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# Fusion protein of single domain antibody and procoagulant


## Abstract

The present invention relates to single domain antibodies (sdAbs) against TREM (triggering receptors expressed on myeloid cells) like transcript-1 (TLT-1) molecules that are present on activated platelets at the site of an injury, and especially on a subset of activated platelets, coated platelets. Furthermore, the present invention relates to fusion proteins comprising sdAbs against TLT-1 and an extracellular (soluble) domain of tissue factor (sTF), to direct targeting of such fusion proteins to activated platelets at the site of injury through binding of the sdAbs to TLT-1, a membrane protein receptor that is only present on activated platelets. Specific interaction of sdAbs with the TLT-1 receptor positions the sTF domain of the fusion to interact with, and activate, FVII. As a result, a targeted procoagulant effect is achieved at the site of injury via activated platelets. The fusion proteins are useful to treat individuals that have a bleeding disorder, such as hemophilia A, hemophilia B, or acute bleeding due to traumatic injury.



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## Field of Classification Search

**USPC:** None

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## Background/Summary

(1) This application is a continuation of PCT/US2020/029599, filed Apr. 23, 2020; which claims the benefit of U.S. Provisional Application No. 62/844,610, filed May 7, 2019. The contents of the above-identified applications are incorporated herein by reference in their entirety.

REFERENCE TO SEQUENCE LISTING, TABLE OR COMPUTER PROGRAM
(1) The Sequence Listing is concurrently submitted herewith with the specification as an ASCII

formatted text file via EFS-Web with a file name of Sequence Listing.txt with a creation date of May 7, 2020, and a size of 87,400 bytes. The Sequence Listing filed via EFS-Web is part of the specification and is hereby incorporated in its entirety by reference herein.

## FIELD OF THE INVENTION

(2) The present invention relates to single domain antibodies (sdAbs) against TREM (triggering receptors expressed on myeloid cells) like transcript-1 (TLT-1) molecules that are present on activated platelets at the site of an injury, and especially on a subset of activated platelets, coated platelets. Furthermore, the present invention relates to fusion proteins comprising sdAbs and the extracellular (soluble) domain of tissue factor (sTF). Such fusion proteins direct sTF to activated platelets at the site of injury. Individuals that have a bleeding disorder, such as hemophilia A, hemophilia B, or acute bleeding due to traumatic injury are benefited from the treatment using such fusion proteins.

## BACKGROUND OF THE INVENTION

(3) Platelets normally circulate in blood flow in their resting stage. When blood vessels are injured, platelets interact with the damaged subendothelial cells via platelet glycoproteins (GP), such as GP Ib-IX-V and GP IIb/IIIa receptors, as well as tissue factor expressed there. This interaction initiates platelet adhesion, aggregation and activation at the site of injury as well as platelet shape change, and subsequent alpha- and dense-granule release. In addition to other membrane proteins, activated platelets express both P-selectin, that mediates interactions with leukocytes, and TLT-1 receptor, that enhances Ca.sup.++ influx and promotes platelet aggregation on the surface when platelets get activated. However, TLT-1 receptor is found to be expressed exclusively on the surface of activated platelets, making it an ideal target molecule for coagulation factor localization, since activated platelets are almost exclusively found at a site of injury, though they have been implicated in some other disease states. Activated, ‘coated’ platelets (Dale, 2005, S. Thromb. Haemost. Volume 3 pp. 2185-2192) can be defined as expressing P-selectin, GPIIb/IIIa, and CD40L proteins, among others, on the platelet surface. (Yun et al., 2016, Biomed Res. Inter., volume 2016, e9060143). This population of activated platelets also induces flipping and exposure of membrane phosphatidylserine (PS) to their surface that serves to mediate interaction with coagulation proteins. This negatively-charged surface of PS-containing platelet-derived membranes plays a critical role in activating prothrombinase complex formation, the final step in the coagulation pathway that drives thrombin production, with subsequent fibrin formation.

(4) Hemostasis is a natural clotting mechanism that takes place at the site of an injury to prevent excessive bleeding. The ideal therapeutic molecules for treating a bleeding disorder should only act at the site of injury and therefore localize coagulation factors there—this principle is a key to therapeutic practices in hemostasis. NOVOSEVEN® (Novo Nordisk, Denmark), a recombinant FVIIa (recFVIIa) molecule produced in cultured mammalian cells, has been a mainstay of biological molecules to treat patients with inhibitors of the coagulation factors FVIII and FIX; as such, molecules like recombinant FVIIa are referred to as “by-pass agents”. Under normal hemostatic conditions, FVII circulating in blood is exposed to cell-bound tissue factor (TF) at sites of injury on the vascular adventitia, is activated by TF by their cooperative binding, and as part of the resulting complex, then cleaves FX to FXa. Recombinant FVIIa administration, as a stand-alone molecule, essentially “by-passes” the normal interaction of FVII and TF and acts on FX independently of tissue factor (that is primarily present and exposed only at the site of injury). However, to achieve this effect, FVIIa needs to be administered in pharmacologically large amounts in order to mimic the effects of natural FVII-TF activation (i.e. FX activation). Binding of recFVIIa to cell membranes, without interaction of TF, appears to be mediated by the exposure to phosphatidylserine in the lipid layer of activated platelets, and, much as plasma-derived FVII, mediates activation of FX through FVIIa active-site proteolysis. The high amounts of recFVIIa required for therapeutic efficacy in hemophilia A and B patients is believed to be due, at least in part, to low PS binding of the protein to platelets and a lack of cooperativity with TF. Other

“bypass agents”, like FEIBA (Baxter International), is composed of a mixture of plasma-derived coagulation factors, that includes only a small fraction of activated coagulation factors, like FVIIa, and can be used to treat hemophilia A and B patients with inhibitors; however, it is difficult to characterize this product due to the nature and variability of its diverse contents.

(5) Rather than relying strictly on the properties of coagulation factors themselves, phosphatidylserine-binding proteins, such as annexin V and lactadherin C-2 proteins, have been considered as potential targeting vehicles to direct coagulation factors and other molecules to the lipid bilayers of activated platelets at a site of injury in order to accelerate clot production. Annexin V, for example, has high-affinity and high-specificity for PS in membranes (Thiagarajan and Tait, 1991, J. Biol. Chem., volume 266, pp. 24302-24307; Rescher and Gerke, 2004, J. Cell Sci., volume 117, pp. 2631-2639) making it ideal for targeting activated platelets. Fusion proteins that incorporate these domains with coagulation factors represent an alternative method for interaction with activated platelets but with a higher affinity than might be achieved with recFVIIa alone, for example. The extracellular domain of tissue factor fused to annexin V has been shown to be extremely potent in stemming blood flow in bleeding models (Huang et al., 2006, Blood, volume 107, pages 980-986) and represents a potential “by-pass” agent. Unfortunately, despite their potential utility, molecules like annexin V that specifically bind to PS have several downsides: phosphatidylserine can be expressed on non-platelet surfaces like apoptotic or dying cells, as well as other cell types, in addition to activated platelets, and PS-binding proteins or their fusions can compete with other coagulation factors for binding to PS on activated platelet surfaces and thereby limit coagulation processes (Thiagarajan and Tait, 1991, J. Biol. Chem., volume 266, pp. 24302-24307).

(6) An alternative means for achieving high-affinity and high-specificity targeting to specific cell types is through antibodies. Monoclonal antibodies are used extensively to target therapeutic molecules to variety of the cells and platelets. These include both delivery of specific drugs to cancer targets (e.g., Yang et al., 2018, Biotechnol. Lett., volume 40, pp. 789-795; Khongorzul et al., 2020, Mol. Cancer Res., volume 18, pp. 3-19) or to damaged tissue (Runge et al., 1987, Proc. Natl. Acad. Sci. (USA), volume 84, pp. 7659-7662). In general, their large molecular size (150 kDa; even larger size as a fusion protein) and the constraints to their flexibility as a function of their complex heavy and light chain architecture and post-translational modifications, can lead to lower accessibility of some relevant target epitopes and relatively high production and purification costs, respectively, thereby limiting their use in developing therapeutically-useful fusion protein derivatives. In addition, their long plasma half-lives can be a detriment where short-lived and self-regulating attributes may be desired. In fact, few molecular fusions involving monoclonal antibodies have successfully been produced or used.

(7) By contrast, single-domain antibodies (sdAbs), also known as nanobodies or domains, are antibodies that derive from heavy-chain-only antibodies present in sera of members of the family Camelidae (FIG. **1**); similar sdAbs have also been identified in some members of the class Chondrichthyes. Camelid antibodies are devoid of the heavy-chain CH1 domain and thus do not support binding to a cognate light chain fragments as do other mammals. The variable domain of the heavy chain immunoglobulin (so-called VHH) is the smallest available intact antigen-binding domain derived from a functional immunoglobulin, ranging from 1.2-15 kDa in molecular weight. The VHH, unlike variable regions of other mammalian heavy and light chains, are able to intercalate or penetrate into domain clefts that are otherwise inaccessible to conventional antibodies or their derivatives that generally bind to epitopes on the surface of proteins (e.g., Schmitz et al., 2013, Structure, volume 21, pp. 1214-1224).

(8) Tissue factor (TF), the primary initiator of coagulation, is a membrane-bound protein not normally expressed on the surface of cells in contact with the bloodstream. With vascular injury, subendothelial TF becomes exposed to blood flow and binds plasma factor VII. The resulting complex initiates an extrinsic cascade of coagulation activation steps, and specific enzymatic

reactions, that ultimately culminate in clot formation and vascular sealing. Neither full-length TF, nor its soluble extracellular domain (sTF), can be used as a therapeutic molecule on its own. This is because, on the one hand, the potent and generalized activation of the coagulation system by full-length TF causes massive and disseminated thrombus formation that was already noted early in the twentieth century (Howell, 1912, Am. J. Physiol., volume 31, pages, p. 1-21). On the other hand, sTF is orders of magnitude less potent than the full-length form: membrane anchoring of TF is essential to support full proteolytic activity of FVIIa (Paborsky, 1991; Petrillo, 2010); as a result, sTF itself is essentially non-functional, especially at lower doses (Morrissey, U.S. Pat. No. 5,504,067).

(9) Molecular agents to stem bleeding are critical for patients suffering from genetic diseases, like hemophilia A or B, but also from severe injuries, due to accidents, surgery or other traumatic events. Over the years, only an exceedingly small number of molecular entities have been created that are able to demonstrate efficacy in use in bleeding diathases, and even then, concern about potential excessive thrombotic side-effects, as well as drug costs, have made their use impractical.

(10) There remains a considerable need to identify affordable and efficacious biological entities for treating bleeding disorders. Such entities will need to demonstrate critical attributes to fill in the areas of need beyond those served by normal or extended half-life coagulants, like long-acting FVIII or FIX, or to newer molecules, like more potent recombinant FVIIa molecules that appear to have untoward side-effects.

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## Description

BRIEF DESCRIPTION OF THE DRAWINGS

(1) FIG. **1** illustrates the structures of conventional antibody, Camelidae antibody, and VHH. The conventional antibody is a four-polypeptide unit consisting of two identical heavy chains (H) and two identical light chains (L) held together by disulfide bonds to form the Y shape of the antibody and the N-terminal variable region (VH-VL) binds to the antigens. Camelid antibodies lacks a light chain and are composed of only two identical heavy chains, where the VHH domain (also known as sdAb or nanobody) binds the antigen.

(2) FIG. **2** shows the amino acid sequences of the 38 anti-TLT-1 sdAb sequences, in which complementary determining regions, CDR1, CDR2 and CDR3, are highlighted.

(3) FIG. **3**A shows the amino acid sequences of CDR1, CDR2 and CDR3, of the 38 anti-TLT-1 sdAbs. FIG. **3**B shows the specific CDR1, CDR2 and CDR3, of the 10 preferred anti-TLT-1 sdAbs.

(4) FIG. **4** shows the amino acid sequence (1-209) of the extracellular domain of tissue factor (SEQ ID NO: 100), and the same sequence plus amino acids derived from the plasmid expression vector at N-terminal (lowercase letters) and a C-terminal His-6 tag at C-terminal (SEQ ID NO: 101)

(5) FIG. **5**A shows the amino acid sequences of sdAb-based proteins. Two anti-TLT-1 sdAb antibodies with a C-terminal His tag are shown: (1) sdAb-2-33-His (SEQ ID NO: 102) and (2) sdAb-2-90-His (SEQ ID NO: 103). Two fusion proteins with His tag are shown: (3) sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His (SEQ ID NO: 104) and (4) sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His (SEQ ID NO: 105). The linker sequence between the anti-TLT-1 sdAb and sTF.sub.209 cassette includes a 22 amino acid Gly-Ser linker (underlined) from human transthyretin and a thrombin cleavage site (bolded) derived from human FVIII. In all cases, lowercase letters at the N-terminus indicate amino acids derived from the plasmid expression vector; uppercase letters indicate the primary sequence of the said protein.

(6) FIG. **5**B shows preferred amino acid sequences of TF fusions with anti-TLT-1 sdAbs. (1) sTF.sub.209-PC1-sdAb 2-33.sub.TLT (SEQ ID NO: 106) and (2) sTF.sub.209-PC1-sdAb 2-33.sub.TLT (SEQ ID NO: 107) containing a thrombin cleavage site proximal to the sdAb.

(7) FIG. **5**C shows the preferred sequences of the tissue factor-sdAb fusion proteins. (1)

sTF.sub.209-sdAb 2-33.sub.TLT (SEQ ID NO: 108) and (2) sTF.sub.209-sdAb 2-33.sub.TLT (SEQ ID NO: 109), that do not contain a thrombin cleavage site.
(8) FIG. **5**D shows a full-length human tissue factor (SEQ ID 110) with the transmembrane domain highlighted.
(9) FIG. **5**E shows two fusion proteins with factor Xa cleavage site shown. (1) sTF.sub.209-PC2-sdAb 2-33.sub.TLT-His (SEQ ID NO: 111) and (2) sTF.sub.209-PC2-sdAb 2-90.sub.TLT-His (SEQ ID NO: 112). The linker sequence between the anti-TLT-1 sdAb and sTF.sub.209 cassette includes a 22 amino acid Gly-Ser linker from human transthyretin and a human factor Xa cleavage site derived from human prothrombin.
(10) FIGS. **6**A-**6**B show schematic representation of fusion proteins of soluble domain of tissue factor (sTF) and single domain antibody (sdAB). (6A) The C-terminus of the sTF.sub.209 is fused to the N-terminus of an sdAb through a flexible polypeptide sequence containing a Gly-Ser linker and FVIII thrombin cleavage site; the figure is based on crystal structures of sTF and a camelid sdAb. (6B) Stick figure representation of the similar structure in (A) but indicating the interaction of sTF with FVII after binding of sdAb to TLT-1 protein on the surface of activated platelets. 'PC' indicates the position of a proteolytic cleavage site.
(11) FIGS. **7**A-**7**C represent plasmid maps for the expression of anti TLT-1 sdAbs, sTF and sTF-sdAb fusion proteins. DNAs corresponding to each protein was subcloned into specified restriction enzyme cleavage sites and expressed under a T7 promoter (stippled box). (7A) Plasmid map for the expression vectors pNT-sdAb 2-33.sub.TLT and pNT-sdAb 2-90.sub.TLT. The expression cassettes contain the DNA sequence encoding sdAb 2-33.sub.TLT-His or sdAb 2-90.sub.TLT-His with a C-terminal His-tag. The cloning sites are Nco I and Bam HI. (7B) Plasmid map for the expression vector pNT-sTF.sub.209-His. The expression cassette contains the DNA sequence encoding extracellular domain of tissue factor amino acid 1-209 (sTF.sub.209) with a C-terminal His-tag. The cloning sites are Nhe I and Bam HI. (7C) Plasmid map for the expression vector pNT-sTF.sub.209-PC-sdAb 2-33.sub.TLT-His and pNT-sTF.sub.209-PC-sdAb 2-90.sub.TLT-His. The expression cassette contains the DNA sequence encoding sTF.sub.209 and either sdAb 2-33.sub.TLT or sdAb 2-90.sub.TLT proteins containing a C-terminal His-tag; 'PC' indicates the presence of a proteolytic cleavage site at the C-terminal side of the Gly-Ser linker. The cloning sites for the DNA cassettes are Nhe I and Bam HI.
(12) FIGS. **8**A-**8**D demonstrate the purity and molecular weight for recombinant proteins. (**8**A) A gel electropherogram of recombinantly-expressed sdAb 2-33 TLT-His (lane 2), sdAb 2-90 TLT-His (lane 4), sTF209-His (lane 1), sTF209-PC1-dAb 2-33 TLT-His (lane 3) and sTF209-PC1-sdAb 2-90 TLT-His (lane 5). Two micrograms of each protein were run onto a 15% SDS-PAGE gel and stained with Coomassie Brilliant Blue stain. (**8**B) A corresponding Western Blot for protein lanes 1, 2, 3, 4, and 5 in FIG. **8**A. An anti-TF tag antibody was used to detect the protein in the Western blot. (**8**C) A corresponding Western Blot for protein lanes 1, 2, 3, 4, and 5 in FIG. **8**A. An anti-His antibody was used to detect the protein in the Western blot. Lane M, Molecular weight marker (MW); Lane 1, sTF209-His; Lane 2, sdAb-2-33TLT-His; Lane 3, sTF209-PC1-sdAb 2-33TLT-His; Lane 4, sdAb 2-90TLT-His; Lane 5, STF209-PC1-sdAb 2-90TLT-His. (8D) A gel electropherogram of all ten recombinantly-expressed sdAbs shown in FIG. **3**. Lane 1, sdAb 2-3TLT-His; Lane 2, sdAb 2-25TLT-His; Lane 3, sdAb 2-33TLT-His; Lane 4, sdAb 2-64TLT-His; Lane 5, sdAb 2-90TLT-His, Lane 6, sdAb 2-127TLT-His, Lane 7, sdAb 2-132TLT-His, Lane 8, sdAb 3-32TLT-His, Lane 9, sdAb 3-38TLT-His, Lane 10, sdAb 2-69TLT-His.
(13) FIGS. **9**A and **9**B show binding-affinity determinations of proteins to the extracellular domain of human TLT-1. The extracellular domain of human TLT-1 protein was coated onto a 96-well plate for ELISA. After 24 hours incubation at 4° C. and 2 hours of blocking at room temperature (RT) with the blocking buffer, increasing concentrations of ten anti-TLT-1 sdAbs, sTF.sub.209-PC1sdAb 2-33.sub.TLT-His and sTF.sub.209-PC2sdAb 2-90.sub.TLT-His proteins were added to the respective wells for 1-hour incubation at RT. Anti-His tag—HRP-labeled antibody was used to

evaluate the binding. The binding affinity of all ten anti-TLT-1 sdAbs (FIG. **9**A) and sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His and sTF.sub.209-PC1sdAb 2-90.sub.TLT-His (FIG. **9**B) are <10 nM. By this criterion, sTF.sub.209-His does not appear to affect the ability of sdAb.sub.TLT to bind to TLT-1.

(14) FIGS. **10**A and **10**B show the binding of proteins to activated platelets. Human and mouse whole blood were used to characterize whether sdAb-2-33.sub.TLT-His, sdAb 2-90.sub.TLT-His, sTF.sub.209-sdAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His would exclusively bind to both activated human (FIG. **10**A) and mouse platelets (FIG. **10**B). To prepare activated human platelets, ADP (5 and 20 μM) were preincubated with human whole blood and the above proteins were then added to the ADP-treated whole blood. To prepare activated mouse platelets, collagen, at either 5 μg/ml or 10 μg/ml, was preincubated with mouse whole blood and the above proteins were then added to the ADP-treated whole blood. The binding of the proteins with platelets was detected with FITC-labeled—anti-His tag antibody. The results clearly demonstrated that the sdAb-2-33.sub.TLT-His, sdAb 2-90.sub.TLT-His, sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His proteins bind to activated both human and mouse platelet exclusively and fused sTF to sdAbs do not alter their binding to platelet TLT-1 receptors. The results provide a basis for using a mouse bleeding model to demonstrate the efficacy of human sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His fusion proteins to stem blood loss.

(15) FIG. **11** shows a characterization of FVIIa amidolytic activity. sTF.sub.209-PC1-dAb 2-33.sub.TLT-His, sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His; sTF.sub.209-His, sdAb-2-33.sub.TLT-His and sdAb 2-90.sub.TLT-His proteins were used as test articles in the assay. The binding curve indicates a similar TF-mediated, concentration-dependent FVIIa amidolytic activity as is seen with both sTF.sub.209-PC1-dAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His. This demonstrates that the function of sTF.sub.209-His was not affected by fusing it to the nanobodies; by contrast, sdAb-2-33.sub.TLT-His and sdAb 2-90.sub.TLT-His alone had no effect on FVIIa amidolytic activity.

(16) FIG. **12** shows the effect of sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His, sTF.sub.209-His, sdAb-2-33.sub.TLT-His and sdAb 2-90.sub.TLT-His in an APTT-like clotting assay. Human FVIII-deficient plasma from a hemophilia A patient was mixed with transfected CHO cells that stably expressed human TLT-1 receptor on the surface, and each of the five proteins at a final concentration of 1 nM were tested in the assay. The result clearly demonstrated the procoagulant activity of both sdAb-2-33.sub.TL-His and sdAb 2-90.sub.TLT-His fusion proteins in hemophilia A patient plasma, as there was a dramatic decrease in clotting time observed only with the two fusion proteins.

(17) FIG. **13** illustrates thrombin generation promoted by the fusion proteins. A thrombin generation assay (TGA) was used to demonstrate the effects of sTF.sub.209-PC1-dAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His, sTF.sub.209-His, sdAb-2-33.sub.TLT-His and sdAb 2-90.sub.TLT-His proteins on thrombin generation. Citrated human platelet-rich plasma (PRP) was mixed with the above five proteins, each present at a final concentration of 25 nM. The TGA results demonstrated that the sTF-sdAb fusions, but not the single-chain antibodies (sdAbs), targeted to platelets markedly reduced the lag-time for peak thrombin generation.

(18) FIGS. **14**A-**14**B demonstrates that the procoagulant effect of fusion proteins in a mouse bleeding model. The mouse bleeding model was established by injecting sodium enoxaparin (30 mg/kg) subcutaneously. Test articles administered in the presence of enoxaparin, namely, STF209-PC1-dAb 2-33 TLT-His, sTF209-PC1-sdAb 2-90 TLT-His, and controls, were administered at a dose of 90 g/kg of mouse body weight. Blood loss was measured by weighing blood collected during the tail bleeding assay. Time to clot formation was determined by directed visualization when bleeding stopped. FIG. **14**A shows the effect of fusion proteins on bleeding time and FIG.

**14**B shows the effect of fusion proteins for blood loss.

## DETAILED DESCRIPTION OF THE INVENTION

### Definitions

(19) “CDR”s are complementary-determining Regions of VH or VL chains of antibody which are critical for binding with antigen.

(20) A “domain” means one region in a polypeptide which is folded into a particular structure independently of other regions.

(21) A “single domain antibody” (sdAb), or a “variable domain of heavy chain of heavy-chain antibody” (VHH), also known as a nanobody, is an antibody fragment consisting of a single monomeric variable antibody, i.e., a variable domain of a heavy chain of an antibody. A single domain antibody is typically derived from the Camelidae family. VHH and sdAb are used interchangeably in this application.

(22) A “tissue factor” (TF), also called platelet tissue factor, factor III, or CD142, is a membrane-bound protein encoded by the F3 gene, present in subendothelial tissue and leukocytes.

(23) Its role in the clotting process is the initiation of thrombin formation from the zymogen prothrombin.

(24) “TREM (triggering receptors expressed on myeloid cells) like transcript-1” (TLT-1), as used herein, is a membrane protein receptor found only in alpha-granules of platelets and megakaryocytes. TLT-1 contains an extracellular V-set Ig domain, a proline-rich region, and an immune receptor tyrosine-based inhibitory motif in its cytoplasmic tail. Upon platelet activation, TLT-1 is rapidly brought to the surface of platelets where it can enhance Ca.sup.++ influx and promote platelet aggregation.

(25) The present invention is directed to high-affinity single-domain antibodies (sdAb) that specifically bind both mouse and human TLT-1 proteins on activated, but not resting, platelets. Due to their smaller size, elevated stability, larger number of accessible epitopes, relatively low production costs and improved robustness, the inventors selected sdAb as targeting agents to prepare fusion proteins.

(26) The present invention is also directed to fusion proteins comprising an extracellular (soluble) domain of tissue factor (sTF) linked to these single-domain antibodies for efficiently targeting sTF to sites of vascular injury. The targeting is through binding of the sdAbs to TLT-1, a membrane protein receptor confined exclusively to the alpha-granules of resting platelets and megakaryocytes that then translocates to the surface of platelets upon their activation; positioning of sTF for interaction with FVII is achieved on activated platelet membranes to promote procoagulant activity. This targeting maximizes TF's ability to function as a strong hemostatic agent, while minimizing the chance of inducing disseminated intravascular coagulation (DIC) by excess thrombin formation. The fusion proteins of present invention fulfill the needs to treating patients with severe bleeding disorders.

(27) Single-Domain Antibodies (sdAb) against TLT-1 (TREM-Like Transcript 1)

(28) TLT-1 (TREM-like transcript 1) protein is expressed selectively on the surface of activated platelets and contains a number of described grooves on its surface (Gattis et al., 2006, Proc. Natl. Acad. Sci. USA, volume 281, pp. 13396-13403). The inventors discovered that such characteristics making TLT-1 ideally suited for interacting with the single-domain antibodies. These surface grooves appear to contain amino acid residues with both negatively-charged and uncharged electrostatic properties that allow interaction with selected amino acids distinctly- and conformationally-displayed on sdAbs.

(29) The inventors have prepared high-affinity single domain antibodies, that target TLT-1 protein. The inventors have generated a total of 103 sdAb, in which 38 sdAb sequences were identified. FIG. **2** shows the amino acid sequences of the 38 anti-TLT-1 sdAb sequences, in which complementary determining regions, CDR1, CDR2 and CDR3, are highlighted. FIG. **3**A shows the CDRs (CDR1, CDR2, and CDR3) of the 38 anti-TLT-1 sdAb sequences. Ten preferred sdAb

sequences with highest activities were selected by solid phase ligand binding assay and their sequences are SEQ ID NOs. 62, 64, 65, 68, 70, 71, 74, 76, 80, and 95. FIG. **3**B shows the specific CDR1, CDR2 and CDR3, of the 10 preferred anti-TLT-1 sdAbs.

(30) The present invention is directed to a single-domain antibody against TLT-1, comprising CDR1 selected from the group consisting of: SEQ ID NOs: 1-30, CDR2 selected from the group consisting of: SEQ ID NOs: 31-39, and CDR3 selected from the group consisting of: SEQ ID NOs: 40-61.

(31) The present invention is also directed to a single domain antibody against TLT-1, comprising: (a) CDR1 being SEQ ID NO: 6, CDR2 being SEQ ID NO: 32, CDR3 being SEQ ID NO: 43; (b) CDR1 being SEQ ID NO: 8, CDR2 being SEQ ID NO: 31, CDR3 being SEQ ID NO: 44; (c) CDR1 being SEQ ID NO: 3, CDR2 being SEQ ID NO: 31, CDR3 being SEQ ID NO: 41; (d) CDR1 being SEQ ID NO: 3, CDR2 being SEQ ID NO: 31, CDR3 being SEQ ID NO: 42; (e) CDR1 being SEQ ID NO: 1, CDR2 being SEQ ID NO: 31, CDR3 being SEQ ID NO: 40; (f) CDR1 being SEQ ID NO: 25, CDR2 being SEQ ID NO: 35, CDR3 being SEQ ID NO: 45; (g) CDR1 being SEQ ID NO: 3, CDR2 being SEQ ID NO: 31, CDR3 being SEQ ID NO: 46; (h) CDR1 being SEQ ID NO: 11, CDR2 being SEQ ID NO: 31, CDR3 being SEQ ID NO: 47; (i) CDR1 being SEQ ID NO: 16, CDR2 being SEQ ID NO: 31, CDR3 being SEQ ID NO: 50; or (j) CDR1 being SEQ ID NO: 25, CDR2 being SEQ ID NO: 35, CDR3 being SEQ ID NO: 59. (FIG. **3**B)

(32) The present invention is further directed to a single domain antibody comprising the sequence selected from the group consisting of SEQ ID NOs: 62-99, or a sequence having at least 95%, or 96%, or 97%, or 98%, or 99% sequence identify thereof, provided that the sequence variations are in the non-CDR framework regions. Preferred single domain antibodies include those comprising the sequence selected from the group consisting of SEQ ID NOs: 62, 64, 65, 68, 70, 71, 74, 76, 80, and 95, preferably SEQ ID NOs. 68 and 70, or a sequence having at least 95%, or 96%, or 97%, or 98%, or 99% sequence identify thereof, provided that the sequence variations are in the non-CDR framework regions. The sequence variation, i.e., the amino acid changes are preferably of a minor amino acid change such as a conservative amino acid substitution. A conservative amino acid substitution is well known to a person skilled in the art.

(33) The present invention provides single domain antibodies that interact both with human and mouse forms of the TLT-1 protein. Such antibodies are suitable for testing in both human and mouse models of bleeding, such as in transgenic mouse models of hemophilia or in acquired bleeding though inhibitors of coagulation pathways.

(34) Fusion Proteins

(35) The second aspect of the invention is directed to a fusion protein comprising (a) an extracellular domain of tissue factor, (b) a single domain antibody against TLT-1, and (c) a linker.

(36) Activated platelets, and in particular, “coated” platelets, are substrates for numerous coagulation cascade components that, in combination with fibrinogen, are able to generate a fibrin-based clot needed to seal a vascular injury. By fusing sdAbs with the soluble domain of human tissue factor (sTF), the inventors have demonstrated the targeting of these protein fusions to activated platelets directly and specifically. This specific targeting thus “bypasses” the normal coagulation cascade much in the way of a recombinant FVIIa. Mechanistically, however, the two ‘bypass agents’ are very different. For the chimeric sTF-sdAb fusions, the extracellular portion of TF becomes anchored to activated platelets through the insertion of a high-affinity sdAb fusion partner into relevant epitope folds of the TLT-1 protein; in the correct surface orientation, the sTF domain is thermodynamically-favored to bind to circulating plasma FVII, and activates it in situ to FVIIa; factor VIIa in turns activates FX to FXa, and further stimulates and promotes the common coagulation cascade. This mechanism is considerably different than the mechanics of recombinant FVIIa activation of coagulation factors and direct platelet binding.

(37) The amino acid sequence of full length of human tissue factor protein is shown in FIG. **5**D (SEQ ID NO: 110). In the fusion protein of the present invention, the extracellular domain of tissue

factor (sTF) is selected from amino acid residues 1-208 to 1-221, or 1-209 to 1-220 of (38) SEQ ID NO: 110. For example, sTF is 1-208, 1-209, 1-210, 1-211, 1-212, 1-213, 1-214, 1-215, 1-216, 1-217, 1-218, 1-219, 1-220, or 1-221 of SEQ ID NO: 110. A preferred sTF is 1-209 of SEQ ID NO: 110.

(39) In the fusion protein of the present invention, the sdAb is any sdAb described above in the preceding sections.

(40) In the fusion protein of the present invention, the sdAb may be C-terminal or N-terminal to the sTF, and a flexible linker is used to connect the sdAb with the soluble tissue factor. A flexible linker can be any length that links the two proteins, spaces the two protein properly, and does not affect the functionality of the two proteins. The length of linker sequence can be optimized in order to allow ideal positioning sTF of the fusion molecule on the surface of the platelet, as a function of its insertion into the TLT-1 molecule, to efficiently bind FVII, which is the first step in propagating the extrinsic coagulation pathway. The length of the linker sequence is in general 5-40, 10-30, or 15-30 amino acids, preferably the length of the linker is 18-26 amino acids.

(41) A flexible linker may contain a variety of amino acids. In one embodiment, a flexible linker comprises various combinations of glycine and serine, as well as other amino acids, such as threonine. For example, a flexible linker can be a natural amino acid sequence derived from a human transthyretin protein such as GSGGGTGGGSGGSGGGTGGGSG (SEQ ID NO: 113). For example, the flexible linker can be an artificial sequence such as GGGGSGGGGSGGGGS (SEQ ID NO: 114).

(42) In one embodiment, the fusion protein of the present invention may further comprise a protease cleavage site. In this embodiment, the fusion protein comprises: (a) an extracellular domain of tissue factor, (b) a single domain antibody against TLT-1, (c) a linker, and (d) a polypeptide sequence that can be proteolytically-cleaved by a protease. The polypeptide sequence of (d) includes, but not limited to, a thrombin cleavage site, a FXa cleavage site, or a FXIa cleavage site, to allow auto-regulation of thrombin production (FIG. **6**). For example, a thrombin cleavage site may comprise the amino acid sequence of AIEPRSFSQN (SEQ ID NO: 115). For example, a FXa cleavage site may comprise the amino acid sequence of LESYIDGRIVEG (SEQ ID NO: 116) or SDRAIEGRTATS (SEQ ID 117). The proteolytic cleavage site may be located at the C-terminus or N-terminal of the flexible linker. The proteolytic cleavage site may also be located inside of the flexible linker. Introduction of a protease cleavage site allows thrombin generated by FXa/FII complex in the vicinity of the sTF-sdAb fusion to access this linker and separate the two fusion partners, namely, the TLT-1 sdAb from the sTF domain; neither fusion partner alone is functionally-active. This self-limiting mechanism will prevent excess thrombin generation and dramatically increase the safety margin upon administration of the fusion protein to patients.

(43) In one embodiment, the present invention provides nucleotide sequences encoding the fusion proteins of the present invention. The nucleotide sequences allow inclusion as part of a prokaryotic, fungal, or eukaryotic expression vector for expression in bacterial cells (like *Escherichia coli*), yeast (like *Saccharomyces cerevisiae*), insect cells (like Sf9, Sf21 and High Five), or mammalian cells (like CHO, HEK, BHK, for example), respectively. Due to the small size of the sdAb, the fusion protein can be expressed in bacteria, yeast, insect cells or other eukaryotic cells, such as mammalian cells.

(44) In a further aspect, the present invention provides a pharmaceutical composition comprising the fusion protein of the present invention and a pharmaceutically acceptable carrier. In a further aspect, the present invention provides a method for treating bleeding disorders, such as those of congenital or acquired coagulopathies, traumatic bleeding due to injury, or other uses where bleeding cannot easily be controlled. The method comprises the step of administering an effective amount of the fusion protein of the present invention to a patient in need thereof with. The fusion protein, for example, can be administered by injection or other parenteral administration, or by oral administration.

(45) The fusion protein of the present invention avidly, and specifically, binds to TLT-1 molecules on activated platelets. This binding to TLT-1 then conformationally-promotes interaction of sTF to FVII, the molecule that, when activated, further facilitates the downstream common coagulation cascade leading to thrombin formation. The resulting fusion protein exhibits the desired properties of a functional procoagulant: high-affinity binding to activated platelets, high-affinity binding to FVII and conversion to FVIIa, conversion of factor X to factor Xa, and incorporation of a proteolytic (thrombin) cleavage site to self-limit excess thrombin formation. Cleavage allows selective dissociation of the sTF domain (domain responsible for FVII activation but only when bound as a fusion) from the sdAb antibody domain that binds TLT-1 on the activated platelet (these domains do not promote coagulation or platelet aggregation in any case). The fusion proteins optionally have a hexanucleotide His tag incorporated at their C-terminus to facilitate purification and detection.

(46) The inventors have demonstrated two high-affinity sdAb domains, sdAb 2-33.sub.TLT (SEQ ID: 68) and sdAb 2-90.sub.TLT (SEQ ID: 70), to act as fusion partners with the extracellular domain of tissue factor (amino acid 1-209 of SEQ ID NO: 100). The resulting preferred fusion molecules, named sTF.sub.209-PC1-sdAb 2-33.sub.TLT (SEQ ID: 106) and sTF.sub.209-PC1-sdAb 2-90.sub.TLT SEQ ID: 107), bind efficiently to both mouse and human platelets through the interaction with the platelet TLT-1 receptor. They effectively bind to FVIIa to promote the generation of FXa from FX, to generate formation of thrombin (FIIa) from prothrombin, and to reduce blood loss in a mouse model of bleeding. On the other hand, neither the sTF domain alone, nor the sdAb antibody domain alone, nor sTF-sdAb fusion protein is able to mediate platelet aggregation or activation at the tested dose.

(47) The following examples further illustrate the present invention. These examples are intended merely to be illustrative of the present invention and are not to be construed as being limiting.

EXAMPLES

(48) TABLE-US-00001 TABLE 1 Abbreviations Abbreviation Name Description TLT-1 TREM-like transcript-1 sdAb Single domain antibody sTF Tissue factor extracellular (soluble) domain VHH Variable domain of the heavy chain immunoglobulin FVIIa Activated factor VII CHO Chinese hamster ovary BHK Baby Hamster Kidney HEK Human embryonic kidney SEQ Sequence CDR Complementary determining regions PBMC Peripheral blood mononuclear cell GLY Glycine SER Serine HIS Histidine PC1 Thrombin cleavage site PC2 Factor Xa cleavage site

Example 1: Human TLT-1 Amino Acid Sequence

(49) The amino acid sequence of human TLT-1, an abundant platelet type I transmembrane receptor with an immunoglobulin-like structure, is presented below; the underlined sequence is the signal sequence at the N-terminus of the protein and the highlighted sequence is the TLT-1 transmembrane domain. The extracellular domain of human ILT-1 that was used in generation of the anti-TLT-1 single domain antibodies (sdAbs) is a 147 amino acid protein between the end of the signal sequence and the beginning of the transmembrane domain (underlined), UniProt sequence Q86YW5:

(50) TABLE-US-00002 (SEQ ID NO: 118)

MGLTLLLLLLLGLEGQGIVGSLPEVLQAPVGSSILVQCHYRLQDVKAQKV
WCRFLPEGCQPLVSSAVDRRAPAGRRTFLTDLGGGLLQVEMVTLQEEDAG
EYGCMVDGARGPQILHRVSLNILPPEEEEETHKIGSLAENAFSDPAGSAN
PLEPSQDEKSIPLIWGAVLLVGLLVAAVVLFAVMAKRKQGNRLGVCGRFL
SSRVSGMNPSSVVHHVSDSGPAAELPLDVPHIRLDSPPSFDNTTYTSLPL
DSPSGKPSLPAPSSLPPLPPKVLVCSKPVTYATVIFPGGNKGGGTSCGPA QNPPNNQTPSS

Example 2. Generation of Anti-Human TLT-41 Single Domain Antibodies (sdAbs)

(51) In order to create coagulation co-factors targeting specific proteins on platelets, the inventors first identified sdAb antibodies specific for human TLT-1 by immunizing llamas five times with recombinant, soluble human TLT-1 protein and recovering the mRNAs coding for the heavy-chain

antibody fragments from the B-cells of the immunized llamas. Messenger RNAs were converted into complementary DNA (cDNA) and cloned into a major coat protein gene (pIII) of bacteriophage M13 for expression. VHH domains of interest were selected by phage display methods (e.g., Kushwaha et al., 2014, J. Vis. Exp., volume 84, e50685; Saw and Song, 2019, Protein Cell, volume 10, pp. 787-807). The recovered sdAbs were selected from the pool of antibody fragments by binding repeatedly to immobilized human TLT-1 protein, as well as to unrelated proteins; repeated selection by this method identified only those that were true and high-affinity binders to TLT-1, while non-specific binders were discarded. DNA sequencing and sequence alignment were used to validate the structure and sequence of the resulting specific sdAb. Further characterization of potential candidates was made by testing of binding of individual sdAb expressed in, and purified from, bacterial cells to full-length, membrane-bound human recombinant TLT-1 that was transfected into, and expressed on the surface of, Chinese hamster ovary (CHO) cells and on both resting and activated platelets using Flow cytometry to demonstrate specificity and functional binding of sdAbs. Finally, select sdAbs were tested as fusions with sTF to determine their ability to reduce blood loss in animal models of bleeding.

(52) Animal Immunization

(53) One llama was immunized subcutaneously at 3-week intervals and at multiple site of injection. Over the course of 5 rounds of injections with 0.5-1.0 mg of human TLT-1-His antigen (encompassing the TLT-1 extracellular domain shown in FIG. **1**, but with the addition of hexahistidine tag; Sino Biological US, PA), the antibody titer against this antigen increased from undetectable to 1:12,800 which indicates a high-titer response to the injected antigen.

(54) Immune Library Construction and Screening

(55) After the immunization protocol was completed, whole blood was collected from the immunized llama for PBMC isolation. RNA was extracted and tested by gel electrophoresis to be intact. The VHH genes of immunoglobulin RNA were amplified by two rounds of PCR after reverse transcription using unique primers to camelid variable and constant region domain sequences. The PCR products and the phagemid DNA were digested with Sfi I restriction site endonuclease and ligated together with T4 DNA ligase. The ligation mix was transformed into *E. coli* TG1 cells. The final constructed library consisted of 5.2×10.sup.8 independent members. Three rounds of bio-panning for single-domain binders against TLT-1-His protein was then performed and an enriching factor of about a thousand-fold was achieved.

(56) Binder Validation

(57) Based on the bio-panning strategy, a total of 300 clones were validated against TLT-1 using ELISA and, of these, 147 clones were identified as positive. DNA sequence indicated that 145 of 147 clones were correctly identified as authentic camelid antibodies. A total of 103 unique clones have been identified at the amino acid level. As some groups of the identified unique clones present the same CDR3 region but have differences in their CDR1 and/or CDR2 regions, these unique sequences were further analyzed based on their CDR3 regions (the CDR regions are predicted via IMGT database). A total of 38 unique sdAb sequences with different CDR1, CDR2, and/or CDR3 were identified (FIG. **2** and FIG. **3**). From the 38 sdAbs, the top 10 clones with the highest clone frequency were re-tested by ELISA (Table 1). Data demonstrated strong positive signals compared with the negative control protein.

(58) ELISA Ligand Binding Assay

(59) The top 10 clones were then confirmed in the final soluble ELISA validation. Soluble TLT-1 extracellular domain His tag (sTLT-1-His) protein was coated (0.1 μg/well) onto a 96-well plate and incubated overnight at 4° C. An irrelevant protein with His tag and an no coating group were used in the assay as negative controls. On the next day, the coated plate was washed 3 times with 200 μL PBST buffer per well and blocked with 300 μL blocking buffer per well for 1 h at 37° C. The blocking buffer was then removed and the plate was washed 3 times with the washing buffer. After washing, 100 μL of HRP-anti-TLT-1 sdAb antibody in blocking buffer was added to each

well and incubated at 37° C. for 1 h. The plate was washed three times with the washing buffer and then 100 μL of TMB substrate solution was added per well and incubated at room temperature for 15 minutes; 100 μL of 2M H.sub.2SO.sub.4 were then added to stop the reaction and the plate was analyzed using a microplate reader at 490 nm. According to the results, consistent results were obtained. In the meantime, the negative control groups present expected low signal, which indicated all the Top 10 clones did not cross-react with His tag and can bind to the target specifically.

(60) TABLE-US-00003 TABLE 2 [OD 490 nm] Coating: Coating: No Clone TLT-1 protein (3 μg/mL) Irrelevant protein (3 μg/mL) Coating 2-2 0.796 0.103 0.096 2-25 0.512 0.073 0.104 2-33 1.501 0.070 0.095 2-64 0.911 0.066 0.104 2-69 0.494 0.076 0.099 2-90 0.762 0.081 0.132 2-127 0.760 0.076 0.102 2-132 0.981 0.114 0.079 3-32 0.858 0.097 0.106 3-38 0.818 0.087 0.099

Example 3. Development of pNT-sdAb 2-33.SUB.TLT.-His, pNT-sdAb 2-132.SUB.TLT.His, pNT-sdAb 2-25.SUB.TLT.-His, pNT-sdAb 2-64.SUB.TLT.-His, pNT-sdAb 2-90.SUB.TLT.-His, pNT-sdAb 2-127.SUB.TLT.-His, pNT-sdAb 2-2.SUB.TLT.-His, pNT-sdAb 3-32.SUB.TLT.-His, pNT-sdAb 3-38.SUB.TLT.-His and pNT-sdAb 2-69.SUB.ILT.-His Expression Constructs

(61) In order to evaluate the utility of these novel antibodies, DNAs corresponding to ten selected single-domain antibodies identified in TABLE 1 were synthesized and codon-optimized for bacterial expression (GenScript, Piscataway N.J.); corresponding amino acid sequences and SEQ ID numbers are shown in FIG. **2**. A Nco I restriction enzyme site at the 5′-end and a Bam HI restriction enzyme site at the 3′-end were included for cloning purposes. To facilitate recombinant sdAb purification, a sequence encoding six histidine amino acids (His) was also incorporated at the 3′-end of the synthesized genes upstream of the Bam HI site. The synthesized genes were inserted into Nco I and Bam HI restriction enzyme sites of a pNT-based plasmid expression vector. The resulting vectors were designated as pNT-sdAb 2-33.sub.TLT-His, pNT-sdAb 2-132.sub.TLT-His, pNT-sdAb 2-25.sub.TLT, pNT-sdAb 2-64.sub.TLT-His, pNT-sdAb 2-90.sub.TLT-His, pNT-sdAb 2-127.sub.TLT-His, pNT-sdAb 2-2.sub.TLT-His, pNT-sdAb 3-32.sub.TLT-His, pNT-sdAb 3-38.sub.TLT-His and PNT-sdAb 2-69.sub.TLT-His (TABLE 2). A representative illustration of the plasmid expression vector for the anti-TLT-sdAbs and sTF-sdAbs fusions is shown in FIG. **7**A.

Example 4. Development of pNT-sTF.SUB.209.-His Expression Construct

(62) DNA corresponding to the extracellular domain of tissue factor (sTF) amino acid 1-209 was synthesized as previously described and codon-optimized for expression in bacteria. A Nhe I restriction enzyme site at the 5′-end and a Bam HI restriction enzyme site at the 3′-end were included for cloning purposes. To facilitate recombinant sTF purification, a sequence encoding six histidine amino acids (His) was also incorporated at the 3′-end of the synthesized genes upstream of the Bam HI site. The synthesized sTF209-His was inserted into Nhe I and Bam HI restriction enzyme sites of a pNT-based expression vector and the resulting vector was designated as pNT-sTF.sub.209-His (TABLE 2). A representative illustration of the plasmid expression vector for the extracellular domain of soluble tissue factor (sTF) is shown in FIG. **7**B.

Example 5. Development of pNT-sTF.SUB.209.-PC1-sdAb 2-33.SUB.TLT .and pNT-sTF.SUB.209.-PC1-sdAb 2-90.SUB.TLT .Expression Constructs

(63) The expression cassettes encoding sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His were synthesized (GenScript, Piscataway N.J.) and codon-optimized for bacterial expression. A Nhe I restriction enzyme site at the 5′-end and a Bam HI restriction enzyme site at the 3′-end were included for cloning purposes. To facilitate purification of the recombinant fusion proteins, a sequence encoding six histidine amino acids (His) was also incorporated at the 3′-end of the synthesized genes upstream of the Bam HI site. To properly position sTF on the surface of the cell surface and to limit thrombin overexpression, a Gly-Ser linker sequence from human transthyretin (encoding 22 amino acids) and a thrombin cleavage site from human factor VIII (‘PCI’), respectively, were inserted between the sTF and sdAb sequences. The synthesized genes were inserted into Nhe I and Bam HI restriction enzyme sites of a pNT

expression vector, itself based on the pET9d plasmid vector. The resulting vectors were designated as pNT-sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His and pNT-sTF209-PC1-sdAb 2-90.sub.TLT-His, respectively (Table 3). A representative illustration of the plasmid expression vector for the sTF-sdAb fusions is shown in FIG. **7**C.

(64) TABLE-US-00004 TABLE 3 Expression Construct Name Coding Protein Description 1 pNT-sdAb 2-33.sub.TLT-His sdAb 2-33.sub.TLT-His 2 pNT-sdAb 2-132.sub.TLT-His sdAb 2-132.sub.TLT-His 3 pNT-sdAb 2-25.sub.TLT-His sdAb 2-25.sub.TLT-His 4 pNT-sdAb 2-64.sub.TLT-His sdAb 2-64.sub.TLT-His 5 pNT-sdAb 2-90.sub.TLT-His sdAb 2-90.sub.TLT-His 6 pNT-sdAb 2-127.sub.TLT-His sdAb 2-127.sub.TLT-His 7 pNT-sdAb 2-2.sub.TLT-His sdAb 2-2.sub.TLT-His 8 pNT-sdAb 3-32.sub.TLT-His sdAb 2-32.sub.TLT-His 9 pNT-sdAb 3-38.sub.TLT-His dAb 2-38.sub.TLT-His 10 pNT-sdAb 2-69.sub.TLT-His sdAb 2-69.sub.TLT-His 11 pNT-sTF.sub.209-His sTF.sub.209-His 12 pNT- sTF.sub.209-PC1-sdAb sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His 2-33.sub.TLT-His fusion 13 pNT- sTF.sub.209-PC1-sdAb sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His 2-90.sub.TLT-His fusion

Example 6. Expression and Purification of Recombinant sTF.SUB.209., TLT-1 sdAbs and sTF.SUB.209.-sdAb Fusion Proteins Expressed in Bacteria

(65) All ten sdAbs, as well as sTF.sub.209, and the two sTF.sub.209-sdAb fusion protein DNA sequences described in TABLE 2 were chemically-transformed into an *E. coli* BL21-based bacteria strain and expressed in LB medium. The bacteria were harvested after protein expression and sonicated in lysis buffer (20 mM HEPES pH 8.0, 300 mM KCl and 10% glycerol). The supernatants were then collected by high-speed centrifugation and applied to a His-Trap HP column (GE) for His-tag protein purification using GE AKTA chromatography system. After washing with 20 column volumes of washing buffer (20 mM HEPES pH 8.0, 20 mM imidazole, 300 mM KCl and 10% glycerol), the absorbed proteins were eluted by using gradient elution buffer (20 mM HEPES pH 8.0, 40-300 mM imidazole, 300 mM KCl and 10% glycerol). Fluted proteins were then concentrated and buffer exchanged into PBS buffer. The purified proteins were analyzed using 10% SDS-PAGE method and confirmed with Western blot. FIG. **5**A (SDS-PAGE), FIG. **8**B (Western blot of FIG. **8**A with anti-TF antibody) and FIG. **8**C (Western blot of FIG. **8**A with anti-His antibody) demonstrate the quality of the purified sdAb 2-33.sub.TLT-His, sdAb 2-90.sub.TLT-His, sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His, sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His and sTF.sub.209-His proteins; all proteins are present as single bands and display the expected molecular weight. FIG. **8**D demonstrates purified ten sdAbs on SDS-PAGE.

Example 7. Binding Affinity (Kd) Determination of TLT-1 sdAbs and sTF.SUB.209.-sdAb Fusion Proteins to Extracellular Domain of TLT-1 Receptor

(66) The binding of sdAb 2-33TLT-His, sdAb 2-132 TLT-His, sdAb 2-25 TLT-His, sdAb 2-64 TLT-His, sdAb 2-90 TLT-His, sdAb 2-127 TLT-His, sdAb 2-2 TLT-His, sdAb 3-32 TLT-His, sdAb 3-38 TLT-His and sdAb 2-69 TLT-His, sTF209-PC1-sdAb 2-33TLT-His and sTF209-PC1-sdAb 2-90TLT-His proteins to the human extracellular domain of TLT-1-Fc tagged protein (sTLT-1-Fc) was analyzed using ELISA. STLT-1-Fc (3 μg/ml) was immobilized onto a 96-well plate for 24 hours at 4° C. and each well immobilized with sTLT-1-Fc was blocked with 2% BSA PBST (PBS plus 0.1% Tween 20) for 2 hours at room temperature (RT). Serial dilution (1000 nM to 0.001 nM) of TLT-sdAbs and sTF-sdAb fusion proteins was performed and diluted proteins were then added to the coated 96-well platelet and incubated for 1 hour. After 3 times of washing with PBST, anti-His HRP antibody was added and incubated for 1 hour at RT. The plate was then washed for 3 times to remove the excess HRP conjugate and 100 μL TMB substrate was then added and incubated for 10-15 mins. To stop the reaction of color development, 2M sulfuric acid was added to the well. The binding affinity (Kd) was calculated based on OD450 nm measurement using GRAPHPAD PRISM® 8.0, computer software for analyzing and graph scientific data (FIGS. **9**A and **9**B). The data indicate that the Kd of sdAb 2-33TLT-His, sdAb 2-90TLT-His, sTF209-PC1-sdAb 2-33TLT-His and sTF209-PC1-sdAb 2-90TLT-His proteins are all in the low nanomolar range (<10 nM).

Example 8. Binding to Activated Human and Mouse Platelets

(67) The binding capability of sdAb 2-33.sub.TLT-His, sdAb sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His proteins to both human and mouse resting and activated platelets was tested by FACS assay. Citrated human (3 donors) and mouse (12 mice) whole blood were collected at room temperature (RT) and 10 μL of whole blood was used for each sample. To activate human platelets, ADP (5 and 20 μM) was used, and incubated with whole blood for 10 min at room temperature (RT). To activate mouse platelet, Type I fibrillary collagen (5 and 10 ug/ml) was used and incubated with whole blood for 10 min at room temperature (RT). Both ADP and collagen used were from Helena Laboratory, Beaumont Tex., Then, for each sample, 10 μg/ml of test article (i.e., sdAb 2-33.sub.TLT-His, sdAb 2-90.sub.TLT-His, sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His or sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His proteins) was added, followed by one or the other labeled antibodies, either APC-anti-CD41a antibody that was used in gating platelet population, or FITC anti-His antibody, that was used in detecting TLT-1 binding of TLT-sdAbs or sTF.sub.209-sdAb fusion proteins on activated platelets. APC-anti-CD62P antibody was used as an activated platelet binding control antibody in the assay. After incubation for 30 min at room temperature, all samples were fixed with 500 μL of 5% paraformaldehyde for 10 min at RT and analyzed by FACS (LSR II, Beckon Dickinson, San Jose, CA). Data in FIG. **10**A are presented as % of positive platelets collected during a fixed time and demonstrated that sdAb 2-33.sub.TLT-His, sdAb 2-90.sub.TLT-His, sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His proteins exclusively bind to activated human platelets. FIG. **10**B shows the equivalent experiment using mouse platelets. No significant binding difference between sdAbs and sTF-sdAb was observed. These observations demonstrate the novelty of these sdAbs in their ability to bind both mouse and human TLT-1 on activated platelets. This observation further indicates that testing of sdAbs and their fusion counterparts can proceed directly in mouse bleeding models without resorting to the use of transfused human platelets to facilitate binding (Example 13).

Example 9. Binding of sTF-sdAb Fusion Proteins to FVIIa

(68) sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His fusion proteins are designed to target sTF to the surface of activated platelets through sdAb/TLT-1 receptor interaction. To verify whether fusing TLT-1 sdAb to sTF would affect its binding to FVIIa, are FVIIa amidolytic activity assay was performed. Various concentrations (0-100 nM) of sTF.sub.209 and sTF.sub.209-sdAb fusion proteins were incubated with factor FVIIa (5 nM) in a butler containing 100 nM NaCl, 50 mM HEPES, pH 7.4, 5 mM CaCl.sub.2, 0.1% BSA at 37° C. for 5 minutes. FVIIa amidolytic activity was assayed with the addition of a 5 mM Chromozym tPA substrate and the absorbance were measured at 405 nm at room temperature. Both sdAb 2-33.sub.TLT-His and sdAb 2-90.sub.TLT-His were included in the assay as negative controls. The data (FIG. **11**) demonstrated that the FVIIa amidolytic activities induced by sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His fusion proteins are indistinguishable from that induced by sTF.sub.209-His in a concentration-dependent manner. These results are consistent with and support observations made for alternate constructs based on sTF and sTF-annexin V (Huang et al., 2006, Blood, volume 107, pp. 980-986).

Example 10. Procoagulant Effect of Targeted sTF.SUB.209 .in a One-Stage Clotting Assay

(69) Targeting sTF.sub.209 to TLT-1 receptor is expected to promote coagulant activity. To confirm the hypothesis, the procoagulant activity of sTF.sub.209-PC1-sdAb 2-33.sub.TLT and sTF.sub.209-PC1-sdAb 2-90.sub.TLT fusion proteins were evaluated in a modified one-stage activated partial thromboplastin time (APTT) clot assay. The APTT clotting times were measured using a STar 4 Hemostasis Analyzer (Diagnostica Stago). Fifty microliters of hemophilia A patient plasma (George King Bio-Medical, Overland Park, KS), 50 μL containing 0.5×10.sup.6 CHO-K1 cells expressing human TLT-1 protein and 1 nM of test article (sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His or sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His) were added to the sample cuvette with total volume of 100 μL. After 200 seconds incubation at 37° C., 50 μL calcium chloride (20 mM) was added to

initiate the clot formation. The data (FIG. **12**) shows that the clotting time of hemophilia A patient plasma with 1 nM sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His fusion protein could be completely normalized compared to sTF.sub.209, sdAb 2-33.sub.TLT-His and sdAb-2-90.sub.TLT-His. sTF209-PC1-sdAb 2-90.sub.TLT-His fusion protein also markedly reduced the clotting time, but potency is less than sTF299-sdAb 2-33.sub.TLT-His in this type of assay.

Example 11. Targeted sTF to the Surface of Activated Platelets Promotes Thrombin Generation

(70) Human platelet-rich plasma (PRP) was prepared by centrifugation of human whole blood containing 0.32% Sodium Citrate at 150×g for 20 min. Thrombin generation assay was performed by adding 20 μL of PRP reagent (Diagnostica Stago), 80 μL of PRP and 25 nM of testing samples. The reaction was started by the addition of 20 μL FluCa substrate (Diagnostica Stago) to U-bottom 96-well plates (ThermoFisher) and the fluorescent signal from the substrate was detected in a Fluoroskan Ascent plate reader (ThermoFisher). The results showed that STF.sub.209-PC1-sdAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His have an increased potency in thrombin generation compared to s TF.sub.209-His, sdAb 2-33.sub.TLT-His and sdAb 2-90.sub.TLT-His (FIG. **13**). The lag time of thrombin generation for sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His was approximately 2-3 times shorter than sTF.sub.209-His. These results support the hypothesis that interaction of the selected sdAbs, only when directly fused with sTF as described, promotes binding to TLT-1 and conformational-positioning of sTF with endogenous FVII, its activation to FVIIa, and subsequent thrombin formation.

Example 12. sTF209-sdAb Fusion Proteins Reduced Tail-Bleeding in Enoxaparin Treated Mice

(71) The procoagulant effect of sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.FLT-His fusion proteins were tested in an enoxaparin-induced tail-bleeding model in mice (Washington Biotechnology Inc, Baltimore Md.). Mice (4 per group) were injected subcutaneously with sodium enoxaparin (30 mg/kg) and two hours later were anaesthetized by intraperitoneal injection of ketamine/xylazine (10 mg/kg). The baseline bleeding time and blood loss were determined by transecting the mouse tail at a point 10 mm from tail tip. The time required for bleeding to stop was recorded, and blood loss was determined by collecting blood in a warmed (37° C.) normal saline solution. An intravenous injection of sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.FLT-His fusion proteins (90 μg/kg) were performed immediately after the first bleeding time determination. A second bleeding time was then measured 5 minutes after the injection of the above proteins, and bleeding time and blood loss was determined in a similar manner as described. The results show that administration of the sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His fusion proteins completely normalize the bleeding time to that of control animals in this bleeding model (FIG. **14**A) and significantly reduced blood loss (FIG. **14**B).

Example 13. Targeted sTF to the Surface of Activated Platelets Promotes Fibrin Clot Formation in Human Whole Blood (Prophetic Example)

(72) Citrated human whole blood (HWB) is drawn from normal donors. Clot formation is measured by thrombelastography (TEG5000) analyzer (Haemonetics, Boston, MA). The final concentrations (0-100 nM) of sdAb 2-33.sub.TLT-His, sdAb 2-90.sub.TLT-His, sTF.sub.209-His, sTF.sub.209-PC1-sdAb 2-33.sub.TLT-His and sTF.sub.209-PC1-sdAb 2-90.sub.TLT-His are added to 340 μL of whole blood containing the kaolin activator. Clotting formation measurement is initiated with addition of 20 μL of 0.2 M CaCl.sub.2. The TEG trace is followed continuously for up to 60 min. The R-time (clotting time) is recorded for potency comparison of testing samples. The data are expected to demonstrate that sTF.sub.209-sdAb fusion proteins shortened R-time (clotting time) in a concentration dependent manner compared to sTF.sub.209-His and sdAb 2-33.sub.TLT-His and sdAb 2-90.sub.TLT-His proteins. The results are expected to further demonstrate that the enhanced thrombin generation seen in Example 11 generates bona fide fibrin formation necessary to generate a functional clot.

## Claims

1. A single domain antibody against triggering receptors expressed on myeloid cells (TREM)-like transcript-1 (TLT-1), comprising: (a) CDR1 being SEQ ID NO: 6, CDR2 being SEQ ID NO: 32, CDR3 being SEQ ID NO: 43; (b) CDR1 being SEQ ID NO: 8, CDR2 being SEQ ID NO: 31, CDR3 being SEQ ID NO: 44; (c) CDR1 being SEQ ID NO: 3, CDR2 being SEQ ID NO: 31, CDR3 being SEQ ID NO: 41; (d) CDR1 being SEQ ID NO: 3, CDR2 being SEQ ID NO: 31, CDR3 being SEQ ID NO: 42; (e) CDR1 being SEQ ID NO: 1, CDR2 being SEQ ID NO: 31, CDR3 being SEQ ID NO: 40; (f) CDR1 being SEQ ID NO: 25, CDR2 being SEQ ID NO: 35, CDR3 being SEQ ID NO: 45; (g) CDR1 being SEQ ID NO: 3, CDR2 being SEQ ID NO: 31, CDR3 being SEQ ID NO: 46; (h) CDR1 being SEQ ID NO: 11, CDR2 being SEQ ID NO: 31, CDR3 being SEQ ID NO: 47; (i) CDR1 being SEQ ID NO: 16, CDR2 being SEQ ID NO: 31, CDR3 being SEQ ID NO: 50; or (j) CDR1 being SEQ ID NO: 25, CDR2 being SEQ ID NO: 35, CDR3 being SEQ ID NO: 59.

2. The single domain antibody according to claim 1, comprising the sequence selected from the group consisting of SEQ ID NOs: 68, 70, 64, 65, 62, 71, 74, 76, 80, and 95, or a sequence having at least 95% identity thereof, provided that the sequence variation is in the non-CDR framework region.

3. A fusion protein comprising (i) an extracellular domain of a tissue factor protein having the amino acid sequence of 1-208, 1-209, 1-210, 1-211, 1-212, 1-213, 1-214, 1-215, 1-216, 1-217, 1-218, 1-219, 1-220, or 1-221 amino acid residues of SEQ ID NO: 110, (ii) a single domain antibody according claim 1, and (iii) a linker.

4. The fusion protein according to claim 3, wherein the linker has a length of 15-30 amino acids.

5. The fusion protein according to claim 3, further comprises a protease cleavage site.

6. The fusion protein according to claim 5, wherein the protease cleavage site is a thrombin cleavage site or a FXa cleavage site.

7. A pharmaceutical composition comprising the fusion protein of claim 3 and a pharmaceutically acceptable carrier.

8. A single domain antibody comprising the sequence selected from the group consisting of SEQ ID NOs: 62-99, or a sequence having 95% identity thereof, provided that the sequence variation is in a non-CDR framework region.

9. A fusion protein comprising the amino acid sequence of SEQ ID NO: 106, 107, 108, 109, 111, or 112.

10. A pharmaceutical composition comprising the fusion protein of claim 9 and a pharmaceutically acceptable carrier.