asn 15 (140), Ala 16 (141), Phe 17 (142), Ser 18 (143), Asp 19 (144), Pro 20 (145), Ala 21 (146) where numbers in parenthesis refer to the corresponding residues in SEQ ID NO: 2 (Tables 12 and 13).

The resulting paratope included residues His 31, Asn 33, Tyr 37, His 39, Tyr 54, Phe 60, Ser 96, Thr 97, Val 99 and Tyr 101 of the Fab0100 light chain corresponding to SEQ ID NO: 153 (Table 12), and residues Val 2, Phe 27, Arg 31, Tyr 32, Trp 33, Glu 50, Thr 57, Asn 59, Ser 98, Gly 99, Val 100 and Thr 102 of the Fab0100 heavy chain corresponding to SEQ ID NO: 152 (Table 13). The TLT-1 epitope residues involved in hydrogen-binding are also indicated in Tables 12 and 13.

Table 13: Results from the X-ray model refinement to the observed data of the Fab0100:hTLT-1.126-162 complex by the software program refmac5.

REMARK 3 REFINEMENT.

REMARK 3 PROGRAM : REFMAC 5.5.0109

REMARK 3 AUTHORS : MURSHUDOV,VAGIN,DODSON

REMARK 3

REMARK 3 REFINEMENT TARGET : MAXIMUM LIKELIHOOD
REMARK 3
REMARK 3 DATA USED IN REFINEMENT.
REMARK 3 RESOLUTION RANGE HIGH (ANGSTROMS) : 1.85
5 REMARK 3 RESOLUTION RANGE LOW (ANGSTROMS) : 34.18
REMARK 3 DATA CUTOFF (SIGMA(F)) : NONE
REMARK 3 COMPLETENESS FOR RANGE (%) : 99.89
REMARK 3 NUMBER OF REFLECTIONS : 46512
REMARK 3
10 REMARK 3 FIT TO DATA USED IN REFINEMENT.
REMARK 3 CROSS-VALIDATION METHOD : THROUGHOUT
REMARK 3 FREE R VALUE TEST SET SELECTION : RANDOM
REMARK 3 R VALUE (WORKING + TEST SET) : 0.17330
REMARK 3 R VALUE (WORKING SET) : 0.17070
15 REMARK 3 FREE R VALUE : 0.22260
REMARK 3 FREE R VALUE TEST SET SIZE (%) : 5.0
REMARK 3 FREE R VALUE TEST SET COUNT : 2463
REMARK 3
REMARK 3 FIT IN THE HIGHEST RESOLUTION BIN.
20 REMARK 3 TOTAL NUMBER OF BINS USED : 20
REMARK 3 BIN RESOLUTION RANGE HIGH : 1.850
REMARK 3 BIN RESOLUTION RANGE LOW : 1.898
REMARK 3 REFLECTION IN BIN (WORKING SET) : 3409
REMARK 3 BIN COMPLETENESS (WORKING+TEST) (%) : 99.81
25 REMARK 3 BIN R VALUE (WORKING SET) : 0.266
REMARK 3 BIN FREE R VALUE SET COUNT : 195
REMARK 3 BIN FREE R VALUE : 0.309
REMARK 3
REMARK 3 NUMBER OF NON-HYDROGEN ATOMS USED IN REFINEMENT.
30 REMARK 3 ALL ATOMS : 3993
REMARK 3
REMARK 3 B VALUES.
REMARK 3 FROM WILSON PLOT (A**2) : NULL
REMARK 3 MEAN B VALUE (OVERALL, A**2) : 14.967
35 REMARK 3 OVERALL ANISOTROPIC B VALUE.
REMARK 3 B11 (A**2) : -0.06

REMARK 3 B22 (A**2) : 0.23
REMARK 3 B33 (A**2) : -0.24
REMARK 3 B12 (A**2) : 0.00
REMARK 3 B13 (A**2) : -0.36
5 REMARK 3 B23 (A**2) : 0.00
REMARK 3
REMARK 3 ESTIMATED OVERALL COORDINATE ERROR.
REMARK 3 ESU BASED ON R VALUE (A): 0.116
REMARK 3 ESU BASED ON FREE R VALUE (A): 0.123
10 REMARK 3 ESU BASED ON MAXIMUM LIKELIHOOD (A): 0.084
REMARK 3 ESU FOR B VALUES BASED ON MAXIMUM LIKELIHOOD (A**2): 6.165
REMARK 3
REMARK 3 CORRELATION COEFFICIENTS.
REMARK 3 CORRELATION COEFFICIENT FO-FC : 0.963
15 REMARK 3 CORRELATION COEFFICIENT FO-FC FREE : 0.939
REMARK 3
REMARK 3 RMS DEVIATIONS FROM IDEAL VALUES COUNT RMS WEIGHT
REMARK 3 BOND LENGTHS REFINED ATOMS (A): 3538 ; 0.027 ; 0.022
REMARK 3 BOND ANGLES REFINED ATOMS (DEGREES): 4833 ; 2.132 ; 1.958
20 REMARK 3 TORSION ANGLES, PERIOD 1 (DEGREES): 473 ; 6.972 ; 5.000
REMARK 3 TORSION ANGLES, PERIOD 2 (DEGREES): 137 ; 35.607 ; 24.453
REMARK 3 TORSION ANGLES, PERIOD 3 (DEGREES): 583 ; 14.216 ; 15.000
REMARK 3 TORSION ANGLES, PERIOD 4 (DEGREES): 14 ; 23.096 ; 15.000
REMARK 3 CHIRAL-CENTER RESTRAINTS (A**3): 552 ; 0.180 ; 0.200
25 REMARK 3 GENERAL PLANES REFINED ATOMS (A): 2664 ; 0.013 ; 0.021
REMARK 3
REMARK 3 ISOTROPIC THERMAL FACTOR RESTRAINTS. COUNT RMS WEIGHT
REMARK 3 MAIN-CHAIN BOND REFINED ATOMS (A**2): 2262 ; 1.399 ; 1.500
REMARK 3 MAIN-CHAIN ANGLE REFINED ATOMS (A**2): 3679 ; 2.333 ; 2.000
30 REMARK 3 SIDE-CHAIN BOND REFINED ATOMS (A**2): 1276 ; 3.462 ; 3.000
REMARK 3 SIDE-CHAIN ANGLE REFINED ATOMS (A**2): 1139 ; 5.231 ; 4.500
REMARK 3
REMARK 3 NCS RESTRAINTS STATISTICS
REMARK 3 NUMBER OF NCS GROUPS : NULL
35 REMARK 3
REMARK 3 TWIN DETAILS

REMARK 3 NUMBER OF TWIN DOMAINS : NULL
REMARK 3
REMARK 3
REMARK 3 TLS DETAILS
5 REMARK 3 NUMBER OF TLS GROUPS : 2
REMARK 3 ATOM RECORD CONTAINS RESIDUAL B FACTORS ONLY
REMARK 3
REMARK 3 TLS GROUP : 1
REMARK 3 NUMBER OF COMPONENTS GROUP : 3
10 REMARK 3 COMPONENTS C SSSEQI TO C SSSEQI
REMARK 3 RESIDUE RANGE : L 1 L 109
REMARK 3 RESIDUE RANGE : H 1 H 113
REMARK 3 RESIDUE RANGE : P 7 P 21
REMARK 3 ORIGIN FOR THE GROUP (A): -4.1790 48.4400 34.3450
15 REMARK 3 T TENSOR
REMARK 3 T11: 0.1731 T22: 0.1937
REMARK 3 T33: 0.1093 T12: -0.0155
REMARK 3 T13: -0.0164 T23: -0.0192
REMARK 3 L TENSOR
20 REMARK 3 L11: 1.9367 L22: 0.4840
REMARK 3 L33: 3.8383 L12: -0.1522
REMARK 3 L13: -1.2215 L23: -0.1172
REMARK 3 S TENSOR
REMARK 3 S11: 0.0447 S12: -0.2657 S13: 0.0758
25 REMARK 3 S21: 0.0958 S22: -0.0414 S23: -0.0674
REMARK 3 S31: 0.0036 S32: 0.0098 S33: -0.0032
REMARK 3
REMARK 3 TLS GROUP : 2
REMARK 3 NUMBER OF COMPONENTS GROUP : 2
30 REMARK 3 COMPONENTS C SSSEQI TO C SSSEQI
REMARK 3 RESIDUE RANGE : L 114 L 219
REMARK 3 RESIDUE RANGE : H 116 H 215
REMARK 3 ORIGIN FOR THE GROUP (A): -24.4360 51.7710 5.9920
REMARK 3 T TENSOR
35 REMARK 3 T11: 0.0252 T22: 0.0170
REMARK 3 T33: 0.0735 T12: 0.0161

REMARK 3 T13: 0.0018 T23: 0.0048
 REMARK 3 L TENSOR
 REMARK 3 L11: 2.0324 L22: 1.6905
 REMARK 3 L33: 0.8461 L12: 0.7328
 5 REMARK 3 L13: 0.0695 L23: 0.3337
 REMARK 3 S TENSOR
 REMARK 3 S11: -0.0068 S12: 0.0156 S13: 0.0515
 REMARK 3 S21: -0.0127 S22: -0.0101 S23: 0.1316
 REMARK 3 S31: -0.0077 S32: -0.0763 S33: 0.0168
 10 REMARK 3
 REMARK 3
 REMARK 3 BULK SOLVENT MODELLING.
 REMARK 3 METHOD USED : MASK
 REMARK 3 PARAMETERS FOR MASK CALCULATION
 15 REMARK 3 VDW PROBE RADIUS : 1.40
 REMARK 3 ION PROBE RADIUS : 0.80
 REMARK 3 SHRINKAGE RADIUS : 0.80
 REMARK 3
 REMARK 3 OTHER REFINEMENT REMARKS:
 20 REMARK 3 HYDROGENS HAVE BEEN ADDED IN THE RIDING POSITIONS
 REMARK 3 U VALUES : RESIDUAL ONLY
 REMARK 3
 LINKR SG CYS L 139 SG ACYS L 199 SS
 LINKR SG CYS H 22 SG ACYS H 96 SS
 25 LINKR SG ACYS H 141 SG ACYS H 197 SS
 CISPEP 1 THR L 7 PRO L 8 0.00
 CISPEP 2 VAL L 99 PRO L 100 0.00
 CISPEP 3 TYR L 145 PRO L 146 0.00
 CISPEP 4 PHE H 147 PRO H 148 0.00
 30 CISPEP 5 GLU H 149 PRO H 150 0.00

Table 14

hTLT-1.126-162 "P" (SEQ ID NO 7) interactions with the Fab0100 light chain (SEQ ID NO: 163). A cut-off of 4.0 Å was used. The contacts were identified by the
 35 CONTACT computer program of the CCP4 suite. In the last column "****" indicates a strong possibility for a hydrogen bond at this contact (distance < 3.3 Å) as calculated by

CONTACT, " *" indicates a weak possibility (distance > 3.3 Å). Blank indicates that the program considered there to be no possibility of a hydrogen bond. Hydrogen-bonds are specific between a donor and an acceptor, are typically strong, and are easily identifiable.

<i>hTLT-1.126-162</i>			<i>Anti-TLT-1</i>			<i>Distance [Å]</i>	<i>Possibly H- bond</i>
<i>Res. Type</i>	<i>Res. # and Chain</i>	<i>Atom name</i>	<i>Res. Type</i>	<i>Res. # and Chain</i>	<i>Atom name</i>		
Ile	9P	CB	Tyr	101L	OH	3.76	
Ile	9P	CD1	Ser	96L	O	3.58	
			Thr	97L	O	3.68	
Ile	9P	CG2	Val	99L	CG2	3.51	
Ile	9P	C	Tyr	101L	OH	3.95	
Gly	10P	N	Tyr	101L	OH	3.06	***
Gly	10P	CA	Tyr	101L	OH	3.48	
Gly	10P	C	Tyr	101L	OH	3.67	
Ser	11P	N	Tyr	101L	OH	3.09	***
Ser	11P	CB	Ser	96L	OG	3.89	
			Tyr	37L	CD1	3.94	
			Ser	96L	O	3.37	
Ser	11P	OG	Ser	96L	OG	2.88	***
			Ser	96L	CA	3.76	
			Ser	96L	CB	3.05	
			Ser	96L	C	3.33	
			Ser	96L	O	2.45	***
			Tyr	101L	CE1	3.94	
			Tyr	101L	CZ	3.59	
			Tyr	101L	OH	3.37	*
Ser	11P	O	Tyr	37L	CE1	3.39	
			Tyr	37L	CZ	3.66	
			Tyr	37L	OH	3.55	*
Leu	12P	CG	Asn	33L	ND2	3.41	
			Tyr	37L	OH	3.86	
Leu	12P	CD1	His	31L	CE1	3.73	
			His	31L	NE2	3.38	
			His	31L	CD2	3.63	

<i>hTLT-1.126-162</i>			<i>Anti-TLT-1</i>			<i>Distance [Å]</i>	<i>Possibly H- bond</i>
<i>Res. Type</i>	<i>Res. # and Chain</i>	<i>Atom name</i>	<i>Res. Type</i>	<i>Res. # and Chain</i>	<i>Atom name</i>		
			Tyr	37L	CE2	3.65	
			Asn	33L	ND2	3.68	
			Tyr	37L	CZ	3.84	
			Tyr	37L	OH	3.51	
Leu	12P	CD2	His	31L	CE1	3.57	
			His	31L	NE2	3.91	
			Asn	33L	ND2	3.39	
Phe	17P	CB	Tyr	54L	CG	3.79	
			Tyr	54L	CE1	3.70	
			Tyr	54L	CD1	3.45	
Phe	17P	CG	Tyr	54L	CG	3.98	
			Tyr	54L	CD1	3.63	
Phe	17P	CD1	Tyr	54L	CB	3.74	
Phe	17P	CE1	His	39L	ND1	3.88	
			His	39L	CE1	3.32	
			His	39L	NE2	3.60	
Phe	17P	CZ	His	39L	CE1	3.36	
			His	39L	NE2	3.66	
			Tyr	37L	CD1	3.98	
Phe	17P	CE2	Tyr	37L	CD1	3.79	
			Tyr	37L	CE1	3.84	
Phe	17P	O	Phe	60L	CD1	3.84	
			Phe	60L	CE1	3.31	
Asp	19P	N	Phe	60L	CE1	3.88	
Asp	19P	CA	Phe	60L	CZ	3.80	
Asp	19P	CB	Phe	60L	CZ	3.85	

Table 15

hTLT-1.126-162 "P" (SEQ ID NO 7) interactions with Fab0100 heavy chain (SEQ ID NO: 162). A cut-off of 4.0 Å was used. The contacts were identified by the CONTACT computer program of the CCP4 suite. In the last column "****" indicates a strong possibility for a hydrogen bond at this contact (distance < 3.3 Å) as calculated by CONTACT, " *" indicates a weak possibility (distance > 3.3 Å). Blank indicates that the

program considered there to be no possibility of a hydrogen bond. Hydrogen-bonds are specific between a donor and an acceptor, are typically strong, and are easily identifiable.

<i>hTLT-1.126-162</i>			<i>Anti-TLT-1</i>			<i>Distance [Å]</i>	<i>Possibly H- bond</i>
<i>Res. Type</i>	<i>Res. # and Chain</i>	<i>Atom name</i>	<i>Res. Type</i>	<i>Res. # and Chain</i>	<i>Atom name</i>		
Lys	8P	C	Asn	59H	OD1	3.95	
Lys	8P	O	Asn	59H	CG	3.69	
			Asn	59H	ND2	3.78	*
			Trp	33H	CH2	3.86	
			Asn	59H	OD1	2.85	***
			Thr	57H	CG2	3.45	
Ile	9P	CA	Trp	33H	CH2	3.91	
			Glu	50H	OE2	3.52	
			Asn	59H	OD1	3.79	
Ile	9P	CB	Glu	50H	OE2	3.81	
Ile	9P	C	Trp	33H	CZ3	4.00	
			Trp	33H	CH2	3.65	
			Glu	50H	OE2	3.62	
			Trp	33H	CZ2	3.87	
Ile	9P	O	Trp	33H	CZ2	3.92	
Gly	10P	N	Glu	50H	CD	3.40	
			Glu	50H	OE1	3.38	*
			Trp	33H	CZ3	3.55	
			Trp	33H	CH2	3.68	
			Glu	50H	OE2	2.77	***
			Trp	33H	CE3	3.85	
			Trp	33H	CZ2	3.99	
Gly	10P	CA	Glu	50H	CD	3.79	
			Glu	50H	OE1	3.40	
			Trp	33H	CZ3	3.80	
			Glu	50H	OE2	3.61	
			Trp	33H	CE2	3.75	
			Trp	33H	CD2	3.55	
			Trp	33H	CE3	3.58	

<i>hTLT-1.126-162</i>			<i>Anti-TLT-1</i>			<i>Distance [Å]</i>	<i>Possibly H- bond</i>
<i>Res. Type</i>	<i>Res. # and Chain</i>	<i>Atom name</i>	<i>Res. Type</i>	<i>Res. # and Chain</i>	<i>Atom name</i>		
			Trp	33H	CZ2	3.99	
Gly	10P	C	Val	100H	CG2	3.82	
Gly	10P	O	Val	100H	CG2	3.86	
Ser	11P	N	Val	100H	CG2	3.78	
Ser	11P	CA	Val	100H	CG2	3.78	
Ala	13P	CB	Trp	33H	CD1	3.60	
			Trp	33H	NE1	3.52	
Asn	15P	CG	Tyr	32H	CD1	3.88	
			Tyr	32H	CE1	3.83	
Asn	15P	ND2	Arg	31H	C	3.94	
			Arg	31H	O	3.09	***
			Tyr	32H	CG	3.98	
			Tyr	32H	CD1	3.76	
			Tyr	32H	CE1	3.70	
			Tyr	32H	CZ	3.88	
			Arg	31H	NH1	3.93	*
Ala	16P	O	Val	100H	CB	3.86	
			Val	100H	CA	3.80	
			Val	100H	N	2.91	***
			Thr	102H	CG2	3.46	
			Gly	99H	CA	3.74	
			Gly	99H	C	3.78	
Phe	17P	CA	Thr	102H	CG2	3.64	
Phe	17P	CD1	Val	100H	O	3.93	
Phe	17P	CE1	Val	100H	CB	3.46	
			Val	100H	CG1	3.52	
			Val	100H	O	3.86	
Phe	17P	CZ	Val	100H	CB	3.81	
			Val	100H	CG1	3.89	
Phe	17P	C	Thr	102H	CG2	3.59	
Phe	17P	O	Thr	102H	CG2	3.79	
Ser	18P	C	Thr	102H	CG2	3.94	

<i>hTLT-1.126-162</i>			<i>Anti-TLT-1</i>			<i>Distance [Å]</i>	<i>Possibly H- bond</i>
<i>Res. Type</i>	<i>Res. # and Chain</i>	<i>Atom name</i>	<i>Res. Type</i>	<i>Res. # and Chain</i>	<i>Atom name</i>		
Ser	18P	O	Thr	102H	CB	3.48	
			Thr	102H	OG1	3.64	*
			Thr	102H	CG2	3.26	
Pro	20P	CA	Tyr	32H	OH	3.47	
			Tyr	32H	CZ	3.90	
Pro	20P	CB	Val	2H	CG2	3.92	
			Phe	27H	CB	3.93	
			Phe	27H	CD1	3.86	
			Phe	27H	CG	3.78	
Pro	20P	CG	Thr	102H	OG1	3.88	
			Ser	98H	OG	3.54	
Pro	20P	CD	Thr	102H	CB	3.95	
			Thr	102H	OG1	3.83	
Pro	20P	C	Val	2H	CG2	3.98	
Pro	20P	O	Phe	27H	CB	3.52	
Ala	21P	N	Val	2H	CG2	3.51	
Ala	21P	CA	Val	2H	CG2	3.97	
Ala	21P	CB	Val	2H	CG2	3.77	

Example 25: Epitope mapping by peptide walk.

The peptide walking ELISA defined the minimal binding region of the peptide.

- 5 This was established by coating biotinylated peptides with one residue frameshift in the stalk region of TLT-1 in streptavidin plates followed by binding of the antibody of interest (mAb0061). A secondary antibody was added for detection and binding was measured at 450 nm. Positive control: binding to biotinylated TLT-1.

10 Materials

- | | |
|----------|---|
| 10X PBS: | 10X GPBS 14200 Gibco |
| Tween20: | Aldrich Cat# 27,434-8, Lot#S30950-315 |
| Plate: | 96-well Streptavidin coated plate Nunc#466014 |
| 15 BSA: | A7030-100g Lot# 057K0737 |

- Blocking/Dilute Buffer: 1X PBS pH=7.4
2% BSA
0.5% Tween20
- Wash Buffer: 1xPBS + 0.5% Tween20
- 5 Standard: Biotinylated TLT-1 04/09-08 1 mg/ml
mAb: 0197-0000-0061-4A - 0.55 mg/ml
Detecting Ab: Goat anti-Human IgG HRP-labeled 1mg/ml Prod. no. NEF802001EA
- TMB Substrate: Ready to use Cat# 4390L lot.# 70904
- 10 Stop Solution: 2M H₃PO₄

Dilution of Biotinylated TLT-1

1mg/ml -> 6.3ng/ml (158500x Dilution)

Concentration in well: 0.63 ng

15

Dilution of Biotinylated peptides

Approx conc 2-5 mg/ml (2.5 mg/ml)

2.5mg/ml -> 10000x Dilution (25ng/well): 100µl of each peptide in each well.

20 Dilution of mAb0061

0.55mg/ml -> 100ng/ml (5500x Dilution)

Concentration in well: 10 ng

Dilution of mAb Goat Anti-human IgG HRP

25 1mg/ml -> 0.2µg/ml (5000x Dilution)

Synthesis of biotinylated peptides in 96 well format

The biotinylated peptides were synthesised using standard solid phase peptide synthesis. Solutions of 0.3M Fmoc-protected amino acids in 0.3 M 1-

- 30 hydroxybenzotriazole (HOBt) in N-methylpyrrolidinone (NMP) were coupled using diisopropylcarbodiimide (DIC) for 1-4 hours. As solid support the Rink amide LL resin (Merck) was used in a 96 microtiter filterplate (Nunc) and ca. 20 mg resin pr well was used. The synthesis was performed using the Multi pep RS peptide synthesiser from Intavis, Germany and manufacture protocol was used. The removal of Fmoc was done
- 35 using 25% piperidin in NMP. All peptides were coupled with biotin at the N-terminal and 8-amino-3,6-dioxaoctanoic acid was used as a spacer between biotin and the peptides.

This spacer was also coupled as a Fmoc-protected building block according to the synthesis protocol (IRIS biotech, Germany)

Final deprotection and workup

- 5 The final deprotection was done using 90% trifluoroacetic acid (TFA), 5% triisopropylsilan and 5% H₂O for 3 hours. A total of 1 ml TFA was used per well. The TFA was filtered to 96 deep well (Nunc) and the TFA was reduced in volume by evaporation to ca. 100- 200 ul per well and diethylether was added to all wells in order to precipitate the peptides. The suspension of peptide in diethylether was transferred to solvinert 96 well
10 filter plate (0.47 um, Millipore) and the peptides were washed twice with diethylether and dried. The peptides were redissolved in 80% DMSO and 20% water giving a stock solution of ca. 1-3 mg/ml.

Biotinylated 20mer peptides from stalk region of TLT-1 (SEQ ID NO 6)

- 15 2-5mg/ml in 75% DMSO/H₂O (biotinylated in N-terminal):

Number of peptide shown at the left:

20	2	A 2	2619.8	bio-Oeg-L-N-I-L-P-P-E-E-E-E-E-T-H-K-I-G-S-L-A-E
	3	A 3	2620.7	bio-Oeg-N-I-L-P-P-E-E-E-E-E-T-H-K-I-G-S-L-A-E-N
	4	A 4	2577.7	bio-Oeg-I-L-P-P-E-E-E-E-E-T-H-K-I-G-S-L-A-E-N-A
	5	A 5	2611.7	bio-Oeg-L-P-P-E-E-E-E-E-T-H-K-I-G-S-L-A-E-N-A-F
25	6	A 6	2585.6	bio-Oeg-P-P-E-E-E-E-E-T-H-K-I-G-S-L-A-E-N-A-F-S
	7	A 7	2603.6	bio-Oeg-P-E-E-E-E-E-T-H-K-I-G-S-L-A-E-N-A-F-S-D
	8	A 8	2603.6	bio-Oeg-E-E-E-E-E-T-H-K-I-G-S-L-A-E-N-A-F-S-D-P
	9	A 9	2545.6	bio-Oeg-E-E-E-E-T-H-K-I-G-S-L-A-E-N-A-F-S-D-P-A
30	10	A10	2473.6	bio-Oeg-E-E-E-T-H-K-I-G-S-L-A-E-N-A-F-S-D-P-A-G
	11	A11	2431.6	bio-Oeg-E-E-T-H-K-I-G-S-L-A-E-N-A-F-S-D-P-A-G-S
	12	A12	2373.6	bio-Oeg-E-T-H-K-I-G-S-L-A-E-N-A-F-S-D-P-A-G-S-A
	13	B 1	2358.6	bio-Oeg-T-H-K-I-G-S-L-A-E-N-A-F-S-D-P-A-G-S-A-N
35	14	B 2	2354.6	bio-Oeg-H-K-I-G-S-L-A-E-N-A-F-S-D-P-A-G-S-A-N-P
	15	B 3	2330.7	bio-Oeg-K-I-G-S-L-A-E-N-A-F-S-D-P-A-G-S-A-N-P-L
	16	B 4	2331.6	bio-Oeg-I-G-S-L-A-E-N-A-F-S-D-P-A-G-S-A-N-P-L-E
	17	B 5	2315.5	bio-Oeg-G-S-L-A-E-N-A-F-S-D-P-A-G-S-A-N-P-L-E-P
	18	B 6	2345.5	bio-Oeg-S-L-A-E-N-A-F-S-D-P-A-G-S-A-N-P-L-E-P-S
	19	B 7	2386.5	bio-Oeg-L-A-E-N-A-F-S-D-P-A-G-S-A-N-P-L-E-P-S-Q
	20	B 8	2388.4	bio-Oeg-A-E-N-A-F-S-D-P-A-G-S-A-N-P-L-E-P-S-Q-D

	21	B 9	2446.4	bio-Oeg-E-N-A-F-S-D-P-A-G-S-A-N-P-L-E-P-S-Q-D-E
	22	B10	2445.5	bio-Oeg-N-A-F-S-D-P-A-G-S-A-N-P-L-E-P-S-Q-D-E-K
	23	B11	2418.5	bio-Oeg-A-F-S-D-P-A-G-S-A-N-P-L-E-P-S-Q-D-E-K-S
	24	B12	2460.6	bio-Oeg-F-S-D-P-A-G-S-A-N-P-L-E-P-S-Q-D-E-K-S-I
5	25	C 1	2410.5	bio-Oeg-S-D-P-A-G-S-A-N-P-L-E-P-S-Q-D-E-K-S-I-P
	26	C 2	2436.6	bio-Oeg-D-P-A-G-S-A-N-P-L-E-P-S-Q-D-E-K-S-I-P-L
	27	C 3	2434.7	bio-Oeg-P-A-G-S-A-N-P-L-E-P-S-Q-D-E-K-S-I-P-L-I

Biotinylated 16mer peptides from the stalk region of hTLT-1 (SEQ ID NO 6)**10 2-5mg/ml in 75% DMSO/H2O:**

	29	C 5	2219.3	bio-Oeg-L-N-I-L-P-P-E-E-E-E-E-T-H-K-I-G
	30	C 6	2193.2	bio-Oeg-N-I-L-P-P-E-E-E-E-E-T-H-K-I-G-S
	31	C 7	2192.3	bio-Oeg-I-L-P-P-E-E-E-E-E-T-H-K-I-G-S-L
	32	C 8	2150.2	bio-Oeg-L-P-P-E-E-E-E-E-T-H-K-I-G-S-L-A
15	33	C 9	2166.1	bio-Oeg-P-P-E-E-E-E-E-T-H-K-I-G-S-L-A-E
	34	C10	2183.1	bio-Oeg-P-E-E-E-E-E-E-T-H-K-I-G-S-L-A-E-N
	35	C11	2157.1	bio-Oeg-E-E-E-E-E-E-T-H-K-I-G-S-L-A-E-N-A
	36	C12	2175.2	bio-Oeg-E-E-E-E-E-T-H-K-I-G-S-L-A-E-N-A-F
	37	D 1	2133.2	bio-Oeg-E-E-E-T-H-K-I-G-S-L-A-E-N-A-F-S
20	38	D 2	2119.2	bio-Oeg-E-E-T-H-K-I-G-S-L-A-E-N-A-F-S-D
	39	D 3	2087.2	bio-Oeg-E-T-H-K-I-G-S-L-A-E-N-A-F-S-D-P
	40	D 4	2029.2	bio-Oeg-T-H-K-I-G-S-L-A-E-N-A-F-S-D-P-A
	41	D 5	1985.2	bio-Oeg-H-K-I-G-S-L-A-E-N-A-F-S-D-P-A-G
	42	D 6	1935.2	bio-Oeg-K-I-G-S-L-A-E-N-A-F-S-D-P-A-G-S
25	43	D 7	1878.1	bio-Oeg-I-G-S-L-A-E-N-A-F-S-D-P-A-G-S-A
	44	D 8	1879	bio-Oeg-G-S-L-A-E-N-A-F-S-D-P-A-G-S-A-N
	45	D 9	1919	bio-Oeg-S-L-A-E-N-A-F-S-D-P-A-G-S-A-N-P
	46	D10	1945.1	bio-Oeg-L-A-E-N-A-F-S-D-P-A-G-S-A-N-P-L
	47	D11	1961	bio-Oeg-A-E-N-A-F-S-D-P-A-G-S-A-N-P-L-E
30	48	D12	1987	bio-Oeg-E-N-A-F-S-D-P-A-G-S-A-N-P-L-E-P
	49	E 1	1945	bio-Oeg-N-A-F-S-D-P-A-G-S-A-N-P-L-E-P-S
	50	E 2	1959	bio-Oeg-A-F-S-D-P-A-G-S-A-N-P-L-E-P-S-Q
	51	E 3	2003	bio-Oeg-F-S-D-P-A-G-S-A-N-P-L-E-P-S-Q-D
	52	E 4	1984.9	bio-Oeg-S-D-P-A-G-S-A-N-P-L-E-P-S-Q-D-E
35	53	E 5	2026	bio-Oeg-D-P-A-G-S-A-N-P-L-E-P-S-Q-D-E-K
	54	E 6	1998	bio-Oeg-P-A-G-S-A-N-P-L-E-P-S-Q-D-E-K-S

55	E 7	2014.1	bio-Oeg-A-G-S-A-N-P-L-E-P-S-Q-D-E-K-S-I
56	E 8	2040.1	bio-Oeg-G-S-A-N-P-L-E-P-S-Q-D-E-K-S-I-P
57	E 9	2096.2	bio-Oeg-S-A-N-P-L-E-P-S-Q-D-E-K-S-I-P-L
58	E10	2122.3	bio-Oeg-A-N-P-L-E-P-S-Q-D-E-K-S-I-P-L-I

5

Method

The epitope mapping involved binding of mAb0061 to two series of biotinylated peptides from the stalk region of TLT-1. The biotinylated peptides were bound to streptavidin plates.

10

Stalk peptide:

LNILPPEEEEEETHKIGSLAENAFSDPAGSANPLEPSQDEKSIPL

1) 20mer peptide mapping with one residue frameshift (20,1) (see materials)

2) 16mer peptide mapping with one residue frameshift (16,1) (see materials)

15

1. Plate was prre-washed 3x with 250µl wash buffer
2. 100µl Biotinylated peptide-solution was added (from Masterplate 10000 X diluted, one peptide pr well)
3. Incubated at RT for 1 hour or +5°C over night
- 20 4. Washed 3x with wash buffer
5. 100µl Primary antibody was added (dilution see above)
6. Incubated at RT for 1 hour
7. Washed 3x with wash buffer
8. 100µl Secondary antibody was added (dilution see above)
- 25 9. Incubated at RT for 1 hour
10. Washed 3x with wash buffer
11. 100µl Substrate/Develop buffer was added (Reaction time 3 min)
12. 100µl 2M H3PO4 was added
13. Endpoint was read at 450 nm

30

Binding to biotinylated peptide in a well was recorded as "binding" when the absorption at 450 nm was above 3. "No binding" was recorded when signal was below 1. A signal in between was recorded as "weak binding".

35

Results

The biotinylated peptides were put into wells in the following way:

Row A: peptide 2-12 (20mers)

Row B: peptide 13-24 (20mers)

Row C: peptide 25-27 (20mers)

Row C: peptide 29-36 (16mers)

5 Row D: peptide 37-48 (16mers)

Row E: peptide 49-58 (16mers)

Result from triple determination:

	1	2	3	4	5	6	7	8	9	10	11	12
A		<0.1	<0.1	<0.1	>3	>3	>3	>3	>3	>3	>3	>3
B	>3	>3	>3	>3	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
C	<0.1	<0.1	<0.1		<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	>3
D	>3	>3	>3	>3	>3	>3	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
E	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1		
F												
G												
H												

- 10 In summary, the 20mer-peptides (5-16) give rise to strong positive signals (<3) corresponding to amino acids: IGSLAENAF. The 16mer peptides 36-42 give rise to a strong positive signals (<3) corresponding to KIGSLAENAF.

Conclusion

- 15 The peptide walking ELISA has defined the minimal binding area of the epitope for binding to mAb0061 as the following stretch of amino acid residues: KIGSLAENAF. This stretch is indeed part of the epitope defined above by the crystal structure: KIGSLA-NAFSDPA.

20 **Example 26: Factor VIIa polypeptide *in vitro* hydrolysis assay.**

- Native (wild-type) Factor VIIa and Factor VIIa variant (both hereafter referred to as "Factor VIIa") are assayed in parallel to directly compare their specific activities. The assay is carried out in a microtiter plate (MaxiSorp, Nunc, Denmark). The chromogenic substrate D-Ile-Pro-Arg-*p*-nitroanilide (S-2288, Chromogenix, Sweden), final
- 25 concentration 1 mM, is added to Factor VIIa (final concentration 100 nM) in 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM CaCl₂ and 1 mg/ml bovine serum albumin. The absorbance at 405 nm is measured continuously in a SpectraMax™ 340 plate reader

(Molecular Devices, USA). The absorbance developed during a 20-minute incubation, after subtraction of the absorbance in a blank well containing no enzyme, is used to calculate the ratio between the activities of variant and wild-type Factor VIIa:

$$\text{Ratio} = (A_{405 \text{ nm}} \text{ Factor VIIa variant}) / (A_{405 \text{ nm}} \text{ Factor VIIa wild-type}).$$

Example 27: Factor VIIa polypeptide *in vitro* proteolysis assay.

Native (wild-type) Factor VIIa and Factor VIIa variant (both hereafter referred to as "Factor VIIa") are assayed in parallel to directly compare their specific activities. The assay is carried out in a microtiter plate (MaxiSorp, Nunc, Denmark). Factor VIIa (10 nM) and Factor X (0.8 µM) in 100 µL 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM CaCl₂ and 1 mg/ml bovine serum albumin, are incubated for 15 min. Factor X cleavage is then stopped by the addition of 50 µL 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 20 mM EDTA and 1 mg/ml bovine serum albumin. The amount of Factor Xa generated is measured by addition of the chromogenic substrate Z-D-Arg-Gly-Arg-*p*-nitroanilide (S-2765, Chromogenix, Sweden), final concentration 0.5 mM. The absorbance at 405 nm is measured continuously in a SpectraMax™ 340 plate reader (Molecular Devices, USA). The absorbance developed during 10 minutes, after subtraction of the absorbance in a blank well containing no FVIIa, is used to calculate the ratio between the proteolytic activities of variant and wild-type Factor VIIa:

$$\text{Ratio} = (A_{405 \text{ nm}} \text{ Factor VIIa variant}) / (A_{405 \text{ nm}} \text{ Factor VIIa wild-type}).$$

Example 28: Factor VIIIa activity assay: chromogenic assay.

The FVIII activity (FVIII:C) of the rFVIII compound is evaluated in a chromogenic FVIII assay using Coatest SP reagents (Chromogenix) as follows: rFVIII samples and a FVIII standard (e.g. purified wild-type rFVIII calibrated against the 7th international FVIII standard from NIBSC) are diluted in Coatest assay buffer (50 mM Tris, 150 mM NaCl, 1% BSA, pH 7.3, with preservative). Fifty µl of samples, standards, and buffer negative control are added to 96-well microtiter plates (Nunc) in duplicates. The factor IXa/factor X reagent, the phospholipid reagent and CaCl₂ from the Coatest SP kit are mixed 5:1:3 (vol:vol:vol) and 75 µl of this added to the wells. After 15 min incubation at room temperature, 50 µl of the factor Xa substrate S-2765/thrombin inhibitor I-2581 mix is added and the reagents incubated for 10 minutes at room temperature before 25 µl 1 M citric acid, pH 3, is added. The absorbance at 415 nm is measured on a Spectramax microtiter plate reader (Molecular Devices) with absorbance

at 620 nm used as reference wavelength. The value for the negative control is subtracted from all samples and a calibration curve prepared by linear regression of the absorbance values plotted vs. FVIII concentration. Specific activity is calculated by dividing the activity of the samples with the protein concentration determined by HPLC. The concentration of the sample is determined by integrating the area under the peak in the chromatogram corresponding to the light chain and compare with the area of the same peak in a parallel analysis of a wild-type unmodified rFVIII, where the concentration is determined by amino acid analyses.

Example 29: Factor VIIIa activity assay: one-stage clot assay.

FVIII activity (FVIII:C) of the rFVIII compounds is further evaluated in a one-stage FVIII clot assay as follows: rFVIII samples and a FVIII standard (e.g. purified wild-type rFVIII calibrated against the 7th international FVIII standard from NIBSC) are diluted in HBS/BSA buffer (20 mM hepes, 150 mM NaCl, pH 7.4 with 1% BSA) to approximately 10 U/ml, followed by 10-fold dilution in FVIII-deficient plasma containing VWF (Dade Behring). Samples are subsequently diluted in HBS/BSA buffer. The APTT clot time is measured using an ACL300R or an ACL5000 instrument (Instrumentation Laboratory) using the single factor program. FVIII-deficient plasma with VWF (Dade Behring) is used as assay plasma and SynthASil, (HemosIL™, Instrumentation Laboratory) as aPTT reagent. In the clot instrument, the diluted sample or standard is mixed with FVIII-deficient plasma and aPTT reagents at 37°C. Calcium chloride is added and time until clot formation is determined by measuring turbidity. The FVIII:C in the sample is calculated based on a standard curve of the clot formation times of the dilutions of the FVIII standard.

Example 30: Factor Xa polypeptide in vitro protolysis assay.

Preactivated native (wild-type) Factor Xa and preactivated Factor Xa variant (both hereafter referred to as "Factor Xa") are assayed in parallel to directly compare their specific activities. The assay is carried out in a microtiter plate (MaxiSorp, Nunc, Denmark). Factor Xa (10 nM) and Prothrombin (0.8 microM) in 100 µL 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM CaCl₂ and 1 mg/ml bovine serum albumin, are incubated for 15 min. Prothrombin cleavage is then stopped by the addition of 50 µL 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 20 mM EDTA and 1 mg/ml bovine serum albumin. The amount of Thrombin generated is measured by addition of the chromogenic substrate H-D-Phenylalanyl-L-pipecolyl-L-Arg-*p*-nitroanilide (S-2738, Chromogenix, Sweden), final concentration 0.5 mM. The absorbance at 405 nm is measured

continuously in a SpectraMax™ 340 plate reader (Molecular Devices, USA). The absorbance developed during 10 minutes, after subtraction of the absorbance in a blank well containing no FXa, is used to calculate the ratio between the proteolytic activities of variant and wild-type Factor FXa:

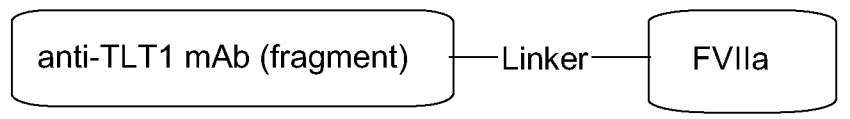
5

$$\text{Ratio} = (A_{405 \text{ nm}} \text{ Factor Xa variant}) / (A_{405 \text{ nm}} \text{ Factor Xa wild-type}).$$

Example 31: Chemical conjugation of an anti-TLT-1 antibody (fragment) with a coagulation factor.

10

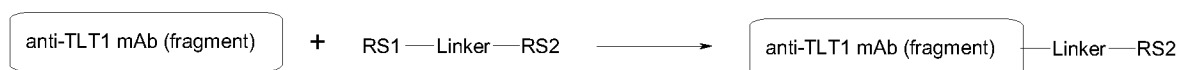
A compound of the general formula



wherein "anti-TLT-1 mAb (fragment)" may be a full size mAb against TLT-1 or a fragment or an analogue intellectually derived thereof such as but not limited to, a FAB-fragment or a sc-FAB with none, one or more point mutations, the linker may be a water soluble polymer such as but not limited to e.g. PEG, polysialic acid, or hydroxyethyl starch, and FVIIa is any molecule with a sequence similarity of >50% to native FVIIa with any retained activity of FVIIa may, for example, be prepared in a two step procedure.

During the first step, a linker, with two different reactive groups RS1 and RS2 may be attached to the anti-TLT-1 mAb (fragment). The reaction may be run with low site selectivity or in a selective way, such that RS1 only reacts at one or few position of the anti TLT-1 mAb (fragment). As a non-exclusive example, RS1 could be an aldehyde and react by reductive amination only with N-termini of the anti TLT-1 mAb (fragment) by reductive amination, known to a person trained in the art. Another non-exclusive example RS1 could be a maleimide group, which may react with a free thiol on the anti-TLT-1 mAb (fragment).

30



During the second step, the reactive group RS2 may be reacted with low site selectivity or site selectivity with a FVIIa molecule. As a non-exclusive example, a site

selective reaction at FVIIa may be obtained when RS2 is a sialic acid derivative, which can react in the presence of a suitable enzyme such as but not limited to ST3Gal-III with N-linked glycans, which do not end exclusively with sialic acids.

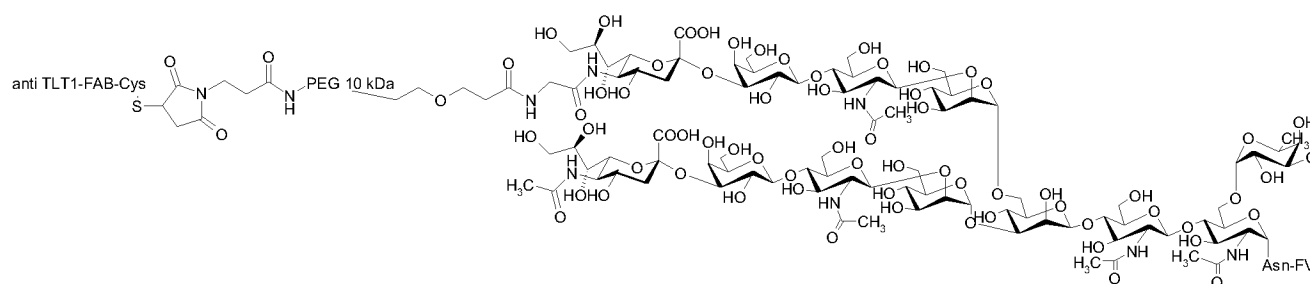
5



The order of attachment of the linker to the two proteins, namely the anti-TLT-1 mAb (fragment) and the FVIIa molecule may be switched, thereby attaching the RS1-Linker-RS2 molecule first to the FVIIa molecule and then to the anti TLT-1 mAb (fragment).

10

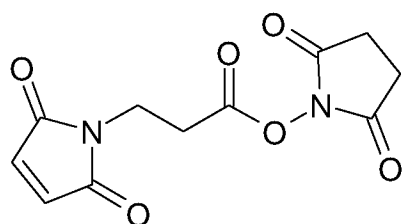
Example 32: Conjugation of anti TLT-1-FAB fragment (Fab0084) to FVIIa for production of FVIIa-Fab1029.



15

Step 1: 3-(2,5-Dioxo-2,5-dihydropyrrol-1-yl)propionic acid 2,5-dioxopyrrolidiny-1-yl ester.

20



3-Maleimidopropionic acid (1.0 g, 5.9 mmol) was dissolved in tetrahydrofuran (20 ml). 2-Succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU, 2.14 g, 7.1 mmol) and ethyldiisopropylamine (1.24 ml, 7.1 mmol) were added subsequently. *N,N*-Dimethylformamide (5 ml) was added. The reaction mixture was stirred at room

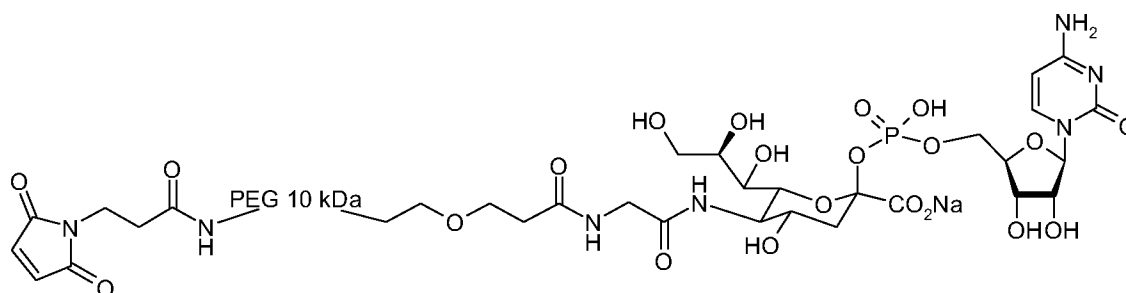
25

temperature, while it was turning sluggish. The mixture was stirred for 2 min. *N,N*-Dimethylformamide (5 ml) was added. The mixture was stirred for 2.5 h at room temperature. It was diluted with dichloromethane (150 ml) and was washed subsequently with a 10% aqueous solution of sodium hydrogensulphate (150 ml), a saturated aqueous solution of sodium hydrogencarbonate (150 ml) and water (150 ml). It was dried over magnesium sulphate. The solvent was removed in vacuo. The crude product was recrystallized from ethyl acetate to give 1.20 g of 3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propionic acid 2,5-dioxopyrrolidiny-1-yl ester.

MS: $m/z = 289$, required for $[M+Na]^+$: 289

$^1\text{H-NMR}$ (CDCl_3) δ 2.82 (m, 4 H); 3.02 (t, 2 H); 3.94 (t, 2 H), 6.73 (s, 2 H).

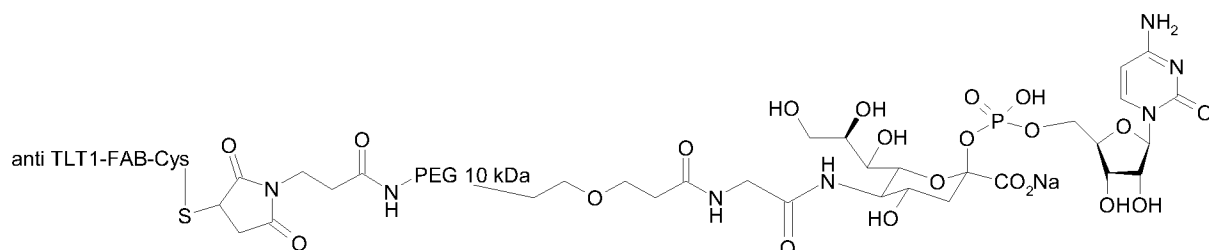
Step 2: *N*-((3-(ω -(3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propionylamino)10kDa PEGyl)propionylamino)acetyl)- O^2 -[5']cytidyl- ξ -neuraminic acid (NNC 0129-0000-3259).



N-((3-(ω -Amino10 kDa PEGyl)propionylamino)acetyl)- O^2 -[5']cytidyl- ξ -neuraminic acid (100 mg, 0.009 mmol) was dissolved in a mixture of tetrahydrofuran (2 ml) and dichloromethane (10 ml). A solution of 3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propionic acid 2,5-dioxopyrrolidiny-1-yl ester (50 mg, 0.18 mmol) in dichloromethane (3 ml) was added. Ethyldiisopropylamine (0.005 ml, 0.028 mmol) was added. The reaction mixture was stirred at room temperature for 16 h. Dichloromethane (2 ml) and ethyldiisopropylamine (0.5 ml) were added. Amionomethylated polystyrene resin (commercially available at e.g. Novabiochem, loading 0.85 mmol/g, 438 mg, 0.372 mmol) was added. The mixture was slowly stirred at room temperature for 1 h. The resin was removed by filtration. The solvent was removed in vacuo with a bath temperature of 25°C. The residue was dissolved in dichloromethane (4 ml). Ether (200 ml) was added. The mixture was left at room temperature for 2 h in order to let the formed precipitation grow old. The precipitation was isolated by filtration and dried in vacuo to give 38 mg of

the title compound. The ^1H -NMR spectrum in DMSO-d_6 showed the presence of a maleimide group.

Step 3: Attachment of N-((3-(ω -(3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propionylamino)10 kDa PEGyl)propionylamino)acetyl)-O²-[5']cytidyl- ξ -neuraminic acid to an anti-TLT-1 FAB.



A LC-MS analysis of an anti-TLT-1 FAB fragment with parts of the hinge region wherein an unpaired Cys was incorporated indicated that the unpaired Cys was capped with a cysteine. Therefore, prior reaction with *N-((3-(ω -(3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propionylamino)10 kDa PEGyl)propionylamino)acetyl)-O²-[5']cytidyl- ξ -neuraminic acid* decapping of the unpaired Cys by reaction with tris(2-carboxyethyl)phosphine hydrochloride had to be performed.

A solution of an anti-TLT-1 FAB fragment with parts of the hinge region wherein an unpaired Cys was incorporated (1 mg) in a buffer of 20 mM HEPES, 5.0 mM EDTA, 100 mM NaCl, which had been adjusted to pH 7.5 was placed in amicon ultracentrifugation device with a cut off of 10 kDa. The buffer was changed to a buffer composed of 20 mM imidazole, 10 mM CaCl_2 , 0.02% Tween 80, 1 M glycerol, which had been adjusted to pH 7.35 by repeated ultracentrifugation at 4000 rpm. After the buffer was changed, a solution of the protein in the buffer composed of 20 mM imidazole, 10 mM CaCl_2 , 0.02% Tween 80, 1 M glycerol, which had been adjusted to pH 7.35 (4 ml) was obtained. A 1 mg/ml solution of tris(2-carboxyethyl)phosphine hydrochloride (0.40 ml) was added. The reaction mixture was shaken at 300 rpm at 20°C for 1 h. The reaction mixture was placed in an ultracentrifugation device with a cut off of 10 kDa and was concentrated by ultracentrifugation at 4000 rpm for 7 min, leaving a solution of 0.700 ml behind. This was applied to a PD-10 column (Amersham Bioscience), which had been equilibrated with a buffer of 25 mM HEPES, which had been adjusted to pH 7.00. The protein was eluted from the column using a buffer of 25 mM HEPES, which had been adjusted to pH 7.00 (3.2 ml). This solution was concentrated by ultracentrifugation at

4000 rpm in an Amicon ultracentrifugation device with a cut off of 10 kDa for 7 min to yield a solution of 0.750 ml. The solution was placed in a vial and buffer of 25 mM HEPES, which had been adjusted to pH 7.00 (0.360 ml) was added. A 1 mg/ml solution of *N*-((3-(ω -(3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propionylamino)10 kDa PEGyl)-propionylamino)acetyl)-*O*²-[5']cytidyl- ξ -neuraminic acid (0.89 ml) was added. The reaction mixture was gently shaken at 300 rpm at 20 °C for 3.5 h. It was placed in an Amicon ultracentrifugation device with a cut off of 10 kDa and concentrated by ultracentrifugation at 4000 rpm for 10 min to a volume of 0.120 ml. The solution was subjected to a size exclusion chromatography on a Superose 75 10/300 GL column (GE Healthcare) at a flow of 0.50 ml/min, utilizing a buffer composed of 25 mM TRIS, 150 mM NaCl, which had been adjusted to pH 8.00 as eluent. Fractions were pooled on the basis of the UV-trace at 280 nm of the chromatogram. The pool (0.148 mg, 1.4 ml), containing the desired compound as judged by SDS-PAGE and which were devoid of free PEG reagent as judged by SDS-PAGE utilizing a PEG-specific staining method (described in Kurfürst, M. M. *Analyt. Biochem.* 1992, 200, 244-248.) was used in the following step.

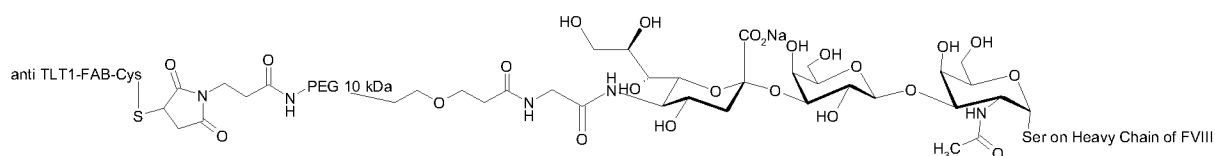
Step 4: Immobilized sialidase. (C. Perfringens type VI-A immobilized on agarose, Sigma: N-5254, 0.6-1.8 U/ml gel, 0.180 ml) was washed with water (2 x 0.50 ml) and subsequently with a buffer of 25 mM MES, 20 mM CaCl₂, 100 mM NaCl, which had been adjusted to pH 6.1 (3 x 0.50 ml). A solution of FVIIa (0.50 mg) in a buffer composed of 25 mM Gly-Gly, 10 mM CaCl₂, which had been adjusted to pH 6.0 was added to the immobilized sialidase. The reaction mixture was left at room temperature, while it was mixed carefully every 20 min. After 3 h, the immobilized resin was removed by filtration through a Pierce spin column by centrifugation at 2000 rpm for 2 min.

The product mixture of the attachment of *N*-((3-(ω -(3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propionylamino)10 kDa PEGyl)propionylamino)acetyl)-*O*²-[5']cytidyl- ξ -neuraminic acid to an anti TLT-1 FAB as described in a preceding step was placed in an Amicon ultracentrifugation device with a cut off of 10 kDa. The buffer was changed by repeated ultracentrifugation to a buffer composed of 25 mM MES, 20 mM CaCl₂, 100 mM NaCl, which had been adjusted to pH 6.1.

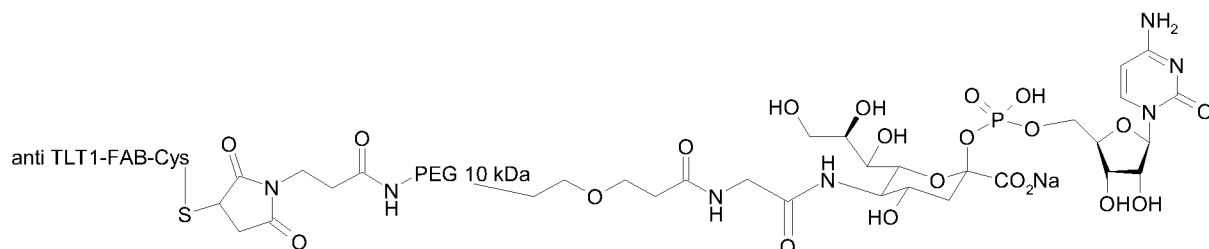
To this solution, a part of the solution of the FVIIa derivative (0.092 ml) was added. The buffer was changed into a buffer composed of 25 mM MES, 20 mM CaCl₂, 100 mM NaCl, which had been adjusted to pH 6.1 and a total volume of (0.20 ml). A solution of ST3-Gal-III (0.015 ml) was added. The reaction mixture is gently shaken at 32 °C for 25 min and thereafter left at 32 °C for 16 h. A 10 mg/ml solution of CMP-*N*-acetylneuraminic acid (CMP NeuNAc, 0.70 mg, 0.070 ml) in a buffer of 25 mM MES, 20 mM CaCl₂, 100 mM NaCl, which had been adjusted to pH 6.1 was added. The reaction

mixture was gently shaken at 32 °C for 15 min and thereafter left at 32 °C for 1 h. The reaction mixture was subjected to a size exclusion chromatography on a Superdex 200 10/300 GL column (GE Healthcare) with a flow of 0.5 ml/min utilizing a buffer of 10 mM Histidine, 20 mM CaCl₂, 150 mM NaCl which had been adjusted to pH 6.1 as eluent. The fractions containing the desired product as judged by SDS-PAGE on a TRIS-Acetate gel were pooled. Using a molar absorption of 11.22 at 280 nm on a Nandrop® apparatus, a yield of 0.022 mg was found. The result of a SDS-PAGE analysis was in accordance with the expectation for the desired product. The product was named FVIIa-Fab1029.

Example 33: Conjugation of an anti-TLT-1-FAB fragment (Fab0084) to FVIII, FVIII-Fab3002.



Step 1: Attachment of *N*-((3-(ω-(3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propionylamino)10 kDa PEGyl)propionylamino)acetyl)-O²-[5']cytidyl-ξ-neuraminic acid to an anti TLT-1 FAB fragment



A LC-MS analysis of an anti-TLT-1 FAB fragment with parts of the hinge region wherein an unpaired Cys was incorporated indicated that the unpaired Cys may be capped with a cysteine. Therefore, prior reaction with *N*-((3-(ω-(3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propionylamino)10 kDa PEGyl)propionylamino)acetyl)-O²-[5']cytidyl-ξ-neuraminic acid decapping of the unpaired Cys by reaction with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) had to be performed.

Said TLT-1-FAB-fragment (1.35 mg, 27.4 nmol) in a 0.27 mg/ml solution in a buffer composed of 20 mM HEPES, 5 mM EDTA, 100 mM NaCl which had been adjusted to pH 7.5 was placed in an Amicon ultracentrifugation device with a cut off of 10 kDa. It

was subjected to ultracentrifugation at 4000 rpm at 20°C for 10 min. Buffer composed of 20 mM imidazole, 10 mM CaCl₂, 0.02% Tween 80, 1 M glycerol which had been adjusted to pH 7.35 (4 ml) was added. The mixture was subjected to ultracentrifugation at 4000 rpm at 20 °C for 8 min. Buffer composed of 20 mM imidazole, 10 mM CaCl₂, 0.02% Tween 80, 1 M glycerol which had been adjusted to pH 7.35 (4 ml) was added. The mixture was subjected to ultracentrifugation at 4000 rpm at 20°C for 8 min. Buffer composed of 20 mM imidazole, 10 mM CaCl₂, 0.02% Tween 80, 1 M glycerol which had been adjusted to pH 7.35 (3 ml) was added. The mixture was subjected to ultracentrifugation at 4000 rpm at 20°C for 10 min. The mixture was placed in a reaction vial. Buffer composed of 20 mM imidazole, 10 mM CaCl₂, 0.02% Tween 80, 1 M glycerol which had been adjusted to pH 7.35 (4 ml) was added, so that the total volume of the reaction mixture was at this time 4.7 ml. A 1 M solution of TCEP (0.54 ml) in a buffer of imidazole, 10 mM CaCl₂, 0.02% Tween 80, 1 M glycerol which had been adjusted to pH 7.35 was added. The reaction mixture was shaken at 300 rpm for 1 h. The mixture was divided into two parts each of which was applied to a PD-10 column (GE Healthtech) which had been equilibrated with a buffer of 25 mM HEPES with a pH 7.0. The eluates were combined (7 ml in total) and were concentrated to 1 ml by ultracentrifugation at 4000 rpm for at 20°C for 6-8 min in an Amicon ultracentrifugation device with a cut off of 10 kDa.

The solution was placed in a reaction vial. A buffer of 25 mM HEPES which had been adjusted to pH 7.0 (0.50 ml) was added to obtain a total volume of 1.5 ml. A freshly prepared 1 mg/ml solution of *N*-((3-(ω-(3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propionylamino)10 kDa PEGyl)propionylamino)acetyl)-O²-[5']cytidyl-ξ-neuraminic acid (1.2 ml, 1.2 mg, 109 nmol) in a buffer of 25 mM HEPES which had been adjusted to pH 7.0 was added. The reaction mixture was gently shaken at 300 rpm at 20°C for 3.2 h. It was concentrated to a volume of 0.30 ml by ultracentrifugation at 4000 rpm at 19 °C for 11 min in an Amicon ultracentrifugation device with a cut off of 10 kDa. It was applied to a size exclusion chromatography on a Superdex 75 10/300 GL column (GE Healthtech) at a flow of 0.50 ml/min utilizing a buffer of 25 mM TRIS, 150 mM NaCl which had been adjusted to pH 8.0 as eluent. The fraction containing the desired product in an acceptable purity as judged by SDS-PAGE analysis in the presence of N-methylmaleimide (NEM) and which were devoid of unreacted *N*-((3-(ω-(3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propionylamino)10 kDa PEGyl)propionylamino)acetyl)-O²-[5']cytidyl-ξ-neuraminic acid as judged by SDS-PAGE in combination with a PEG-sensitive staining ((described in Kurfürst, M. M. *Analyt. Biochem.* 1992, 200, 244-248.)) was used in the following step. The SDS-PAGE analysis under reducing conditions was in compliance with the expected

result for the desired product. The SDS-PAGE analysis under non-reducing conditions showed some material, in which one chain of the anti-TLT-1-FAB fragment had been lost. However, it remained unsolved, whether this finding was due to problems in the analysis or whether a chain had really been lost either during the described reaction or even earlier. Using a molar absorbance of 10.44 at 280 nm on a Nanodrop® apparatus, a concentration of 0.1 mg/ml was found giving a yield of 0.287 mg.

Step 2: The solution of the product of the attachment of *N*-(((3-(ω-(3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propionylamino)10 kDa PEGyl)propionylamino)acetyl)-O²-[5']cytidyl-ξ-neuraminic acid to an anti TLT-1 FAB fragment as described in the

preceding example and a solution of B-domain deleted FVIII which had a residual B-domain sequence of SFSQNSRHPSQNPPVLKRHRQR at the C-terminus of the heavy chain (0.780 mg, 5.64 μmol) in a buffer composed of 20 mM imidazole, 10 mM CaCl₂, 150 mM NaCl, 0.02% Tween80 and 1 M glycerol which had been adjusted to pH 7.35 (0.018 ml) were mixed and placed in an Amicon ultracentrifugation device with a cut off of 10 kDa. The solution was subjected to a buffer change to 20 mM histidine, 10 mM CaCl₂, 20% glycerol, 0.02% Tween 80, 500 mM NaCl which had been adjusted to pH 6.05 by repeating ultracentrifugation and addition of the buffer. A total volume of 0.40 ml was obtained. A 0.4 mg/ml (242 U/mg, 98 U/ml, 0.0055 ml) solution of sialidase from *A. Urifaciens* and a 2.5 mg/ml solution of ST3Gal-III (0.033 ml) were added subsequently. The reaction mixture was gently shaken for 15 min at 300 rpm at 32 °C, left for 2 h at 32 °C during which it was occasionally shaken carefully, and finally left at 32 °C for 18 h. The reaction mixture was diluted with water (0.030 ml). It was subjected to a size exclusion chromatography using a Superose 6 10/300 GL column (GE Healthcare) and utilizing a buffer of 10 mM Histidine, 1.7 mM CaCl₂, 0.01% Tween80, 0.3 M NaCl, 8.8 mM sucrose which had been adjusted to pH 7 at a flow of 0.50 ml/min. The fractions containing the desired product as judged by SDS-PAGE analysis were pooled. The pool was subjected to a buffer change using an Amicon ultracentrifugation device with a cut off of 10 kDa to a buffer composed of 20 mM histidine, 10 mM CaCl₂, 1 M glycerol, 0.02% Tween 80, 500 mM NaCl which had been adjusted to pH 6.07. A total volume of 0.250 ml was obtained. Using a molar absorption of 14.6 at 280 nm on a Nanodrop apparatus, a concentration of 0.59 mg/ml was found, corresponding to a yield of 0.148 mg. A 10 mg/ml solution of CMP-*N*-acetylneuraminic acid (CMP NeuNAc, 0.26 mg, 0.026 ml) in a buffer of 20 mM histidine, 10 mM CaCl₂, 20% glycerol, 0.02% Tween 80, 500 mM NaCl which had been adjusted to pH 6.05 and a solution of ST3Gal-III (0.015 ml) was added. The reaction mixture was shaken gently at 300 rpm at 32 °C for 15 min and then left at 32 °C for another 45 min. The reaction mixture was diluted with water (0.10 ml). It was

subjected to a size exclusion chromatography using Superose 6 10/300 GL column (GE Healthcare) and utilizing a buffer of 10 mM Histidine, 1.7 mM CaCl_2 , 0.01% Tween80, 0.3 M NaCl, 8.8 mM sucrose which had been adjusted to pH 7 at a flow of 0.50 ml/min. The fractions containing the desired product as judged by SDS-PAGE analysis were pooled giving and concentrated by ultracentrifugation in an Amicon ultracentrifugation device to a total volume of approximately 0.275 ml. Using a molar absorption at 280 nm of 14.6 on a Nanodrop® apparatus, a concentration of 0.180 ml/ml was found corresponding to a yield of 0.0495 mg. The SDS-PAGE analysis of the product under reduced conditions is in accordance with the expectation for the desired product. The SDS-PAGE analysis under non-reduced conditions shows changing amounts of a band which corresponds to a product where the FAB-fragment has lost one chain. The appearance of a band corresponding to a may be due to the presence of such compound in the product or may be due to decomposition during denaturizing prior SDS-PAGE analysis.

Example 34: Binding to TLT-1.

Table 16: Reagents

Reagent	Source
TLT-1	Example 2
FVIIa-Fab1029	Example 32
FIX-Fab0135	Example 16
All other reagents	Biacore

Method:

TLT-1 was immobilized directly to a CM5 chip to a level of approx 2000 RU (50 ug/ml diluted in Na-acetate, pH 4.0) using the standard procedure recommended by the supplier and reagents provided in Table 16. Two-fold dilutions of FVIIa-Fab1029 and FIX-Fab0135 from 20 nM to 0.3 nM were tested for binding to TLT-1. Running and dilution buffer: 10 mM HEPES, 150 mM, 0.005% p20, pH 7.4. Regeneration was obtained by 10 mM Glycine, pH 1.7. Determination of kinetic and binding constants (k_{on} , k_{off} , K_D) was obtained assuming a 1:1 interaction of TLT-1 and FVIIa-Fab1029 or FIX-Fab0135 using the Biacore T100 evaluation software.

Result:

Table 17

TLT-1 binding	k_a (1/M)	k_d (1/s)	K_D (M) TLT-1
---------------	-------------	-------------	-----------------

FVIIa-Fab1029	3.97E+05	0.007259	1.83E-08
FIX-Fab0135	4.28E+05	8.71E-04	2.04E-09

Conclusion:

Binding constants for FVIIa-Fab1029 and FIX-Fab0135 binding to TLT-1 was estimated by biacore analysis and binding to TLT-1 was confirmed (Table 17).

5

Example 35: FVIIa-Fab1029 promotes fibrin clot formation in hemophilia-like whole blood.

The TEG traces obtained with normal HWB (NWB), "hemophilia" blood, and "hemophilia" blood supplemented with (0; 0.25; 0.5; 1.0; nM) of FVIIa-Fab1029 or rFVIIa are shown in Figure 11A. Shown in Figure 11B are the R-time values obtained for the depicted TEG traces. Figure 11B, in addition to R-time values for the FVIIa-Fab1029 or rFVIIa protein, also includes R-time values for equivalent concentrations of rFVIIa. All data are obtained from one representative donor. The FVIIa-Fab1029 is observed to efficiently normalize clotting of hemophilia-like HWB. In addition the results show that the known pro-coagulant effect of rFVIIa is further potentiated by conjugation of rFVIIa to a Fab fragment of an antibody against TLT-1. Thus, the example with the FVIIa-Fab1029 protein demonstrates that targeting of rFVIIa to TLT-1 on platelets further potentiates the FVIII bypassing activity of rFVIIa.

Example 36: FIX-Fab0135 has FVIII-bypassing activity and promotes fibrin clot formation in hemophilia A-like whole blood.

Citrated-stabilized human whole blood (HWB) is drawn from normal donors. Clot formation is measured by thrombelastography (5000 series TEG analyzer, Haemoscope Corporation, Niles, IL, USA). Hemophilia A-like conditions are obtained by incubation of normal citrate-stabilized human whole blood (HWB) with 10 µg/ml anti-FVIII antibody (Sheep anti-Human Factor VIII; Hematologic Technologies Inc) for 30 min at room temp. Various concentrations (0.1; 0.2; 1.0; 5.0; 10 nM) of the FIX-Fab0135 are added to hemophilia A-like citrated HWB. Clotting is initiated when 340 µl of normal or premixed HWB is transferred to a thrombelastograph cup containing 20 µl 0.2 M CaCl₂ with 0.03 pM lipidated TF (Innovin®, Dade Behring GmbH (Marburg, Germany)). The TEG trace is followed continuously for up to 120 min. TEG traces obtained with normal HWB (HWB), "hemophilia" blood, and "hemophilia" blood supplemented with (0.1; 0.2; 1.0; 5.0; 10 nM) of FIX-Fab0135 are shown in Figure 12. Also shown for comparison are the TEG

traces obtained when FIX-Fab0135 is replaced by 1 nM rFVIIa or 10 nM rFIX. All data are obtained from one representative donor.

Surprisingly the results show that fusion of FIX to a Fab fragment of an antibody against TLT-1 produces a protein with FVIII bypassing activity. The FIX-Fab0135 is observed to efficiently normalize clotting of hemophilia A-like HWB. FIX dependent propagation of the coagulation requires assembly of a FIXa/FVIIIa complex on the surface of activated platelets, and the resulting FX activation (tenase) activity is prevented by inhibitory FVIII antibodies. The present example with FIX-Fab0135 demonstrates that targeting of FIX to TLT-1 on the surface of platelets generates a FIX containing complex with pro-coagulant activity even when FVIII is blocked by an inhibitory antibody.

Example 37: FIX-Fab0135 fusion protein targeted to TLT-1 enriched phospholipid vesicles markedly promotes FX activation induced by FVIIa in absence of FVIII.

Methods: TLT-1 enriched phospholipid vesicles, prepared as described in Example 20 are applied in the experiment shown in Figure 13 to mimic TLT-1 on the surface of activated platelets. Various concentrations (0.05 – 100 nM) rFVIIa is incubated at room temperature for 15 min i) in Hepes buffer (50 mM Hepes, 0.1 M NaCl, 5 mM CaCl₂, 1 mg/ml BSA pH 7.4) or in Hepes buffer with ii) 10 nM FIX, iii) 10 nM FIXa or iv) 10 nM FIX-Fab0135. This is then mixed with 100 nM FX (Enzyme Research Laboratories, UK) and TLT-1 enriched phospholipid vesicles at 1:4,000 dilution in absence or presence of 5 nM FVIII and incubated for 3 min. The reaction is stopped by addition of an equal volume stop buffer (50 mM Hepes, 0.1 M NaCl, 20 mM EDTA, 1 mg/ml BSA pH 7.5). The amount of FXa generated in the samples is then determined in a chromogenic assay by transferring 50 µl of the mixture to a microtiter plate well and adding 25 µl Chromozyme X (final 0.42 mg/ml) to the well. The absorbance at 405 nm is measured continuously in a microplate Spectramax photometer (Molecular Devices, Sunnyvale CA, USA).

FVIIa is capable of activating FIX (Østerud and Rappaport, 1977) and the resulting FIXa is then designated to combine with FVIIIa and form the proteolytic component of the so-called tenase complex. Formation of this complex takes place on the surface of activated platelets. The tenase complex is responsible for a massive FX activation that plays a key role in the propagation phase of the coagulation process. Inability to form an active tenase complex is the central diathesis in both hemophilia A and B patients.

In the present example, TLT-1 enriched phospholipid vesicles are applied to mimic the surface of activated platelets. This system is used to test whether targeting of FIX to TLT-1 on such vesicles by means of the FIX-Fab0135 can promote FX activation in analogy with the tenase complex on activated platelets. Furthermore it is of interest to examine whether the presence of FVIII is obligatory or not for this activity.

Figure 13 shows: 1) that very little FXa is generated at all FVIIa concentrations tested in absence and presence of 5 nM FVIII under the conditions of the experiment without FIX derivatives added (○); 2) that this is the case also when 10 nM FIX is present in the reaction mixture (□); 3) that a marked FX activation is observed when 10 nM FIX is replaced by 10 nM FIXa, however, only in the presence of FVIII (■); 4) that targeting of FIX to TLT-1 enriched phospholipid vesicles by means of the FIX-Fab0135 provides a complex which is activated by FVIIa in a concentration dependent manner; 5) that the resulting activated tenase-like complex markedly promotes FX activation both in presence and in absence of FVIII.

These results, as well as those of example 36, suggest that the FIX-Fab0135 FIX fusion protein provides a FVIII bypassing agent which markedly promotes FVIIa-mediated FXa activation by a mechanism which involves FVIIa activation of FIX targeted to TLT-1 and formation of a complex with a considerable FVIII-independent tenase-like activity.

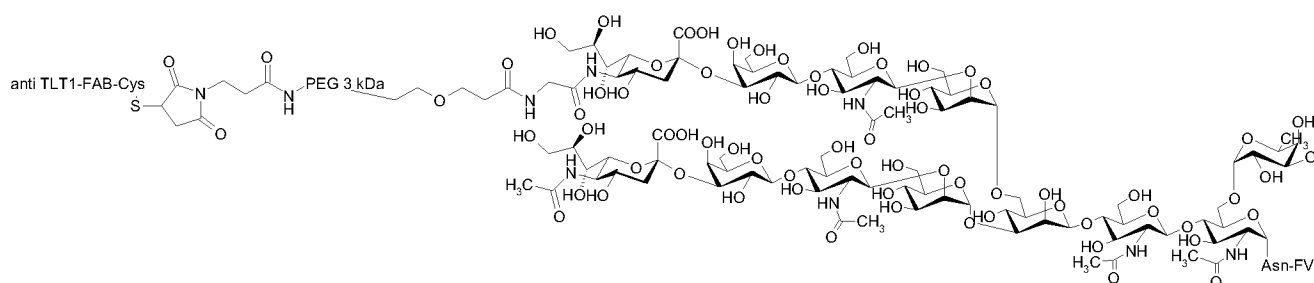
Example 38: TLT-1 expression on human platelets.

TLT-1 has been reported to only be expressed on activated platelets (Washington et al. (2004) Blood. 2004 Aug 15;104(4):1042-7). To verify this and estimate the specific copy number of TLT-1 on the platelet surface we used the "Platelet calibrator kit" from Biocytex (Marseille, France) in which platelets are stained by no wash indirect immunofluorescence with specific monoclonal antibodies and analyzed by quantitative flow cytometry. The expression level of the tested antigen is determined using calibration beads with a definite number of binding sites for the detecting antibody. According to manufacturers instruction platelets in citrated human whole blood were activated by different concentrations (0.3-30 μ M) of protease activated receptor (PAR) -1 activating peptide with amino acid sequence SFLLRN. The samples were diluted 1:4 in a saline buffer provided in the kit before labeled with TLT-1 binding antibodies. The TLT-1 binding antibodies used were either mAb0123 (IgG1 subtype of the mAb0023 antibody) or mAb0136 (IgG1 subtype of the mAb0012 antibody). After 15 min incubation with either of the TLT-1 antibodies (10 μ g/ml) followed by 15 min incubation with FITC labeled detecting antibody (provided by the manufacturer) the samples were diluted 1:50 before

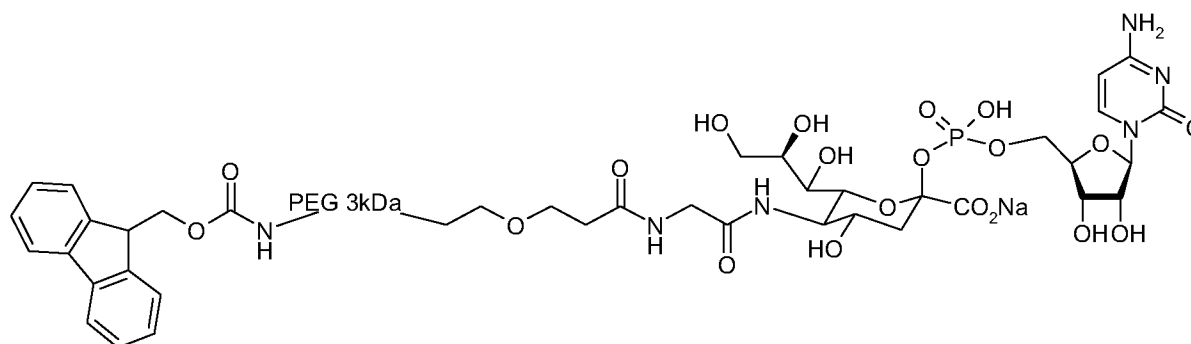
immediately flow cytometry analysis. The absolute number of TLT-1 on the platelet surface was obtained by using the bead derived standard curve.

Unactivated platelets show no expression of TLT-1 with none of the two antibodies used. However, when the platelets were activated with SFLLRN an increased TLT-1 expression was observed with a maximal expression of 9685 ± 1696 and 12981 ± 2083 surface molecules detected by mAb0123 and mAb0136 respectively (Figure 14). Maximal platelet TLT-1 expression was achieved by double stimulation by SFLLRN (30 μ M) and Convulxin (100 ng/ml).

Example 39: Conjugation of anti TLT-1-FAB fragment (Fab0084) to FVIIa via a 3 kDa PEG linker, FVIIa-Fab1001.



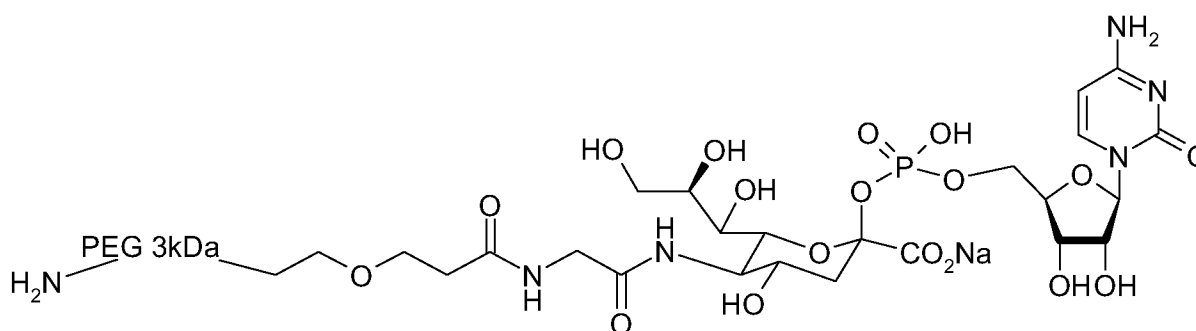
Step 1: *N*-((3-(ω -(Fluorenylmethoxycarbonylamino)3 kDa PEGyl)propionylamino)acetyl)- O^2 -[5']cytidyl- ξ -neuraminic acid



3-(ω -(Fluorenylmethoxycarbonylamino)3 kDa PEGyl)propionic acid pyrrolidin-2,5-dion-1-ylester (purchased at Rapp Polymere GmbH, 1 g, 0.292 mmol) was dissolved in tetrahydrofuran (80 ml). A solution of cytidine-5'-monophospho-N-glycylneuraminic acid disodium salt (0.276 g, 0.438 mmol) in a buffer (20 ml) composed of 50 mM TRIS, which had been adjusted to pH 8.9 was added. The reaction mixture was stirred at room temperature for 16 h. The THF was removed in vacuo, having a bath temperature of

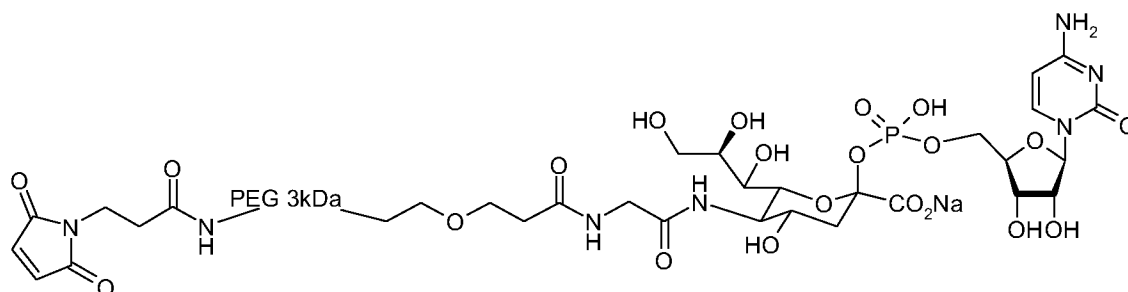
25°C. The remaining mixture was diluted with water up to 60 ml and was filtered through a 0.45 μ m filter. The solution was divided into three parts each of which was subjected to an HPLC chromatography on a C4 column, using a flow of 20 ml/min and a gradient of 0-60% acetonitrile in an aqueous buffer of 50 mM ammonium hydrogencarbonate over 50 min after it had washed with an aqueous buffer of 50 mM ammonium hydrogencarbonate for 10 min. Fractions were combined, having no cytidine-5'-monophospho-N-glycylneuraminic acid and having an absorption at 214 nm greater than 15-20% of the maximum absorption. The combined fractions were freeze dried to give 532 mg of *N*-((3-(ω -(fluorenylmethoxycarbonylamino)3 kDa PEGyl)propionylamino)acetyl)-*O*²-[5']cytidylyl- ξ -neuraminic acid. The ¹H-NMR spectrum was in accordance with the expectation.

Step 2: *N*-((3-(ω -Amino3 kDa PEGyl)propionylamino)acetyl)-*O*²-[5']cytidylyl- ξ -neuraminic acid



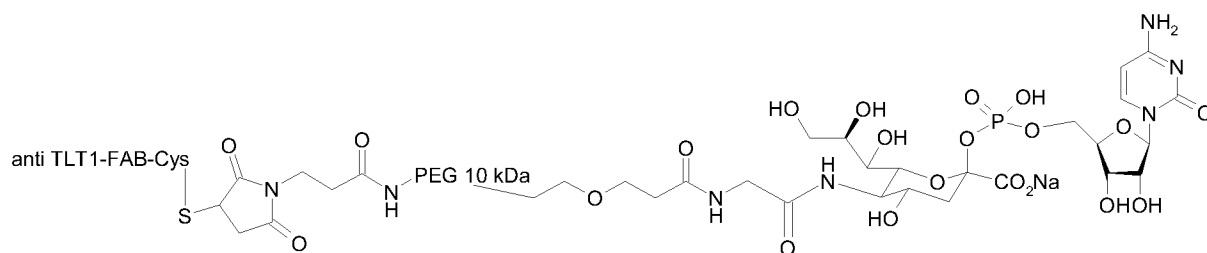
N-((3-(ω -(Fluorenylmethoxycarbonylamino)3 kDa PEGyl)propionylamino)acetyl)-*O*²-[5']cytidylyl- ξ -neuraminic acid (532 mg, 0.135 mmol) was dissolved in *N,N*-dimethylformamide (9 ml). Piperidine (2.25 ml) was added. The reaction mixture was stirred for 20 min at room temperature. Ether (150 ml) was added. The mixture was left at room temperature for 1 h. The formed precipitate was isolated by decantation and centrifugation. It was dissolved in dichloromethane (10 ml). Ethyldiisopropylamine (2.4 ml) was added. The mixture was stirred for 2 min. Ether (150 ml) was added. The mixture was left for 1.5 h in order to let the precipitate grow old. The precipitate was isolated by decantation and filtration. It was dried in vacuo with a bath temperature of 25°C to give 343 mg of *N*-((3-(ω -amino3 kDa PEGyl)propionylamino)acetyl)-*O*²-[5']cytidylyl- ξ -neuraminic acid. The ¹H-NMR was in accordance with the expectation.

Step 3: *N*-((3-(ω -(3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propionylamino)3 kDa PEGyl)propionylamino)acetyl)-*O*²-[5']cytidylyl- ξ -neuraminic acid (NNC 0129-0000-3259)



N-((3-(ω-Amino3 kDa PEGyl)propionylamino)acetyl)-*O*²-[5']cytidylyl-ξ-neuraminic acid (343 mg, 0.009 mmol) was dissolved in a mixture of dichloromethane (15 ml). Ethyldiisopropylamine (0.05 ml, 0.028 mmol) was added. 3-(2,5-Dioxo-2,5-dihydropyrrol-1-yl)propionic acid 2,5-dioxopyrrolidiny-1-yl ester (492 mg, 1.85 mmol) was added as a solid. The reaction mixture was stirred at room temperature for 16 h. Dichloromethane (140 ml) was added. Amionomethylated polystyrene resin (commercially available at e.g. Novabiochem, loading 0.85 mmol/g, 4.3 g, 3.69 mmol) was added. The mixture was slowly stirred at room temperature for 1 h. The resin was removed by filtration. The solvent was removed in vacuo with a bath temperature of 25°C. Amberlyst 15 resin (2 g) was added. The reaction mixture was slowly stirred for 20 min. The resin was removed by filtration. The solvent was removed in vacuo with a bath temperature of 25°C. The residue was dissolved in dichloromethane (10 ml). Ether (200 ml) was added. The mixture was left at room temperature for 6 h in order to let the formed precipitate grow old. The precipitate was isolated by decantation and centrifugation. It was dried in vacuo to give 180 mg of the title compound. The ¹H-NMR spectrum in DMSO-*d*₆ showed the presence of a maleimide group.

Step 4: Attachment of *N*-((3-(ω-(3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propionylamino)3 kDa PEGyl)propionylamino)acetyl)-*O*²-[5']cytidylyl-ξ-neuraminic acid to an anti TLT-1 FAB



A solution of an anti-TLT-1 FAB fragment with parts of the hinge region wherein an unpaired Cys was incorporated (10 mg) in a phosphate buffer was placed in amicon ultracentrifugation device with a cut off of 10 kDa. The buffer was changed to a buffer

composed of 100 mM HEPES which had been adjusted to pH 7.3, by repeated ultracentrifugation at 4000 rpm. After the buffer was changed, a solution of the protein in the buffer composed of 100 mM HEPES which had been adjusted to pH 7.3, (36 ml) was obtained. A 1 mg/ml solution of tris(2-carboxyethyl)phosphine hydrochloride (4 ml) was added. The reaction mixture was shaken at 300 rpm at 20°C for 15 min and left at 20°C for 45 min. The reaction mixture was placed in an ultracentrifugation device with a cut off of 10 kDa. The buffer was changed to a buffer composed of 25 mM HEPES which had been adjusted to pH 7.0 by repeated ultracentrifugation at 4000 rpm to give a solution of 13.2 ml. A 1 mg/ml solution of *N*-((3-(ω -(3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propionylamino)3 kDa PEGyl)propionylamino)acetyl)-*O*²-[5']cytidyl- ξ -neuraminic acid (6.4 ml) was added. The reaction mixture was gently shaken at 300 rpm at 20 °C for 15 min and left at 20°C for 16 h. It was placed in an Amicon ultracentrifugation device with a cut off of 10 kDa and concentrated by ultracentrifugation at 4000 rpm for 10 min to a volume < 5 ml. The solution was subjected to a size exclusion chromatography on a Superose 75 16/60 GL column (GE Healthcare) at a flow of 1 ml/min, utilizing a buffer composed of 25 mM TRIS, 150 mM NaCl, which had been adjusted to pH 8.00 as eluent. Fractions were pooled on the basis of the UV-trace at 280 nm of the chromatogram. The pool (9.9 mg, 13.7 ml), containing the desired compound as judged by SDS-PAGE and which were devoid of free PEG reagent as judged by SDS-PAGE utilizing a PEG-specific staining method (described in Kurfürst, M. M. *Analyt. Biochem.* 1992, 200, 244-248.) was used in the following step.

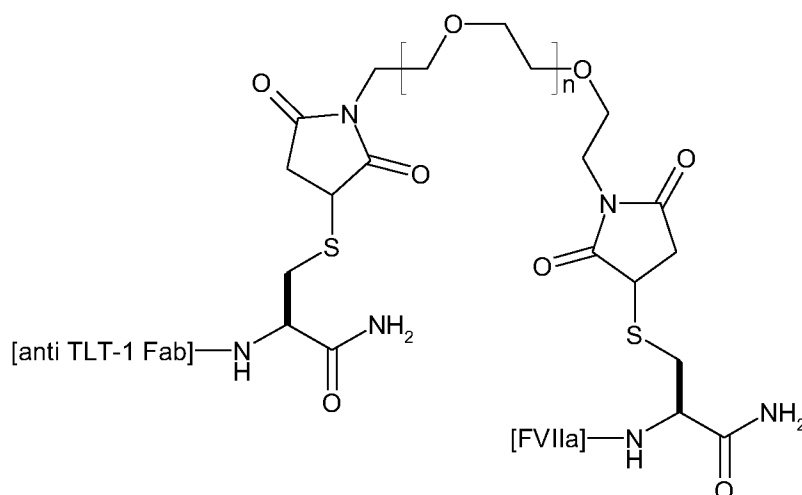
Step 5: Immobilized sialidase. (*C. Perfringens* type VI-A immobilized on agarose, Sigma: N-5254, 0.6-1.8 U/ml gel, 1.52 ml) was washed with water (3 x 9 ml) and subsequently with a buffer of 20 mM HEPES, 10 mM CaCl₂, 0.005% Tween80, 100 mM NaCl, which had been adjusted to pH 7.5 (3 x 9 ml). A solution of FVIIa (8.9 mg) in a buffer (6.59 ml) composed of 25 mM Gly-Gly, 10 mM CaCl₂ which had been adjusted to pH 6.0 was placed in an Amicon ultracentrifugation device with a cut off of 10 kDa. The buffer was changed to a buffer of 20 mM HEPES, 10 mM CaCl₂, 0.005% Tween80, 100 mM NaCl, which had been adjusted to pH 7.5 by repeated centrifugation at 4000 rpm to give a final volume of 6.5 ml. This solution was added to the immobilized sialidase. The reaction mixture was rolled at room temperature for 3.5 h.

The product of the attachment of *N*-((3-(ω -(3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propionylamino)3 kDa PEGyl)propionylamino)acetyl)-*O*²-[5']cytidyl- ξ -neuraminic acid to an anti TLT-1 FAB (10 mg) obtained as described in a preceding step in a buffer (13.5 ml) consisting of 25 mM TRIS, 150 mM NaCl, which had been adjusted to pH 8.00 was placed in an Amicon ultracentrifugation device with a cut off of 10 kDa. Buffer composed

of 20 mM histidine, 10 mM CaCl₂, 20% glycerol, 0.02% Tween 80, 500 mM NaCl which had been adjusted to pH 6.0 was added. An ultracentrifugation at 4000 rpm for 2 min was applied. Another portion of buffer composed of 20 mM histidine, 10 mM CaCl₂, 20% glycerol, 0.02% Tween 80, 500 mM NaCl which had been adjusted to pH 6.0 was added.

5 An ultracentrifugation at 4000 rpm for 10 min was applied. The reaction product of the reaction with the immobilized sialidase was added, by filtration to remove the immobilized sialidase. Another portion of buffer composed of 20 mM histidine, 10 mM CaCl₂, 20% glycerol, 0.02% Tween 80, 500 mM NaCl which had been adjusted to pH 6.0 was added. An ultracentrifugation at 4000 rpm for 10 min was applied to obtain a total
10 volume of 9 ml. A solution of ST3Gal-III (500 μ l) was added. The reaction mixture is gently shaken at 32°C for 15 min and thereafter left at 32°C for 16 h. A 10 mg/ml solution of CMP-*N*-acetylneuraminic acid (CMP NeuNAc, 0.70 mg, 0.89 ml) in a buffer of 20 mM histidine, 10 mM CaCl₂, 20% glycerol, 0.02% Tween 80, 500 mM NaCl which had been adjusted to pH 6.0 was added was added. The reaction mixture was gently shaken
15 at 32°C for 15 min and thereafter left at 32°C for 1 h. The reaction mixture was subjected to a size exclusion chromatography on a Superdex 200 26/60 GL column (GE Healthcare) with a flow of 2 ml/min utilizing a buffer of 10 mM Histidine, 10 mM CaCl₂, 0.01% Tween 80, 200 mM NaCl which had been adjusted to pH 6 as eluent. The fractions containing the desired product as judged by SDS-PAGE on a TRIS-Acetate gel were pooled. Using a
20 molar absorption of 12.86 at 280 nm on a Nandrop® apparatus, a yield of 3.2 mg was found. The result of a SDS-PAGE analysis was in accordance with the expectation for the desired product.

Example 40: Conjugation of anti TLT-1-FAB fragment (Fab0084) to FVIIa 407C via 3 kDa PEG linker, FVIIa-Fab9015.



FVIIa 407C (5 mg, 0.55 mg/ml, 9 ml) in 20mM HEPES, 100mM NaCl, 10mM CaCl₂, pH 7.0 was mixed with solutions of glutathione (reduced, 40 mM, 125 microliter, in HEPES buffer), glutathione (oxidised, 1.6 mM, 125 microliter, in HEPES buffer), *para*-aminobenzamidine (0.5 M, 500 microliter, in HEPES buffer), and glutaredoxin (Grx2, EC 1.20.4.1, 96 micromolar, 200 microliter). The volume was adjusted to 10.0 ml, pH was 7.0.

The resulting mixture was incubated at 32 degrees Celsius for 5 h.

EDTA in water (800 microliter, 0.25 M, pH 7.0) was added. The solution was diluted with desalted water until the conductivity was reduced to 8.3 mS/cm (20 ml).

The solution was injected on a pre-conditioned HiTrap Q FF column (pre-conditioned in Buffer A, 5 ml column volume).

Buffer A: 50 mM HEPES, 100 mM NaCl, 1 mM EDTA, 0.01% Tween-80, pH 7.0

Buffer B: 50 mM HEPES, 100 mM NaCl, 10 mM CaCl₂, 0.01% Tween-80, pH 7.0

The immobilised protein was washed with Buffer A (5 CV) using a Äkta purifier 100 chromatography station. The protein was eluted with buffer B (10 CV) at 2 ml/min. Elution of the protein of interest was done by monitoring the absorbance at 280 nm.

Seven fractions were pooled. Protein concentration in the combined pool was estimated to be 0.49 mg/ml (abs. 280 nm), volume 7.5 ml, 3.8 mg FVIIa (77.6 nmol).

A solution of bis-maleimide polyethylene glycol linker (3 kDa, Rapp Polymere GmbH, Tübingen, Germany, prod. no. 11300-45, lot no. 1210.764, 70 mg, 23 micromol) in 20mM HEPES, 100mM NaCl, 10mM CaCl₂, pH 7.0 (3.5 ml) was added. The resulting mixture was incubated at room temperature for 1 h.

A solution of EDTA in water (250 mM, 900 microliter) was added to the mixture. pH was adjusted to 7.0. The resulting mixture was diluted with water until the conductivity was 8.3 mS/cm reaching a volume of 17 ml.

The solution was injected on a pre-conditioned HiTrap Q FF column (pre-conditioned in Buffer A, 5 ml column volume).

Buffer A: 50 mM HEPES, 100 mM NaCl, 1 mM EDTA, 0.01% Tween-80, pH 7.0

Buffer B: 50 mM HEPES, 100 mM NaCl, 10 mM CaCl₂, 0.01% Tween-80, pH 7.0

The immobilised protein was washed with Buffer A (5 CV) using a Äkta purifier 100 chromatography station. The protein was eluted with buffer B (10 CV) at 2 ml/min. Elution of the protein of interest was done by monitoring the absorbance at 280 nm.

The fractions of interest were pooled resulting in a total volume of 12 ml. The protein concentration was measured (Abs. at 280 nm) to 0.30 mg/ml, 3.6 mg of protein in total.

A solution of antibody fragment, Fab protein ID 0084 (6.7 mg, 3.21 mg/ml) in HEPES buffer (20 mM HEPES, 1.0 mM CaCl₂, 100 mM NaCl, 0.005% (v/v) Tween-80, pH 7.5) was mixed with a solution of *tris*(3-sulfonatophenyl)phosphine hydrate sodium salt (techn. grade 85% pure, 10 mg/ml, 5 ml, same buffer). The resulting mixture was
5 incubated for 2 h at room temperature. The mixture was placed in an Amicon Ultracentrifugal filter device (Millipore corp., MWCO 10 kDa) and the buffer was exchanged by repetitive additions of buffer (20 mM HEPES, 1.0 mM CaCl₂, 100 mM NaCl, 0.005% (v/v) Tween-80, pH 7.5) followed by centrifugation.

The buffer exchanged sample of antibody fragment was mixed with the linker
10 conjugated FVIIa sample and the resulting solution was bufferexchanged into a buffer (50 mM HEPES, 100 mM NaCl, 35 mM CaCl₂, 50 mM benzamidine, 0.01% Tween-80, pH 7.5) and subsequently concentrated to 7 ml. The mixture was incubated over night at room temperature. The resulting mixture was analysed using SDS-PAGE gel electrophoresis.

15 Water (8.5 ml), EDTA solution (5.5 ml, 0.25 M), and sodium hydroxide (1 M) was added to the mixture until pH was 7.2 and the conductivity measured to 11.0 mS/cm, total volume: 21ml

The solution was injected on a pre-conditioned HiTrap Q FF column (pre-conditioned in Buffer A, 5 ml column volume).

20 Buffer A: 50 mM HEPES, 100 mM NaCl, 1 mM EDTA, 0.01% Tween-80, pH 7.0

Buffer B: 50 mM HEPES, 100 mM NaCl, 10 mM CaCl₂, 0.01% Tween-80, pH 7.0

The immobilised protein was washed with Buffer A (5 CV) using a Äkta purifier 100 chromatography station. The protein was eluted with buffer B (10 CV) at 2 ml/min. The selected fractions were concentrated in an Amicon Ultracentrifugal filter device
25 (Millipore corp., MWCO 10 kDa) and the buffer was exchanged by repetitive additions of buffer (20 mM HEPES, 1.0 mM CaCl₂, 100 mM NaCl, 0.005% (v/v) Tween-80, pH 7.5) followed by centrifugation. The concentrated sample (5 ml) was injected on a pre-conditioned Superdex Hiloal 16/60 column (GE Healthcare, pre-conditioned in the applied buffer).

30 Buffer: 10 mM L-Histidine, 10 mM CaCl₂, 100mM NaCl, 0.01% Tween80, pH 6.0

The protein was purified by elution at a flow of 0.8 ml/min over 2 CV.

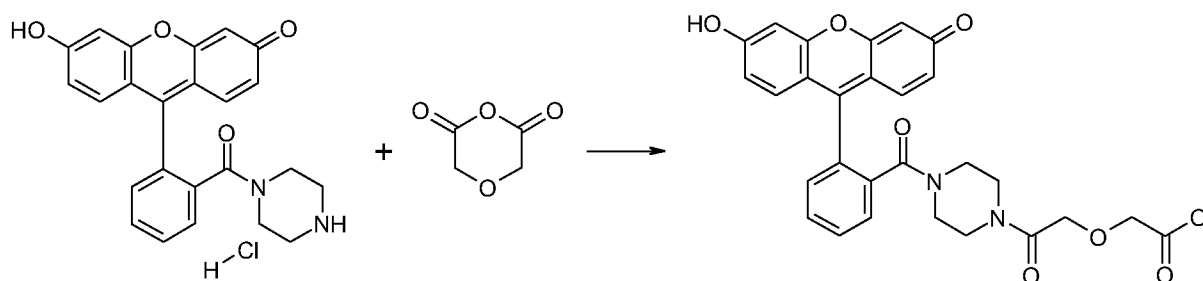
Fractions were selected based on analysis by SDS PAGE gel electrophoresis (4-12% Bis-Tris acetate, MES running buffer).

The pool of selected fractions was concentrated using an Amicon Ultracentrifugal
35 filter device (Millipore corp., MWCO 10 kDa) to a total volume: 2.25ml. The amount of protein was measured (abs. 280 nm) to be 1.2 mg (protein conjugate).

Analysis by SDS PAGE gel electrophoresis (4-12% Bis-Tris acetate) and (HPC4) Western Blotting against

1. Primary - ProteinC-tag Antibody (HPC4) pAb, Rabbit, cot no: A00637 (40 ug). was dissolved in 80 ul milliQ water to concentration of 0.5mg/ml. Diluted 1:1000 during the blot.
2. Secondary - Goat anti-Rabbit IgG antibody (H&L) (HRP), pAb, cat. No A00098, Lot No 11B000259. Diluted 1:1000 during the blot.

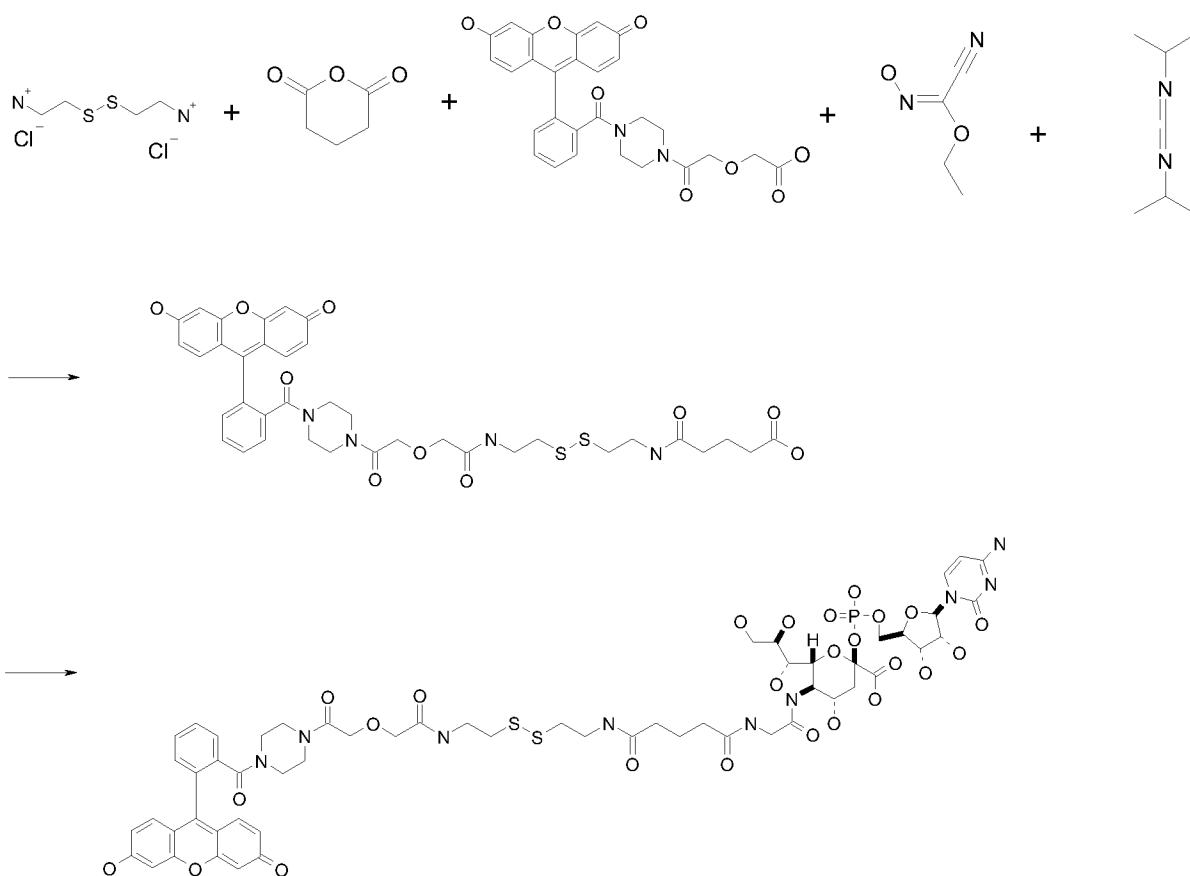
Example 41: Synthesis of 2-[2-[4-[2-(3-hydroxy-6-oxo-xanthen-9-yl)benzoyl]piperazin-1-yl]-2-oxo-ethoxy]acetic acid.



Name	Dens	Mw	Mol	N	W	V
	[g/ml]	[g/mol]	Ratio	[mmol]	[g]	[ml]
1		436.899	1	121.31	53	
2		116.074	1	121.31	14.081	
Product: C ₂₈ H ₂₄ N ₂ O ₈		516.512				

6-hydroxy-9-[2-(piperazin-4-ium-1-carbonyl)phenyl]xanthen-3-one (prepared as described in Chang et al., *J. Am. Chem. Soc.*, 2007, 129, 8400) was suspended in a mixture of sat. aq. sodium bicarbonate (50 ml) and tetrahydrofuran (50 ml). The mixture was stirred for 10 minutes. Diglycolic anhydride is added. After 3 h, additional diglycolic anhydride (500 mg) was added. The mixture is stirred for 20 h. The mixture was acidified with fuming hydrochloric acid to pH 1. Dichloromethane (100 ml) and hydrochloric acid (1 M, 100 ml) were added. Brine (100 ml) and solid sodium chloride was added. A massive amount of solid was observed. The solid was isolated by filtration, washed with water, and dried under vacuum for several days. LC-MS: 517.1641 [M+H]⁺.

Example 42: Synthesis of fluorescent cytidyl monophosphate neuraminic acid derivative, (2R, 5R, 6R)-2-[[[(2R, 3S, 4R, 5R)-5-(4-amino-2-oxo-pyrimidin-1-yl)-3, 4-dihydroxy-tetrahydrofuran-2-yl]methoxy-hydroxy-phosphoryl]oxy-4-hydroxy-5-[[2-[[5-[2-[2-[2-[2-[4-[2-(3-hydroxy-6-oxo-xanthen-9-yl)benzoyl]piperazin-1-yl]-2-oxo-ethoxy]acetyl]amino]ethyl]disulfanyl]ethylamino]-5-oxo-pentanoyl]amino]acetyl]amino]-6-[(2R)-1,2,3-trihydroxypropyl]tetrahydropyran-2-carboxylic acid.



10

Name	Dens	Mw	Mol	n	W	V
	[g/ml]	[g/mol]	Ratio	[mmol]	[g]	[ml]
Cysteamine dihydrochloride		225,204	1	8,881	2	
Glutaric anhydride		114,102	1	8,881	1,013	
Fluorescent		516,512	0,218	1,936	1	
Oxyma Pure		114,105	0,3	2,664	0,304	
diisopropylcarbodiimide	0,815	126,203	0,5	4,44	0,56	0,688
Product: C37H40N4O10S2		764,88				
Product: C57H70N9O25PS2		1376,341				

Cystamine dihydrochloride was dissolved in 1 M NaOH (aq.), 50 ml. The solution was extracted with DCM (5x30 ml). The combined org. phases were dried (Na₂SO₄), filtered, and concentrated in vacuo.

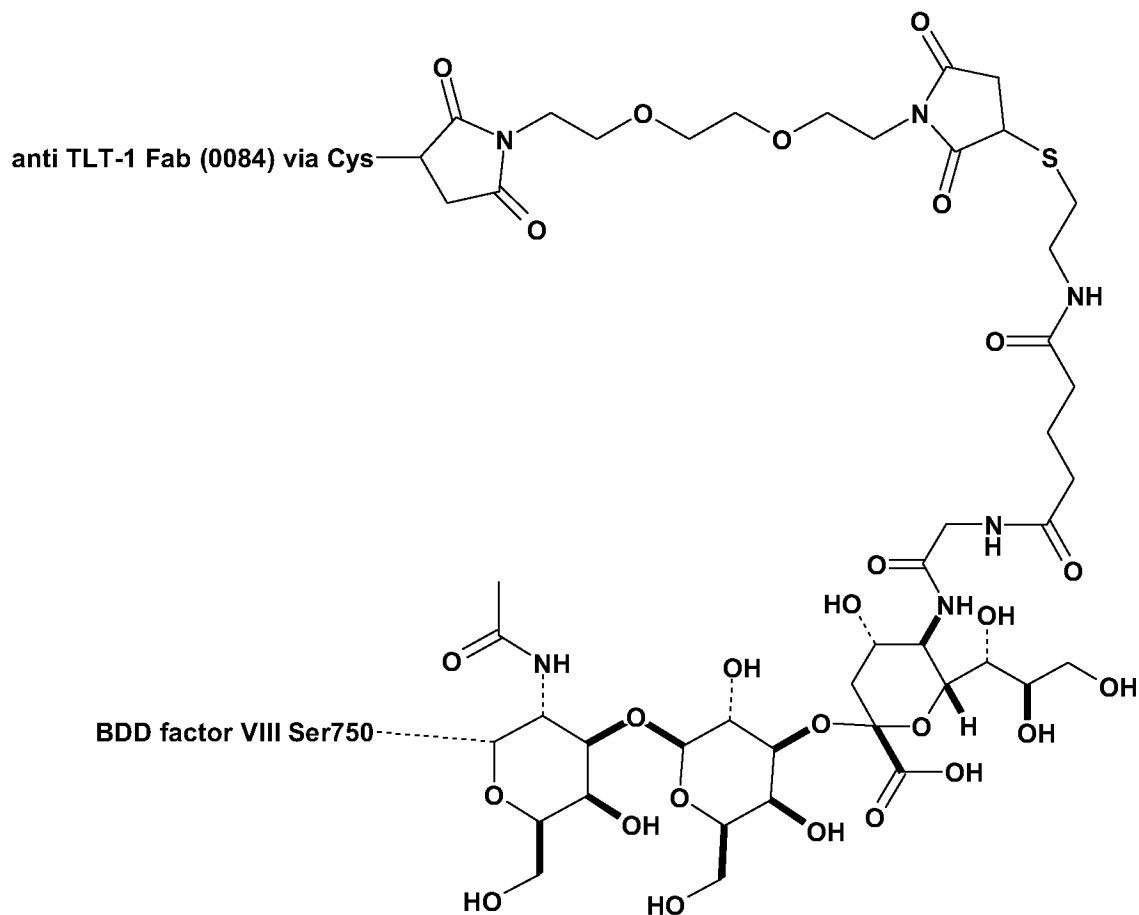
The diamine was dissolved in acetonitril (30 ml). A solution was added dropwise solution of anhydride in acetonitril (30 ml) to the solution. The resulting mixture was stirred for 15 minutes. The formed solid was allowed to settle for 1 h. The solvent was decanted off.

2-[2-[4-[2-(3-hydroxy-6-oxo-xanthen-9-yl)benzoyl]piperazin-1-yl]-2-oxo-ethoxy]acetic acid, Oxyma, and DIC were mixed in DMF (25 ml). The mixture was stirred for 1 h.

The formed amino acid was dissolved in sat. aq. sodium bicarbonate (25 ml). The resulting mixture was stirred over night. DCM (50 ml) and aq. sodium hydroxide (50 ml) were added. The phases were separated. The organic phase was extracted with aq. sodium hydroxide (3x50 ml). The combined aqueous extracts were acidified by addition of hydrochloric acid (fuming) causing extensive precipitation. The mixture was filtered. The isolated slurry was redissolved in DMF and concentrated in vacuo.

The crude isolated compound and Oxyma were dissolved in DMF (20 ml). DIC (1.5 ml) was added. The mixture was stirred for 2 h. A solution of GSC in sat. aq. sodium bicarbonate (10 ml) was added. The mixture was stirred over night. DCM (50 ml) was added. The phases were separated. The org. phase was extracted with sat. aq. sodium bicarbonate (2x5 ml). The combined aqueous phases were purified using reversed phase HPLC (0-50% MeCN in water, 50 mM NH₄HCO₃, 5 cm column). LC-MS: 688.6752 [M+H]²⁺. Analytical HPLC and LC-MS indicated that the compound was not pure. It was, however, used as is.

Example 43: Conjugation of anti TLT-1-FAB fragment (Fab0084) to FVIII, FVIII-Fab0247

(0084)

B-domain deleted factor VIII (turoctocoq alpha, Novo Nordisk A/S, 1.92 ml, 4.2 mg/ml) in imidazol buffer (20 mM Imidazol, 10 mM CaCl_2 , 0.02% Tween 80, 150 mM NaCl, 1 M glycerol, pH 7.3) and sialidase (recombinant, *Arthrobacter Ureafaciens* sialidase, 3.2 U) were mixed and left for 1 hour at ambient temperature. The sample was diluted to 25 ml with buffer.

The solution was injected on a pre-conditioned monoQ column (pre-conditioned in Buffer A, 5 ml column volume).

Buffer A: 20 mM Imidazol, 10 mM CaCl_2 , 0.02% Tween 80, 25mM NaCl, 1M glycerol, pH 7.3

Buffer B: 20 mM Imidazol, 10 mM CaCl_2 , 0.02% Tween 80, 1M NaCl, 1 M glycerol, pH7.3.

The immobilised protein was washaed with Buffer A (5 CV) using a Äkta purifier 100 chromatography station. The protein was eluted with a gradient of buffer B (2 CV eq + 5 wash out unbound sample + 2 CV 0-20% B + 10 CV 20% B + 10 CV 100% B) at 1 ml/min. Elution of the protein of interest was done by monitoring the absorbance at 280 nm.

The isolated N,O-asialo BDD-FVIII (0.98mg/ml, 5 mg) was mixed with Fluorescent cytidyl monophosphate neuraminic acid derivative and recombinant sialyltransferase (His-ST3Gal1). The resulting mixture was incubated over night at room temperature in the dark.

5 The sample was diluted to 40ml with buffer A: 20 mM Imidazol, 10 mM CaCl₂, 0.02% Tween 80, 25mM NaCl, 1M glycerol, pH 7.3.

The solution was injected on a pre-conditioned monoQ column (pre-conditioned in Buffer A, 5 ml column volume).

10 Buffer A: 20 mM Imidazol, 10 mM CaCl₂, 0.02% Tween 80, 25mM NaCl, 1M glycerol, pH 7.3.

Buffer B: 20 mM Imidazol, 10 mM CaCl₂, 0.02% Tween 80, 1M NaCl, 1 M glycerol, pH 7.3.

15 The immobilised protein was washed with Buffer A (5 CV) using a Äkta purifier 100 chromatography station. The protein was eluded with a gradient of buffer B (2 CV eq + 5 wash out unbound sample + 2 CV 0-20% B + 10 CV 20% B + 10 CV 100% B) at 1 ml/min. Elution of the protein of interest was done by monitoring the absorbance at 280 nm.

20 The selected fractions were pooled and incubated with cytidylmonophosphate N-acetylneuraminic acid and sialyltransferase (ST3GalIII) for 30 minutes. The mixture was diluted to 35 ml with buffer A.

The solution was injected on a pre-conditioned monoQ column (pre-conditioned in Buffer A, 5 ml column volume).

Buffer A: 20 mM Imidazol, 10 mM CaCl₂, 0.02% Tween 80, 25mM NaCl, 1M glycerol, pH 7.3.

25 Buffer B: 20 mM Imidazol, 10 mM CaCl₂, 0.02% Tween 80, 1M NaCl, 1 M glycerol, pH 7.3.

30 The immobilised protein was washed with Buffer A (5 CV) using a Äkta purifier 100 chromatography station. The protein was eluded with a gradient of buffer B (2 CV eq + 5 wash out unbound sample + 2 CV 0-20% B + 10 CV 20% B + 10 CV 100% B) at 1 ml/min. Elution of the protein of interest was done by monitoring the absorbance at 280 nm.

The selected fractions were pooled and evaluated by SDS PAGE gel electrophoresis (4-12% Bis-Tris acetate, reduced and non-reduced).

35 The isolated fractions were mixed with buffer (15 ml) containing *tris*(carboxyethyl)phosphine, TCEP (20 mM Imidazol, 10 mM CaCl₂, 0.02% Tween 80, 25 mM NaCl, 1M glycerol, pH 7.3, 0.7 mM TCEP). The resulting mixture was incubated for 20

minutes. The solution was injected on a pre-conditioned monoQ column (5/50 GL, pre-conditioned in Buffer A with TCEP).

Buffer A1: 20 mM Imidazol, 10 mM CaCl_2 , 0.02% Tween 80, 25mM NaCl, 1M glycerol, pH 7.3, 0.7 mM TCEP).

5 Buffer A2: 20 mM Imidazol, 10 mM CaCl_2 , 0.02% Tween 80, 25mM NaCl, 1M glycerol, pH 7.3).

Buffer B1: 20 mM Imidazol, 10 mM CaCl_2 , 0.02% Tween 80, 1 M NaCl, 1M glycerol, pH 7.3).

10 10 CV eq i A1, 5 wash out unbound sample i A1, 30CV in 100% A1, 10 CV in 100% A2 , 15 CV 100% B1

The immobilised protein was washed with Buffer A1 (45 CV) followed by Buffer A2 (10 CV) using a Äkta purifier 100 chromatography station. The protein was eluted with buffer B (15 CV 100% B) at 1 ml/min. Elution of the protein of interest was done by monitoring the absorbance at 280 nm.

15 The selected fractions were pooled and analysed by SDS-PAGE (Tris-acetate).

The isolated factor VIII compound was diluted with Buffer A and injected on a pre-conditioned monoQ column (5/50 GL, pre-conditioned in Buffer A).

20 A flow of Buffer A containing 4.8mM BM(PEG)2 (1,8-Bismaleimidodiethyleneglycol, Pierce/Thermo scientific) was maintained for 100 minutes. The protein was washed and eluted using the following protocol: 10 CV A1, 25CV in buffer A2 (bismaleimide) at 0.25ml/min(100min), 10CV buffer A1, 10CV 100% B1.

25 Anti-TLT-1 antibody fragment, 0084, was buffer exchanged using an Amicon Ultracentrifugal filter device MWCO 30kDa into HEPES buffer (20mM HEPES + 1mM CaCl_2 , 100mM NaCl + 0.005% Tween80, pH: 7.50). Concentration measured to be: 3.44mg/ml, 2mg): *Tris*(3-sulfonatophenyl)phosphine hydrate sodium salt (Alfa Aesar, technical grade 85%) was added to a resulting concentration of 12.5 mM. The resulting mixture was incubated at room temperature for 2 h. The antibody fragment was buffer exchanged using an Amicon Ultracentrifugal filter device MWCO 30kDa into imidazol buffer (20 mM Imidazol, 10 mM CaCl_2 , 0.02% Tween 80, 25mM NaCl, 1M glycerol, pH 7.3).

The solutions of factor VIII and antibody fragment were mixed and concentrated to 2 ml. The resulting solution was incubated over night at room temperature.

The solution was injected on a pre-conditioned monoQ column (pre-conditioned in Buffer A, 5 ml column volume).

35 Buffer A: 20 mM Imidazol, 10 mM CaCl_2 , 0.02% Tween 80, 25mM NaCl, 1M glycerol, pH 7.3.

Buffer B: 20 mM Imidazol, 10 mM CaCl_2 , 0.02% Tween 80, 1M NaCl, 1 M glycerol, pH 7.3.

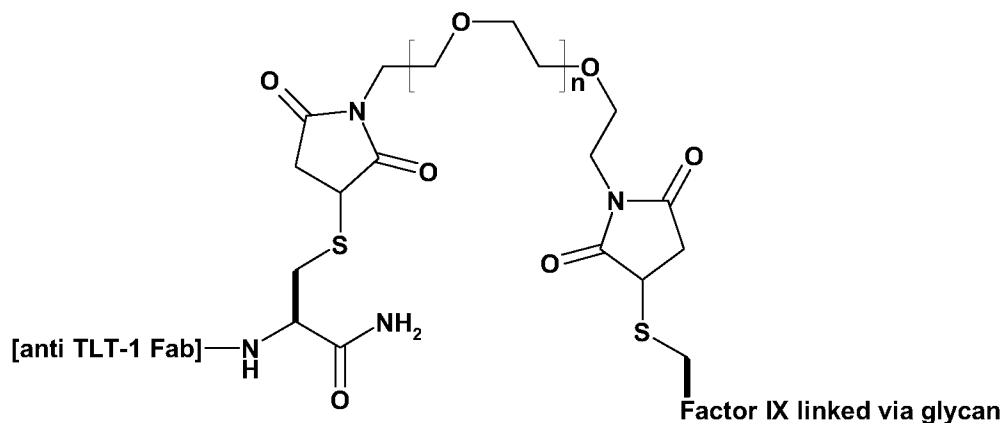
The immobilised protein was washed with Buffer A (5 CV) using a Äkta purifier 100 chromatography station. The protein was eluted with a gradient of buffer B (2 CV eq + 5 wash out unbound sample + 2 CV 0-20% B + 10 CV 20% B + 10 CV 100% B) at 1 ml/min. Elution of the protein of interest was done by monitoring the absorbance at 280 nm.

The selected fractions were pooled and concentrated. The protein was injected on a pre-conditioned Superdex 200 16/60 PG column (pre-conditioned in Buffer A).

Buffer A: Histidine (1.5g , 1.5 mg/ml), $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ (376 mg, 0.37mg/ml), NaCl (18g, 18 mg/ml), Sucrose (3g, 3 mg/ml), Tween 80 (100mg, 0.1mg/ml), Diluted to 1000 ml with MQ, adjust to pH 7.0.

Fractions were selected based on analysis by SDS-PAGE and anti-HPC4 Western blotting.

Example 44: Conjugation of anti TLT-1-FAB fragment (Fab0084) to FIX.



Factor IX (50 microliter), Sialyltransferase3 (10 microliter), Fluorescent cytidyl monophosphate neuraminic acid derivative; tip of a spatula) are mixed. The mixture is incubated at 32 degrees Celsius for 24 hours.

The end concentrations are: Factor IX: 0.33 mg/ml, ST3Gal3: 0.08 mg/ml

Analysed by SDS-PAGE (fluorescence respons and coomassie blue stained).

Factor IX and an antibody fragment are conjugated using the methods described herein, i.e, reduction mediated by a phosphine or glutathion, coupling to a linker entity, and conjugation followed by purification and analysis.

Example 45: FVIIa-Fab9015 is superior to rFVIIa in reducing tail-bleeding in transient haemophilic mice.

Humanized TLT-1 knock-out/knock-in (KOKI) mice were made transiently haemophilic by administration of a monoclonal FVIII-antibody. Five minutes before induction of tail-bleeding, the mice were pre-treated with 20, 5 or 0.8 nmol/kg FVIIa-Fab9015 (3.625 ml/kg), 20 nmol/kg rFVIIa or vehicle. Tail-bleeding was induced by transection 4 mm from the tail-tip, and the resulting bleeding was observed for 30 minutes. Platelet counts were obtained initially and 30, 60 and 120 minutes after induction of tail-bleeding.

In order to be able to show superiority of FVIIa-Fab9015, we used a dose of rFVIIa (20 nmol/kg ~ 1 mg/kg) that was not expected to have significant effect on the bleeding.

TLT-1-Fab-FVIIa dose-dependently reduced blood loss and bleeding time, reaching statistical significance at 20 and 5 nmol/kg. Moreover 20 nmol/kg FVIIa-Fab9015 was significantly more efficacious compared to 20 nmol/kg rFVIIa (Figure 15).

No significantly changes in platelet count was observed within 2 hours of treatment in any of the treatment groups (Figure 16).

In conclusion, FVIIa-Fab9015 was superior to rFVIIa in reducing haemophilic tail-bleeding in TLT-1 KOKI mice. No signs of adverse effects, eg. decrease in platelet counts, were observed.

Example 46: Enhancement of thrombin generation by localization of rFVIIa to the surface of activated platelets through binding to surface expressed TLT-1 under haemophilia A like conditions.

FVIIa-Fab9015 was tested in a thrombin generation assay. In brief, human platelet rich plasma (PRP) obtained by centrifugation of citrated human whole blood at 220g for 20 min. The upper phase containing platelets was collected and the remaining sample was centrifuged at 2500g for 10 min to obtain platelet poor plasma (PPP) used to adjust the platelet concentration to be used at a final concentration of 150000 plts/ μ l.

The PRP was made haemophilic by 30 min incubation with a sheep anti-human FVIII polyclonal antibody (0.1 mg/ml) (HTI #Z0429). Platelets were activated with either protease activated recertor-1 activation peptide (SFLLRN; Bachem #H-2936) or a combination of the peptide and the GPVI activating snake venom Convulxin (Pentapharm #119-02). Generated thrombin was measured using a fluorogenic method from Thrombinoscope®. PRP, FluCa reagent and compound were mixed and added to 96-well Nunc Microwell round bottom well plates. The reaction was started by the addition of

platelet activator and the fluorescent signal from the substrate was detected in a ThermoFisher Fluoroskan plate reader (Fisher Scientific). The thrombin concentration was calculated using a Thrombin calibrator provided by Thrombinoscope according to their instructions.

5 The results showed an increased potency of FVIIa-Fab9015 compared to rFVIIa (Figure 17A). Furthermore the results revealed that this increase in potency was dependent on the activation stage of the platelets. Figure 18 shows the increased thrombin generation capacity when the platelets were activated with SFLLRN (10 μ M) and that full potential was reached when the platelets were activated with a combination of SFLLRN
10 (30 μ M) and Convulxin (100ng/ml). When activating the platelets with this combination of activators FVIIa-Fab9015 showed an approximately four times increased potency, measured as peak thrombin generation, compared to rFVIIa at the two concentrations tested (5 and 25 nM) (Figure 17A and 17B, respectively). This increase in thrombin generation capacity was completely dependent on FVIIa-Fab9015 binding to TLT-1 on the surface of
15 the activated platelet since pre-incubation with soluble TLT-1 fully reversed the enhancement (Figure 19).

**Example 47: Enhancement of thrombin generation by localization of rFIX to the surface of activated platelets through binding to surface expressed TLT-1 under
20 haemophilia B like conditions.**

FIX-Fab0155 was tested in a thrombin generation assay and compared to rFIX (Benefix®). Human platelets were isolated from fresh citrate stabilized whole blood. One part ACD-solution (2.5% tri-sodium citrate, 1.5% citric acid and 2% D-glucose) was added to five parts of blood before centrifugation at 220g for 20 min to obtain platelet rich
25 plasma. The upper phase was collected and transferred to a new cone shaped tube and spun at 500g for 15 min. The plasma was removed and the pellet was dissolved in Hepes-buffer (10 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 1.7 mM MgCl₂, 5 mM D-glucose, 0.4 mM NaH₂PO₄; pH 6.5) supplemented with prostaglandin E1 (5 μ g/ml). After a second centrifugation at 500g for 15 min the supernatant was discarded and the
30 washed platelets were dissolved in factor IX deficient plasma (Geroge King Bio-medical, Inc.) and the platelet concentration was adjusted to 300000 plts/ μ l. In the thrombin generation assay when agonists, factor IX variants and FluCa reagent (Thrombinoscope®) were added to 96-well Nunc Microwell round bottom well plates together with the factor IX deficient plasma containing platelets the final platelet concentration was
35 150000 plts/ μ l. The platelets were activated with a combination of protease activated receptor-1 activation peptide (SFLLRN; Bachem #H-2936) and the GPVI activating snake

venom Convulxin (Pentapharm #119-02). Generated thrombin was measured by a fluorogenic method from Thrombinoscope® in which the fluorescent signal from the thrombin cleaved substrate was detected in a ThermoFisher Fluoroskan plate reader (Fisher Scientific). The thrombin concentration was calculated using a thrombin calibrator provided by Thrombinoscope® according to their instructions.

The results showed an increased potency at 1 nM of FIX-Fab0155 compared to rFIX (Figure 20) when the platelets were activated with a combination of SFLLRN (30µM) and Convulxin (100ng/ml). Furthermore the results revealed that this increase in potency was dependent on expression of TLT-1 on the platelets since soluble TLT-1 (100 nM) antagonised the enhanced effect.

Example 48: Purification and characterization of wild-type FVIIa (SEQ ID NO: 156), FVIIa 407C (SEQ ID NO: 181) and FVIIa-Fab5001 (SEQ ID NO: 180).

Purification of FVIIa proteins were conducted using a capture affinity chromatography method based on the anti-FVIIa F1A2-Sepharose 4B resin, which is a resin (base affinity gel from GE Healthcare) with a coupled antibody, developed at Novo Nordisk, that binds specifically the FVIIa Gla domain (for details see reference Jurlander B, Thim L, Klausen NK, Persson E, Kjalke M, Rexen P, Jørgensen TB, Østergaard PB, Erhardtsen E, Bjørn SE (2001) Recombinant activated factor VII (rFVIIa): characterization, manufacturing, and clinical development. Semin Thromb Hemost. 27:373-84). The purification was conducted using an ÄktaExplorer chromatography system (GE Healthcare, cat. no. 18-1112-41). The buffer systems used for the purification step were an equilibration buffer composed of 10 mM Histidine, pH 6.0, 5 mM CaCl₂, 25 mM NaCl and 0.01% (v/v) Tween-80, a wash buffer composed of 10 mM Histidine, pH 6.0, 5 mM CaCl₂, 1.0 M NaCl and 0.01% (v/v) Tween-80, and an elution buffer composed of 50 mM Histidine, pH 6.0, 15 mM EDTA. Cell supernatants were adjusted with 5 mM CaCl₂ final concentration and applied onto a pre-equilibrated anti-HPC4 column. The column was washed with 5-10 column volumes of equilibration buffer, 5-10 column volumes of wash buffer and last with 5-10 column volumes of equilibration buffer. The FVIIa proteins were eluted isocratically in approximately 5 column volumes of elution buffer.

Further purifications of capture eluates with low purities of the FVIIa variants (< 90% based on a SEC-HPLC method setup on an Agilent 1100/2100 system and using a TSK G3000SW_{XL} column (From Tosho) and a PBS running buffer) were conducted using 1) an anion-exchange chromatography (AIEC) based on a Poros HQ50 resin (from Applied Biosystems) and 2) preparative gel filtrations using pre-packed Superdex200 columns (from GE Healthcare).

The buffer systems used for the AIEC purification step was an equilibration buffer composed of 10 mM Hepes, pH 5.9 and 150 mM NaCl, a wash buffer composed of 10 mM Hepes, pH 5.9 and 50 mM NaCl, and an elution buffer composed of 10 mM Hepes, pH 5.9, 50 mM NaCl and 30 mM CaCl₂. The capture eluate was diluted 1:6 (v:v) before applying it onto a pre-equilibrated Poros HQ50 column. The column was washed with 5-10 column volumes of equilibration buffer and 5-10 column volumes of wash buffer before eluting the FVIIa proteins isocratically in approximately 2-5 column volumes of elution buffer.

The buffer systems used for the gel filtration steps was a running buffer composed of 10 mM Histidine, pH 6.0, 10 mM CaCl₂, 100 mM NaCl and 0.01% Tween 80. The FVIIa proteins were collected as symmetric peaks in approx 0.02-0.08 column volumes.

Activations of the purified FVIIa preparations were conducted using a resin composed of plasma-derived FXa (from Enzyme Research Lab.) coupled to activated CNBr-Sepharose 4 FF bead (from GE Healthcare).

The FVIIa proteins were analyzed using SDS-PAGE/Coomassie and intact molecular mass determinations performed using a Liquid Chromatography Electrospray Ionisation Time-of-Flight Mass Spectrometry method setup on an Agilent 6210 instrument and a desalting column MassPREP (from Waters) with an equilibration buffer composed of 0.1% Formic acid in LC-MS graded-H₂O and an elution buffer composed of 0.1% Formic acid in LC-MS graded-ACN. All FVIIa variants and wild-type purified displayed intact molecular masses of ~50 kDa. Based on intact-mass SDS-PAGE/Coomassie and LC-MS analyses using reductive conditions, chain identifications of FVIIa-Fab5001 fusion were performed.

Based on SEC-HPLC analyses, all FVIIa preparations displayed a purity of >90-99% and appeared highly homogenous.

Example 49: Autoactivation of FVII-Fab5001 using a proteolytic assay in the presence of soluble Tissue Factor.

Autoactivation was determined as the ability to activate FX in the presence of soluble Tissue Factor (sTF). The protein was diluted in 50 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM CaCl₂, 1 mg/mL BSA, and 0.1% (w/v) PEG8000. The kinetic parameters for FX activation were determined by pre-incubating 5 pM of the FVII-Fab5001 (n=2) with 100 nM sTF and 25 µM PC:PS phospholipids (Haematologic technologies) for 10 min. 30 nM FX was then added in a total reaction volume of 100 µL in a 96-well plate and the reaction allowed to incubate for 20 min at room temperature. After incubation, the reaction was quenched by adding 50 µL stop buffer (50 mM HEPES (pH 7.4), 100 mM NaCl, 80 mM EDTA) followed by the addition of 50 µL 2 mM S-2765. Finally, the

absorbance increase was measured continuously at 405 nM in a Spectramax 190 microplate reader. The k_{cat}/K_m values were determined by fitting the data to a revised form of the Michaelis Menten equation ($[S] < K_m$) using linear regression. The amount of FXa generated was estimated from a FXa standard curve.

- 5 The zymogen fusion protein FVII-Fab5001 showed 38% proteolytic activity relative to that of wtFVIIa, being a result of autoactivation of the protein in the presence of sTF, see Figure 21).

Example 50: Lack of anti-aggregatory effect of anti-TLT-1 antibodies, mAb0023, mAb0051, mAb0061 and mAb0062.

- 10 The anti-aggregatory effect of anti-TLT-1 antibodies was tested in human platelet rich plasma (PRP). The PRP was obtained by a 200g centrifugation for 15 min of heparin stabilized human whole blood. The upper phase containing platelets was collected and the remaining sample was centrifuged at 1500g for 10 min to obtain platelet poor plasma (PPP) which was used as a reference sample in the aggregation
15 measurements. The PRP was incubated 3 min at 37°C in the Platelet Aggregation Profiler (PAP-8) instrument (Bio/Data Corporation, Horsham, PA). A stable baseline was recorded before the addition of anti-TLT-1 antibodies (10 nM) or irrelevant control antibody (10 nM) to the PRP. The platelets were activated 3 min after the addition of the antibodies with protease activated receptor-1 (PAR-1) activating peptide SFLLRN (1 or 10 µM)
20 (Bachem).

- The results showed no inhibitory effect of the antibodies (10 nM) (mAb0023, mAb0051, mAb0061 and mAb0062) compared to irrelevant control antibody. Platelet aggregation was initiated with either a high (10 µM) or an intermediate (1 µM) concentration of SFLLRN. The data shows that the antibodies did not inhibit aggregation at neither
25 of the SFLLRN concentrations. In conclusion, these data shows that the antibodies do not induce nor inhibit platelet function measured as aggregation.

- 30 **Example 51. Effect of FIX-mAb0145 on TF-induced fibrin clot formation in human whole blood (HWB) from a normal donor measured by thrombelastography (TEG).**

- Citrated-stabilized human whole blood (HWB) is drawn from normal donors. Hemophilia-like conditions are obtained by incubation of HWB with 10 µg/ml anti-FVIII antibody (Sheep anti-Human Factor VIII; Hematologic Technologies Inc) for 30 min at room temp. Clot formation is measured by thrombelastography (5000 series TEG analyzer,
35 Haemoscope Corporation, Niles, IL, USA). Various concentrations (0; 0.2; 1.0; 5.0 and 10 nM) of FIX-mAb0145 or (0; 0.2; 5.0 and 10 nM) of rFIX (Novo Nordisk A/S) are added

to "hemophilia-like" citrated HWB. Clotting in is initiated when 340 µl of normal or "hemophilia-like" HWB is transferred to a thrombelastograph cup containing 20 µl 0.2 M CaCl₂ with 0.03 pM lipidated TF (Innovin®, Dade Behring GmbH (Marburg, Germany)). The TEG trace is followed continuously for up to 120 min. The following TEG variables are recorded: R time (clotting time i.e. the time from initiation of coagulation until an amplitude of 2 mm was obtained), α-angle (clot development measured as the angle between the R value and the inflection point of the TEG trace), K (speed of clot kinetics to reach a certain level of clot strength, amplitude = 20 mm), and MA (maximal amplitude of the TEG trace reflecting the maximal mechanical strength of the clot). Figure 23 shows R time values determined from individual TEG traces.

The R time obtained from TEG traces with normal HWB and "hemophilia" blood supplemented with various concentrations of FIX-mAb0145 or rFIX are shown in Figure 23. HWB from a normal donor showed a R time of 980 sec which was prolonged to 5475 sec when the blood was made "haemophilia like" by the addition of a FVIII neutralizing antibody. FIX-mAb0145 shortened R-time clotting in a concentration dependent manner in contrast to rFIX which was without a significant effect when added as a control at equivalent concentrations. All data are obtained from one representative donor.

FIX-mAb0145 is observed to normalize clotting of "hemophilia-like HWB" in a concentration dependent manner. Thus surprisingly the results show that conjugation of rFIX to an antibody against TLT-1, which target rFIX to the surface of activated platelets, provides it with a FVIII-independent by-passing activity resulting in improved procoagulant activity.

Example 52. Enhancement of thrombin generation by localization of rFVIIa to the surface of activated platelets through binding to surface expressed TLT-1 under haemophilia A like conditions.

FVIIa-Fab9015, FVIIa-Fab1029 and FVIIa-Fab1001 at 25 nM were tested in a thrombin generation assay. In brief, human platelet rich plasma (PRP) was obtained by centrifugation of citrated human whole blood at 220g for 20 min. The upper phase containing platelets was collected and the remaining sample was centrifuged at 2500g for 10 min to obtain platelet poor plasma (PPP) used to adjust the platelet concentration to a final concentration of 150000 plts/µl. The PRP was made haemophilic by 30 min incubation with a sheep anti-human FVIII polyclonal antibody (0.1 mg/ml) (HTI #Z0429). Platelets were activated with a combination of protease activated receptor-1 (PAR-1) activation peptide (30 µM) (SFLLRN; Bachem #H-2936) and the GPVI activating snake venom Convulxin (100 ng/ml) (Pentapharm #119-02). Generated thrombin was

measured using a fluorogenic method from Thrombinoscope® in which PRP, FluCa reagent and test compound were mixed and added to 96-well Nunc Microwell round bottom well plates (Nunc #268152). The reaction was started by the addition of platelet activators and the fluorescent signal from the substrate was detected in a ThermoFisher Fluoroskan plate reader (Fisher Scientific). The thrombin concentration was calculated using a Thrombin calibrator provided by Thrombinoscope® according to their instructions.

The results showed an increased potency of all three proteins, FVIIa-Fab9015, FVIIa-Fab1029 and FVIIa-Fab1001, compared to rFVIIa. FVIIa-Fab9015 and FVIIa-Fab1001 (25 nM) showed an approximately four times increased potency whereas FVIIa-Fab1029 (25 nM) had approximately a twofold increased potency measured as peak thrombin generation, compared to rFVIIa (25 nM) (Figure 24).

Example 53. Purification and characterization of recombinantly expressed FIX (SEQ ID 161), FIX-anti-TLT-1 Fab and Mab fusion protein (FIX-Fab0155 and FIX-Mab0145).

Purification of said FIX proteins were conducted using a capture affinity chromatography method based on the anti-FIX A3B6-Sepharose 4 FF resin, which is a resin (base affinity gel from GE Healthcare) with a coupled antibody, developed at Novo Nordisk, that binds specifically to the FIX Gla domain (see reference Østergaard et al. (2011), Blood 118: 2333-41). The purification was conducted using an ÄktaExplorer chromatography system (GE Healthcare, cat. no. 18-1112-41). The buffer systems used for the purification step were an equilibration buffer composed of 20 mM Tris, 1 mM CaCl₂, 100 mM NaCl, 0.01% (v/v) Tween 80 pH 7.5, a wash buffer composed of 20 mM Tris, 1 mM CaCl₂, 2.0 M NaCl, 0.01% (v/v) Tween 80 pH 7.5 and an elution buffer composed of 20 mM Tris, 20 mM EDTA, 50 mM NaCl, 0.01% (v/v) Tween 80 pH 7.5. Cell supernatants were adjusted with 5 mM CaCl₂ final concentration and applied onto a pre-equilibrated A3B6-Seph 4 FF column. The column was washed with 5-10 column volumes of equilibration buffer, 5-10 column volumes of wash buffer and last with 5-10 column volumes of equilibration buffer. The FIX fusion proteins were eluted isocratically in approximately 5 column volumes of elution buffer.

The FIX proteins were analyzed using SDS-PAGE/Coomassie and intact molecular mass determinations performed using a Liquid Chromatography Electrospray Ionisation Time-of-Flight Mass Spectrometry method setup on an Agilent 6210 instrument and a desalting column MassPREP (from Waters) with an equilibration buffer composed of 0.1% Formic acid in LC-MS graded-H₂O and an elution buffer composed of 0.1% Formic acid in

LC-MS graded-ACN. Based on intact-mass SDS-PAGE/Coomassie and LC-MS analyses using reductive conditions, chain identifications of the FIX proteins were performed. Based on SEC-HPLC analyses, all FIX fusion protein preparations displayed a purity of >85–99% and appeared highly homogenous.

5

Example 54. Production of recombinant expression of FVIIa wild-type (SEQ ID NO: 157) and FVIIa-407C (SEQ ID NO:181).

Said variant and wild-type FVIIa were produced as described in patent 02/077218 and Jurlander et al. (2001), *Semin Thromb Hemost.* 27:373-84. More specifically, the FVIIa molecules were expressed in adherent BHK cell lines. The growth medium employed was a DMEM/F12 medium variant with 5 mg/L vitamin K1 and 10% fetal bovine serum (2% during production). Briefly, the cells were propagated in vented T-175 flasks, 2-layer and 10-layer cell factories incubated at 37°C and 5% CO₂. At confluency, cells were dissociated using TrypLE™ Express (GIBCO cat. no. 12604-013) prior to passaging to the next step. The production phase was performed as a repeated batch culture in a 15 L bioreactor with microcarriers (5 g/L, Cytodex 3, GE Life Sciences). pH was controlled at an upper limit of 7.2 by adding CO₂ and at a lower limit of 6.8 by adding Na₂CO₃. Dissolved oxygen concentration was kept above 50% of saturation in air by sparging with oxygen. Temperature was maintained at 36.5°C. Agitation was at 50-70 rpm. Harvesting/medium exchanges were performed to maintain a glutamine concentration above 1 mM. One hour before a medium exchange, agitation was stopped to allow microcarriers (with cells attached) to settle at the bottom of the reactor. Thereafter, approximately 80% of the volume was harvested before filling up with fresh medium to 100% working volume. Cell harvests were withdrawn and clarified by a filter train consisting of two disposable capsule filters (3 µm Clarigard, Opticap XL10, Millipore, cat. no. K030A10HH1; 0.22 µm Durapore, Opticap XL10, Millipore, cat. no. KVGLS10HH1) prior to purification.

Example 55. Enhancement of thrombin generation by localization of rFVIIa to the surface of activated platelets through binding to surface expressed TLT-1 under haemophilia A like conditions.

A FVIIa-Fab5001 was tested in a thrombin generation assay and compared to rFVIIa in factor VIII deficient plasma containing washed human platelets. To isolate human platelets, one part ACD-solution (2.5% tri-sodium citrate, 1.5% citric acid and 2% D-glucose) was added to five parts of blood before centrifugation at 220g for 20 min to obtain platelet rich plasma. The upper phase was collected and transferred to a new

cone shaped tube and spun at 500g for 15 min. The plasma was removed and the pellet was dissolved in Hepes-buffer (10 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 1.7 mM MgCl₂, 5 mM D-glucose, 0.4 mM NaH₂PO₄; pH 6.5) supplemented with prostaglandin E1 (5 µg/ml). After a second centrifugation at 500g for 15 min the supernatant was discarded and the washed platelets were dissolved in factor VIII deficient plasma (Geroge King Bio-medical, Inc.) and the platelet concentration was adjusted to 300000 plts/µl. In the thrombin generation assay when agonists, factor IX variants and FluCa reagent (Thrombinoscope®) were added to 96-well Nunc Microwell round bottom well plates together with the factor IX deficient plasma containing platelets the final platelet concentration was 150000 plts/µl. The platelets were activated with a combination of protease activated recertor-1 activation peptide (SFLLRN; Bachem #H-2936) and the GPVI activating snake venom Convulxin (Pentapharm #119-02). Generated thrombin was measured by a fluorogenic method from Thrombinoscope® in which the fluorescent signal from the thrombin cleaved substrate was detected in a ThermoFisher Fluoroskan plate reader (Fisher Scientific). The thrombin concentration was calculated using a thrombin calibrator provided by Thrombinoscope® according to their instructions. The results showed an increased potency of the FVIIa-Fab5001 compared to wild-type rFVIIa (Figure 25). Furthermore the results revealed that this increase in potency was dependent on platelet activation. Activating the platelets with a combination of SFLLRN (30µM) and Convulxin (100ng/ml), FVIIa-Fab5001 (25 nM) showed an approximately four times increased potency, measured as peak thrombin generation, compared to wild-type rFVIIa (25 nM).

Example 56. SPR-analysis of FVIIa-Fab1001 and FVIIa-Fab5001 binding to TLT1.

SPR-analysis of FVIIa-Fab1001 binding to TLT1. FVIIa-Fab1001 binds TLT-1 as tested by SPR analysis in a Biacore T200 instrument.

Materials used are shown in Table 18.

Table 18

Reagent	Source
His tagged human TLT1	Example 1
FVIIa-Fab1001	Example 39
Anti 6X his mAb	R&D #MAB050 0.5 mg/ml in PBS
All other reagents	Biacore GE Healthcare

Method: An anti 6Xhis antibody was immobilised to a level of approx 9000 RU on a CM5 chip (0.5 mg/ml diluted in Na-acetate, pH 5.0) using the standard procedure recommended by the supplier. Human his-tagged TLT-1 in a concentration of 100 ng/ml was used as ligand. FVIIa-Fab1001 in two-fold dilutions from 29.29 nM to 0.45 nM was used as analytes. The running and dilution buffer was made from: 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% p20, pH 7.4. Regeneration was obtained by 3 M MgCl₂. The experiment was run at 25 degree Celsius. Determination of kinetic and binding constants (k_{on} , k_{off} , K_D) was obtained assuming a 1:1 interaction of TLT1 and FVIIa-Fab1001 using the Biacore T200 evaluation software (Table 19).

Table 19: FVIIa-Fab1001 binding to TLT-1

	Ka (1/Ms)	kd (1/s)	KD (M)
FVIIa-Fab1001 binding to TLT-1	8.01E+05	3.19E-03	3.99E-09

Conclusion: Binding constants for FVIIa-Fab1001 binding to TLT-1 was estimated by SPR-analysis and binding to TLT-1 was confirmed.

SPR-analysis of FVIIa-Fab5001 binding to TLT1. FVIIa-Fab5001 binds TLT-1 as tested by SPR analysis in a Biacore T200 instrument.

Materials used are shown in Table 20.

Table 20

Reagent	Source
His tagged human TLT1	Example 1
FVIIa-Fab5001	Example 48
Anti 6X his mAb	R&D #MAB050 0.5 mg/ml in PBS
All other reagents	Biacore GE Healthcare

Method: An anti 6Xhis antibody was immobilised to a level of approx 9000 RU on a CM5 chip (0.5 mg/ml diluted in Na-acetate, pH 5.0) using the standard procedure recommended by the supplier. Human his-tagged TLT-1 in a concentration of 100 ng/ml was used as ligand. FVIIa-Fab5001 in two-fold dilutions from 15.77 nM to 0.49 nM was used as analytes. The running and dilution buffer was made from: 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% p20, pH 7.4. Regeneration was obtained by 3 M MgCl₂. The experiment was run at 25 degree Celsius. Determination of kinetic and binding constants

(k_{on} , k_{off} , K_D) was obtained assuming a 1:1 interaction of TLT1 and FVII-fab using the Biacore T200 evaluation software (Table 21).

Table 21: FVIIa-Fab5001 binding to TLT-1

	k_a (1/Ms)	k_d (1/s)	K_D (M)
FVIIa-Fab5001 binding to TLT-1	2.384E+05	0.001696	7.11E-09

5

Conclusion: Binding constants for FVIIa-Fab5001 binding to TLT-1 was estimated by SPR-analysis and binding to TLT-1 was confirmed.

Example 57: Recombinant production of human FVIII.

10

The production of the human Factor VIII variants as used herein has been described in patent number WO2009108806, example 1.

CLAIMS

1. A procoagulant protein comprising (i) at least one coagulation factor, covalently attached to (ii) a monoclonal antibody or a fragment thereof that is capable of specifically binding (iii) TLT-1, and/or a fragment or variant thereof.
5
2. The procoagulant protein according to claims 1, wherein (i) is a serine protease or a derivative thereof.
- 10 3. The procoagulant protein according to claim 2, wherein (i) is a FVII polypeptide.
4. The procoagulant protein according to claim 2, wherein (i) is a FIX polypeptide.
- 15 5. The procoagulant protein according to any one of claims 1-4, wherein (ii) is a Fab fragment.
6. The procoagulant protein according to any one of claims 1-5, wherein the epitope of (ii) comprises one or more residues selected from the group consisting of V17, Q18, C19, H20, Y21, R22, L23, Q24, D25, V26, K27, A28, L63, G64, G65, G66, L67, L68, G89, A90, R91, G92, P93, Q94, I95 and L96 of SEQ ID NO: 5.
20
7. The procoagulant protein according to any one of claims 1-5, wherein the epitope of (ii) comprises one or more residues selected from the group consisting of L36, P37, E38, G39, C40, Q41, P42, L43, V44, S45, S46, A47, V73, T74, L75, Q76, E77, E78, D79, A80, G81, E82, Y83, G84, C85, M86, R91, G92, P93, Q94, I95, L96, H97, R98, V99, S100 and L101 of SEQ ID NO: 5.
25
8. The procoagulant protein according to any one of claims 1-5, wherein the epitope of (ii) comprises one or more residues selected from the group consisting of V17, Q18, C19, H20, Y21, R22, L23, Q24, D25, V26, K27, A28, R91, G92, P93, Q94, I95, L96, H97, R98, V99, S100 and L101 of SEQ ID NO: 5.
30
9. The procoagulant protein according to any one of claims 1-5, wherein the epitope of (ii) comprises one or more residues selected from the group consisting of K118, I119, G120, S121, L122, A123, E124, N125 and A126, F127 of SEQ ID NO: 6.
35

10. The procoagulant protein according to any one of claims 1-5, wherein the epitope of (ii) comprises one or more residues selected from the group consisting of E5, T6, H7, K8, I9, G10, S11, L12, A13, E14, N15, A16, F17, S18, D19 and P20 of SEQ ID NO: 7.

5

11. The procoagulant protein according to any one of claims 1-5, wherein the epitope of (ii) comprises one or more residues selected from the group consisting of K133, I134, G135, S136, L137, A138, N140, A141, F142, S143, D144, P145 and A146 of SEQ ID NO: 2.

10

12. The procoagulant protein according to any one of claims 1-5, wherein the epitope of (ii) comprises K133, I134, G135, S136, L137, A138, N140, A141, F142, S143, D144, P145 and A146 of SEQ ID NO: 2.

15

13. The procoagulant protein according to claim 11 or 12, wherein the heavy chain of (ii) comprises:

- a CDR1 sequence of amino acids 49 to 53 (RYWMT) of SEQ ID NO: 40, wherein one of these amino acids may be substituted by a different amino acid; and/or
- a CDR2 sequence of amino acids 68 to 84 (EINPDSSTINYNP SLKD) of SEQ ID NO: 40, wherein one, two, three or four of these amino acids may be substituted by a different amino acid; and/or
- a CDR3 sequence of amino acids 117 to 121 (GVFTS) of SEQ ID NO: 40, wherein one, two or three of these amino acids may be substituted by a different amino acid;

20

and wherein the light chain of (ii) comprises:

- a CDR1 sequence of amino acids 43 to 58 (RSSQSLVHRNGNTYFH) of SEQ ID NO: 41, wherein one, two, three or four of these amino acids may be substituted with a different amino acid; and/or
- a CDR2 sequence of amino acids 74 to 80 (KVS NRFS) of SEQ ID NO: 41, wherein one or two of these amino acids may be substituted with a different amino acid; and/or
- a CDR3 sequence of amino acids 113 to 121 (SQSTHVPYT) of SEQ ID NO: 41, wherein one or two of these amino acids may be substituted with a different amino acid.

25

30

14. The procoagulant protein according to any one of claims 1-13, which is a conjugate of (i) and (ii).

35

15. A process for preparing the conjugate of claim 13, comprising chemically conjugating (i), the TLT-1 monoclonal antibody or fragment thereof, with one reactive group (RS1) of a linker and reacting (ii), the coagulation factor, with another reactive group (RS2) of said linker.

5

16. The process as defined in any one of claims 11-13, wherein said linker is a polymer.

17. The procoagulant protein of any one of claims 1-14 for use as a medicament.

10

Fig. 1

M G L T L L L L L L L L G L E G Q G I
 1 ATGGGCCTC ACCCTGCTC TTGCTGCTG CTCCTGGGA CTAGAAGGT CAGGGCATA

 V G S L P E V L Q A P V G S S I L V
 55 GTTGGCAGC CTCCTGAG GTGCTGCAG GCACCCGTG GGAAGCTCC ATTCTGGTG

 Q C H Y R L Q D V K A Q K V W C R F
 109 CAGTGCCAC TACAGGCTC CAGGATGTC AAAGCTCAG AAGGTGTGG TGCCGGTTC

 L P E G C Q P L V S S A V D R R A P
 163 TTGCCGGAG GGGTGCCAG CCCCTGGTG TCCTCAGCT GTGGATCGC AGAGCTCCG

 A G R R T F L T D L G G G L L Q V E
 217 GCGGGCAGG CGTACGTTT CTCACAGAC CTGGGTGGG GGCCTGCTG CAGGTGGAA

 M V T L Q E E D A G E Y G C M V D G
 271 ATGGTTACC CTGCAGGAA GAGGATGCT GGCGAGTAT GGCTGCATG GTGGATGGG

 A R G P Q I L H R V S L N I L P P E
 325 GCCAGGGGG CCCAGATT TTGCACAGA GTCTCTCTG AACATACTG CCCCAGAG

 E E E E T H K I G S L A E N A F S D
 379 GAAGAAGAA GAGACCCAT AAGATTGGC AGTCTGGCT GAGAACGCA TTCTCAGAC

 P A G S A N P L E P S Q D E K S I P
 433 CCTGCAGGC AGTGCCAAC CCTTTGGAA CCCAGCCAG GATGAGAAG AGCATCCCC

L I W G A V L L V G L L V A A V V L
 487 TTGATCTGG GGTGCTGTG CTCCTGGTA GGTCTGCTG GTGGCAGCG GTGGTGCTG

F A V M A K R K Q G N R L G V C G R
 541 TTTGCTGTG ATGGCCAAG AGGAAACAA GGGAACAGG CTTGGTGTC TGTGGCCGA

 F L S S R V S G M N P S S V V H H V
 595 TTCTGAGC AGCAGAGTT TCAGGCATG AATCCCTCC TCAGTGGTC CACCACGTC

 S D S G P A A E L P L D V P H I R L
 649 AGTGACTCT GGACCGGCT GCTGAATTG CCTTTGGAT GTACCACAC ATTAGGCTT

 D S P P S F D N T T Y T S L P L D S
 703 GACTACCA CCTTCATTT GACAATACC ACCTACACC AGCCTACCT CTTGATTCC

 P S G K P S L P A P S S L P P L P P
 757 CCATCAGGA AAACCTTCA CTCCCAGCT CCATCCTCA TTGCCCCCT CTACCTCCT

 K V L V C S K P V T Y A T V I F P G
 811 AAGGTCTG GTCTGCTCC AAGCCTGTG ACATATGCC ACAGTAATC TTCCCGGA

 G N K G G G T S C G P A Q N P P N N
 865 GGGAAACAAG GGTGGAGGG ACCTCGTGT GGGCCAGCC CAGAATCCA CCTAACAA

 Q T P S S
 919 CAGACTCCA TCCAGC

Fig. 2

```

      HindIII           M   G   L   T   L   L   L   L   L   L   L   G   L
1  AAGCTTGCC GCCACCATG GGCCTCACC CTGCTCTTG CTGCTGCTC CTGGGACTA
   E   G   Q   G   I   V   G   S   L   P   E   V   L   Q   A   P   V   G
55 GAAGGTCAG GGCATAGTT GGCAGCCTC CCTGAGGTG CTGCAGGCA CCCGTGGGA
   S   S   I   L   V   Q   C   H   Y   R   L   Q   D   V   K   A   Q   K
109 AGCTCCATT CTGGTGCAG TGCCACTAC AGGCTCCAG GATGTCAAA GCTCAGAAG
   V   W   C   R   F   L   P   E   G   C   Q   P   L   V   S   S   A   V
163 GTGTGGTGC CGGTTCTTG CCGGAGGGG TGCCAGCCC CTGGTGTCC TCAGCTGTG
   D   R   R   A   P   A   G   R   R   T   F   L   T   D   L   G   G   G
217 GATCGCAGA GCTCCGGCG GGCAGGCGT ACGTTTCTC ACAGACCTG GGTGGGGGC
   L   L   Q   V   E   M   V   T   L   Q   E   E   D   A   G   E   Y   G
271 CTGCTGCAG GTGGAATG GTTACCCTG CAGGAAGAG GATGCTGGC GAGTATGGC
   C   M   V   D   G   A   R   G   P   Q   I   L   H   R   V   S   L   N
325 TGCATGGTG GATGGGGCC AGGGGGCCC CAGATTTTG CACAGAGTC TCTCTGAAC
   I   L   P   P   E   E   E   E   E   T   H   K   I   G   S   L   A   E
379 ATACTGCCC CCAGAGGAA GAAGAAGAG ACCCATAAG ATTGGCAGT CTGGCTGAG
   N   A   F   S   D   P   A   G   S   A   N   P   L   E   P   S   Q   D
433 AACGCATTC TCAGACCCT GCAGGCAGT GCCAACCCT TTGGAACCC AGCCAGGAT

      E   K   S   I   P   H   H   H   H   H   H   *   EcoRI
487 GAGAAGAGC ATCCCCCAC CATCACCAT CACCATTAA GAATTC

```


1	2	3	4	5	6	
123456789012345678901234567890	12345AB678901234567890	012ABC34567890				Kabat
EVKLLSEGGGLVQPGGSLKLSCAASGFD	FSRYWMT--WVRQAPGKGLEWIGEIN--	PDSSTINYT				0012HC-V

7	8	9	10	11	
1234567890123456789012ABC345678901234	567890ABCDEF	GHIJK12	34567890123		Kabat
PSLKDKFIIISRDNAKNTLYLQMS	EVRS	EDALYYCASGVFTS-----	WGQGT	LVTVSA	0012HC-V

Fig. 4



1 M K L P V G L L V L M F W I P A S S
ATGAAGTGT CCTGTTGGG CTGTTGGTG CTGATGTTT TGGATTCCA GCTTCCAGC

55 S D V V M T Q T P L S L P V S L G D
AGTGATGTT GTGATGACC CAAACTCCA CTCTCCCTG CCTGTCAGT CTTGGAGAT

109 Q A S I S C R S S Q S L V H R N G N
CAAGCCTCC ATCTCTTGC AGATCTAGT CAGAGCCTT GTACACAGA AATGGAAAC

163 T Y F H W C L Q K P G Q S P K L L I
ACCTATTTT CATTGGTGC CTGCAGAAA CCAGGCCAG TCTCCAAAG CTCCTGATC

217 Y K V S N R F S G V P D R F S G S G
TACAAAGTT TCCAACCGA TTTTCTGGG GTCCCAGAC AGGTTCAGT GGCAGTGGA

271 S G T D F T L K I S R V E A E D L G
TCAGGGACA GATTTCACA CTCAAGATC AGCAGAGTG GAGGCTGAG GATCTGGGA

325 V Y F C S Q S T H V P Y T F G G G T
GTTTATTTT TGCTCTCAA AGTACACAT GTTCCGTAC ACGTTCGGA GGGGGGACC

379 K L E I K R T V A A P S V F I F P P
AAGCTGGAA ATAAAACGT ACGGTGGCT GCACCATCT GTCTTCATC TTCCCGCCA

433 S D E Q L K S G T A S V V C L L N N
TCTGATGAG CAGTTGAAA TCTGGAAC TGCCTCTGTT GTGTGCCTG CTGAATAAC

487 F Y P R E A K V Q W K V D N A L Q S
TTCTATCCC AGAGAGGCC AAAGTACAG TGGAGGTG GATAACGCC CTCCAATCG

541 G N S Q E S V T E Q D S K D S T Y S
GGTAACTCC CAGGAGAGT GTCACAGAG CAGGACAGC AAGGACAGC ACCTACAGC

595 L S S T L T L S K A D Y E K H K V Y
CTCAGCAGC ACCCTGACG CTGAGCAAA GCAGACTAC GAGAAACAC AAAGTCTAC

649 A C E V T H Q G L S S P V T K S F N
GCCTGCGAA GTCACCCAT CAGGGCCTG AGCTCGCCC GTCACAAAG AGCTTCAAC

703 R G E C E D Q V D P R L I D G K
AGGGGAGAG TGTGAGGAC CAGGTGGAC CCCAGACTG ATCGACGGC AAG

Fig. 5



```

      M D F G L I F F I V A L L K G V Q C
1  ATGGATTTT GGGCTGATT TTTTATT GTTGCTCTT TTAAAGGG GTCCAGTGT

      E V K L L E S G G G L V Q P G G S L
55 GAGGTGAAA CTCTCGAG TCTGGAGGT GGCCTGGTG CAGCCTGGA GGATCCCTG

      K L S C A A S G F D F S R Y W M T W
109 AAACCTCTCC TGTGCAGCC TCAGGATTC GATTTTAGT AGATACTGG ATGACTTGG

      V R Q A P G K G L E W I G E I N P D
163 GTCCGGCAG GCTCCAGGG AAAGGGCTA GAATGGATT GGAGAAATT AATCCAGAT

      S S T I N Y T P S L K D K F I I S R
217 AGCAGTACG ATAAACTAT ACGCCATCT CTAAAGGAT AAATTCATC ATCTCCAGA

      D N A K N T L Y L Q M S E V R S E D
271 GACAACGCC AAGAATACG CTGTACCTG CAAATGAGC GAAGTGAGA TCTGAGGAC

      T A L Y Y C A S G V F T S W G Q G T
325 ACAGCCCTT TATTACTGT GCAAGCGGG GTGTTTACT TCCTGGGGC CAAGGGACT

      L V T V S A A S T K G P S V F P L A
379 CTGGTCACT GTCTCTGCA GCTAGCACC AAGGGCCCA TCCGTCTTC CCCCTGGCG

      P C S R S T S E S T A A L G C L V K
433 CCCTGCTCC AGGAGCACC TCCGAGAGC ACAGCCGCC CTGGGCTGC CTGGTCAAG

      D Y F P E P V T V S W N S G A L T S
487 GACTACTTC CCCGAACCG GTGACGGTG TCGTGGAAC TCAGGCGCC CTGACCAGC

      G V H T F P A V L Q S S G L Y S L S
541 GGCGTGCAC ACCTTCCCG GCTGTCCTA CAGTCCTCA GGACTCTAC TCCCTCAGC

      S V V T V P S S S L G T K T Y T C N
595 AGCGTGGTG ACCGTGCCC TCCAGCAGC TTGGGCACG AAGACCTAC ACCTGCAAC

      V D H K P S N T K V D K R V E S K Y
649 GTAGATCAC AAGCCAGC AACACCAAG GTGGACAAG AGAGTTGAG TCCAAATAT

      G P P C P P C P A P E F L G G P S V
703 GGTCCCCCA TGCCACCA TGCCAGCA CCTGAGTTC CTGGGGGGA CCATCAGTC

      F L F P P K P K D T L M I S R T P E
757 TTCCTGTTC CCCCCAAA CCAAGGAC ACTCTCATG ATCTCCCGG ACCCCTGAG

      V T C V V V D V S Q E D P E V Q F N
811 GTCACGTGC GTGGTGGTG GACGTGAGC CAGGAAGAC CCCGAGGTC CAGTTCAAC

      W Y V D G V E V H N A K T K P R E E
865 TGGTACGTG GATGGCGTG GAGGTGCAT AATGCCAAG ACAAGCCG CGGGAGGAG

      Q F N S T Y R V V S V L T V L H Q D
919 CAGTTCAAC AGCACGTAC CGTGTGGTC AGCGTCCTC ACCGTCTG CACCAGGAC

      W L N G K E Y K C K V S N K G L P S
973 TGGCTGAAC GGCAAGGAG TACAAGTGC AAGGTCTCC AACAAAGGC CTCCCGTCC

      S I E K T I S K A K G Q P R E P Q V
1027 TCCATCGAG AAAACCATC TCCAAAGCC AAAGGGCAG CCCGAGAG CCACAGGTG

      Y T L P P S Q E E M T K N Q V S L T
1081 TACACCCCTG CCCCATCC CAGGAGGAG ATGACCAAG AACCAGGTC AGCCTGACC

```

1135 C L V K G F Y P S D I A V E W E S N
TGCCTGGTC AAAGGCTTC TACCCAGC GACATCGCC GTGGAGTGG GAGAGCAAT

1189 G Q P E N N Y K T T P P V L D S D G
GGGCAGCCG GAGAACAAC TACAAGACC ACGCCTCCC GTGCTGGAC TCCGACGGC

1243 S F F L Y S R L T V D K S R W Q E G
TCCTTCTTC CTCTACAGC AGGCTAACC GTGGACAAG AGCAGGTGG CAGGAGGGG

1297 N V F S C S V M H E A L H N H Y T Q
AATGTCTTC TCATGCTCC GTGATGCAT GAGGCTCTG CACAACCAC TACACACAG

1351 K S L S L S L G K
AAGAGCCTC TCCCTGTCT CTGGGTAAA

Fig. 6



Fig. 7

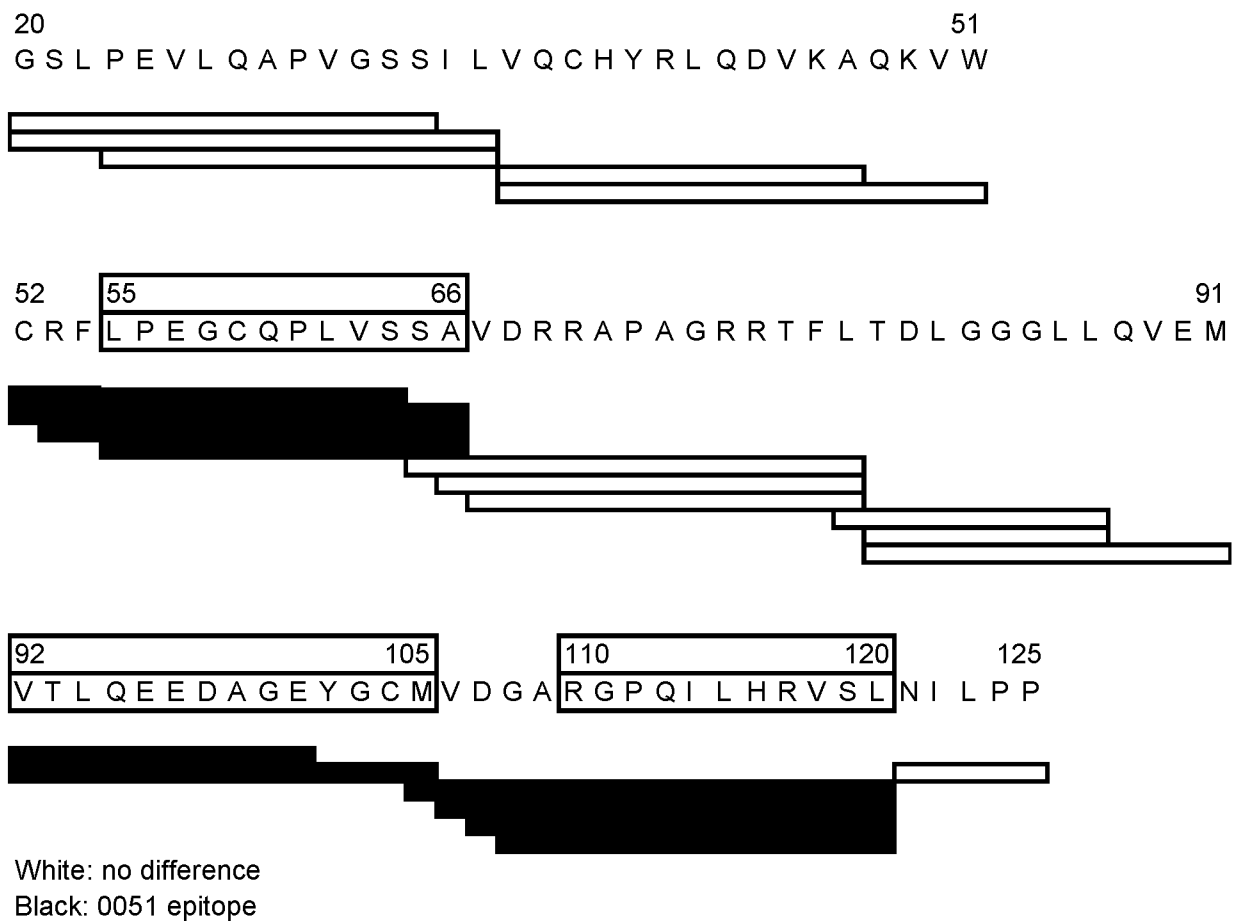
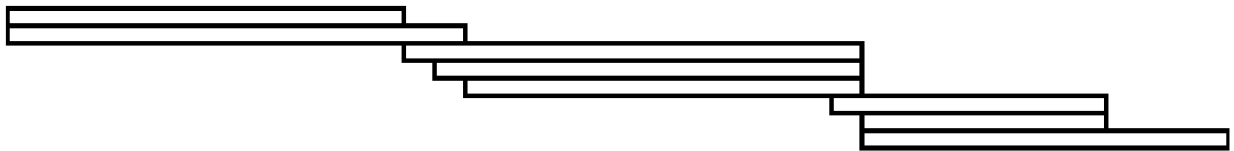


Fig. 8

20 36 47 51
G S L P E V L Q A P V G S S I L V Q C H Y R L Q D V K A Q K V W



52 91
C R F L P E G C Q P L V S S A V D R R A P A G R R T F L T D L G G G L L Q V E M

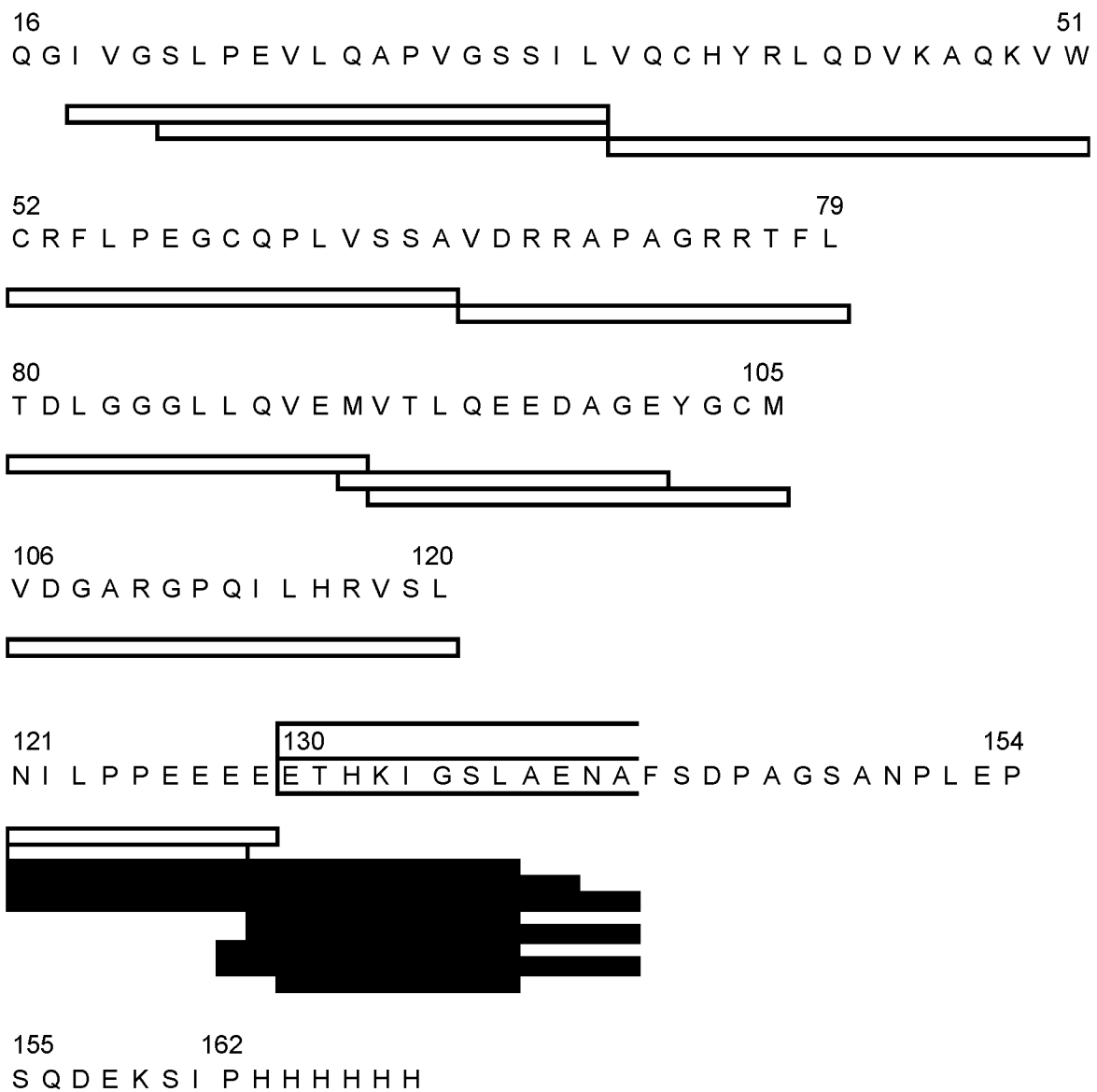


92 110 120 125
V T L Q E E D A G E Y G C M V D G A R G P Q I L H R V S L N I L P P



White: no difference
Black: 0062 epitope

Fig. 9



White: no difference

Black: 0061 epitope

Fig. 10

126 130 145 162
E E E E E T H K I G S L A E N A F S D P A G S A N P L E P S Q D E K S I P



White: no difference
Black: 0061 epitope

Fig. 11A

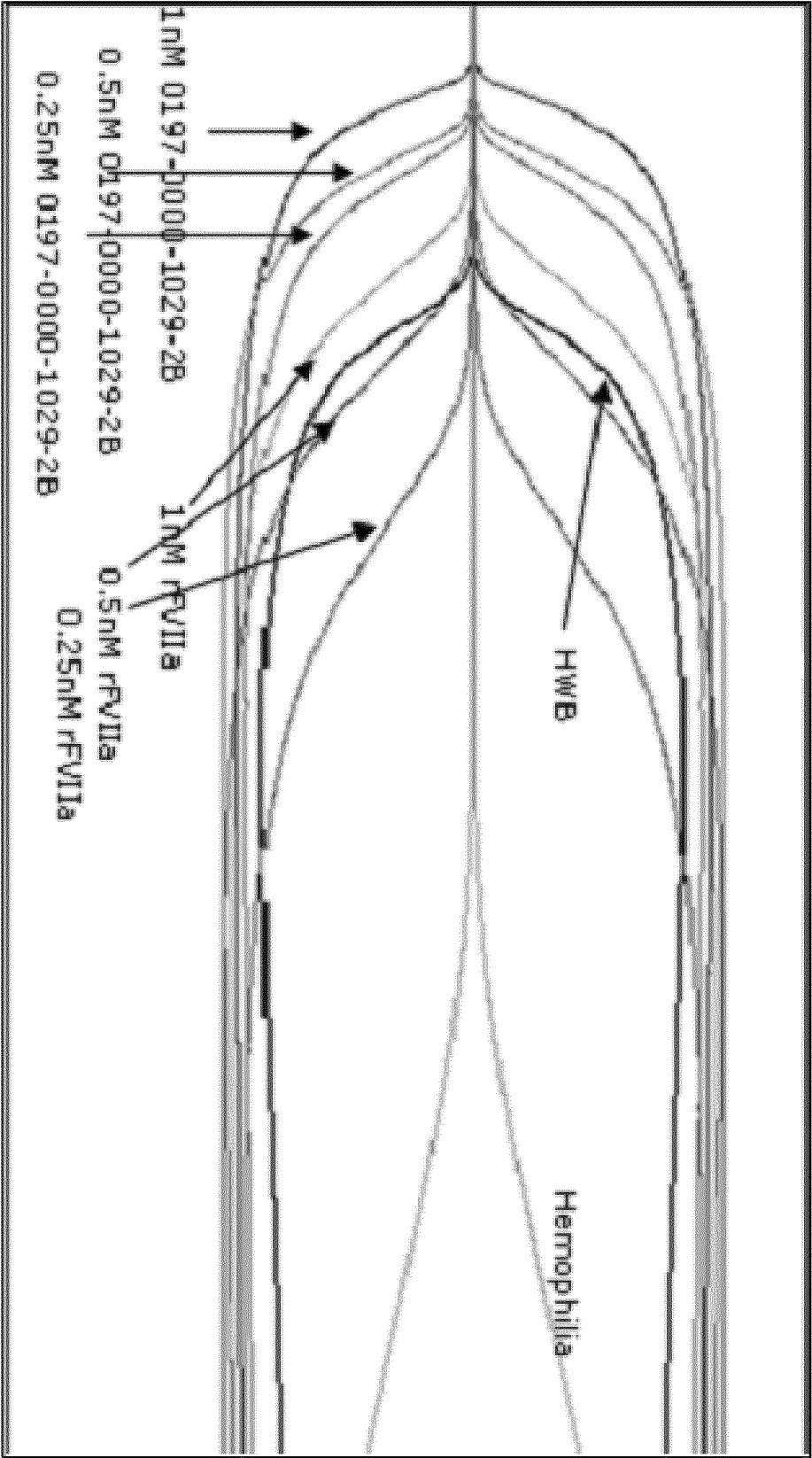


Fig. 11B

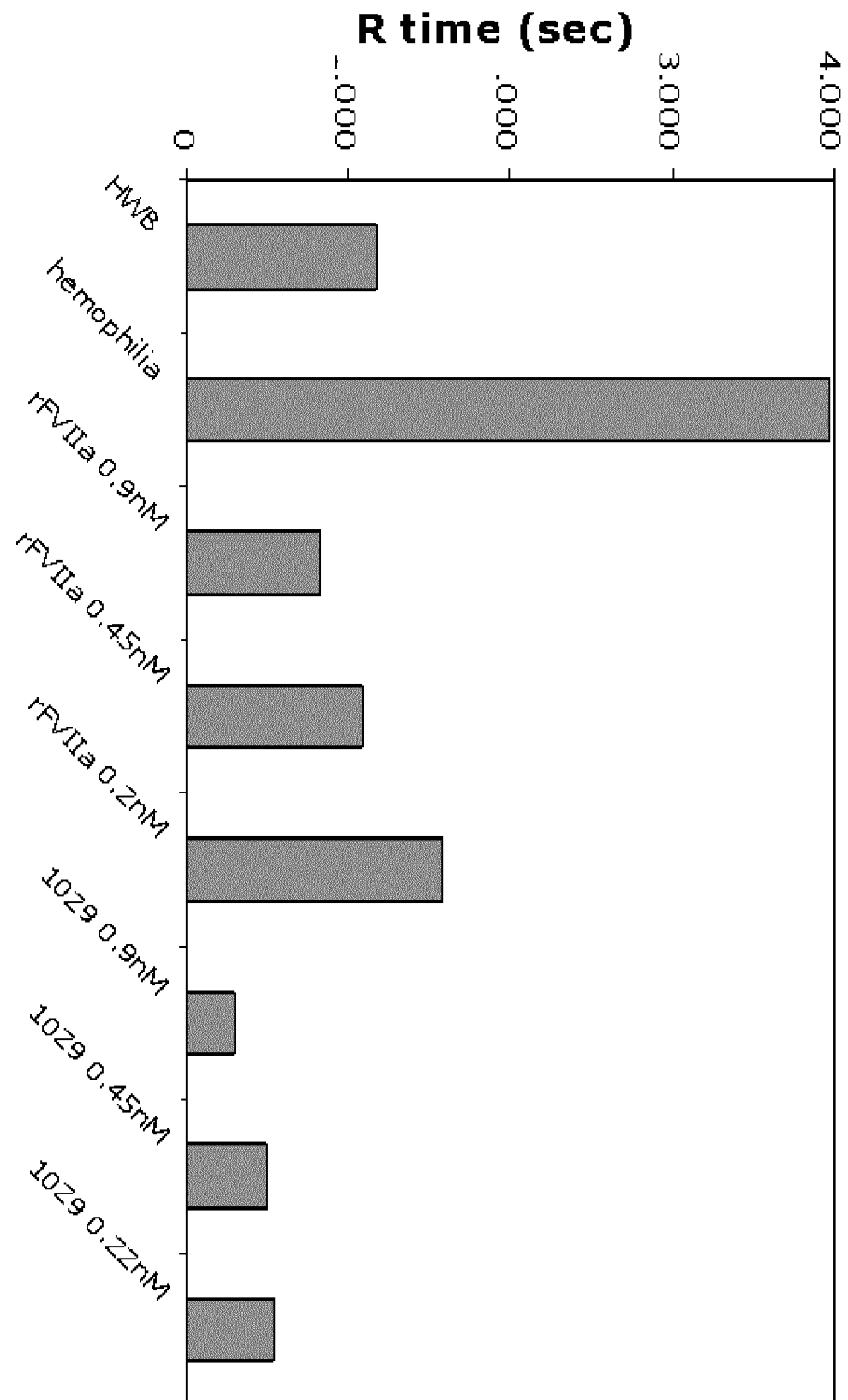


Fig. 12

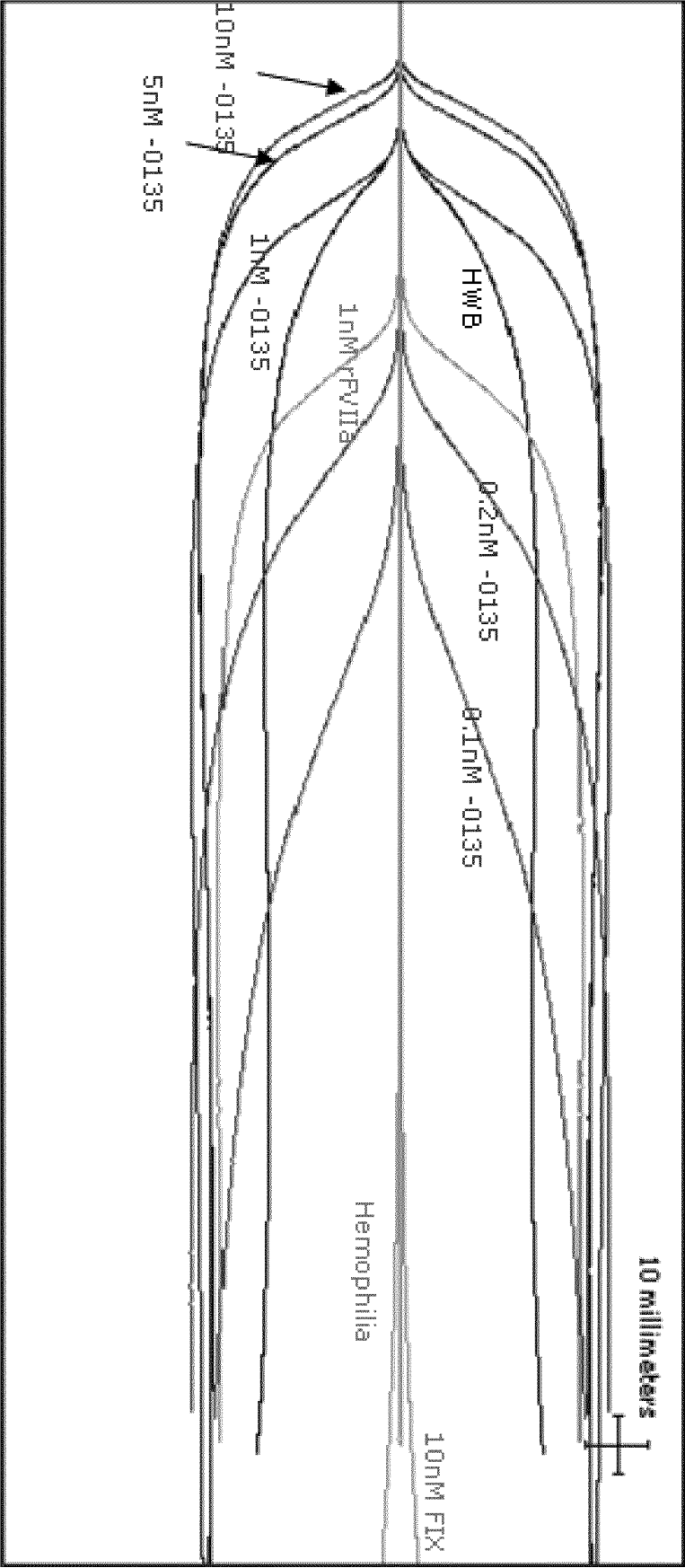


Fig. 13

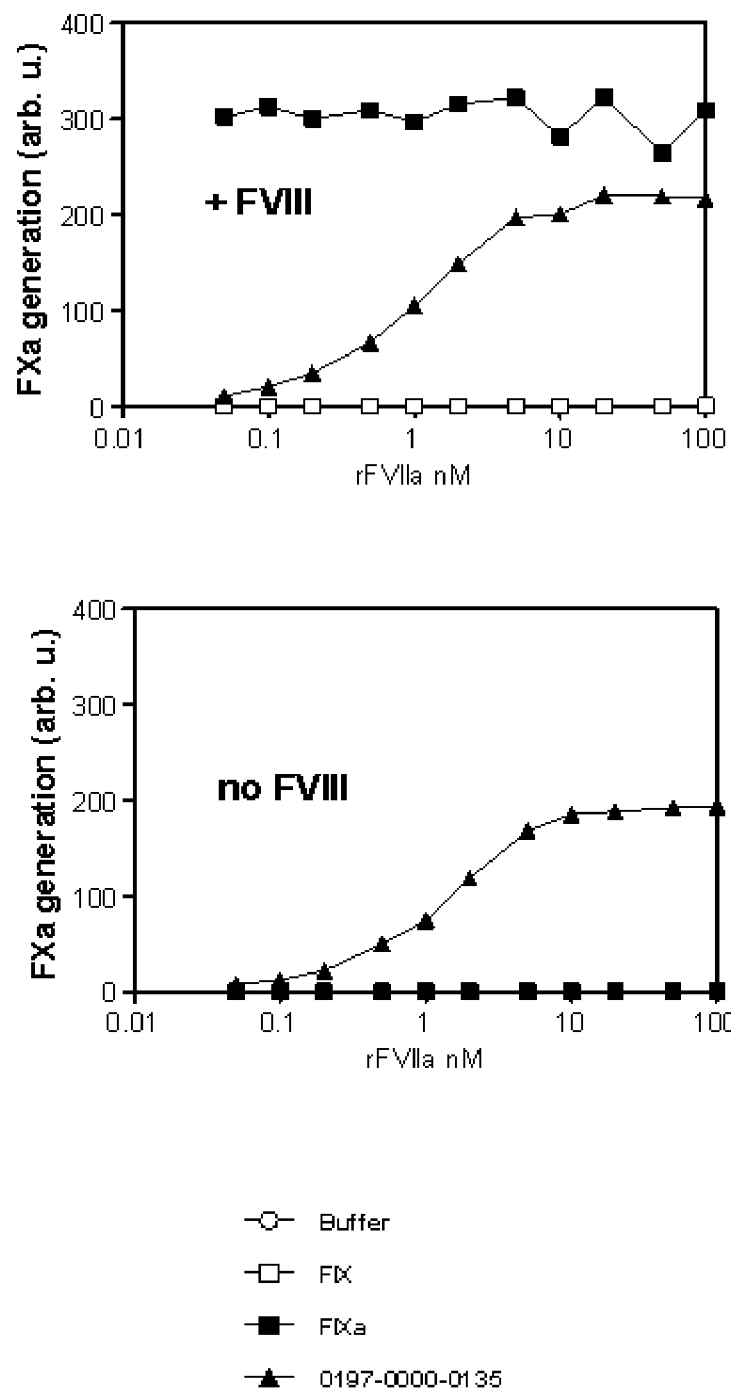


Fig. 14

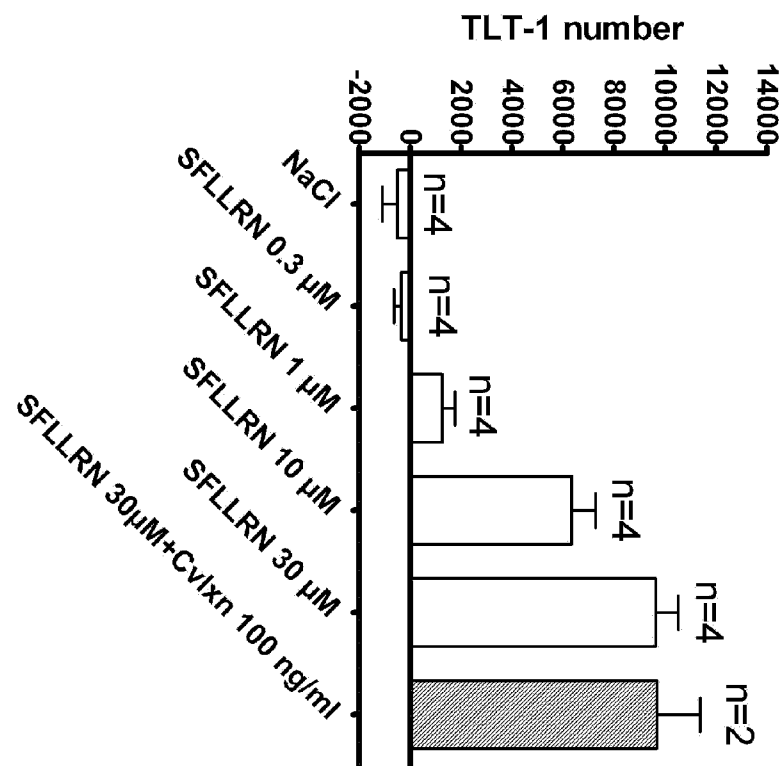
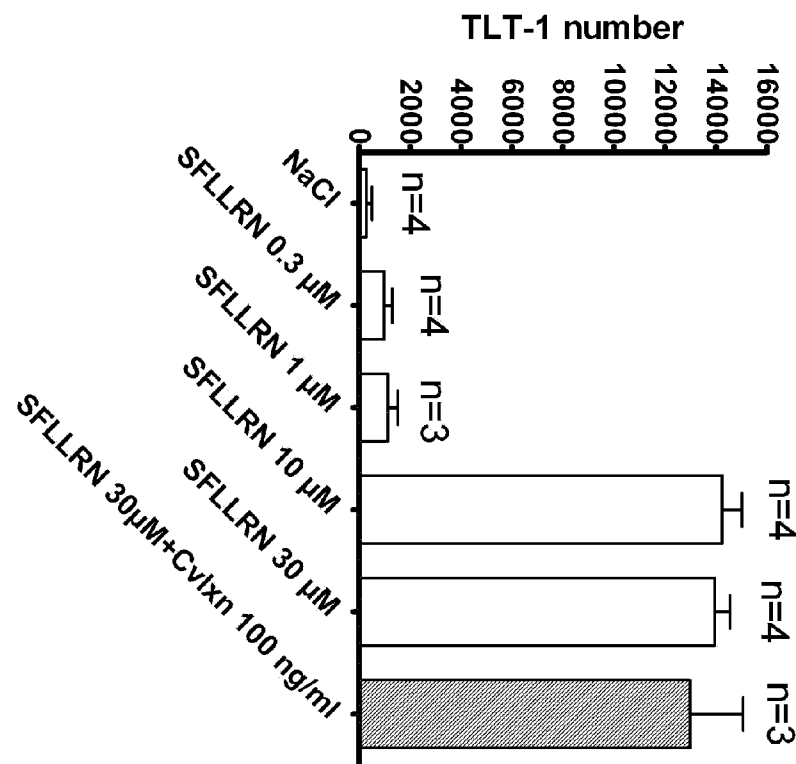


Fig. 15

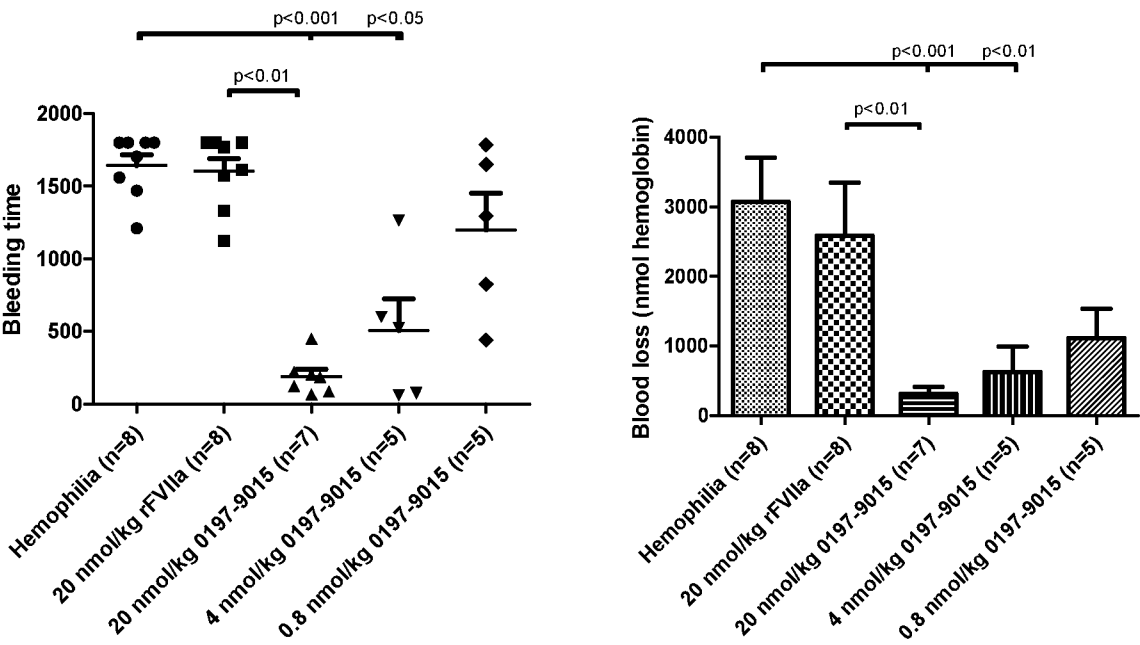


Fig. 16

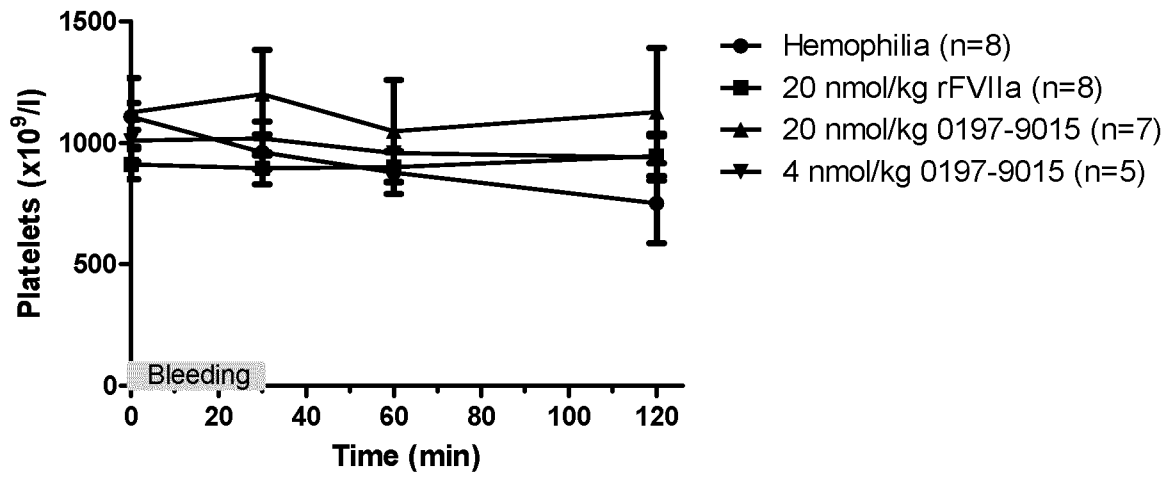


Fig. 17A

25nM FVII variants
Platelets activated with SFLLRN (30 μ M) and Convulxin (100ng/ml)

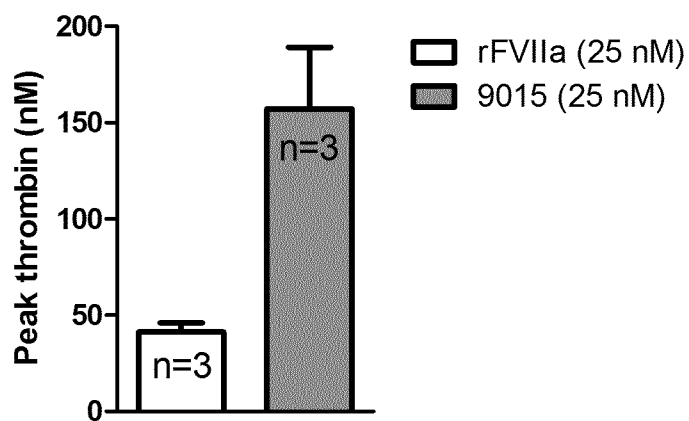


Fig 17B

5nM FVII variants
Platelets activated with SFLLRN (30 μ M) and Convulxin (100ng/ml)

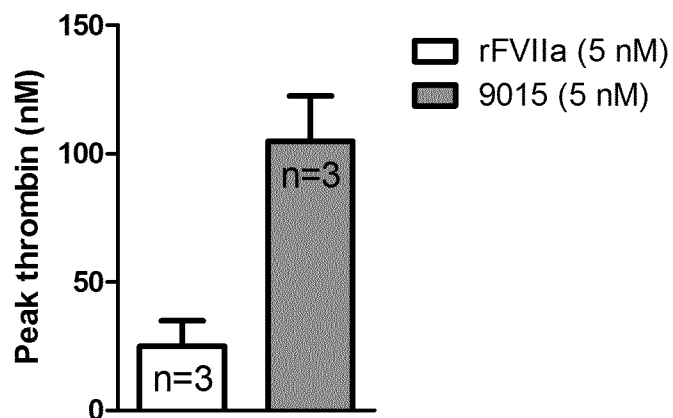


Fig. 18

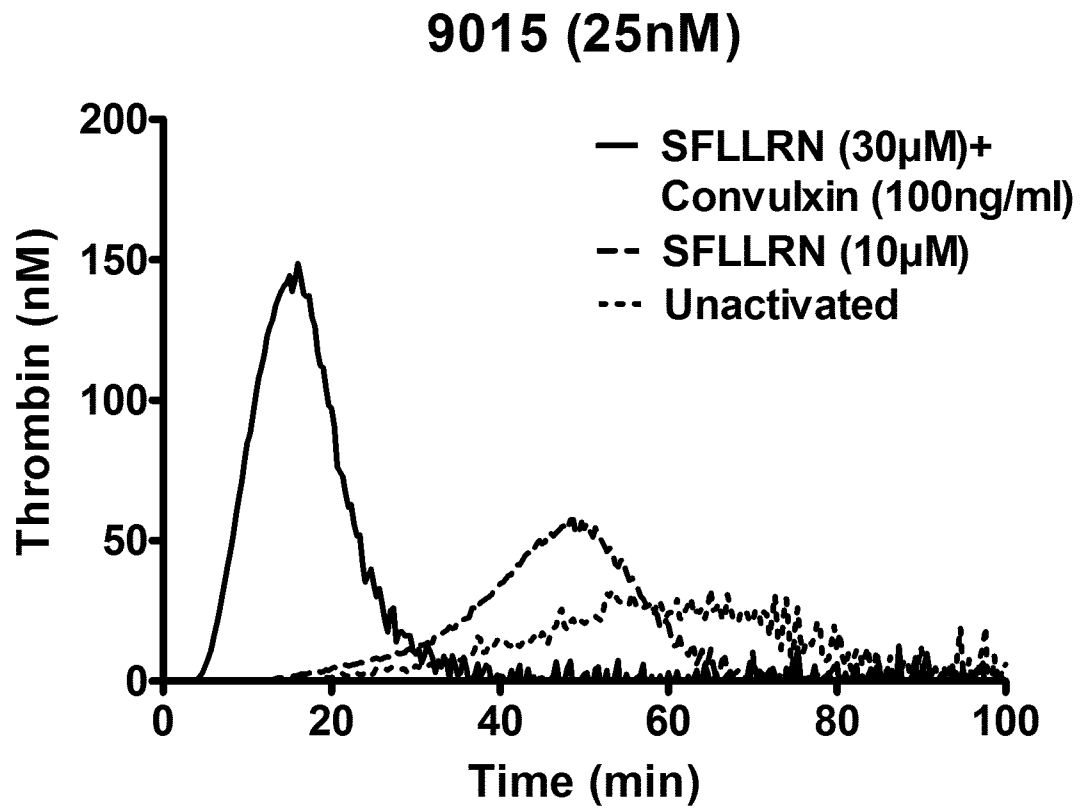


Fig. 19

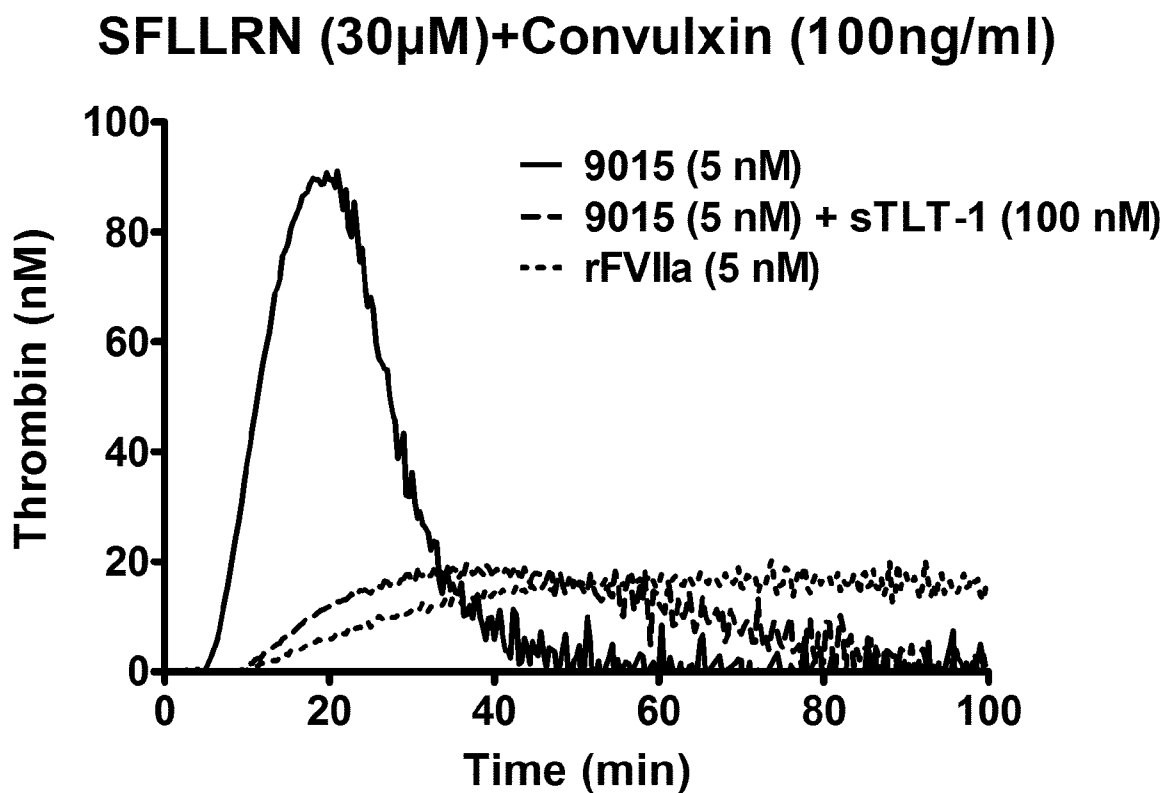


Fig. 20

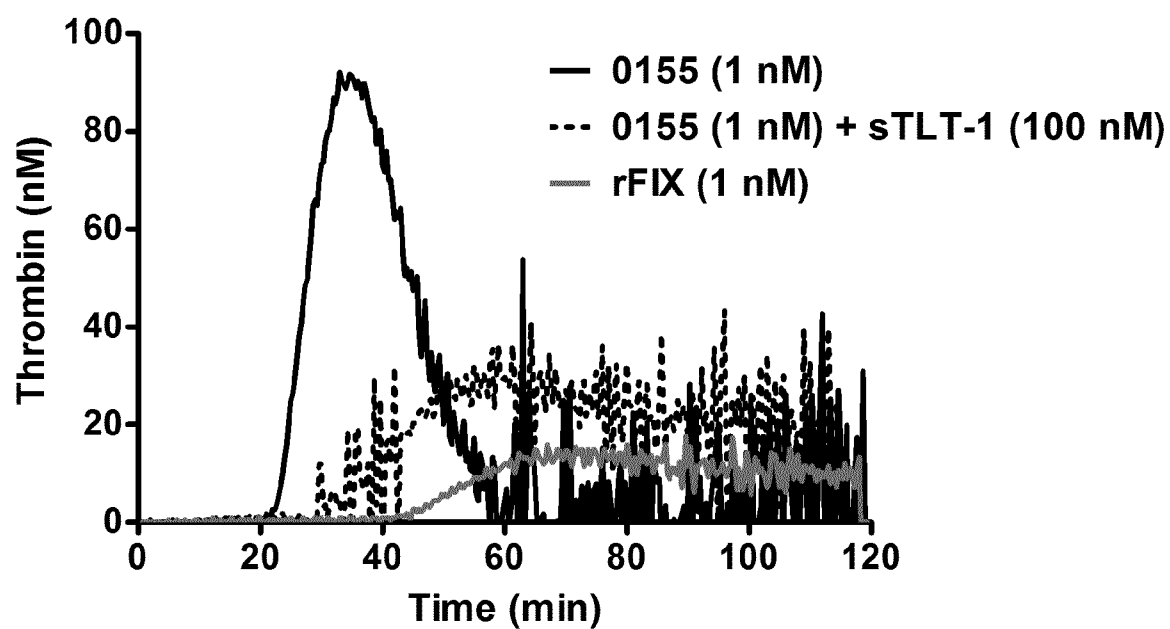


Fig. 21

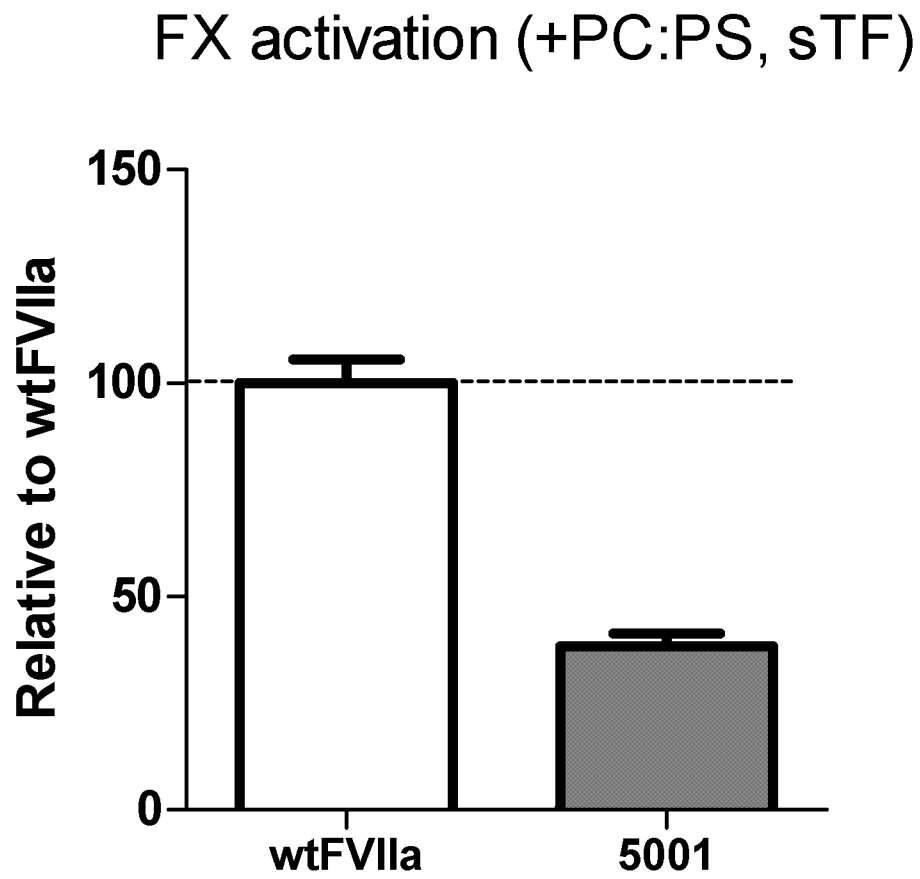


Fig. 22A

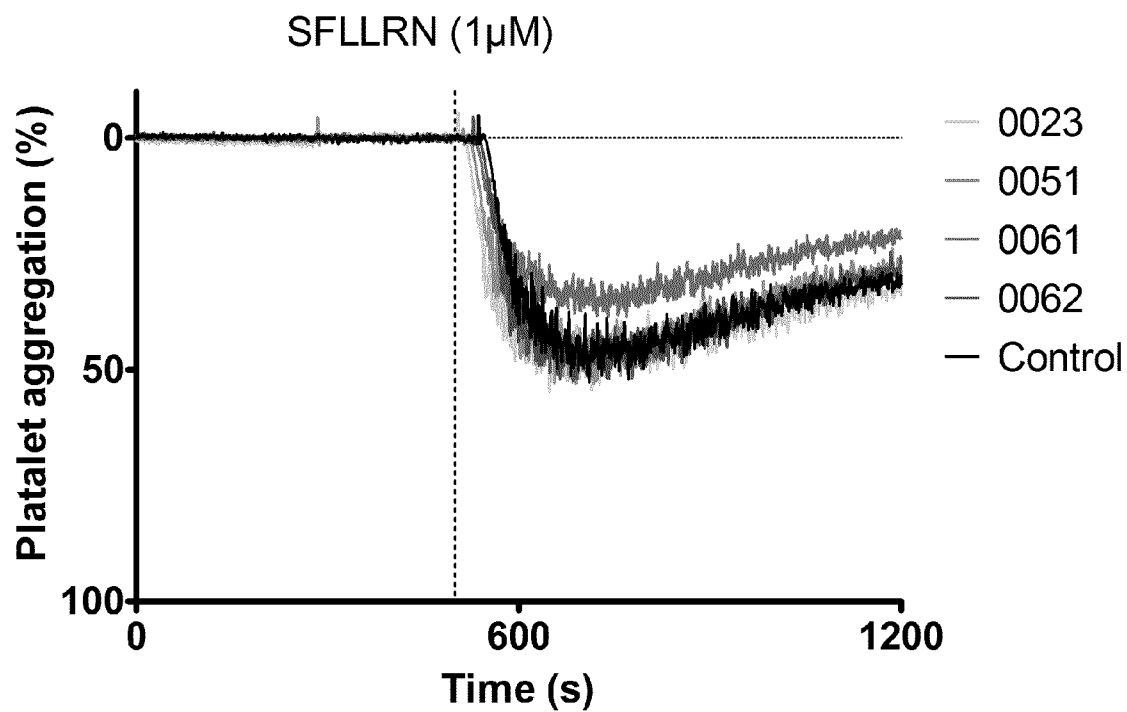


Fig. 22B

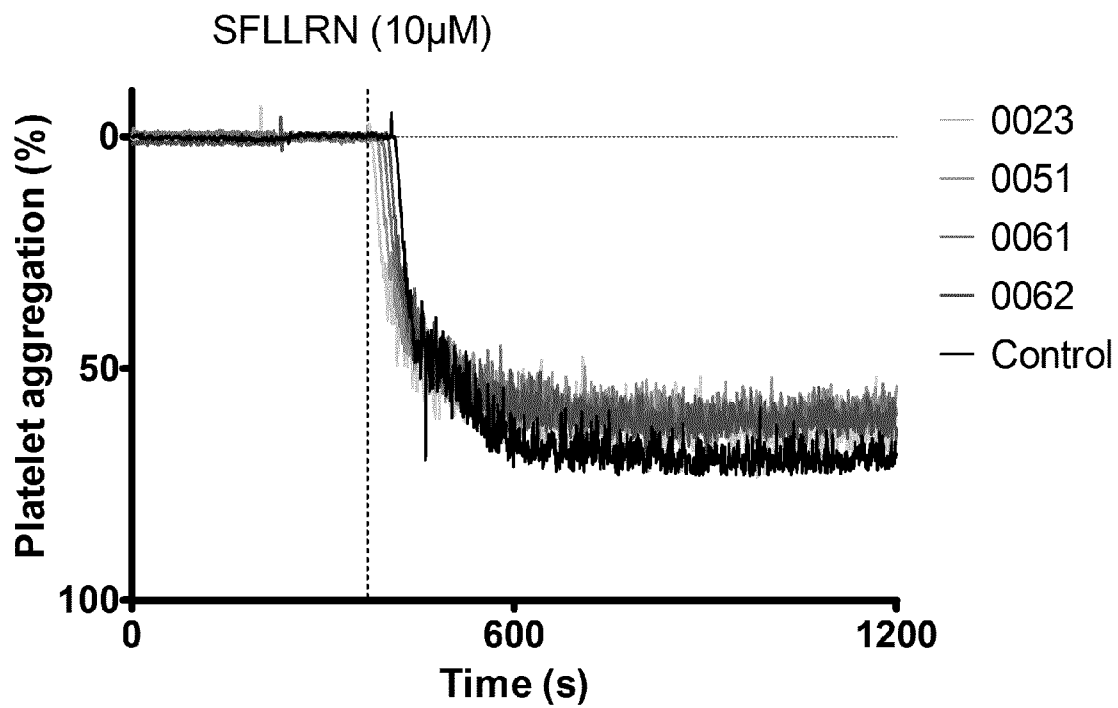


Fig. 23

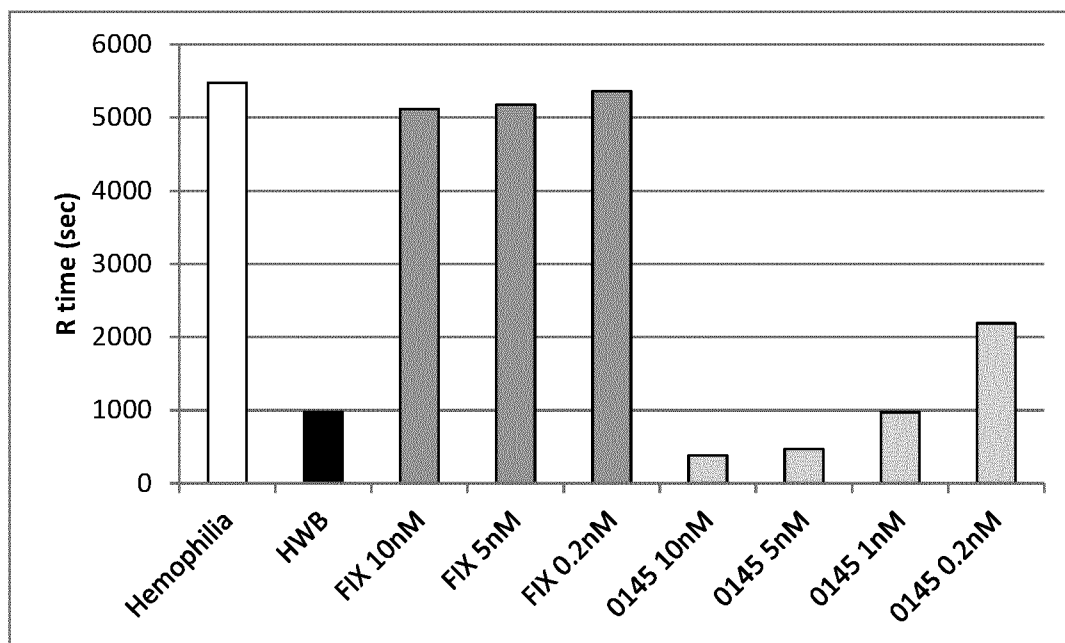


Fig. 24

25nM FVII variants
Platelets activated with SFLLRN (30 μ M)+Convulxin (100ng/ml)

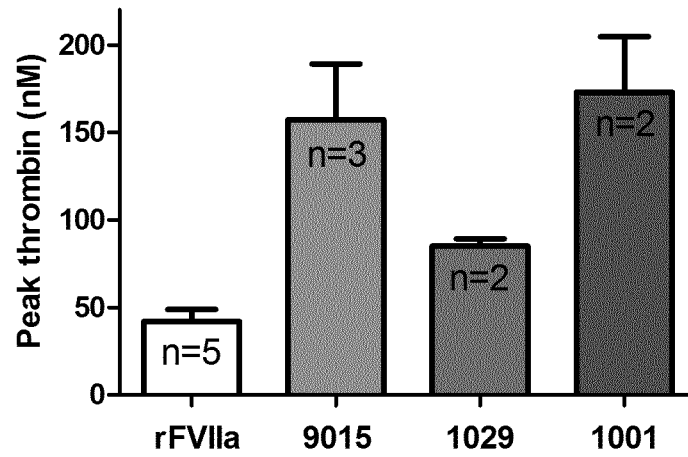
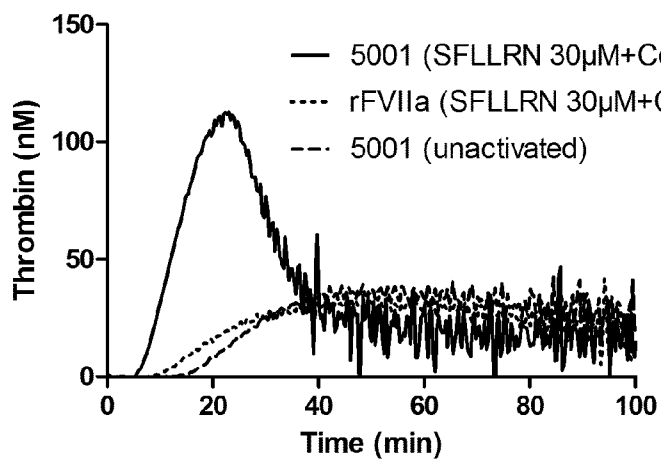


Fig. 25

FVIIa variants (25nM) in factor VIII deficient plasma with human platelets



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/053619

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/36 C07K14/16 C07K14/745
ADD. C07K14/745

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data, CHEM ABS Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GIOMARELLI BARBARA ET AL: "Inhibition of thrombin-induced platelet aggregation using human single-chain Fv antibodies specific for TREM-like transcript-I", THROMBOSIS AND HAEMOSTASIS, SCHATTAUER GMBH, DE; US, vol. 97, no. 6, 1 June 2007 (2007-06-01), pages 955-964, XP008127046, ISSN: 0340-6245, DOI: 10.1160/TH06-08-0456	1
A	the whole document	2-17
X	US 2008/131423 A1 (MORI TOSHIYUKI [JP] ET AL) 5 June 2008 (2008-06-05) cited in the application	1
A	abstract; claims	2-17
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Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

14 May 2012

Date of mailing of the international search report

22/05/2012

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Meyer, Wolfram

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2012/053619

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/096828 A2 (LIND STUART E [US]; DING WEI-QUN [US]; HARRISON ROGER G JR [US]) 14 September 2006 (2006-09-14)	1
A	abstract; claims -----	2-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2012/053619

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2008131423	A1	05-06-2008	NONE

WO 2006096828	A2	14-09-2006	AU 2006220513 A1 14-09-2006
			CA 2608070 A1 14-09-2006
			CN 101405017 A 08-04-2009
			EP 1877437 A2 16-01-2008
			WO 2006096828 A2 14-09-2006
