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(54) Title: SOLUBLE TREM-1 FAMILY PEPTIDES AND METHODS OF USE

(57) Abstract: The invention provides compositions including sTLT-1 and sTREM-1 polypeptides, the use of such polypeptides in therapeutic methods, and kits including such peptides.



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## **SOLUBLE TREM-1 FAMILY PEPTIDES AND METHODS OF USE**

### **CROSS-REFERENCE TO RELATED APPLICATION**

This application claims priority to US Provisional Patent Application Serial No. 61/177,242,  
5 filed on May 11, 2009 which is incorporated herein in its entirety.

### **STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH**

10 This work was supported by the National Cancer Institute of the National Institutes of Health Contract No. N01-Co-12400, the National Center for Cancer Research under NIH Grant No. 2G12RR3035, and the National Institute of General Medical Sciences under NIH Grant SC2GM081237. The government has certain rights in the invention.

### **BACKGROUND**

15 Septic shock claims over 200,000 people a year in the United States and is a leading cause of morbidity and mortality. The clinical manifestations associated with a hypovolemic shock from sepsis or viral infection include hemoconcentration and low blood pressure, resulting from an acute increase in capillary permeability. Release of cytokines, such as tumor necrosis factor (TNF- $\alpha$ ) and interleukin 1b (IL-1b), enhances neutrophil extravagation into the tissues,  
20 subsequently leading to vascular leakage. Both platelets and endothelial cells are called upon to control the loss of blood and plasma from the vessels through receptor engagement, release of granule contents, and remodeling of their actin cytoskeletons. Storage granules in endothelial cells (weibel palade bodies), and in platelets (alpha and dense granules) play a major role in maintaining vascular integrity. Upon activation, platelets and endothelial cells release proteins  
25 from their storage granules into the plasma that enhance their ability to stop blood leakage.

The morbidity of sepsis begins with an inflammatory response that causes endothelial dysfunction, vascular leakage, and a subsequent systemic activation of the hemostatic system manifested as profound thrombocytopenia and disseminated intravascular coagulation (DIC). Death from hypovolemic shock occurs when the deposition of microthrombi, together with  
30 vasodilation, results in loss of perfusion leading to multiple organ failure. Platelets play an integral part in the thrombin generation and thrombus formation that lead to organ failure and death.

### **SUMMARY OF THE INVENTION**

The invention provides compositions and methods including soluble fragments of TLT-1 and  
35 TREM-1.

The invention provides for the use of an active sTLT-1 peptide for the preparation of a medicament for the treatment of inflammation. In certain embodiments, the inflammation is related to a coagulation disorder. In certain embodiments, active sTLT-1 family peptide promotes coagulation. In certain embodiments, the inflammation is related to sepsis or hypovolemic shock. In certain  
5       embodiments, the inflammation is related to infection. In certain embodiments, the inflammation is related to a wound. In certain embodiments, the inflammation is related to trauma. In certain embodiments, inflammation is related to vascular damage. In preferred embodiments, the active sTLT-1 peptide reduces inflammation.

10       The invention further provides for the use of an active sTLT-1 peptide for the preparation of a medicament for the treatment of a coagulation disorder. In certain embodiments, the coagulation disorder is at least one of sepsis, hypovolemic shock, stroke, vascular occlusion, and thrombosis.

The invention provides for the use if an active sTLT-1 peptide including an amino acid sequence at least 80%, 85%, 90%, 95%, 98%, or 100% identical to: CHYRLQDVKAQKVWCR FLPEGCQPLVSSAVDRRAPAGRRFTLTDLGGGLLQVEMVTLQEEDAGEYGC.

15       The invention provides a kit to detect a sTLT-1 peptide in a subject sample, wherein presence of the sTLT-1 is indicative of the subject suffering from sepsis or hypovolemic shock. In certain embodiments, the presence of the sTLT-1 peptide in the sample comprises a concentration of at least 15 µg/ml, 20 µg/ml, 25 µg/ml, 30 µg/ml, 35 µg/ml, 40µg/ml, 45 µg/ml, or 50 µg/ml. In certain  
20       embodiments, the test provided by the kit is performed at a first time and at a later second time, e.g., a time interval of about 30 minutes, one hour, 2 hours, 3 hours, 4, hours, 6, hours, 8 hours, 12 hours, 24 hours, or more, wherein an increase in the amount of sTLT-1 peptide in the sample from the first time to the later second time is indicative of the subject suffering from sepsis or hypovolemic shock; and wherein a decrease in the amount of sTLT-1 family peptide in the sample from the first time to the later second time is indicative of the subject not suffering from sepsis or hypovolemic shock. The kit  
25       can include, for example, an antibody, such as a polyclonal antibody, to recognize one or more sTLT-1 sequences.

The invention provides methods of diagnosing sepsis or hypovolemic shock in a subject comprising:

- a) providing a serum sample from a subject;
- 30       b) detecting a sTLT-1 family peptide in the serum,

wherein a sTLT-1 family peptide in the serum is indicative of sepsis or hypovolemic shock is diagnosed. In certain embodiments, the amount of sTLT-1 peptide in the serum is at least 50 µg/ml of sTLT-1 polypeptide. In certain embodiments, the test is performed at a second time, after a time interval of about 30 minutes, one hour, 2 hours, 3 hours, 4, hours, 6, hours, 8 hours, 12 hours, 24

hours, or more, and the amount of sTLT-1 peptide in the serum from the first test is compared to the amount of sTLT-1 peptide in the serum from the second test, and an increase in the amount of the sTLT-1 peptide in the second sample as compared to the first sample is indicative of sepsis or hypovolemic shock, and a decrease in the amount of sTLT-1 in the second sample as compared to the first sample is indicative of not having sepsis or hypovolemic shock.

The invention provides methods of treatment of inflammation including administration of an active sTLT-1 peptide. In certain embodiments, the inflammation is related to a coagulation disorder. In certain embodiments, active sTLT-1 family peptide promotes coagulation. In certain embodiments, the inflammation is related to sepsis or hypovolemic shock. In certain embodiments, the inflammation is related to infection. In certain embodiments, the inflammation is related to a wound. In certain embodiments, the inflammation is related to trauma. In certain embodiments, inflammation is related to vascular damage. In preferred embodiments, the active sTLT-1 peptide reduces inflammation.

The invention provides method of treatment of a coagulation disorder by administration of an sTLT-1 peptide. In certain embodiments, the coagulation disorder is at least one of sepsis, hypovolemic shock, stroke, vascular occlusion, and thrombosis

In the methods, the active sTLT-1 peptide includes an amino acid sequence at least 80% identical to: CHYRLQDVKAQKVWCRFLPEGCQPLVSSAVDRRAPAGRRTFLTDLGGGLLQVEMVTLQEEDAGEYGC.

The invention provides pharmaceutical composition including an active sTLT-1 peptide having an amino acid sequence at least 80% identical to: CHYRLQDVKAQKVWCRFLPEGCQPLVSSAVDRRAPAGRRTFLTDLGGGLLQVEMVTLQEEDAGEYGC in a pharmaceutically acceptable carrier. In certain embodiments, the amino acid sequence consists essentially of CHYRLQDVKAQKVWCRFLPEGCQPLVSSAVDRRAPAGRRTFLTDLGGGLLQVEMVTLQEEDAGEYGC.

The invention provides for the use of an active sTREM-1 peptide for the preparation of an adjuvant for administration in conjunction with an antigen to stimulate an immune response. In certain embodiments, the sTREM-1 polypeptide includes an amino acid sequence at least 80% identical to: CDYTLEKFASSQKAWQIIRDGEMPKTLACTERPSKNSHPVQVGRILEDYHDHGLLRVRMVNLQVEDSGLYQC.

The invention provides kits including a polypeptide having an amino acid sequence at least 80% identical to: CDYTLEKFASSQKAWQIIRDGEMPKTLACTERPSKNSHPVQVGRILEDYHDHGLLRVRMVNLQVEDSGLYQC.

The invention provides pharmaceutical compositions including an active sTREM-1

polypeptide, the polypeptide having an amino acid sequence at least 80% identical to:

CDYTLKFASSQKAWQIIRDGEMPKTLACTERPSKNSHPV QVGRILEDYHDHGLLRVRM  
VNLQVEDSGLYQC. In certain embodiments, the polypeptide consisting essentially of an amino  
acid sequence of: CDYTLKFASSQKAWQIIRDGEMPKTLACTERPSKNSHPVQVGRILE  
5 DYHDHGLLRVRM VNLQVEDSGLYQC.

The invention provides method of immunization of a subject comprising co-administration of  
an active sTREM-1 polypeptide and an adjuvant.

The invention provides kits for practicing the methods of the invention.

## DEFINITIONS

10 “Adjuvant” as used herein is understood as an agent that increases the immune response, and  
thereby the efficacy of a vaccine.

An “agent” is understood herein to include a therapeutically active compound or a potentially  
therapeutic active compound. An agent can be a previously known or unknown compound. As used  
herein, an agent is typically a non-cell based compound, however, an agent can include a biological  
15 therapeutic agent, e.g., peptide or nucleic acid therapeutic, cytokine, antibody, etc.

As used herein “amelioration” or “treatment” is understood as meaning to lessen or decrease  
at least one sign, symptom, indication, or effect of a specific disease or condition. For example,  
amelioration or treatment of sepsis or hypovolemic shock can be determined using the methods  
provided herein, or any other clinically acceptable indicators of disease state or progression.  
20 Amelioration or treatment of a coagulation disorder can be determined by determining bleed time/  
clotting time. Amelioration and treatment can require the administration of more than one dose of an  
agent or therapeutic. Amelioration and treatment can include the prevention or a limitation of  
exacerbation of sepsis or hypovolemic shock by, for example, early detection of DIC or other  
exacerbations of sepsis or hypovolemic shock. As used herein, “prevention” is understood as to limit,  
25 reduce the rate or degree of onset, or inhibit the development of at least one sign or symptom of a  
disease or condition. For example, by treatment of a subject who has undergone a trauma or an  
immunocompromised subject having an infection who is susceptible to shock. Prevention can require  
the administration of more than one dose of an agent or therapeutic. Prevention can require  
administration of a combination of therapeutics.

30 As used herein, an “antigen” is understood as a molecule that can stimulate an immune  
response, e.g., protein, nucleic acid, small molecule, etc. Antigens can be used, for example, in  
vaccines to stimulate an immune response for prophylaxis, e.g., seasonal flu, measles, tetanus,  
hepatitis, that are a threat to the general population. Tumor antigens can be used, for example, to treat  
diseases already ongoing in a subject, e.g., Alphafetoprotein (AFP), Carcinoembryonic antigen

(CEA), CA-125, MUC-1, epithelial tumor antigen (ETA), Melanoma-associated antigen (MAGE), and abnormal products of ras, p53, etc. Tumor antigens can also be obtained from a tumor present in a subject to make a specific antigen for immunization of the subject. The adjuvants of the instant invention can be co-administered with molecules that are typically not sufficiently immunogenic to produce a robust immune response. The amount of antigen to be administered will depend on any of a number of factors including, for example, the strength of the antigen and the adjuvant.

As used herein, "changed as compared to a control" sample or subject is understood as having a level of the analyte or diagnostic or therapeutic indicator to be detected at a level that is statistically different than a sample from a normal, untreated, or control sample. Control samples include, for example, cells in culture, one or more laboratory test animals, or one or more human subjects. Methods to select and test control samples are within the ability of those in the art. An analyte can be a naturally occurring substance that is characteristically expressed or produced by the cell or organism (e.g., detection of sTLT-1 in serum) or a substance produced by a reporter construct (e.g.,  $\beta$ -galactosidase or luciferase). Depending on the method used for detection the amount and measurement of the change can vary. For example, a change in the amount of cleavage of analyte present will depend on the exact reaction conditions and the amount of time after exposure to the agent the sample is collected. Changed as compared to a control reference sample can also include decreased binding of a ligand to a receptor in the presence of an antagonist or other inhibitor. Determination of statistical significance is within the ability of those skilled in the art.

As used herein, "co-administration" is understood as providing two or more agents to a subject such that they are active at the same time within the subject and does not require, but does not exclude, the administration of an admixture of the agents. The time during which agents are active in a subject can be readily determined and are known in the art. Co-administration can also include contacting cells ex vivo with two or more agents, e.g., obtaining dendritic cells from a subject, contacting the cells with an antigen and an adjuvant, e.g., sTREM-1. It is understood that it may be necessary to administer one agent more frequently than the other agent.

"Consisting essentially of" is understood to have the meaning assigned in US patents as limiting the scope of a claim to the specified materials or steps "and those that do not materially affect the basic and novel characteristic(s)" of the claimed invention. As used herein in reference to antigenic polypeptides, "consisting essentially of" is understood as a polypeptide sequence including the claimed sequence, and optionally further containing other elements or optionally having shorter amino acid sequences than presented that do not materially affect the basic and novel characteristics of the polypeptide. That is, other elements or deletion of sequences that neither substantially inhibit the anti-inflammatory activity and/or its activity as an inhibitor or sepsis or shock. In certain embodiments, antigenic fragments of longer polypeptides can be expressed to include an initiator

methionine, a signal sequence for translocation of the protein, or may include sequences at the N- or C-terminus after cleavage with a protease not present in the native sequence.

“Contacting a cell” is understood herein as providing an agent or isolated cell to a test cell or cell to be treated in culture or in an animal, such that the agent or isolated cell can interact with the surface of the test cell or cell to be treated, potentially be taken up by the test cell or cell to be treated, and have an effect on the test cell or cell to be treated. The agent or isolated cell can be delivered to the cell directly (e.g., by addition of the agent to culture medium or by injection into the cell or tissue of interest), or by delivery to the organism by an enteral or parenteral route of administration for delivery to the cell by circulation, lymphatic, or other means.

“Contiguous” is understood as touching or connected to through an unbroken sequence.

As used herein, “detecting”, “detection” and the like are understood that an assay performed for identification of a specific analyte in a sample (e.g., a sTLT-1 or sTREM-1 peptide, e.g., in serum) or a product from a reporter construct in a sample. Detection can also include identification of activation of a kinase or other enzyme, or a change in cytokine level, level of a protein in serum or plasma, etc. Detection can include the identification of a mutation in a gene sequence, such as a point mutation, a deletion of all or part of the coding sequence or transcriptional/ translational regulatory sequences of the gene, a truncation of the gene sequence, or any other alteration that can alter the expression level or the sequence of the protein expressed by the gene, particularly when the alteration of the sequence results in a phenotypic change in the subject. The amount of analyte detected in the sample can be none or below the level of detection of the assay or method.

As used herein, a “diagnosing” is understood as the process of recognizing a disease or condition by observation, either directly or using devices such as x-ray, MRI, CT-scan, thermometer, sphygmomanometer, etc; and/ or analyzing a sample from a subject e.g., using clinical laboratory methods, to identify a subject suffering from or suspected of suffering from a disease or condition.

Diagnosing typically includes observation of a number of signs or symptoms of disease in combination with one or more diagnostic tests performed, for example, in a clinical laboratory. It is not required that any single test, or any single test performed one time be sufficient to provide a conclusive diagnosis of the disease or condition from which the subject is suffering.

As used herein, the terms “effective” and “effectiveness” includes both pharmacological effectiveness and physiological safety. Pharmacological effectiveness refers to the ability of the treatment to result in a desired biological effect in the patient. Physiological safety refers to the level of toxicity, or other adverse physiological effects at the cellular, organ and/or organism level (often referred to as side-effects) resulting from administration of the treatment. On the other hand, the term “ineffective” indicates that a treatment does not provide sufficient pharmacological effect to be

therapeutically useful, even in the absence of deleterious effects, at least in the unstratified population. (Such a treatment may be ineffective in a subgroup that can be identified by the expression profile or profiles.) “Less effective” means that the treatment results in a therapeutically significant lower level of pharmacological effectiveness and/or a therapeutically greater level of adverse physiological effects, e.g., greater liver toxicity.

Thus, in connection with the administration of a drug, a drug which is “effective against” a disease or condition indicates that administration in a clinically appropriate manner results in a beneficial effect for at least a statistically significant fraction of patients, such as a improvement of symptoms, a cure, a reduction in disease signs or symptoms, extension of life, improvement in quality of life, or other effect generally recognized as positive by medical doctors familiar with treating the particular type of disease or condition.

As used herein, “heterologous” as in “heterologous protein” is understood as a protein not natively expressed in the cell in which it is expressed. The heterologous protein may be, but need not be, from a different species.

As used herein, the terms “identity” or “percent identity”, refers to the subunit sequence similarity between two polymeric molecules, e.g., two polynucleotides or two polypeptides. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, e.g., if a position in each of two peptides is occupied by serine, then they are identical at that position. The identity between two sequences is a direct function of the number of matching or identical positions, e.g., if half (e.g., 5 positions in a polymer 10 subunits in length), of the positions in two peptide or compound sequences are identical, then the two sequences are 50% identical; if 90% of the positions, e.g., 9 of 10 are matched, the two sequences share 90% sequence identity. The identity between two sequences is a direct function of the number of matching or identical positions. Thus, if a portion of the reference sequence is deleted in a particular peptide, that deleted section is not counted for purposes of calculating sequence identity. Identity is often measured using sequence analysis software e.g., BLASTN or BLASTP (available at ([www.ncbi.nih.gov/BLAST](http://www.ncbi.nih.gov/BLAST))). The default parameters for comparing two sequences (e.g., “Blast”-ing two sequences against each other), by BLASTN (for nucleotide sequences) are reward for match = 1, penalty for mismatch = -2, open gap = 5, extension gap = 2. When using BLASTP for protein sequences, the default parameters are reward for match = 0, penalty for mismatch = 0, open gap = 11, and extension gap = 1. Additional, computer programs for determining identity are known in the art.

As used herein, “immunoassay” is understood as any immunoassay format including, but not limited to ELISA, immunoprecipitation assay, dot blot, slot blot, western blot, immunofluorescence assay, and particle based flow cytometric detection; or any other method wherein the antigen is detected by its binding to the antibody, either directly, or indirectly. Immunoassay methods are well

known in the art.

As used herein, "isolated" or "purified" when used in reference to a polypeptide means that a naturally occurring polypeptide or protein has been removed from its normal physiological environment (e.g., protein isolated from plasma or tissue) or is synthesized in a non-natural environment (e.g., artificially synthesized chemically or in a heterologous system). Thus, an "isolated" or "purified" polypeptide can be in a cell-free solution or placed in a different cellular environment (e.g., expressed in a heterologous cell type). The term "purified" does not imply that the polypeptide is the only polypeptide present, but that it is essentially free (about 90-95%, up to 99-100% pure) of cellular or organismal material naturally associated with it, and thus is distinguished from naturally occurring polypeptide. Similarly, an isolated nucleic acid is removed from its normal physiological environment. "Isolated" when used in reference to a cell means the cell is in culture (i.e., not in an animal), either cell culture or organ culture, of a primary cell or cell line. Cells can be isolated from a normal animal, a transgenic animal, an animal having spontaneously occurring genetic changes, and/or an animal having a genetic and/or induced disease or condition.

As used herein, "kits" are understood to contain at least the non-standard laboratory reagents for use in the methods of the invention, such as antibodies, peptides, cDNAs or nucleic acid constructs encoding sTLT-1, or fragments thereof, for the use in the methods of the invention. The kit can further include any other components required to practice the method of the invention, as dry powders, concentrated solutions, or ready to use solutions. In some embodiments, the kit comprises one or more containers that contain reagents for use in the methods of the invention; such containers can be boxes, ampules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable container forms known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding reagents.

The term "label" or "detectable label" as used herein refers to any atom or molecule which can be used to provide a detectable (preferably quantifiable) signal, and which can be attached to a nucleic acid or protein. Labels may provide signals detectable by fluorescence, radioactivity, colorimetry, gravimetry, X-ray diffraction or absorption, magnetism, enzymatic activity, and the like. Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes (e.g.,  $^3\text{H}$ ), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (e.g., horseradish peroxidase, beta-galactosidase, luciferase, alkaline phosphatase), biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric

hindrance. In others, the label is part of the fusion protein, e.g. Green Fluorescent Protein (GFP), Yellow Fluorescent Protein (YFP).

As used herein, “monitoring” as in monitoring the condition of a subject includes performing one or more diagnostic tests repeatedly (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, or more times) over time, at  
5 regular or irregular intervals, to determine if the condition of the subject is improving, worsening, or maintained.

A “non-naturally occurring” polypeptide sequence or nucleic acid sequence and the like is an amino acid or nucleotide sequence that is not present in the proteome or the genome, respectively, of the organism from which the sequence is derived. In certain embodiments, the amino acid or  
10 nucleotide sequence can include one or more mutations that have not been identified as naturally occurring mutations. In certain embodiments, the amino acid or nucleotide sequence can be a truncated sequence or a sequence with one or more internal deletions. In certain embodiments, the amino acid or nucleotide sequence can be fused to another amino acid or nucleotide sequence, e.g., a coding sequence, a regulatory sequence, etc., that confers a new property to the sequence not present  
15 in the naturally occurring sequence. A non-naturally occurring polypeptide sequence or nucleic acid sequence can include one or more non-naturally occurring amino acids or nucleic acids.

As used herein, a “nucleic acid encoding a polypeptide” is understood as any possible nucleic acid that upon (transcription and) translation would result in a polypeptide of the desired sequence. The degeneracy of the nucleic acid code is well understood. Further, it is well known that various  
20 organisms have preferred codon usage, etc. Determination of a nucleic acid sequence to encode any polypeptide is well within the ability of those of skill in the art.

“Obtaining” is understood herein as manufacturing, purchasing, or otherwise coming into possession of.

The phrase “pharmaceutically acceptable carrier” is art recognized and includes a  
25 pharmaceutically acceptable material, composition or vehicle, suitable for administering compounds of the present invention to mammals. The carriers include liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject agent from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be “acceptable” in the sense of being compatible with the other ingredients of the formulation and not  
30 injurious to the patient. For example, pharmaceutically acceptable carriers for administration of cells typically is a carrier acceptable for delivery by injection, and do not include agents such as detergents or other compounds that could damage the cells to be delivered. Some examples of materials which can serve as pharmaceutically acceptable carriers include: sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium

carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; and other non-toxic compatible substances employed in pharmaceutical formulations.

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Examples of pharmaceutically acceptable antioxidants include: water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate,  $\alpha$ -tocopherol, and the like; and metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Formulations of the present invention include those suitable for oral, nasal, topical, transdermal, buccal, sublingual, intramuscular, intravenous, intraarterial, intraperitoneal, rectal, vaginal and/or various parenteral administration routes. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient that can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound that produces a therapeutic effect.

As used herein, "plurality" is understood to mean more than one. For example, a plurality refers to at least two, three, four, five, or more.

A "polypeptide" or "peptide" as used herein is understood as two or more independently selected natural or non-natural amino acids joined by a covalent bond (e.g., a peptide bond). A peptide can include 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more natural or non-natural amino acids joined by peptide bonds. Polypeptides as described herein include full length proteins (e.g., fully processed proteins) as well as shorter amino acids sequences (e.g., fragments of naturally occurring proteins or synthetic polypeptide fragments).

As used herein, "polypeptides" include modified polypeptides including non-natural amino acids, L-amino acids, lipid and/or carbohydrate modifications, etc. Modifications include those to modulate the pharmacokinetic and pharmacodynamic properties of the peptides, including modulation of half-life, peptide targeting, etc. Such considerations are well understood by those of skill in the art.

As used herein, “related to” is understood as one or more of being caused by, being a result of, or being coincident with, as in inflammation related to a wound, or sepsis related to inflammation and/or infection. It is understood that conditions such as inflammation can result in tissue damage at both the initial site of inflammation and at remote sites, e.g., vascular damage, which further stimulates inflammation.

A “sample” as used herein refers to a biological material that is isolated from its environment (e.g., blood or tissue from an animal, cells, or conditioned media from tissue culture) and is suspected of containing, or known to contain an analyte, such as a sTLT-1 or sTREM-1 polypeptide. A sample can also be a partially purified fraction of a tissue or bodily fluid. A reference sample can be a “normal” sample, from a donor not having the disease or condition, or from a normal tissue in a subject having the disease or condition (e.g., normal tissue vs. subject suffering from sepsis). A reference sample can also be from an untreated donor or cell culture not treated with an active agent (e.g., no treatment or administration of vehicle only) and/or stimulus. A reference sample can also be taken at a “zero time point” prior to contacting the cell or subject with the agent or cell to be tested.

“Small molecule” as used herein is understood as a compound, typically an organic compound, having a molecular weight of no more than about 1500 Da, 1000 Da, 750 Da, or 500 Da. In an embodiment, a small molecule does not include a polypeptide or nucleic acid.

As used herein, “soluble (s)TLT-1” peptide or a “recombinant soluble (rs)TLT-1” peptide is understood a sequence of contiguous amino acids of a sequence provided by at least one of GenBank No. AF508193\_1 (human) (encoded by e.g., NM\_178174.2) and NP\_082039.1 (mouse) (encoded by e.g., AY078502) in the version available on the day of filing of the instant application (all sequences incorporated by reference), having a length of at least 40 amino acids, at least 50 amino acids, at least 60 amino acids, at least 65 amino acids, at least 68 amino acids, at least 70 amino acids, at least 72 amino acids, at least 75 amino acids, at least 78 amino acids, at least 80 amino acids, at least 82 amino acids, at least 85 amino acids, at least 87 amino acids, at least 90 amino acids, at least 95 amino acids, or at least 96 amino acids, at least 97 amino acids, or at least 98 amino acids, at least 99 amino acids, or at least 100 amino acids, at least 101 amino acids, or at least 102 amino acids, at least 103 amino acids, or at least 105 amino acids, at least 106 amino acids, or more of TLT-1, preferably human TLT-1, preferably of amino acids 20-105 of human TLT-1, i.e., TLT-1 not including a transmembrane domain, and soluble in normal saline at room temperature at a concentration appropriate for dosage. In an embodiment, sTLT-1 includes at least amino acids 39 to 105 of mouse TLT-1 (GenBank NP\_082039.1) (CHYRLQDVRLK VWCQFLQEGCHPLVTS AVDRRAPGN GRIFLTDL GGLLQVEMVTLQEEDTGEYGC) or amino acids 38 to 104 of human TLT-1 (GenBank AAO37827.1) (CHYRLQDVKAQKVWCRFLPEGCQPLVSS AVDRRAPAGRR TFLTDLGG GLLQVEMVTLQEEDAGEYGC). Transmembrane domains can be predicted using any of a

number of available software packages including, but not limited to, ExPasy ([www.expasy.ch/tools/](http://www.expasy.ch/tools/)). In an embodiment, an “sTLT-1” peptide or an “rsTLT-1” peptide further includes one or more amino acid deletions or substitutions such that the “sTLT-1” peptide or an “rsTLT-1” peptide is at least 80% identical, 85% identical, 90% identical, 95% identical, 97% identical, 98% identical, 99% identical to a contiguous amino acid sequence of at least 40 amino acids, at least 50 amino acids, at least 60 amino acids, at least 65 amino acids, at least 68 amino acids, at least 70 amino acids, at least 72 amino acids, at least 75 amino acids, at least 78 amino acids, at least 80 amino acids, at least 82 amino acids, at least 85 amino acids, at least 87 amino acids, at least 90 amino acids, at least 95 amino acids, or at least 96 amino acids, at least 97 amino acids, or at least 98 amino acids, at least 99 amino acids, or at least 100 amino acids, at least 101 amino acids, or at least 102 amino acids, at least 103 amino acids, or at least 105 amino acids, at least 106 amino acids, or more of an amino acid sequence provided by one of the GenBank numbers set forth above. Mutations can be conservative mutations, or non-conservative mutations. Conservative mutations replace an amino acid with an amino acid having similar structural and/or chemical properties. Amino acids are typically grouped based on the properties of their side chains. For example, lysine, arginine, and histidine are basic amino acids. Aspartic acid and glutamic acid have acidic side chains. Glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan, and cysteine have non-polar side chains. Asparagine, glutamine, serine, threonine, and tyrosine have uncharged side chains. An rsTLT-1 peptide is a subset of the group of sTLT-1 peptides.

An “sTLT-1” peptide or a “rsTLT-1” peptide can be encoded by a native nucleic acid sequence of the *Trem1* genesuch as those provided by GenBank numbers above. Alternatively, an “sTLT-1” peptide or an “rsTLT-1” peptide can be encoded by any nucleotide sequence that provides a polypeptide having the sequence of an “sTLT-1” peptide or an “rsTLT-1” peptide. The degeneracy of the genetic code is well understood such that the native nucleic acid sequence can be substantially modified without altering the sequence of the amino acid encoded. “sTLT-1” peptide or “rsTLT-1” peptide sequences and nucleic acid sequences encoding such peptides are provided, for example in US Patent Publication No. 20040180409 which is incorporated herein by reference.

As used herein, “soluble (s)TREM-1” peptide or a “recombinant soluble (rs)TREM-1” peptide is understood a sequence of contiguous amino acids of a sequence provided by at least one of GenBank No. NP\_061113.1 (human) (encoded by e.g., NM\_018643.2) and NM\_021406 (mouse) (encoded by e.g., NP\_067381.1) in the version available on the day of filing of the instant application (all sequences incorporated by reference), having a length of at least 40 amino acids, at least 50 amino acids, at least 60 amino acids, at least 65 amino acids, at least 68 amino acids, at least 70 amino acids, at least 72 amino acids, at least 75 amino acids, at least 78 amino acids, at least 80 amino acids, at least 82 amino acids, at least 85 amino acids, at least 87 amino acids, at least 90 amino acids, at least 95 amino acids, or at least 96 amino acids, at least 97 amino acids, or at least 98 amino acids, at least

99 amino acids, or at least 100 amino acids, at least 101 amino acids, or at least 102 amino acids, at least 103 amino acids, or at least 105 amino acids, at least 106 amino acids, or more of TREM -1, preferably human TREM -1, preferably of amino acids 51 to 113 of human TREM-1

(CDYTLKFASSQKAWQIIRDGEMPKTLACTERPSKNSHPVQVGRIILEDYHDHGLLRVRM

VNLQVEDSGLYQC) or amino acids 41 to 112 of mouse TREM-1, (CPFNIMKYANSQKAW  
QRLPDGKEPLTLVVTQRPFTRPSEVHMGKFTLKHDPSEAMLQVQMTDLQVTD SGLYRC)

e.g., TREM -1 not including a transmembrane domain, and soluble in normal saline at room temperature at a concentration appropriate for dosage. Transmembrane domains can be predicted using any of a number of available software packages including, but not limited to, ExPasy

(www.expasy.ch/tools/). In an embodiment, an “sTREM -1” peptide or an “rsTREM -1” peptide further includes one or more amino acid deletions or substitutions such that the “sTREM -1” peptide or an “rsTREM -1” peptide is at least 80% identical, 85% identical, 90% identical, 95% identical, 97% identical, 98% identical, 99% identical to a contiguous amino acid sequence of at least 40 amino acids, at least 50 amino acids, at least 60 amino acids, at least 65 amino acids, at least 68 amino acids, at least 70 amino acids, at least 72 amino acids, at least 75 amino acids, at least 78 amino acids, at least 80 amino acids, at least 82 amino acids, at least 85 amino acids, at least 87 amino acids, at least 90 amino acids, at least 95 amino acids, or at least 96 amino acids, at least 97 amino acids, or at least 98 amino acids, at least 99 amino acids, or at least 100 amino acids, at least 101 amino acids, or at least 102 amino acids, at least 103 amino acids, or at least 105 amino acids, at least 106 amino acids, or more of an amino acid sequence provided by one of the GenBank numbers set forth above.

Mutations can be conservative mutations, or non-conservative mutations. Conservative mutations replace an amino acid with an amino acid having similar structural and/or chemical properties.

Amino acids are typically grouped based on the properties of their side chains. For example, lysine, arginine, and histidine are basic amino acids. Aspartic acid and glutamic acid have acidic side chains.

Glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan, and cysteine have non-polar side chains. Asparagine, glutamine, serine, threonine, and tyrosine have uncharged side chains. An rsTLT-1 peptide is a subset of the group of sTLT-1 peptides.

An “sTREM-1” peptide or a “rsTREM-1” peptide can be encoded by a native nucleic acid sequence of the *Trem1* genesuch as those provided by GenBank numbers above. Alternatively, an

“sTREM-1” peptide or an “rsTREM-1” peptide can be encoded by any nucleotide sequence that provides a polypeptide having the sequence of an “sTREM-1” peptide or an “rsTREM-1” peptide.

The degeneracy of the genetic code is well understood such that the native nucleic acid sequence can be substantially modified without altering the sequence of the amino acid encoded. “sTREM-1” peptide or “rsTREM-1” peptide sequences and nucleic acid sequences encoding such peptides.

A “soluble (s)TREM-1 family polypeptide” is understood as an sTLT-1 or an sTREM-1 polypeptide.

A "subject" as used herein refers to living organisms. In certain embodiments, the living organism is an animal. In certain preferred embodiments, the subject is a mammal. In certain embodiments, the subject is a domesticated mammal. Examples of subjects include humans, non-human primates, monkeys, dogs, cats, mice, rats, cows, horses, goats, and sheep. A human subject may also be referred to as a patient.

A subject "suffering from or suspected of suffering from" a specific disease, condition, or syndrome has a sufficient number of risk factors or presents with a sufficient number or combination of signs or symptoms of the disease, condition, or syndrome such that a competent individual would diagnose or suspect that the subject was suffering from the disease, condition, or syndrome. Methods for identification of subjects suffering from or suspected of suffering from conditions such as sepsis, hypovolemic shock, a wound, inflammation or a disease or condition related to inflammation, a coagulation disorder, trauma, or a subject in need of treatment of such conditions, is within the ability of those in the art. Subjects suffering from, and suspected of suffering from, a specific disease, condition, or syndrome are not necessarily two distinct groups.

"Therapeutically effective amount," as used herein refers to an amount of an agent which is effective, upon single or multiple dose administration to the cell or subject, in prolonging the survivability of the patient with such a disorder beyond that expected in the absence of such treatment.

An agent can be administered to a subject, either alone or in combination with one or more therapeutic agents, as a pharmaceutical composition in mixture with conventional excipient, e.g., pharmaceutically acceptable carrier, or therapeutic treatments.

The peptide agents of the instant invention would be administered at a dose determined by the condition to be treated or prevented and other considerations known to those of skill in the art. Peptide agents are typically administered at a dose of about 1 ng/kg to about 1 mg/kg body weight, preferably about 10 ng/kg to about 100 µg/kg body weight of the subject to be treated, e.g., about 1 ng/kg to about 100 ng/kg body weight, about 10 ng/kg to about 10 µg/kg body weight, about 1 µg/kg to about 100 µg/kg body weight, about 10 ng/kg to about 100 ng/kg body weight, or about 10 ng/kg to about 10 µg/kg body weight.

The pharmaceutical agents may be conveniently administered in unit dosage form and may be prepared by any of the methods well known in the pharmaceutical arts, e.g., as described in *Remington's Pharmaceutical Sciences* (Mack Pub. Co., Easton, PA, 1980). Formulations for parenteral administration may contain as common excipients such as sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes and the like. In particular, biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be useful excipients to control the

release of certain agents.

It will be appreciated that the actual preferred amounts of active compounds used in a given therapy will vary according to e.g., the specific compound being utilized, the particular composition formulated, the mode of administration and characteristics of the subject, e.g., the species, sex,  
5 weight, general health and age of the subject. Optimal administration rates for a given protocol of administration can be readily ascertained by those skilled in the art using conventional dosage determination tests conducted with regard to the foregoing guidelines.

The term "wild-type" or "WT" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that  
10 which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene or gene product which displays modifications (e.g. deletions, substitutions, etc.) in sequence and or functional properties (i.e., altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have  
15 altered characteristics when compared to the wild-type gene or gene product.

Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21,  
20 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

"At least" a particular value is understood to mean the specific value provided, optionally including more.

Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive.

25 Unless specifically stated or obvious from context, as used herein, the terms "a", "an", and "the" are understood to be singular or plural.

Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%,  
30 0.1%, 0.05%, or 0.01% of the stated value.

The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in

combination with any other embodiments or portions thereof.

All oligonucleotide sequences are written from the 5'-end to the 3'-end unless otherwise specifically noted. All peptides are written from the N-terminus to the C-terminus.

Nucleic acids encoding the various peptide sequences can readily be determined by one of  
 5 skill in the art, and any sequence encoding any of the peptide sequences of the invention falls within the scope of the invention, as well as the complement of the coding sequence, and double stranded nucleic acid sequences including coding sequences and their complement as well as artificial and non-naturally occurring sequences and their complement.

Any compositions or methods provided herein can be combined with one or more of any of  
 10 the other compositions and methods provided herein.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Elevated levels of D-dimers and sTLT-1 in patients diagnosed with sepsis. Plasma isolated from patients diagnosed with sepsis or healthy individuals was evaluated for the presence of D-dimers by ELISA (A) and sTLT-1 by dot blot analysis (B). Markers represent individual patients.  
 15 Horizontal lines represent the mean. \*\*P values from t-test.

Figure 2. Correlations between sTLT-1, DIC, and outcome in sepsis. (A) Time course of plasma sTLT-1 concentrations in surviving (circles) and non-surviving patients (squares). Fifteen healthy donors served as controls (triangles). By day 4 non-survivors showed higher sTLT-1 concentrations than survivors (ANOVA,  $p < 0.03$ ). (B, C) Correlation between plasma sTLT-1  
 20 concentration and DIC score as calculated using the ISTH criteria (B) or plasma D-dimer levels (C). Rs values were calculated via the Spearman test. (D) Soluble TLT-1 levels in patients with DIC (DIC score 5) compared to those without. P value represents the results of a Mann-Whitney test. (E) Receiver Operative Characteristic curve for sTLT-1 prediction of DIC. AUC = area under the curve.

Figure 3. sTLT-1 augments platelet aggregation. Platelet aggregation was initiated by 0.5  $\mu$ M  
 25  $\text{TxA}_2$  mimetic, U46610 (A,B), 3  $\mu$ M ADP (C), or 0.625  $\mu$ g/ml collagen (D) in the presence or absence of the indicated concentration of rsTLT-1 (A) or 60  $\mu$ g/ml (B,C,D). In (B) a peptide (25  $\mu$ M) comprised of 17 amino acids 94-110 of TLT-1 or a control peptide was added together with the rsTLT-1. These results are representative of at least three experiments.

Figure 4. Fibrinogen interaction with TLT-1. (a) Elution fractions from an AminoLink  
 30 column loaded with nothing (Empty), TLT-1 or TREM-1 after exposure to platelet lysate, washing and elution with decreasing pH. Fractions 3, 5, and 7 from each column were resolved by PAGE and were stained with Coomassie blue. (b) Fractions eluted off nickel column preloaded with purified TLT146-HIS or TREM-1-His (No TLT) and exposed to platelet lysate. Flow through (FT) and

proteins eluted by 250mM imidazole (25%) were resolved by PAGE in non-reduced (Non-red, left panel), or reduced form (Red, two right panels). Gels were stained with coomassie blue (left two panels) or immunoblotted with anti-fibrinogen (IB:  $\alpha$ -Fg, right panel). (C) TLT-1-Ig (Red) or control Ig fusion (Blue) binding to plate-bound vitronectin (VN), fibrinogen (Fg), or BSA. Positive controls (+CNT) were Ig fusions bound directly to plastic. After washing bound Ig fusion was detected using anti-human Ig. Markers represent individual determinations and the horizontal line represents the mean.

Figure 5. The cytoplasmic tail of TLT-1 binds ERMs: (A, Left) Whole cell lysate (WCL) or proteins bound to GST alone, or GST-TLT-1 cytoplasmic tail (GST-TLT-1) were stained with Simply Blue (Invitrogen). The unique TLT-1 binding protein is boxed. (A, Right) Proteins as above were immunoblotted with anti-moesin. Elutes of GST-TLT-1 that were not exposed to platelet lysate are in lane 3. (B) Confocal fluorescent images (633X) of resting platelets (top) and those activated for 2 min with thrombin (bottom). Cells were stained with goat anti-TLT-1 (red) and rabbit anti-moesin (green). (C) Whole cell lysate from highly purified human (lane 1) or mouse (lane 2) platelets were probed with ERM antibodies as indicated. (D) HEK293 cells were mock transfected (MT) or co-transfected with V5-tagged TLT-1 and/or GFP-tagged ezrin as indicated. Lysates were immunoprecipitated and probed as indicated. (E) Cos7 cells transfected (+) or not (-) with either yellow fluorescent protein (YFP) alone or a TLT-1 YFP construct were immunoprecipitated with anti-YFP and probed as indicated. (E) Primary human platelets were either stimulated (+) or not (-) with thrombin then lysed and TLT-1 was immunoprecipitated with anti-TLT-1 or control (C) antibody. Filters were probed with anti-moesin then anti-TLT-1.

Figure 6. Targeting of the *Trem1* locus. (A) A schematic representation of the *Trem1* genomic region and the strategy for replacement of exons (black boxes) 1 and 2 with the NEO cassette (grey rectangle). The positions of restriction sites for EcoRI (E), KpnI (K) are noted as are the positions of the upstream (UP) and downstream (DP) probes used for southern analysis of recombinants. (B) PCR analysis of tail DNA from a WT and two *Trem1* null mice. Primers 1 and 2 amplify products of 947 by (WT), and primers 1 and 3 amplify a produce of 1250 by (null) (C) Southern analysis of tail DNA from a wt and two homozygote null mice digested with KpnI and probed with UP. (D) Western blot of whole cell extracts of platelets from *Trem1*<sup>-/-</sup>, WT, or heterozygote mice (top). Immunoblotting for actin served as a loading control (d, bottom). (E) Confocal analysis of U46619 activated WT and null platelets stained with anti-TLT-1 antibody (left panels) or phalloidin (right panels). Bar = 7.5  $\mu$ M.

Figure 7. Platelet Aggregation Defects and Extended Bleeding Times in *Trem1* Mice. (A, B, C, and D) PRP from WT or *Trem1*<sup>-/-</sup> mice was isolated from citrated blood and aggregation was measured using thrombin (A), Collagen (B), ADP (C), or U46619 (D) as agonists. These results are

representative of at least three experiments. (E) Percent of platelets activated with suboptimal doses of ADP that bind fibrinogen as determined in whole blood flow cytometry analysis. (F) Bleeding time measured by tail snip assay in WT and *Trem1*<sup>-/-</sup> mice. Lower boundary of the whisker box is the 25th percentile and the upper boundary is the 75th percentile. The line within the box is the median and the whiskers represent the highest and lowest values (n=12 for each group, p<0.05 by t-test).

Figure 8. Relationships between sTLT-1, TNF, Platelet, and leukocytes counts during endotoxemia. Blood from naive C57BL/6 mice (TO) or mice injected i.p. with LPS (5mg/kg) was collected at the indicated times after LPS. Plasma sTLT-1(A) and TNF (B) levels were assessed by dotblot or ELISA analysis, respectively. Platelet (C) and leukocyte (D) counts were derived from whole blood analysis. Results of A, B, and C represent the mean +/- SEM. Data in D are median +/- IQR.

Figure 9. Accentuated TNF, thrombocytopenia, D-dimer production and decreased survival in LPS treated *Trem1*<sup>-/-</sup> mice. (A) WT and *Trem1*<sup>-/-</sup> mice (n=18) were challenged i.p. with 6 mg/kg LPS. After 2 hrs blood was collected from the retroorbital plexus and serum TNF was assayed. Markers represent individual animals and horizontal lines represent the mean (p=0.01 by Mann Whitney test). Survival was then monitored over the next 80 hours (C) (p<0.05 by Log Rank test). (B) Mice of the indicated genotype were injected i.p. with LPS. Twenty-four hours later plasma levels of D-dimer were determined. Markers represent individual animals and horizontal lines represent the mean (p<0.05 by student's t test).

Figure 10. Targeted deletion of *Trem1*<sup>-/-</sup> results in increased hemorrhage associated with inflammation. (A) Representative macroscopic views of the Schwartzman lesion from WT and *Trem1*<sup>-/-</sup> mice. (B) Microscopic view of the Schwartzman lesion of WT and *Trem1*<sup>-/-</sup> mice (40x top and 200x bottom). Skin sections from WT or *Trem1*<sup>-/-</sup> mice are shown stained with hemoatoxylin and eosin. Areas of hemorrhage are marked with green arrows. Characteristic thrombi are marked by black arrows. (C and D) The lesions of the Schwartzman reaction were scored for hemorrhage as defined in methods (C), and area of the lesion (D) (WT maroon n=9; *Trem1*<sup>-/-</sup> blue n=8: ' p <0.05, \*"p<0.001 by t test).

Figure 11. Quantification of platelet adherence to endothelial cells. (A-F) Flow cytometric analysis of platelet adherence. FL-1 is shown on the Y-axis (calcein) and FL-2 (PE) is shown on the X-axis. Scatter profile of resting endothelial cells showing gate (PEC) used to identify adherent platelets (a), activated endothelial cells (b), resting platelets (c), and activated platelets (d). (e) Quantification of adherent platelets by flow cytometry. Results are from four independent experiments. PEC (platelet—endothelial cell) BAEC R (BAEC resting), PLTR (resting platelets), PLTA (activated platelets) and BAEC A (BAEC activated).

Figure 12. rsTLT-1 effect on platelet adhesion to resting and activated endothelial cells. (a, b)

Flow cytometric analysis of platelets within the PEC gate shown in Figure 1a after incubation with activated endothelial cells either without rsTLT-1 (a), or with rsTLT-1 (b). Quantification of platelet adhesion to (c) resting BAEC (d) TxA<sub>2</sub> activated BAEC incubated with either resting or thrombin activated human platelets. Binding was expressed as the number of platelets stained with calcein bound to the endothelial cell monolayer. The data presented here is an average of three independent experiments. PEC, platelet—endothelial cell; ER, BAEC resting; PR, resting platelets; PA, activated platelets; EA, BAEC activated; and PA, activated platelets. (\*) stars represent a  $P < 0.05$ .

Figure 13. Actin polymerization and platelet spreading on sTLT-1 and/or fibrinogen matrixes, (a-d) Representative photomicrographs demonstrating changes in platelet spreading on fibrinogen matrixes (100 µg/ml) in the absence of rsTLT-1 (a) or with 25 µg/ml rsTLT-1 (b), 50 µg/ml rsTLT-1 (c), or 100 µg/ml rsTLT-1 (d). (e) Quantification of platelet spreading and (f) average platelet radius - TLT-1 as evaluated and determined using quantification of rhodamine phalloidin. Platelets are counter stained with anti gpIIb IIIa (green). These are representative of at least three independent experiments. (\*) stars represent a  $P < 0.05$ .

Figure 14. Quantification of the binding of human platelets to fibrinogen rsTLT-1 matrixes, (a- d) Representative photomicrographs demonstrating changes in platelet binding on fibrinogen matrixes (100 µg/ml) in the absence of rsTLT-1 (a) or with 25 µg /ml rsTLT-1 (b), 50 µg/ml rsTLT-1 (c), or 100 µg/ml rsTLT-1 (d). (e) Quantification of platelet: rsTLT-1: fibrinogen matrices as evaluated by quantification of rhodamine phalloidin. Platelets are counter stained with anti gpIIb IIIa (green). These are representative of at least three independent experiments.

Figure 15 A-B Human sTREM-1 activates human monocytes. Peripheral blood monocytes were plated at  $1 \times 10^6$  cells/ml and cultured in the absence (untreated) or presence of sTREM-1 (10 µg/ml) or ultrapure LPS (1-2 µg/ml) for 48h. (a) Cells were then collected and the expression of CD80, CD86, CD18, CD29 and CD54 was analyzed by flow cytometry. A representative experiment out of 3 is shown. (b) Supernatants from 48h monocyte cultures were analyzed for the expression of hTNF-a, hIL-6 and hIL-10. One representative experiment out of two is shown.

Fig. 16A-C Human sTREM-1 activates human dendritic cells (DCs). (a) Peripheral blood CD1<sup>+</sup> DCs were freshly purified and plated in the presence of sTREM-1 (10 µg/ml) or ultrapure LPS (1 µg/ml) or left untreated for 48h. Supernatants were collected after 48h and analyzed for IL-6, TNF-alpha, IL-12p70 and IL-10 content. Graphs show the average of three different donors  $\pm$  SD.

\*= $p < 0.01$ ; \*\*= $p < 0.05$ . (b) Immature human mo-DCs were cultured in the presence or absence of sTREM-1 (10 µg/ml) or ultrapure LPS (500 ng/ml-1 µg/ml) for 48h. Membrane expression of CD80, CD83, CD86 and HLA-DR molecules was analyzed by flow cytometry. The left panel shows the profile of a representative donor, with the isotype control (filled) and specific antibody (open) staining in each panel. The average (mean+SD) of 11 donors is represented in the right panel, as the increment

of Mean of Fluorescence Intensity (MFI obtained by subtracting the geometric mean of the isotype control from the specific antibody fluorescence). (c) Supernatants collected after 48h were analyzed for production of IL-6, TNF-alpha, IL-12p70 and IL-10 cytokines (Fig.15C). Data shows the average of 10 independent experiments  $\ast=p<0.01$ ;  $\ast\ast=p<0.05$ .

5 Figure 17A-B. sTREM-1-activated DCs promote Th1/Th17-type polarization in vitro. (a) Immature mo=DCs treated with sTREM-1 (10ug/ml), ultrapure LPS (500ng/ml) or left untreated (Unt) for 48 hours were co-cultured with allogeneic lymphocytes at 1:50 or 1:250 mo-DC:lymphocyte ratio. As a control, lymphocytes alone were plated without moDCs. A) After 4 days, the cultures were pulsed with  $^3\text{H}$ -TdR (1  $\mu\text{Ci}$ /well) for the last 16 h before harvested for the measurement of  $^3\text{H}$ -TdR  
10 incorporation. One representative experiment out of four is shown. (b) Supernatants from MLRs were recovered after 48-72h and analyzed for IL-5, IFN-gamma and IL-17. The fold of increase of cytokine production was plotted as: [specific production of each test] – [cytokine production of the lymphocytes alone]. Data represents the average of four independent experiments  $\pm$  SD.

Fig. 18A-D. TLR-4 is involved in sTREM-1-induced APC activation. (a) Bone Marrow-  
15 derived Dendritic Cells (BMDCs) from C3H/HeN (Left panel) or C3H/HeJ mice (right panel) were treated with sTREM-1 (10 $\mu\text{g}/\text{ml}$ ), ultrapure LPS (1 $\mu\text{g}/\text{ml}$ ) or left untreated for 48h. Cells were then collected and the expression of CD80, CD86, class II and CD11c molecules was analyzed. One representative experiment out of three is shown. Freshly isolated human monocytes were cultured at  $1 \times 10^6$  cells/ml and pretreated with anti-TLR-4 30 minutes before direct addition of sTREM-1 (b). In  
20 other cases, sTREM-1 at 100 $\mu\text{g}/\text{ml}$  was boiled (b) or treated with Proteinase-K (PK) (d) and then used at 10 $\mu\text{g}/\text{ml}$  for monocyte activation. After 48h, supernatants were collected and analyzed for TNF-alpha and IL-10 cytokine production. One representative experiment out of three is shown.

Figure 19A-D. (a) Sequence of Murine TREM-1 extracellular domain (mT1-EC) cloned into pEF6. (b) sTREM-1 levels in the supernatants of 293 cells transfected with mT1-EC or pEF6 control  
25 plasmid. (c) sTREM-1 levels in the serum of mice hydrodynamically injected with 10 $\mu\text{g}$  mT1-EC or control plasmid. (d) Survival curve of mice hydrodynamically injected with 10 $\mu\text{g}$  mT1-EC or control plasmid followed by treatment with LPS at 5 mg/kg.

Figure 20. Amino acid sequences of mouse and human TLT-1 and TREM-1. The immunoglobulin domains are underlined. The predicted transmembrane domains are in italics.

### 30 DETAILED DESCRIPTION

Triggering receptor expressed on myeloid cells (TREM)-like transcript (TLT)-1 is a type-1 single Ig domain orphan receptor specific to the  $\alpha$ -granules of platelets and megakaryocytes. TLT-1 is relocated to the platelet surface upon platelet stimulation. Here we show that in contrast to healthy individuals, patients diagnosed with sepsis have substantial levels of soluble TLT-1 (sTLT-1) in their

plasma that correlate with the presence of disseminated intravascular coagulation (DIC). sTLT-1 binds to fibrinogen and augments platelet aggregation *in vitro* and the cytoplasmic domain of TLT-1 binds the ezrin/radixin/moesin family of proteins suggesting the ability to link fibrinogen to the platelet cytoskeleton. Accordingly, platelets of *Trem1*<sup>-/-</sup> mice (lacking TLT-1 protein) fail to aggregate efficiently extending tail bleeding times. Lipopolysaccharide treated *Trem1*<sup>-/-</sup> mice develop higher plasma levels of TNF and D-dimers than wild type and are more likely to succumb during challenge. Lastly, *Trem1*<sup>-/-</sup> mice are predisposed to hemorrhage associated with localized inflammatory lesions. These data demonstrate that TLT-1 plays a protective role during inflammation by dampening the inflammatory response and facilitating platelet aggregation at sites of vascular injury. Therefore, therapeutic modulation of TLT-1-mediated effects may provide clinical benefit to patients with hyper-coagulatory conditions including those associated with inflammation.

The Triggering Receptors Expressed on Myeloid Cells (TREM) gene cluster includes several type 1, single Ig-domain-containing orphan receptors clustered on human chromosome 6 and mouse chromosome 17 (Klesney-Tait, et al. 2006. The TREM receptor family and signal integration. *Nat. Immunol.* 7:1266-1273). The founding members of the TREM receptor family (TREM-1 and TREM-2) couple to the Immune receptor Tyrosine-based Activation Motif (ITAM)-containing receptor chain, DAP12 and activate various cells of the myeloid lineage including monocytes, macrophages, neutrophils, and dendritic cells. In addition to DAP12-coupled receptors, the TREM gene cluster includes, TREM-like Transcript 1 (TLT-1) (Washington, et al., 2002. Initial characterization of TREM-like transcript (TLT)-1: a putative inhibitory receptor within the TREM cluster. *Blood* 100:3822-3824). Unlike TREM-1 and 2, TLT-1 does not couple to DAP12 and little is known regarding its function. Unlike other TREM, TLT-1 has been reported only in the platelet and megakaryocyte lineage suggesting that it plays a specific role in hemostasis and/or thrombosis and could be an attractive target for modulating platelet function. Along with p-selectin, TLT-1 is sequestered in the platelet  $\alpha$ -granules, and it has been demonstrated that upon platelet activation with thrombin, collagen, or lipopolysaccharide (LPS), it is moved to the platelet surface.

Our characterization of TLT-1 demonstrated that activated platelets release a soluble fragment detectable in serum but not in plasma, of healthy mice or humans. This finding suggests that detection of significant levels of sTLT-1 in the plasma may serve as an important indicator of peripheral platelet activation in specific disease states. In addition, we demonstrated that blocking TLT-1 with TLT-1 specific single chain fragment antibodies (scFv) inhibited platelet aggregation induced by low concentrations of agonists, *in vitro*, implying that TLT-1 may facilitate platelet aggregation during early stages of vessel damage *in vivo*.

Herein we show that patients diagnosed with sepsis have dramatically increased levels of soluble TLT-1 (sTLT-1) in their blood using a polyclonal antibody for detection, and that this level

correlates with the clinical manifestation of DIC. Consistent with this finding we demonstrate that TLT-1 augments platelet aggregation. We further demonstrate that TLT-1 binds fibrinogen and directly couples to the ezrin/radixin/moesin (ERM) family of actin binding proteins providing a potential mechanism for TLT-1-mediated enhancement of platelet aggregation. Accordingly, we  
5 define a defect in platelet aggregation in mice lacking TLT-1 (*Trem1*<sup>-/-</sup> mice) and find sTLT-1 in the plasma of mice challenged with LPS. Further, we demonstrate the inability of these animals to control hemorrhage associated with inflammatory injury. Collectively, these data define TLT-1 as a regulator of hemostasis during sepsis via autocrine stimulation of platelet aggregation. Moreover, these data define TLT-1 as a potentially valuable biomarker for sepsis and imply that the circulating levels  
10 sTLT-1 represent biologically active molecules in the regulation of inflammation and thrombosis.

The potential biological significance of the sTLT-1 fragment is reinforced by the existence of two splice variants with limited or absent intracellular domains. The first is the most abundant TLT-1 mRNA species and possesses an extracellular domain identical to full length TLT-1 but only a 16 aa cytoplasmic domain. The second form was recently identified in our laboratory and encodes contains  
15 only the TLT-1 extracellular domain. From an evolutionary perspective, the presence of these truncated and soluble species in both mice and humans, argues for an important role for the extracellular domain in physiology. Herein we demonstrate that sTLT-1 is released under pathological conditions resulting in changes in platelet function during those disease states. Specifically, during  
rife, unfocused platelet activation associated with sepsis, abnormally high levels of sTLT-1 are  
20 detected.

Data from two independent cohorts confirmed the presence of increased levels of sTLT-1 in sepsis patients. Our initial study incorporated patients in the very early stages of sepsis as indicated by their relatively low SOFA scores as compared to our in depth study. Our original patients also had much higher levels of sTLT than the cohort with higher SOFA scores suggesting that sTLT-1 may  
25 spike early in the septic response. However, clinical characteristics of some patients in our initial study including HIV infection, limit these interpretations.

Our finding that patients who died during sepsis had increasing levels of sTLT-1 in the 2 days following admission to the ICU, whereas those who survived showed declines in sTLT-1 during this same period, suggest that monitoring of sTLT-1 levels could be an important prognostic indicator.  
30 Similarly, the correlation between sTLT-1 and D-dimers indicates association of sTLT-1 with the clinical manifestations of DIC. In support of this conclusion, ROC analysis showed that at 50 µg/ml sTLT-1 has good sensitivity and specificity as an indicator for DIC. In fact, a recent report found that three criteria included in the current ISTH classification of DIC (platelet count, AUC 0.67; prothrombin time, AUC 0.74; and Fibrinogen AUC, 0.70) all had AUC values below what we find for  
35 sTLT-1 as an indicator of DIC (Zarbock and Ley, The role of platelets in acute lung injury. *Front.*

*Biosci.* 14:150-158, 2009). Taken together these data clearly demonstrate the involvement of TLT-1 in the host response to sepsis and indicate that sTLT-1 may provide a significant clinical tool for the diagnosis of DIC associated with sepsis perhaps becoming readily detectable well before other manifestations of DIC.

5           The ability of anti-TLT-1 scFv to block aggregation of washed platelets, suggested that TLT-1 facilitates thrombosis by interacting with a ligand(s) on or in activated platelets. Our finding here that clinically relevant levels of sTLT-1 directly promote platelet aggregation *in vitro* suggests TLT-1 is a novel, platelet specific, secondary activation factor; poised to promote aggregation in situations where only low levels of agonist are present, yet vascular integrity must be maintained. This  
10 conclusion is bolstered by our demonstration of significant platelet aggregation defects and extended bleeding times in *Trem1*<sup>-/-</sup> mice which do not express TLT-1. Surprisingly, we even detected aggregation defects in *Trem1*<sup>-/-</sup> platelets stimulated with ADP, an agonist not normally associated with the release of alpha granules. However, our analysis of TLT-1 expression in whole blood isolated from human donors or mice confirmed the ability of ADP to induce TLT-1 expression on platelets;  
15 this despite the recent confirmation of TLT-1's location within alpha granules via ultrastructural analysis. Oddly enough, our flow cytometrical analysis of sTLT-1-mediated amplification of platelet aggregation failed to demonstrate any direct binding of sTLT-1 to resting or activated platelets (Washington A.V. unpublished data). Instead, we find that TLT-1 binds fibrinogen. These data are consistent with a model where, during platelet activation, stored fibrinogen is secreted and crosslinked  
20 by both soluble and cell surface TLT-1. The inability of TLT-1 to interact with vitronectin or fibronectin suggests that unlike GPIIb/IIIa TLT-1 likely does not interact with RGD type sequences found in fibrinogen; a conclusion not unexpected given the distinct structural properties of integrins and TREM. Rather, the use of unique binding sites suggests that TLT-1 may work in concert with GPIIb/IIIa to facilitate fibrinogen/platelet interactions and/or higher order platelet aggregation. Future  
25 detailed biochemical analysis of the TLT-1/fibrinogen interaction will clarify these possibilities.

Our biochemical analysis also showed that the TLT-1 cytoplasmic tail interacts strongly with the ERM protein moesin. Thus, TLT-1 becomes the second ITIM containing receptor in platelets shown to interact with the ERMs, PECAM being the other. Although others have suggested that moesin is the only ERM in mouse platelets, moesin null mice don't show an aggregation defect like  
30 *Trem1*<sup>-/-</sup> mice. We could readily detect ezrin and radixin in purified mouse platelets and found that both these proteins also interact with TLT-1, leading us to conclude that in the absence of moesin, TLT-1 couples to other members of the ERM family. The ERMs are implicated in the formation of filopodia and lamellipodia in various cell types including platelets. Moesin signaling is regulated downstream of Rho by phosphorylation at threonine 558 in a process controlled by myosin  
35 phosphatase and Rho-kinase, both of which play a role in platelet activation during shape change. Thus, the interaction of TLT-1 with moesin we report here is consistent with our previous scFv

studies that suggested that TLT-1 functions after shape change. Moreover, when we assessed CD62 expression by flow cytometry we found no differences between *Trem1*<sup>-/-</sup> and wild type platelets suggesting that initial agonists signaling is not affected by the lack of TLT-1. Collectively, these findings support a model where platelet activation causes TLT-1 to be brought to the platelet surface facilitating the release of sTLT-1. After shape change and degranulation TLT-1 binds fibrinogen and guides rapid pseudopodia formation in platelets through interaction with moesin and other ERM proteins resulting in enhanced higher order platelet aggregation. This model places TLT-1 in an emerging class of platelet regulatory molecules including CD40L, Gas6, CD36, and the eph kinases that assist thrombin, fibrinogen, and collagen with control of the more subtle aspects of platelet aggregation providing a critical mechanisms allowing for hemostasis without thrombosis.

Our establishment of a direct role for TLT-1 in the regulation of platelet aggregation, together with the elevated sTLT-1 levels in septic patients and its association with DIC, suggested that the release of sTLT-1 during sepsis would be beneficial in maximizing platelet aggregation at sites of vascular damage during the inflammatory response. Alternatively, by virtue of its ability to reduce the aggregation threshold in the periphery, the high systemic levels of sTLT-1 detected in patients might contribute to aberrant platelet aggregation at distal sites potentiating the development of DIC and the subsequent depletion of coagulation factors that contribute to morbidity. When we assessed TLT-1 during endotoxemia in mice we found detectable levels of sTLT-1 within two hours of LPS administration. The levels of sTLT-1 were in strong inverse correlation with platelet counts. Moreover, in these experiments we did not detect an increase in cell surface TLT-1 on platelets remaining in the circulation, and when stimulated *ex vivo* these platelets expressed normal levels of TLT-1. Therefore, sTLT-1 is most likely released only by platelets as they leave the circulation during endotoxemia not from the remaining circulating pool.

In normal mice LPS causes a spike in TNF levels 2 hrs after injection that fall as the sTLT-1 levels increase. This relationship opens the possibility of a direct feedback mechanism between sTLT-1 and TNF producing cells. Indeed, activated platelets have been reported to express a TREM-1 ligand, interact with neutrophils and monocytes, and enhance neutrophil response to LPS via TREM-1. Although these initial reports suggested TLT-1 is not itself the TREM-1 ligand on activated platelets, without wishing to be bound by mechanism, we cannot currently rule out inhibition of these platelet leukocyte interactions as the mechanism whereby TLT-1 tempers inflammation.

Our challenge of *Trem1*<sup>-/-</sup> mice with LPS confirmed a role for TLT-1 in both the inflammatory and consumptive phases of sepsis. Although LPS-induced leukocytopenia was largely unaffected in *Trem1*<sup>-/-</sup> mice, they had higher serum levels of TNF and higher levels of D-dimers following LPS than did wild type mice. However, these changes translated into only a limited survival benefit for the *Trem1*<sup>-/-</sup> mice likely because DIC does not play a significant role in the mortality

associated with endotoxemia in mice. Thus, without being bound by mechanism, we propose that TLT-1 primarily supports platelet aggregation at sites of inflammatory vascular injury thereby controlling vascular integrity during the septic response. This interpretation is strongly supported by the results of our analysis of localized Shwartzman reactions in *Trem1<sup>-/-</sup>* mice. Although not all  
5 models of inflammation induced hemorrhage are dependent on platelet adhesion, the Shwartzman reaction is, as indicated by exacerbated hemorrhage in pSelectin null mice. Accordingly, we found that removal of TLT-1 slightly increased neutrophil influx at the site of injection and that Shwartzman lesions were demonstrably more hemorrhagic in *Trem1<sup>-/-</sup>* mice. Thus, without being bound by mechanism, our data suggest that TLT-1 may minimize vessel damage by regulating the production of  
10 TN F and augment platelet aggregation at the site of vessel injury preventing hemorrhage. Therefore, we suggest that the high levels of sTLT-1 detected in patients who die from sepsis likely indicate the haemostatic system's aggressive attempts at maintaining vascular integrity as well as efforts by the host to contain the inflammatory response.

We performed a platelet-endothelial adherence assay and demonstrated for the first time that  
15 TLT-1 plays a role in platelet adherence to endothelial cells in a static assay. Our results show that the addition of rsTLT-1 to either resting or TxA<sub>2</sub>-activated BAEC increased platelet adherence to the BAEC over controls. Using activated platelets appeared to cause a reduction of platelet adherence compared with incubations with resting platelets. Activated endothelial cells have been shown to shrink and lose cell-to-cell contact and detach. The addition of activated platelets may further increase  
20 the shrinkage and detachment of the endothelial cells, resulting in a greater variation in the numbers of platelets that adhere under those conditions. Although we consistently found lower numbers of adherent platelets when activated platelets were incubated with activated endothelial cells, the addition of rsTLT-1 increased the total amount of adherent platelets to similar levels seen when resting platelets were used. We have explored the possibility of sTLT-1 increasing adherence to  
25 endothelial cells, by investigating the ability of sTLT-1 to directly interact with the extracellular matrix proteins (ECM) vitronectin, collagen, and fibronectin by enzyme by enzyme-linked immunosorbent assay (ELISA). Using the ELISA assay we were, however, unable detect any interactions of sTLT-1 with any of these proteins. Therefore, we do not believe that the increased adhesion induced by sTLT-1 is mediated through any of these ECM proteins. Without being bound by  
30 mechanism, this work also suggests that sTLT-1 may play a role in the maintenance of vascular integrity and that addition of rsTLT-1 to sites of vascular injury may accelerate the cession of bleeding.

The TLT-1 receptor, to date, has only been identified on platelets. The lineage restriction of TLT-1 suggests that TLT-1 plays a specific role in platelet biology. Another interesting point is that  
35 the most significant difference was seen when rsTLT-1 was incubated with resting platelets. Although resting platelets bound activated endothelial cells more effectively than activated platelets, our results

indicate that addition of rsTLT-1 resulted in a statistically significant effect on the interaction between resting platelets and endothelial cells that was not seen with activated platelets under the same conditions. Without being bound by mechanism, based on these results and recent demonstrations of the importance of  $\alpha$ -granule components to platelet function, we hypothesize that rsTLT-1 amplifies activation signals from the endothelial cells and/or processing derived signals that may occur during the treatment. A possible mechanism for the increased adherence with resting platelets may lie in the generation of platelet microparticles. It is generally accepted that even with gentle manipulation of platelet samples, platelets will generate microparticles, which have been shown to lower the natural anticoagulant properties of endothelial cells as well as contain the procoagulant tissue factor. The combination of lower endothelial resistance to platelet adherence and low levels of tissue factor in the presence of sTLT-1 may lead to increased platelet degranulation. Our results are consistent with the finding that rsTLT-1 enhances platelet aggregation and may indicate that TLT-1 may enhance both platelet-platelet and platelet-endothelial interactions by a single mechanism.

Because actin polymerization plays a large role in platelet aggregation, adhesion, and functions downstream of the calcium signal needed for fusion of  $\alpha$  granules to the platelet membrane, we investigated the ability of TLT-1 to effect actin polymerization. Recombinant sTLT-1 increased platelet spreading and the amount of platelets that adhered to fibrinogen matrixes on glass slides. On the fibrinogen-only slides, the beginnings of filopodia extension as evidence of increased actin polymerization at 5 min can be observed (Fig. 13a-d). However, on the slides with rsTLT-1 there were visually detectable increases in the amount of platelet structures such as filopodia and lamellipodia. These differences were most noticeable on the slides that contained 50 and 100  $\mu$ g/ml of rsTLT-1. These results are consistent with the rsTLT-1-mediated adhesion of platelets to the endothelial cells. We attribute the enhanced binding and size of platelets to an increase in the amount of actin structures that were made in the presence of the rsTLT-1 compared with fibrinogen alone. Actin polymerization in platelets is a crucial step in this cascade of events; allowing for a rapid shape change in platelets that leads to the formation of filopodia and lamellipodia on activated platelets. Confocal microscopy revealed that rsTLT-1 promotes platelet cytoskeletal actin polymerization and allows for the formation of filopodia and lamellipodia at the mobile edges of the platelets. Collectively, our studies demonstrate that TLT-1 play a visible role in the maintenance of vascular integrity and demonstrates that the addition of rsTLT-1 to sites of vascular injury may accelerate the cession of bleeding.

Without being bound by mechanism, we conclude that rsTLT-1 functions to augment actin polymerization in platelets leading to increased aggregation and adhesion. Our data suggests that rsTLT-1 mediated actin polymerization may mediate enhanced platelet adhesion to the endothelium in viva. Although more studies are needed to dissect TLT-1 function and its effects in relation with vascular integrity and its association with platelets behavior, these studies give new insights to

platelet-endothelial cell interactions. Since rsTLT-1 modulates actin formation, it could affect not only platelets, but also other cells as well,

In summary our data establish TLT-1 as a molecule capable of fine-tuning platelet aggregation and inflammation for the control of vascular integrity. As such, its characterization has revealed a unique opportunity for the therapeutic separation of the benefits of hemostasis from the detriment of thrombosis. Based on our findings, we predict that specific intervention with TLT-1 or TLT-1-mediated signals might have potential in the therapy of a variety of hyper-coagulatory states including those associated with sepsis.

The invention is also related to sTREM-1 and its use to stimulate the immune system, for example, as an adjuvant during immunization. Antigen presentation is one of the critical steps to initiate adaptive immune responses. Thus, activation of Antigen-Presenting Cells (APCs) is required for them to interact with lymphocytes and initiate immune responses. The type of immune response will depend on the degree of activation, the source, timing, etc the APCs will receive.

Over the last few years, the TREM family of proteins have been reported to be involved in the modulation (activation or inhibition) of different components of the immune system 2. As a relatively new family, all the functions the members exert have not yet been defined, although depending on whether they are linked to an ITAM or an ITIM domain they have been assigned to activation vs inhibition receptor categories.

TREM-1 was among the first of these proteins to be cloned. It is expressed in neutrophils and a subset of monocytes and macrophages. Although it has been reported that platelets present a TREM-1 ligand on their surface, this remains to be characterized. Ligation of TREM-1 is believed to have activation effects, as antibody cross-linking of TREM-1 synergizes with LPS and inflammasome ligands. Its expression is upregulated in membrane and an increase in a soluble form (sTREM-1) is found upon cellular activation with LPS or fungal components. Very little is known about this soluble form, and nothing has been reported so far relating sTREM-1 to antigen presenting cells.

Herein we show for the first time that a soluble form of TREM-1 is able to activate APCs and APC precursors per se, including human monocytes, human monocyte-derived Dendritic Cells (mo-DCs) and peripheral blood CD1c<sup>+</sup> dendritic cells. We have seen no effect on human peripheral blood plasmacytoid Dendritic Cells (not shown). As the cytokine production obtained contained an increase in IL-12p70, IL-6 and IL-10, the type of responses these sTREM-1-treated dendritic cells would be promoting is not clear. But when allogeneic lymphocytes were co-cultured with those DCs, a clear Th1/Th17 polarization was induced. Th1 and Th17 lymphocytic responses are required for bacterial and fungal infection, therefore, as TREM-1 is induced by components of these pathogens, production of sTREM-1 would be helping the immune system to deal with these infections.

The observation that bone marrow-derived DCs from TLR-4 null mice did not respond to sTREM-1 indicated a potential mechanism by which sTREM-1 was exerting its effects on APCs. To corroborate this observation in our human models, anti-TLR-4 blocking mAb used in mo-DC cultures prior to sTREM-1 treatment inhibited its effects.

5           Concern about the presence of LPS in our samples being responsible for the effects observed, led us to ensure that the results were specifically due to sTREM-1 by: 1) The LAL assay showed endotoxin contamination was less than 1EU per µg of protein; 2) Protein boiling resulted in a clear decrease in sTREM-1 activity, and 3) PK treatment clearly decreased the activity of sTREM-1, indicating that there was a protein requirement.

10           Previous studies have reported that the use of a soluble form of TREM-1 extracellular domain fused with the Fc domain of hIgG1 prevents septic shock in vivo, presumably by sequestering TREM-1 ligand. Our in vitro observations point to an active proinflammatory role of sTREM-1 in inflammation instead of acting as an inhibitor, making it a useful vaccine adjuvant. The fact that only at high doses (above 100ng/ml) these observations have been made may indicate that only at the site  
15           of infection, where sTREM-1 is released and its concentration peaks, sTREM-1-mediated DC activation would happen.

              We have not observed an inhibition of LPS-induced activation when both LPS and sTREM-1 we co-cultured with APCs. Preliminary results indicate that sTREM-1 and LPS do not synergize, as low doses of sTREM-1 used in conjunction with either low doses of ultrapure LPS (1-10 ng/ml) or  
20           high doses (100 ng-1µg/ml) have no synergistic effects. What is more, sub-optimal doses of sTREM-1 do not seem to synergize with suboptimal doses of LPS.

              However, the structure of the soluble form of TREM-1 produced in vivo is not known and the location of putative protease sites and folding of the protein are yet to be described. Discrepancies between our results and those indicating a role of sTREM-1 as a scavenger receptor may be due to  
25           those differences in the structure of the proteins used.

              We propose that sTREM-1, instead or in addition of scavenging potential TREM-1 ligands and preventing an exacerbation of the inflammatory response that activated the myeloid cells 2, it may also contribute to an amplification of the response by activating APCs.

              The results provided herein demonstrate that sTREM-1 can be useful as an adjuvant for  
30           immunization, either for co-administration with an antigen, or for priming a subject prior to administration of an antigen.

              When membrane-bound TREM-1 is cross-linked with an antibody, it is reported to co-localize with TLR-4. This interaction might be responsible for the synergistic effects observed between TLR-4 ligands and TREM-1. Therefore, sTREM-1 has also the potential interact with TLR-4

after internalization, acting as a positive feedback loop of the LPS-response machinery. Furthermore, this situation could also be consider as a pro-inflammatory boost in conditions where bacterial components are not present and sTREM-1 is produced, as in arthritis, ankylosing spondylitis, and other inflammatory diseases.

5 Our laboratory has been identifying mediators that recruit and activate antigen presenting cells (alarmins). We have not observed that sTREM-1 induces migration of either antigen presenting cells, human neutrophils or lymphocytes tested. We therefore consider sTREM-1 as a mediator that fits more the definition of a damage-associated molecular pattern (DAMP), being able to induce APC maturation, thus activating both the innate and the adaptive immune system. This novel observation  
10 places sTREM-1 as a potential target for anti-inflammatory treatment, like in RA or septic shock, although these disorders use to rely on multiple factors that overlap, requiring multi-target approaches for an appropriate therapy.

### Example 1: Materials and Methods

*Initial Study Population:* The Universidad Central del Caribe Escuela del Medicina  
15 institutional review board (Federal Wide Assurance number FWA00001103) approved the study and patients or their surrogates provided written informed consent before enrollment (Protocol #200619, P.I Ismael A. Acevedo Valentin). All patients 18 years of age or older who were hospitalized and diagnosed with sepsis during a one month period were invited to participate in the study. Sepsis and their stages were judged according to the established consensus. The following items were recorded  
20 for each patient on admission into the ICU: age, sex, severity of underlying medical condition stratified according to the criteria of the Simplified Acute Physiology Score II (32)(APACHE II: scores can range from 0 to 71, with higher scores indicating a higher risk of death), the Sepsis-related Organ Failure Assessment (SOFA) (33) score (the total score can range from 0 to 24; with scores for each organ system [respiration, coagulation, liver, cardiovascular, central nervous system, and kidney]  
25 ranging from 0 [normal] to 4 [most abnormal]), and the reason for admission to the ICU. The following base-line variables were also recorded at enrollment: body temperature; leukocyte count; ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen ( $PaO_2:FiO_2$ ); and the length and the outcome (death or discharge) of stay in the ICU were also recorded. D-dimers were measured by ELISA according to manufacturer's specifications (Aniara Corporation).

30 *Longitudinal Study Patient Population.* Between July and December 2007, all consecutive patients with septic shock admitted into a 16-bed medical intensive care unit of a teaching hospital were enrolled. Data for this study were derived from pathological waste samples and the NIH Office of Human Subjects Research reviewed and approved inclusion of data from this study (approval #4037).

The diagnosis of septic shock was established on the basis of current definitions. Patients were not enrolled if they were >80 yrs of age or were immunocompromised (treatment with corticosteroids >1mg/kg equivalent prednisone; bone marrow or organ transplant recipients; neutropenia <0.5 x 10<sup>9</sup>/L; hematologic malignancy; or acquired immune deficiency syndrome).

Approval of the institutional review board and informed consent was obtained from patients or their relatives before inclusion. Upon admission into the ICU, age, gender, severity of underlying medical condition stratified according to the criteria of McCabe and Jackson, Simplified Acute Physiology Score (Le et al., 1993. A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study. JAMA 270:2957-2963.) (SAPSII), SOFA score (Vincent et al., 1996. The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. Intensive Care Med 22:707-710.), vital signs, respiratory variables, routine blood tests, and microbial culture results were recorded. The DIC score was calculated according to the ISTH recommendations (Voves et al., 2006. International Society on Thrombosis and Haemostasis score for overt disseminated intravascular coagulation predicts organ dysfunction and fatality in sepsis patients. Blood Coagul. Fibrinolysis 17:445-451.). Outcome was assessed during a 28-day follow-up period.

*Dot blot analysis:* Levels of sTLT-1 in samples of patient plasma were measured by immunoblot technique using a goat polyclonal antibody against human TLT-1 (R&D systems). Patient's samples were subjected to a series of dilutions (1:3) and 100 µl of each dilution was dotted onto a nitrocellulose membrane, dried, and subjected to 5% milk blocking for 1 hour. The nitrocellulose sheet was then incubated for 60 minutes in the presence of antibody (dilution, 1:1000). After thorough washing, the sheet was incubated for another 60 minutes with rabbit anti-goat antibody (dilution, 1:10,000; Pierce) in 5% milk block washed 3 times more with Tris buffered saline with 0.1% Tween® 20, and visualized with substrate (Pierce). Each sheet also contained calibration samples of a known concentration of rsTLT-1 (0 to 10µg per milliliter). Densitometric determination was achieved by means of the Versa doc and Quantity One Quantitation Software (Bio-Rad). The level of sTLT-1 in each sample was determined by comparing the optical densities of the samples with that of the standard curve. All measurements were performed in duplicate, and the results are expressed as µg/ml plasma.

*Nickel and Aminolink affinity columns:* We used AminoLink Plus Coupling Resin kit, TLT107 or TREM-1 was coupled to the column according to manufacturers specifications and platelet lysate was applied and washed until UV absorbance returned to baseline. Bound proteins were eluted according to manufacturers specifications. Three nickel chelating column runs were performed. First with a column preloaded with purified TLT146-HIS. After preloading the column, platelet lysate was passed over the column and flow through was collected. The column was washed as above, then

with 20mM imidazole, and finally with 50mM imidazole. TLT-1 and bound proteins were then eluted with 250mM (25%), imidazole. This same process was repeated preloading the column with TREM1-HIS or without preloading the column as controls.

*TLT-1-Ig binding to Fibrinogen:* One hundred microliters of protein solution was incubated in 96 well Nunc-Immuno Plate with maxiSorp surface (Nalgene) for either 2 hours at 37°C or overnight at 4°C. Plates were washed two times with PBS, blocked with 1 % bovine serum albumin for 30 min at room temperature, washed two times with PBS/0.5% Tween® 20 and the chimeric protein (50ng/ml) was added for 1 hr. Plates were washed three times with PBS/0.5% Tween® 20 and incubated with goat anti-human HRP secondary (Jackson Laboratories) for 1/2 hour, washed four times with PBS/0.5% Tween® 20 and 1 time with PBS then developed using 100 ul TMB substrate (SureBlue; KPL). The reaction was stopped using KPL stop solution and the plates were read at 452 nM.

*Confocal analysis:* Platelets were prepared as described in (Washington, et al., 2004. A TREM family member, TLT-1, is found exclusively in the alpha-granules of megakaryocytes and platelets. Blood 104:1042-1047). Confocal analysis was completed on an Olympus FV1000 SIM scanner inverted microscope system equipped with a 60x/1.43 oil objective (Olympus). Images were analyzed using Olympus Fluorview software

*Mice.* *Trem1*<sup>-/-</sup> and control C57BL/6 mice of the same gender and age were maintained under specific pathogen-free conditions at the National Cancer Institute (NCI)-Frederick, MD and at the Universidad Central del Caribe animal facility. Animal care was provided in accordance with the procedures outlined in, "A Guide for the Care and Use of Laboratory Animals". All mice were between 18 and 30 gm weight. Ethical approval for the animal experimentation detailed in this article was received from the Institutional Animal Care and Use Committee at the NCI Frederick (OLAW assurance number A4159-01) and/or Animal Welfare Assurance/UCC Institutional Universidad Central del Caribe School of Medicine (OLAW assurance number A3566-1).

*Whole blood flowcytometry.* Aliquots of murine blood (5 µl) was added to microcentrifuge tubes containing HEPES-Tyrode's buffer (5 mM HEPES, 137 mM NaCl, 2.7 mM NaHCO<sub>3</sub>, 0.36 mM NaH<sub>2</sub>PO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 4 mM MgCl<sub>2</sub>, and 5 mM glucose, pH 7.4) and fluorochrome-labeled ligands (all from Becton Dickinson Biosciences). For assessment of platelet function, the microcentrifuge tubes contained either no platelet agonist (for assessment of baseline activation) or ADP (3 µM or 5 µM) to activate platelets. A PE conjugated antibody to GPIIb (CD41, Becton Dickinson Biosciences) was used as an activation independent marker of platelets. Alexa 594 conjugated fibrinogen (Invitrogen) was added to permit assessment fibrinogen binding. A FITC-conjugated antibody to CD62 was used to delineate platelet surface expression of P-selectin. The reaction mixture (total of 65 µl volume) was incubated for 15 min at room temperature. Subsequently, FACS lysing solution

(Becton Dickinson Biosciences) was added to fix the platelets and lyse erythrocytes. All assays were performed in triplicate and results reported as the averages of the 2 determinations. Flow cytometric analysis was performed with the use of a FACS scan (Becton Dickinson). For analysis of platelet function platelets were identified on the basis of size and binding of anti-CD41. To assess non-activation dependent protein association with platelets, PE-conjugated IgG were used in separate control tubes. Fibrinogen binding was expressed as percentage of the increase in platelets binding fibrinogen compared to unactivated controls. Platelet activation by ADP was confirmed by expression of P-selectin. No differences were noted in expression between null and wild type platelets. Platelet activation during collection or processing in vitro was assumed to have occurred when the P-selectin expression in unstimulated platelets exceeded 5% and these samples were excluded from analysis.

*Platelet lysis, GST pulldowns, and immunoprecipitations:* Purified human platelets were handled and lysed described (Washington et al., 2004. Blood 104:1042-1047.). The entire cytoplasmic tail of TLT-1 was amplified by PCR and cloned into the pGEX-2TK vector (Amersham Biosciences). Fusion proteins were generated in BL-21 E. coli and prebound to GST-beads (Amersham). Lysates were incubated with preloaded beads for 1-2 hr at 4° then washed with lysis buffer and bound proteins were eluted with SDS-PAGE sample buffer. Immunoprecipitations and western blotting were with anti-TLT-1 (svFv C10), Anti-V5 monoclonal antibody (Invitrogen), anti-GFP (Abcam), anti-ezrin and anti-radixin (Sigma-Aldrich), or anti-moesin (Neomarkers).

*Peptide Sequencing:* Protein bands were excised from Coomassie stained gels and digested with bovine sequencing grade trypsin (Roche Diagnostics). The extracted peptides were purified using IIC18 ZipTips (Millipore Corporation). Peptides were eluted with 1 µl of solution containing 2 mg/mL of α-cyano-4-hydroxycinnamic acid in acetonitrile-0.1% TFA (50/50 vol/vol). The purified peptides were spotted on target and analyzed by MALDI-TOF MS (Matrix Assisted Laser Desorption Ionisation Time-of-Flight mass spectrometry). A Voyager-DE Pro mass spectrometer (Perceptive) was used for analysis. The instrument was operated in a positive reflector mode. The accelerating voltage was 20 kV, guide wire 0% and grid voltage 76%. The instrument was operated in reflector mode under positive ion conditions. A nitrogen laser was used at 337 nm with 150 laser shots averaged per spectrum. Calibration was performed internally using trypsin autolytic peptides. Data analysis was carried out using Data Explorer software resident on the instrument. Peptide mass lists were used to search SwissProt database with Mascot search engine 1) with the following settings. Taxonomy: human; missed cleavage: 1; peptide mass tolerance: 100 ppm.

*Construction of the Trem1<sup>-/-</sup> null mouse:* We flanked a neo cassette with -250 base pairs of DNA sequence homologous to the sequences 3' and 5' prime to exons 1 and 2 of Trem1 and the cassette was inserted into a C57BL/6-derived BAC containing the entire Trem1 gene as described (Liu, et al., 2003. A highly efficient recombineering-based method for generating conditional

knockout mutations. Genome Res. 13:476-484.). This construct was designed to completely prevent the expression of the TLT-1 protein because it deleted the leader sequence as well as the splice sites for exons one and two. BACs carrying the modification were screened by PCR using the three primer system (described below), confirmed by Southern hybridization analysis and sequencing of the modified BAC PCR products. The resulting BAC-derived targeting vector contained approximately 190 kb of isogenic homologous flanking arms. The modified BAC was digested with NotI, phenol chloroform extracted, and electroporated into C57BL/6J mouse embryonic stem cells and 96 clones were picked for analyses; 12 clones were screened by fluorescent *in situ* hybridization as described by Seed et al. (Yang and Seed, 2003. Site-specific gene targeting in mouse embryonic stem cells with intact bacterial artificial chromosomes. Nat. Biotechnol. 21:447-451.) We used the whole unmodified BAC as a probe yielding 7 positives. Clones showing only two points of hybridization by the probe were chosen for further evaluation. Their genomic structure was confirmed by Southern hybridization to KpnI or EcoRI digested DNA using upstream and downstream genomic and neo probes. The chosen clone (#5) was further confirmed by real-time PCR. Positive clones were injected into Balb/c blastocysts and chimeric mice bred to C57BL/6J females to obtain heterozygotes in a pure C57BL/6 background. Heterozygous progeny were intercrossed to generate *Trem1*<sup>-/-</sup> mice. Three-primer PCR was carried out for genotyping tail DNA from offspring. Common forward primer 1 (5' ggggtaccttgagaatcagatggccctg 3') lays 5' of the pgk-neo cassette; reverse primer 2 (5' cggcacatgtggcagctcgtccatgccgagagtg 3') is neo cassette-specific and reverse primer 3 (5' gatcatcctgcctacagtgg') was wild-type-specific. The PCR products were 1247 base pairs (mutant) and 942 base pairs (wild-type) (Figure 6).

Absence of the TLT-1 protein was determined by western blot and confocal analysis using rabbit polyclonal antibodies developed by this laboratory (Washington, et al., 2002. Initial characterization of TREM-like transcript (TLT)-1: a putative inhibitory receptor within the TREM cluster. Blood 100:3822-3824; Washington, et al., 2004. Blood 104:1042-1047.).

*Blood counts:* Whole blood was collected via cardiac puncture. Platelet and leukocyte counts were determined using a Sysmex KX-21 automated hematology analyzer cell (Mundelein, IL).

*Platelet aggregation:* Blood was isolated by cardiac puncture from mice anesthetized with CO<sub>2</sub> using a syringe containing 3.8% sodium citrate, and spun at 100 x g for 10 minutes to remove red cells. Blood from 3-5 mice of the same genotype was pooled. The final platelet count was adjusted to 2 x 10<sup>8</sup>/ml with platelet-poor plasma from the same mice. For washed platelets, platelet-rich plasma (PRP) was spun at 2100 x g for 8 minutes, the platelets were washed in 10% ACD in Tyrodes buffer and were resuspended in Tyrodes buffer with 0.02 units/ml of apyrase. Human platelets were prepared as described (Giromarelli, et al., 2007. Inhibition of thrombin-induced platelet aggregation using human single-chain Fv antibodies specific for TREM-like transcript-1. Thromb. Haemost. 97:955-963.).

Aggregation was initiated with various agonists applied to a 400- $\mu$ l aliquot at 37°C with stirring at 800 rpm and measured in a ChronoLog Corp. aggregometer. For the recombinant TLT-1 studies rsTLT-1 was incubated with the platelets at least 3 min with stirring before the addition of agonist.

5 *TNF and D-dimer analysis:* Plasma was isolated by cardiac puncture as described in platelet aggregation studies. Plasma samples were frozen and later analyzed for TNF (mouse TNF- $\alpha$  ELISA; U-Cytech, ANIARA) for human (D-dimer ELISA kit; Hyphen biomed, ANIARA), or mouse d-dimers (Asserachrom D-DI; Diagnostica Stago inc) according to the manufacturers' instructions.

10 *Protein production and peptides:* rsTLT-1 has been described (amino acids 20-125 of human TLT-1) (Gattis, et al., 2006. The structure of the extracellular domain of triggering receptor expressed on myeloid cells like transcript-1 and evidence for a naturally occurring soluble fragment. J. Biol. Chem. 281:13396-13403, incorporated herein by reference.). Peptides used in this study were purchased from Anaspec. TLT-1 peptide was derived from amino acids 94-110, sequence - (LQEEDAGEYGCMVDGAR). The control peptide sequence was derived from previously published work on the TREM family members, sequence - (TDSRCVIGLYHPPLQVY) (Gibot, et al., 2004. A  
15 soluble form of the triggering receptor expressed on myeloid cells-1 modulates the inflammatory response in murine sepsis. J. Exp. Med. 200:1419-1426.).

*Bleeding time assays:* The bleeding time measurements were performed as described (Offermanns, et al., 1997. Defective platelet activation in G alpha(q)-deficient mice. Nature 389:183-186.). Briefly, tails were cut 2mm from the end and immersed in PBS at 37°C. The bleeding time was  
20 defined as the time required for the stream of bleeding to cease. All experiments with excessive bleeding were stopped at 10 min by cauterizing the tail.

*Shwartzman reaction:* Animals were shaved in the priming region with electric clippers. A priming dose of *Escherichia coli* lipopolysaccharide (100  $\mu$ g in 100  $\mu$ l of sterile PBS; Sigma; *Escherichia coli* :LPS 0127:B8) was injected subcutaneously using a 27-gauge needle. Mouse  
25 recombinant TNF (0.3  $\mu$ g in 100  $\mu$ l sterile PBS; Pepro Tech) was injected subcutaneously at the same site 20-24 h later. Lesions were observed 24 hours after the second injection and tissues were harvested for staining. The lesions were scored by individuals blinded to the genotype of the mice and the scores are graded from no lesion (0) to hemorrhagic necrosis (4) (see below). Lesion area was determined by measurement of length and width of each lesion.

30 *Histology:* The skin was fixed in 10% buffered formalin and paraffin sections were stained with hematoxylin and eosin. The sections were examined by light microscopy and scored for thrombosis, hemorrhage, and inflammatory cellular infiltrate on a scale of 0 to 4, in which 0 was no response. The thrombi were graded from 1 to 4 depending on the percentage of vessels occluded (10% to 25%, 26% to 50%, 51% to 75%, and most vessels). Small hemorrhages associated with less than

20% vessels were graded 1, and those associated with 21 % to 50% were graded 2. Moderate hemorrhage limited to the dermis was graded 3, and widespread red blood cells in the tissues extending into the subcutaneous tissue were graded as 4. For the inflammatory infiltrate, grade 1 was assigned for few neutrophils surrounding a minority of vessels, grade 2 was many neutrophils surrounding a minority of vessels, grade 3 was many neutrophils surrounding majority vessels, and grade 4 was numerous neutrophils widely scattered in the field.

*Trem1<sup>-/-</sup> Bovine aortic endothelial cells:* Bovine aortic endothelial cells (BAEC) were prepared as described previously (Katutani et al., 2000, Proc. Natl. Acad. Sci. USA, 97:360-364), briefly the cells were maintained under a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. The cells were seeded on 12 well plates 4 days before each assay. Cells were cultured in RPMI 1640 medium (20% bovine calf serum, 90 ug/ml Heparin, 50 ng/ml endothelial growth factor) and allowed the cells to grow to confluence in 12 well plates.

*Adherence assay:* BAEC were activated with 150 nM of Thromboxane A<sub>2</sub> (TxA<sub>2</sub>) for 15 min at 37°C in RPMI media. After 15 min BAEC were washed twice with RPMI media and incubated with 600 000 of Hepes-Tyrod's buffer with 2 mM CaCl<sub>2</sub>, and 40 µl of the solution of calcein-treated human platelets. The platelets were either resting or thrombin activated and incubation was carried out either in the presence or absence of rsTLT-1 for 30 min at 37°C. Unbound platelets were removed by two washes with PBS. BAEC were harvested mechanically, washed once and then fixed with 80% ethanol on ice for 30 min, The cells were resuspended in 500 µl of PBS containing 0.1% Triton X-100, 5 µg/ml propidium iodine and 50 µg/ml ribonuclease A. We analyzed adherence by flow cytometry: Bound platelets were identified by the increase in events in the endothelial cell platelet (PEC) gate (shown in Fig. 2c). Results are expressed as the average number of events in the PEC gate from at least three experiments. At least 20 000 events were counted per sample.

Platelets were seeded on glass slides with fibrinogen matrixes (100 µg/ml) in the presence of different concentrations of rsTLT-1 (0, 25, 50, and 100 µg/ml). Platelets were allowed to adhere for 5 min then the slides were washed with tyrodes and fixed with Cytotfix/Cytoperm (BD Science) for 20 min at 4°C. Platelets were stained with rhodamine phalloidin to determine the changes in actin polymerization (red) and counter stained with anti-CD41 (the integrin anti also known as platelet GPIIb/3, US Biologicals; Swampscott, Massachusetts, USA cat#C2394-10 clone 711134. Because phalloidin only binds to polymerized actin we correlated platelet spreading with increased rodamine intensity. Ten fields of each slide were counted in each of three experiments. The quantification of platelet binding and spreading was completed by using the Metamorph program (Molecular Devices, Downingtown, Pennsylvania, USA).

*Antibodies:* anti-human CD4, CD18, CD80, CD83, CD86 FITC-conjugated, CD3, CD11b, CD14, CD19, CD29, CD56 PE-conjugated, CD54 Cy5.5-conjugated and anti-human HLA-DR

PerCP-Cy5.5-conjugated were purchased from BD Pharmingen (Palo Alto, CA). Anti-human CD1c FITC-conjugated were purchased from Miltenyi Biotech (Bergisch Gladbach, Germany). Anti-mouse CD86, IAIE-FITC, CD80 and CD11c PE were purchased from BD Biosciences. Anti-hTLR-4 was purchased from Ebiosciences.

5           *Protein expression:* Recombinant Soluble TREM-1 (sTREM-1) was produced in *E.coli* using routine methods. Insoluble TREM-1 was solubilized and further purified by reverse phase HPLC.

*Cell culture.* Human peripheral blood enriched in mononuclear cells was obtained from healthy donors by leukapheresis (Transfusion Medicine Department, Clinical Center, National Institutes of Health, Bethesda, MD, with an approved human subjects agreement). The blood was  
10           centrifuged through Ficoll-Hypaque (Sigma), and peripheral blood mononuclear cells (PBMCs) collected at the interface were washed with PBS and centrifuged through isoosmotic Percoll (Pharmacia, Uppsala, Sweden) gradient. The enriched monocyte populations were obtained at the very top of the gradient (top fraction). To obtain monocyte-derived dendritic cells, monocytes were resuspended in RPMI 10%FBS (Gibco) at  $1 \times 10^6$  cells/ml supplemented with IL-4 and GM-CSF  
15           (Peprotech) every two days. After six days, cells were considered as immature DCs (iDCs). CD1c+ PBDCs were purified with a CD1c+ purification kit (Miltenyi).

          To induce maturation, cells were collected, plated at  $1 \times 10^6$  cells/ml and cultured in the presence of ultrapure lipopolysaccharide (LPS; *E. coli* 0111.B4, Invivogen, San Diego, CA) or distinct concentrations of TREM-1. Ultra-pure LPS was used to discard effects through other  
20           bacterial components beside Lipid A, as TLR-2 and Nod ligands.

*Isolation of Bone Marrow-derived dendritic cells (BMDCs).* BDMCs were obtained extracting the bone marrow cells of selected mice from tibias and femurs and culturing them in RPMI 10% FBS at  $1-2 \times 10^6$  cells/ml in the presence of murine GM-CSF (25ng/ml; Peprotech). Media was replaced every other day with fresh GM-CSF in culture media. At day 6, cells were used as immature  
25           DCs. Expression of CD11c was always checked, being higher than 90% in all cases.

*Statistical Analysis:* Descriptive results of continuous clinical variables were expressed as mean ( $\pm$ SD). Non-normally distributed values, as assessed by the Kolmogorov-Smirnov test, were reported as median (interquartile range: IQR). Correlations between sTLT-1 plasma concentration and clinical or biological parameters were investigated by using the Spearman test. Soluble TLT-1 was  
30           also tested for its association with several variables by using Mann-Whitney U test. The time course of sTLT-1 plasma level was assessed by analysis of variance (ANOVA). Analyses were completed with Statview software (Abacus Concepts), and a two-tailed p value of less than 0.05 was deemed significant. Tail bleeding times were expressed as means  $\pm$ SD. We used the paired t-test to evaluate for statistical differences between control and test groups for bleeding times, hemorrhage analysis and

lesion areas. Serum TNF levels were compared using the Mann-Whitney test and D-dimer levels white blood cell counts, and platelet counts were tested using a t test. Survival after LPS challenge was tested using the Log Rank Test. Some analysis was completed with Prism software (version 5.01: Graph-Pad Software, San Diego, California, USA). Platelet binding to endothelial cells, platelet spreading, and platelet binding to the fibrinogen matrixes were expressed as means SD. We used a two-tailed Student's t test and a  $P < 0.05$  was considered statistically significant.

### **Example 2: Detection of sTLT-1 in Septic Patients.**

DIC is prominent in patients with sepsis. Given our previous observation of sTLT-1 in serum but not the plasma of healthy donors, we screened a small cohort of patients admitted to the Ruiz Arnau University Hospital in Bayamon, PR with a diagnosis of clinical sepsis for the presence of plasma sTLT-1. The demographic and clinical profiles of the subjects of this initial study are listed in Table 1. In this initial study, twenty individuals (seven healthy and 13 admitted with the diagnosis of sepsis) were first evaluated for the presence of D-dimers, a degradation product of cross linked fibrin, whose level becomes elevated following fibrinolysis. Healthy individuals had a median (IQR) level of 17.3 ng/ml (4.0-107.8), whereas septic individuals had a level of 1344.0 ng/ml (544.8-2224.0) D-dimers, confirming that the majority of these patients had detectable disseminated activation of their coagulation system (Figure 1A). We then evaluated the levels of sTLT-1 in the plasma of these individuals by dot blot analysis using the commercially available polyclonal antibody from R&D systems (Catalog No. AF2394) raised against the extracellular domain of human TREML1. The median (IQR) level of sTLT-1 in patients diagnosed with sepsis was 320.7  $\mu$ g/ml (145.3-403.8) while those of healthy individuals was only 0  $\mu$ g/ml (0-45.0) (Figure 1 B).

### **Example 3: Correlations Between sTLT-1, DIC and Survival.**

Based on these initial data, we conducted a longitudinal study of sTLT-1 during sepsis at the Hopital Central in Nancy, France. Forty-six septic shock patients were included in this study. Unlike our initial screen for sTLT-1, which included HIV+ individuals, patients were not enrolled in this study if they were immunocompromised. All were seriously ill as witnessed by the universal need for mechanical ventilation and vasopressor therapy. The main clinical and biological characteristics of this cohort are summarized in Table 2. There were no differences in clinical parameters between survivors and non-survivors on admission. Infection originated from the lung (community-acquired pneumonia, 65%), the urinary tract (pyelonephritis, 9%) or the abdomen (peritonitis, 26%). Microbiological documentation was obtained in 32 (69.5%) patients (Table 3). The high 28-day mortality rate (47.8%) was not unexpected considering the elevated SAPSii and SOFA scores of this cohort.

Analysis of plasma sTLT-1 levels in these septic patients showed a median (IQR) sTLT-1

concentration of 36.6 (16.5-79.8)  $\mu\text{g/ml}$  admission, as compared to 5.4 (0-38.4)  $\mu\text{g/ml}$  in 15 healthy volunteer controls ( $p<0.005$ ). Soluble TLT-1 levels did not differ between survivors and non-survivors at admission and we found no correlation with the SOFA score or each of its components taken individually ( $\text{PaO}_2/\text{FiO}_2$ , platelet count, bilirubinemia, mean arterial pressure, creatinemia, Glasgow coma score). Interestingly, while there was a progressive decline of sTLT-1 concentration among the survivors, non-survivors showed an increase in sTLT-1 level between day one and day three (Figure 2A). Thus the two populations diverged after day three ( $p<0.03$ ) with 20 of 24 survivors (83.3%) showing a decrease in sTLT-1 between day one and day three of the study. In contrast sTLT-1 increased in all but 5 (17/22, 77.3%) non-survivors.

Considering the potential implications of TLT-1 in regulation of coagulation, we investigated the relationship between plasma sTLT-1 and DIC. Indeed, we found a strong correlation ( $R_s=0.75$ ,  $p<0.0001$ ) between sTLT-1 and DIC score, as appreciated using the ISTH criteria (Figure 2B). Although no correlation was found with platelet counts or prothrombin time, sTLT-1 was strongly correlated with D-dimer levels ( $R_s=0.82$ ,  $p<0.0001$ , Figure 2C). Consequently, patients suffering from DIC (DIC score  $>5$ ) had a higher plasma sTLT-1 concentration than those without DIC (Figure 2D) (12). Thus we next sought to evaluate the usefulness of sTLT-1 measurement in detecting DIC. To this end, a Receiver Operative Characteristic (ROC) Curve was constructed (Figure 2E). Our Day one sTLT-1 ROC curve showed that at a cut-off set at 50  $\mu\text{g/ml}$ , both the sensitivity and specificity in assessing the presence of DIC were 76%. Taken together these data suggest that sTLT-1 is strongly associated with septicemia and that sTLT-1 could be an important prognostic indicator for DIC.

#### **Example 4: Enhancement of Platelet Aggregation by Recombinant sTLT-1.**

Our previous studies have shown that single chain anti-TLT-1 antibodies reduce platelet aggregation *in vitro* suggesting that in addition to being an indicator of systemic platelet activation, sTLT-1 might directly modulate platelet aggregation during sepsis. To evaluate this possibility we tested whether rsTLT-1 comprised of amino acids 20-126 might modulate human platelet aggregation using *in vitro* aggregation assays (Figure 3). Indeed, addition of rsTLT-1 at concentrations below those found in the plasma of some patients augmented platelet aggregation initiated with 0.5 pM U46619, a thromboxane A<sub>2</sub> (TxA<sub>2</sub>) mimetic, in a dose dependant manner (Figure 3A). The specificity of this activity was confirmed by blocking rsTLT-1-enhanced aggregation with a peptide derived from residues 94 to 110 of TLT-1, a region important in TREM-mediated interactions; a control 17-mer peptide had no effect (Figure 3B). Stimulation of platelets with rsTLT-1 or TLT-1-derived peptides alone had no effect. In addition, rsTLT increased platelet aggregation in response to suboptimal concentrations of either ADP (Figure 3C), or collagen (Figure 3D). Interestingly, due to the large disparity in molecular weight between fibrinogen and sTLT-1 the first doses of sTLT-1 with strong effect (60  $\mu\text{g/ml}$ ) is roughly equal in molarity to plasma fibrinogen. Thus, sTLT-1 at physiologically

relevant concentrations augments platelet aggregation in response to a variety of agonists suggesting that the sTLT-1 detected in patients may contribute to the hypercoagulative state induced by a systemic inflammatory response

**Example 5: TLT-1 binds Fibrinogen and Interacts with ERM Family Proteins.**

5 Our demonstration that antibodies against TLT-1 could inhibit the aggregation of washed platelets suggested that the ligand(s) for TLT-1 were on or in platelets. To gain insight into sTLT-1-mediated increases in aggregation we sought to identify platelet-derived TLT-1 ligands. Lysates generated from purified human platelets were applied to AminoLink columns preloaded with either sTLT-1 or sTREM-1. After extensive washing bound proteins were eluted by decreasing

10 pH and multiple fractions were reduced with DTT, resolved with electrophoresis and visualized by coomassie staining. This approach revealed specific binding of 3 proteins with molecular masses between 50 and 80 kDa (Figure 4A). Mass spectroscopy identified these proteins as the  $\alpha$ ,  $\beta$ , and  $\gamma$  chains of fibrinogen. To confirm these findings we used HIS-tagged TLT-1 and TREM-1 bound to nickel columns. After washing and elution of bound proteins with 25% imidazole, 15 aliquots were resolved by PAGE in either native or reduced conditions (Figure 4B). Consistent with disulfide linked multimers of fibrinogen, our TLT-1 column specifically bound a high molecular weight complex that when reduced resolved into the same three bands detected with our AminoLink columns. Immunoblotting with anti-fibrinogen confirmed the identity of these TLT-1 interacting proteins as fibrinogen (Figure 4B).

20 In order to prove that TLT-1 could bind fibrinogen under more physiologic conditions, we used a chimera the TLT-1 extracellular domain fused with the Fc domain of human IgG (Washington, et al., 2004. Blood 104:1042-1047.). Consistent with TLT-1 interaction with fibrinogen rather than a cell surface protein, multiple attempts failed to detect binding of this TLT-1-Ig fusion to either activated or resting platelets. However, in ELISA-based experiments, TLT-1- Ig, but not a control Ig- 25 fusion, bound well to plate bound fibrinogen but not vitronectin (Figure 4C). Collectively these data establish fibrinogen as a ligand of TLT-1 and suggest that during platelet aggregation TLT-1 crosslinks extracellular fibrinogen stabilizing higher order platelet aggregates.

In order to begin to dissect the signaling pathway utilized by TLT-1 we constructed GST fusions of its cytoplasmic domain and used them in binding assays using lysates from human 30 platelets. GST-TLT-1, but not GST alone, interacted strongly with a 75 kDa protein from the platelets of three independent donors. Mass spectroscopy identified this band as moesin and immunoblotting confirmed this finding (Figure 5A). Immunofluorescent staining of moesin and TLT-1 confirmed their colocalization particularly after thrombin stimulation (Figure 5B). Moesin is part of the ezrin/radixin/moesin (ERM) family of proteins known to link membrane proteins to the actin

cytoskeleton. Current reports suggest that of the ERM proteins, only moesin is expressed human platelets. However, because moesin null mice have no apparent platelet defect and Ezrin and Radixin are approximately 75% homologous to moesin and thought to have redundant functions, we immunoblotted highly purified human and mouse platelets for the presence of all three ERMs.

Although moesin was most abundant, all three proteins were readily detected in mouse platelets and both radixin and moesin were found in human platelets (Figure 5C). Therefore, we addressed the possibility of a TLT-1 interaction with radixin and/or ezrin using a series of reciprocal immunoprecipitations (Figure 5D and E). TLT-1 co-immunoprecipitated with ezrin in co-transfected HEK293 cells and endogenous moesin, ezrin and radixin were detected in TLT-1 immunoprecipitations from COS7 cells. Finally, we detected moesin/TLT-1 interactions in primary human platelets (Figure 5F). Collectively these data suggests that TLT-1 facilitates platelet aggregation by linking fibrinogen to the platelet cytoskeleton via the ERMs.

**Example 6: Defective Platelet Aggregation and Extended Bleeding Times in Mice lacking *Trem11*<sup>-/-</sup>.**

In order to better evaluate TLT-1's role in platelet aggregation and inflammation we deleted exons 1 and 2 of *Trem11* (the gene encoding TLT-1) in C57B1/6 mice. Homozygous *Trem11*<sup>-/-</sup> mice were identified by PCR using a three primer system and confirmed by Southern blot analysis (Figure 6A-C).

Null mice were viable, fertile, and completely devoid of TLT-1 protein as demonstrated using antibodies to either the extracellular or intracellular (Washington A.V. unpublished data) regions of the receptor by western and confocal analysis (Figure 6D and E). Although *Trem11*<sup>-/-</sup> mice had somewhat higher leukocyte counts (13.98 +/- 0.9 vs. 11.88 +/- 1.1, n=7) and slightly lower platelet counts than wild type mice (761.0 +/- 44.5 vs. 949.6 +/- 35.69, n=7, p=0.006), only the platelet differences reached statistical significance.

Our previous in vitro studies have shown that single chain anti-TLT-1 antibodies reduce platelet aggregation, whereas here we show recombinant sTLT-1 facilitates aggregation (Figure 3). Therefore we tested *Trem11*<sup>-/-</sup> platelet aggregation using thrombin, collagen, ADP, or the TxA<sub>2</sub> mimetic U46619. Regardless of the agonist, *Trem11* null platelets were able to commence normal shape change as indicated by the transient decrease in light transmission (Figure 7A-D). However, *Trem11*<sup>-/-</sup> platelets reproducibly aggregated less efficiently when stimulated with thrombin (Figure 7A) or collagen (Figure 7B). Defects in *Trem11*<sup>-/-</sup> platelet aggregation were even more pronounced when ADP (Figure 7C) or U46619 (Figure 7D), were used as agonists. In accordance with our identification of fibrinogen as a TLT-1 ligand, *Trem11*<sup>-/-</sup> platelets bound demonstrably lower amounts of fibrinogen as compared to their wild type counterparts (Figure 7E). Notably, with all four agonists, distinct aggregation defects were evident at all concentrations tested (Washington A.V. unpublished data),

suggesting that TLT-1 plays a fundamental role in platelet aggregation. Finally, to evaluate the potential effects of the TLT-1 aggregation defect *in vivo*, we compared bleeding times between *Trem1<sup>-/-</sup>* and WT mice. Bleeding times in the null mice were double that of controls (controls; 87.33 seconds  $\pm$  13.73 vs *Trem1<sup>-/-</sup>*; 184.33 seconds  $\pm$  57.57; n=12; p=0.05), consistent with the decreased, but not absent, ability of *Trem1<sup>-/-</sup>* platelets to aggregate *in vitro* (Figure 7F).

#### Example 7: TLT-1 regulates the systemic response to LPS.

The elevated levels of sTLT-1 in septic patients and the role of TLT-1 in platelet function prompted us to assess the effects of LPS administration on wild type and *Trem1<sup>-/-</sup>* mice. Consistent with our findings in septic patients i.p. LPS administration led to detectable levels of sTLT-1 within 2 hrs that continued to climb through the 24 hr study (Figure 8A). To assess whether sTLT-1 levels might correlate with other parameters of endotoxemia we measured TNF, platelet counts, leukocyte numbers and D-dimers levels in these mice. As expected, administration of LPS resulted in the production of TNF, severe thrombocytopenia and leukocytopenia over the study period (Figure 8B-D). TNF production peaked 2 hrs post LPS exposure and matched the leukocyte nadir. In contrast, platelet counts fell by about 30% within the first 2 hrs then steadily decreased further thereafter (Figure 8C). Importantly, the pattern of thrombocytopenia closely mirrored the increase in sTLT-1 ( $r^2 = -0.922$ ) suggesting sTLT-1 is released or shed as platelets leave the circulation during endotoxemia. Under these conditions D-dimers were not detected in the blood of wild type mice until 24 hr after LPS injection. Therefore, sTLT-1 is associated with the septic response in mice where it is inversely correlated to platelet count, but readily detectable well before clinically relevant thrombocytopenia. Given the dramatic variability in platelet number across the human population and the lack of detectable D-dimers until endotoxemia is well established, these data demonstrate that sTLT-1 levels represent a more powerful prognostic indicator of developing DIC than these commonly used parameters.

When challenged with LPS *Trem1<sup>-/-</sup>* mice exhibited signs of endotoxemia that included hunching and the appearance of a rough coat similar to wild type mice. We next assessed LPS-induced thrombocytopenia, leukocytopenia, TNF and D-dimers in *Trem1<sup>-/-</sup>* mice. Although LPS-induced leukocytopenia (wt 5.296  $\pm$  1.13  $\times 10^3$ /ml, *Trem1<sup>-/-</sup>* 5.211  $\pm$  0.59  $\times 10^3$ /ml, n=9, p=0.948) and thrombocytopenia (wt 451.4  $\pm$  75.67  $\times 10^6$ /ml, *Trem1<sup>-/-</sup>* 319.7  $\pm$  39.9  $\times 10^6$ /ml, n=9, p=0.143) were unaffected in the null mice, we found increased TNF and D-dimers in *Trem1<sup>-/-</sup>* mice relative to controls (Figure 9A and 9B). From our studies we cannot conclusively determine whether the increased levels of D-dimer in *Trem1<sup>-/-</sup>* mice is due to increased coagulation or decreased fibrinolysis, however, consistent with the increased production of TNF and increased D-dimer production following LPS injection, *Trem1<sup>-/-</sup>* mice died faster than WT with a median survival time of 40 hr as compared to 48 hrs (Log Rank Test, p=0.049) (Figure 9C). In addition, whereas in this and multiple

smaller experiments 15-20% of WT mice survived LPS challenge, all the *Trem1*<sup>-/-</sup> mice routinely succumbed. Taken together, these data demonstrate that TLT-1 provides benefit to the host during endotoxemia, without being bound by mechanism, perhaps by limiting TNF production.

**Example 8: Increased Inflammatory Hemorrhage in *Trem1*<sup>-/-</sup> Mice.**

5 Having established a role for TLT-1 in platelet function and demonstrated its association with sepsis, we evaluated the role of TLT-1 in controlling hemorrhage associated with vascular injury secondary to inflammation. To this end we employed the localized Schwartzman model of hemorrhagic vasculitis. The localized Schwartzman reaction is a surrogate of the septic response and DIC in humans, producing a local lesion amenable to direct evaluation. Consistent with the ability of  
10 TLT-1 to help maintain vascular integrity, the hemorrhagic Schwartzman lesions of *Trem1*<sup>-/-</sup> mice were almost four times the size of those in WT mice (Figure 10A). Microscopic evaluation of these lesions supported these macroscopic observations (Figure 10B). Using a grading system of 1 to 4 (see methods) we compared the lesions from mice of each genotype for microclots, thrombi, lesion size, hemorrhage, and neutrophil influx. Overall, *Trem1*<sup>-/-</sup> mice exhibited fewer occlusive thrombi, more  
15 microclots, and increased neutrophil influx relative to WT although these differences were not significant. In contrast, there was nearly twice the degree of microscopic hemorrhage associated with the Schwartzman lesions of *Trem1*<sup>-/-</sup> mice as compared to those of WT mice (Figure 10C and 10D). These results dramatically demonstrate that TLT-1 is critical in controlling hemorrhage associated with the inflammatory response.

**20 Example 9: Soluble TLT-1 enhances platelet—endothelial cells binding interactions**

We hypothesized that the rsTLT-1 mediated enhanced aggregation would translate to increased platelet adhesion to endothelial cells. To address this possibility, we modified the protocol of Kakutani et al, 2000, which used bovine aortic endothelial cells (BAEC) to measure platelet adhesion to endothelium in a static assay. Recent reports demonstrate that TxA<sub>2</sub> plays a formidable  
25 role in mediating neutrophil and platelet adhesion to endothelial cells. Because we have consistently seen the greatest TLT-1 mediated platelet inhibition when we used TxA<sub>2</sub> to activate platelets and recent reports demonstrate that TxA<sub>2</sub> plays a formidable role in mediating leukocyte and platelet adhesion to and vascular leakage from the endothelium, we used TxA<sub>2</sub> to activate the endothelial cells. TxA<sub>2</sub> causes cellular withdrawal and shrinkage and as expected TxA<sub>2</sub> activation of endothelial  
30 cells caused greater endothelial cell loss during harvesting. The greater cell loss is reflected in the flow cytometry shown in Fig. 11(a and b) where we consistently counted less endothelial cells from wells where activated endothelial cells were harvested.

Platelets were treated with the fluorescent intercellular cell dye calcein before addition to endothelial cells. Resting and activated calcein-treated platelets demonstrated a typical platelet scatter

with a classical subtle shift in fluorescence once activated as shown in Fig. 11 c and d. Resting or activated platelets were incubated with either activated or resting endothelial cells for 30 min at 37°C. Adherent platelets were monitored by flow cytometry, as described in materials and methods. Endothelial cell activation caused a 30% increase in the total numbers of resting platelets that adhered compared with resting platelets that adhered to resting endothelial cells (Fig. 11 g). The addition of previously activated platelets to resting or activated endothelial cells show a 25 and 43% reduction respectively in average total numbers of adherent platelets.

Once we established the parameters of our experimental system, we repeated these experiments in either the presence or absence of rsTLT-1. Figure 12a and b give an example of the changes seen within the PEC gate with the addition of rsTLT-1 (Fig. 12b) to activated BAEC and resting platelets. Examination of platelet-endothelial cell aggregates demonstrated that rsTLT-1 has an augmentative effect on platelet adherence to BAEC in all the conditions tested when compared with controls (Fig. 12c and d). Using resting platelets and resting endothelial cells, addition of rsTLT-1 increased the average observed binding events from  $320 \pm 46$  to  $460 \pm 51$ . Using activated platelets with resting endothelial cells the observed binding events increased from  $297 \pm 109$  to  $456 \pm 248$ . The effect of added rsTLT-1 was much more pronounced on platelet binding to activated BAEC. Using resting platelets, the addition of rsTLT-1 increased platelet numbers from  $423 \pm 43$  to  $676 \pm 148$  and with activated platelets the number of binding events without rsTLT-1 was  $314 \pm 286$ , and  $636 \pm 219$  in the presence of  $50 \mu\text{g/ml}$  rsTLT-1. Although  $\text{TxA}_2$  stimulated endothelial cells incubated with thrombin-activated platelets show an increase in platelet adherence, the increase in binding seen with resting platelets to either resting or activated endothelial cells demonstrated a greater and significant ( $P=0.05$ ) increase in the number of platelets that remained bound to the monolayer.

#### **Example 10: TLT-1 enhances actin polymerization and platelet binding to fibrinogen matrixes**

Previous work with TLT-1 using scFvs to inhibit platelet aggregation showed that the scFv-mediated inhibition fails to stop P-selectin expression suggesting that the TLT-1 mediated inhibition is downstream of the calcium signal required for  $\alpha$ -granule/membrane fusion. These results open the possibility that actin polymerization, which plays an important role downstream of the calcium signal in both platelet activation and adhesion could provide key mechanistic insights to TLT-1 function. To evaluate the potential of actin polymerization playing a role in TLT-1 mediated platelet function, we seeded platelets at  $1 \times 10^8$  cells on glass slides coated with bovine serum albumin (BSA), fibrinogen, TLT-1, and/or a fibrinogen/TLT-1 mixture and slides were washed after 5 min of incubation at 37°C. Slides were subsequently stained with rhodamine phalloidin, which binds only polymerized actin. Slides were examined by confocal microscopy and evaluated for platelet spreading and adhesion, Confocal microscopy revealed that actin polymerization in platelets increased with increasing TLT-1 concentration (25, 50, or  $100 \mu\text{g/ml}$ ) when compared with those allowed to adhere to fibrinogen alone

(Fig. 13). There was no difference in actin polymerization between the slides that contained fibrinogen-only (100 µg/ml), fibrinogen (100 µg/ml)/BSA (50 µg/ml) or BSA-only (100 µg/ml) (data not shown). Recombinant sTLT-1 concentrations of 25, 50, 100 µg/ml yielded a significant difference in the increase of spreading compared with fibrinogen only controls. There was an increase in average platelet radius (Fig. 13f) and area (Fig. 13e). Average platelet areas observed in photomicrographs increased on average from  $33 \mu\text{m}^2 \pm 0.3$  with fibrinogen-only to  $53.38 \mu\text{m}^2 \pm 5.2$ ,  $45.7 \pm 3.1$ , or  $57.8 \mu\text{m}^2 \pm 12.1$  with addition of 25, 50, or 100 µg/ml of rsTLT-1 respectively (Fig. 13e). Platelets allowed to adhere for 15 min did not show the close-dependent difference in actin polymerization suggesting that TLT-1 plays a role early during the process of platelet aggregation and adhesion.

We subsequently measured the amount of platelets that adhered to the fibrinogen and fibrinogen/TLT-1 matrixes (Fig. 14a-d). Platelets were seeded at  $1 \times 10^8$  platelets/ml and allowed to adhere for 5 min. Platelets were then stained with rodaminc phalloidin and subjected to counting using Metamorph. Consistent with the results from the platelet spreading, we saw an increase in the amount of platelets that adhered in the presence of rsTLT-1 compared with fibrinogen alone. Accordingly, the increase was greatest at the rsTLT-1 concentration 50 µg/ml showing an average increase of  $217 \pm 12$  adherent platelets per 10 fields counted ( $P=0.02$ ) (Fig. 14e). The results are consistent with the results from the endothelial cell experiments.

#### **Example 11: Human sTREM-1 activates human monocytes**

As precursors of antigen presenting cells such as macrophages and dendritic cells, we first investigated the effect of sTREM-1 on human peripheral blood monocytes. Freshly isolated monocytes cultured for 48h in the presence of sTREM-1 exhibited an upregulation of membrane Beta1 (CD29) and Beta2 (CD18) integrins, CD54 (ICAM-1) as well as co-stimulatory molecules CD80 and CD86 (Fig. 15A), indicating that these cells had been activated, since a similar profile was obtained when ultrapure LPS was used.

This observation was associated with an increase in the production of cytokines like  $\text{TNF-}\alpha$ , IL-10 and IL-6 (Fig. 15 B), confirming that sTREM-1 treatment not only affects the phenotype, but also the function of human monocytes. These data suggest that sTREM-1 could be useful for stimulating an immune response, e.g., for use as an adjuvant.

This assay can be used as a screening assay to characterize the activity of various sTREM-1 fragments.

#### **Example 12: Human sTREM-1 induces DC maturation**

To test the effect of sTREM-1 on dendritic cells (DCs), freshly isolated peripheral blood myeloid dendritic cells  $\text{CD1c}^+$  DCs were purified from the mononuclear fraction of leukocytes from

healthy donors. After purification, cells were >99% CD11c<sup>+</sup> /HLA-DR<sup>high</sup>. These DCs were cultured in the presence or absence of TREM-1 or LPS. After 48h, supernatants were collected and their cytokine content was determined by ELISA.

There was a clear effect on the function of these DCs, as sTREM-1 was able to induce  
 5 significant increase in the production of IL-1Beta (p<0.05), TNF-alpha (p<0.05), IL-12p70 (p<0.05) and IL-18 (p<0.05) (Fig. 16A). Levels of IL-10 and IL-6 were not increased compared to basal treatment (p=0.2 and p=0.07 respectively).

Peripheral blood (PB) DCs are present in very low numbers within the mononuclear fraction obtained from blood. For further studies of antigen presenting cells, we therefore used IL-4 and GM-  
 10 CSF to derive immature monocyte-derived dendritic cells from monocytes (mo-DCs) in vitro.

Immature mo-DCs were cultured in the presence or absence (untreated) of sTREM-1 for 48h and the expression of different maturation markers was analyzed by flow cytometry using fluorochrome-conjugated antibodies. The presence of sTREM-1 induced a considerable (p<0.05) upregulation of the co-stimulatory molecules CD80 (p=0.001), CD83 (p=0.002), CD86 (p=0.011).  
 15 MHC-II expression was not increased (p=0.24; Fig 16B)

The supernatants of these cultures were also collected to analyze the cytokine production by ELISA (Fig. 16C). sTREM-1 induced an increase in TNF-alpha, IL-6, IL-10 and IL-12p70 levels (p<0.01). Although this effect could be detected at concentrations of 1µg/ml, when 10µg/ml was used, the effect was more marked and consistent.

20 This assay can be used as a screening assay to characterize the activity of various sTREM-1 fragments.

#### **Example 13: DCs primed with human sTREM-1 promote Th1/Th17 polarization.**

The effect of mo-DCs primed with sTREM-1 on lymphocyte activation was tested using mixed leukocyte reaction (MLR) assays. moDCs pre-treated with sTREM-1 were able to promote  
 25 allogeneic T cell proliferation (Fig. 17A). Supernatants from these DC-lymphocyte cultures were collected after 48 or 72h to analyze the type of T cell response induced by the DCs. IL-4, IL-5, IFN-gamma and IL-17 levels were measured for this purpose (Fig. 17B). Allogeneic lymphocytes primed with sTREM-1-DCs had increased IFN-gamma and IL-17 production by an average of 29.9 and 20.3 respectively, (p<0.01) compared to the cytokine level produced by lymphocytes primed with resting  
 30 DCs. IL-4 (data not shown) and IL-5 were not increased (avg=5.1; p>0.05).

This assay can be used as a screening assay to characterize the activity of various sTREM-1 fragments.

#### **Example 14: Human sTREM-1 triggers TLR-4-dependent activation in mice and humans.**

Membrane-bound TREM-1 has been reported to amplify toll-like receptor (TLR)-4 signaling. Further investigation of a TLR-4-null mouse model (C3H/HeJ) in our study revealed that sTREM-1 does not activate bone marrow-derived dendritic cells (BMDCs) from C3H/HeJ mice. Co-stimulatory molecules like CD80, CD86 and class-II MHC were normally upregulated, as well as CD11c was  
5 downregulated when LPS or sTREM-1 were used to stimulate C3H/HeN-derived cells (wild type). In contrast, such changes were not observed in BMDCs from C3H/HeJ mice (Fig. 17A)

To analyze the role of TLR-4 in human cells, peripheral blood monocytes were pretreated with anti-TLR4 neutralizing antibody prior sTREM-1 exposure and the cytokine production was analyzed (Fig. 18B). In the three donors tested, anti-TLR-4 blocking antibody markedly decreased the  
10 production of TNF-alpha and IL-10 induced by sTREM-1.

To ensure that our sTREM-1 preparations were not contaminated with endotoxin, a classical ligand for TLR-4, we analyzed the effect on mo-DC activation of sTREM-1 after boiling or treatment with proteinase K (PK) (Figs. 17C and 17D respectively). These treatments inactivated sTREM-1 but not the ultrapure LPS.

sTREM-1 samples were also tested in a Limulus Amebocyte Lysate (LAL) assay. The results showed levels of endotoxin activity (EA) <1 U/ $\mu$ g protein, whereas amounts of ultrapure LPS as low as 5ng, which were unable to induce cellular activation, yielded 2.6 EA (519.3 EA/ $\mu$ g).  
15

#### **Example 15: sTREM-1 enhances immune response in mice**

Having found that sTLT-1 functions as an agonists in assays of platelet aggregation we asked  
20 whether the soluble portions of other TREM family members might also be agonistic. To this end the entire extracellular domain of murine TREM-1 including the Ig domain and stalk was cloned into a mammalian expression vector (Fig 18a). The ability of this construct to result in production of sTREM-1 was tested by transfecting HEK293 cells with this or control empty vector. Twenty four hours or 72 hours later the tissue culture supernatant was collected and assayed for sTREM-1 by  
25 ELISA. At both time points substantial levels of soluble TREM-1 were detected (Fig 18b).

We next tested the ability of this construct to direct expression of sTREM-1 *in vivo*. A cohort of mice was injected with plasmid DNA encoding sTREM-1 or control vector. Mice were sacrificed 1, 2 or 3 days post injection and their blood was tested for the presence of sTREM-1 by ELISA. High levels of sTREM-1 were detected 24 hours after injection and these levels fell rapidly thereafter (Fig  
30 18c). Of note, even three days post injection the blood levels of sTREM-1 were higher than those reported for patients with inflammatory conditions or sepsis. Injection of mice with large amounts (500 $\mu$ g/mouse) of a TREM-1-human Fc fusion protein protects mice from endotoxemia, however, the Fc portion of this protein may dramatically affect its function *in vivo*.

We tested the ability of sTREM-1 to suppress endotoxemia by administering sTREM cDNA

as above and injecting mice twenty-four hours later with LPS. Rather than protecting animals from endotoxemia, sTREM-1 exacerbated the effect and enhanced the lethality of LPS (Fig 18d).

Together with our findings in platelets and dendritic cells, these data support the notion that rather than solely block interactions, multiple soluble TREM family proteins are agonists and could be likely employed in the amplification of inflammation and coagulation, and act as vaccine adjuvants.

#### **Example 16: Coagulation assay for identifying active sTREM-1 family members**

To identify STEM-1 family member polypeptides having procoagulant activity, any platelet aggregation assay is used. In a preferred method, sequences identified to have procoagulant activity are subsequently tested in other assays for coagulation activity, e.g. the LPS mouse challenge. As a positive control for a peptide having procoagulant activity, the rsTLT-1 peptide comprised of amino acids 20-126 can be used. This peptide has been demonstrated to have procoagulant activity. STEM-1 family peptides should have at least 1%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% 100%, or more of the activity of the rsTLT-1 peptide comprised of amino acids 20-126 on an equimolar basis. Methods of comparing relative activity of peptides using varying amounts of peptides is well known in the art.

A peptide library including a mixture of peptides having various lengths, amino acid sequences, chemical modifications, can be prepared using routine methods (e.g., expressed as recombinant proteins), ordered from a commercial source, etc. The peptides are contacted with platelets in combination with various agents, including but not limited to collagen, thrombin, and/or Thromboxane A<sub>2</sub>, at concentration that lead to suboptimal platelet activation. Platelet activation as assessed by aggregometry is monitored over time. In addition, platelet secretion, an indication of full platelet activation can be monitored.

Any method to analyze platelet aggregation, such as those provided herein or by the references cited herein, can be used to select agonists and inhibitors of aggregation.

#### **Example 17: Use of sTREM-1 as a vaccine adjuvant**

TREM-1 is used a vaccine adjuvant for stimulation of an immune response to a non-relevant protein, ovalbumin. It is understood that methods to promote an immune response to a physiologically relevant antigen can be performed in a similar manner. Dosages are provided as examples and can vary depending, for example, on the size of the subject to be treated (e.g., a lab mouse typically weighs about 20 g), and the immunogenicity of the antigen. Four groups of animals matched animals (e.g., mice) are injected intraperitoneally with 0.2ml of a solution containing one of the following:

- 1) Buffer control + 50µg OVA (Ovalbumin) per mouse (negative control)

2) sTREM1 protein 5µg per mouse + 50µg OVA per mouse

3) sTREM1 protein 0.5µg per mouse + 50µg OVA per mouse

3) LPS 5µg per mouse + 50µg OVA per mouse

On day 10, mice are bleed (e.g., retro orbital bleeding) to analyze the antibody titers against  
5 OVA during the primary immunization. Serum is retained to measure immunoglobulin levels.

On day 14, a boost immunization of 50µg OVA is administered as on day 0.

On day 20, mice are bled again, to measure the antibody levels generated during the  
secondary immunization.

10 On day 21, mice are sacrificed and spleens are collected to harvest the splenocytes to  
determine the number of OVA specific clones. Splenocytes are plated and grown *in vitro* at 2 million  
cells per ml and cultured in duplicate in the presence (or absence -baseline level control-) of OVA at  
different concentrations, ranging from 1 to 100ug per ml, e.g., 1, 5, 10, 20, 50 and 100 µg per ml are  
usual concentrations.

15 After 2 days in culture, supernatant is collected from one of the duplicates to observe the  
cytokine produced by the splenocytes at time point. Cytokine levels can be determined using routine  
methods, e.g., commercially available ELISA assays. This allows for characterization of the type of  
T-helper cell response.

After 5 days, from the second plate, radioactive H<sup>3</sup>-labelled thymidine is added to the wells,  
20 to assay cell proliferation. After 8-10 hours, cells harvested into appropriate pads and radioactivity is  
counted using a beta-counter. A larger amount of radioactivity is found in the cells from animals in  
groups 2 and 3 as compared to group 1. This demonstrates OVA-specific cells proliferate in response  
to OVA.

Based on the results, it is determined that sTREM-1 acts as an adjuvant and that the immune  
25 response of the mice in groups 2 and 3 are higher than the mice in group 1.

Those skilled in the art will recognize, or be able to ascertain using no more than routine  
experimentation, many equivalents to the specific embodiments of the invention described herein.  
Such equivalents are intended to be encompassed by the following claims.

## 30 References

All references, patents, patent publications, and sequence reference numbers cited herein are

incorporated herein by reference.

Cohen J. TREM-1 in sepsis. *Lancet* 2001; 358:776-778.

Cohen J. The immunopathogenesis of sepsis. *Nature* 2002; 420:885 - 891,

5 Bente and Rico-Hesse Models of dengue virus infection. *Drug Discov Today Dis Models* 2006; 3:97-103.

Krishnamurti et al., Platelet adhesion to dengue-2 virus-infected endothelial cells. *Am J Trop Med Hyg* 2002; 66:435- 441.

Noisakran and Perng. Alternate hypothesis on the pathogenesis of dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) in dengue virus infection. *Exp Biol Med (Maywood)* 2008;  
10 233:401 -408.

Colonna and Facchetti. TREM-1 (triggering receptor expressed on myeloid cells): a new player in acute inflammatory responses. *J Infect Dis* 2003; 187 (Sup, 2):S397-S401.

Fox JE. The platelet cytoskeleton. *Thromb Haemost* 1993; 70:684-893,

Fox JE. Cytoskeletal proteins and platelet signaling. *Thromb Haemost* 2001; 86:198-213.

15 Hartwig and, Italiano. Cytoskeletal mechanisms for platelet production. *Blood Cells Mol Dis* 2006; 36:99-103.

George JN. Platelets. *Lancet* 2000; 355:1531-1539.

Frenette et al. P-Selectin glycoprotein ligand 1 (PSGL-1) is expressed on platelets and can mediate platelet-endothelial interactions in vivo. *J Exp Med* 2000; 191:1413-1422.

20 Geng et al., P-selectin cell adhesion molecule in inflammation, thrombosis, cancer growth and metastasis. *Curr Med Chem* 2004; 11:2153-2160.

Washington et al., A TREM family member, TLT-1, is found exclusively in the alpha-granules of megakaryocytes and platelets. *Blood* 2004; 104:1042 - 1047.

Ford and McVicar. TREM and TREM-like receptors in inflammation and disease. *Curr Opin. Immunol* 2009; 21:38-46.  
25

Washington et al., Initial characterization of TREM like transcript (TLT)-1: a putative inhibitory receptor within the TREM cluster. *Blood* 2002; 100:3822-3824.

Goerge, et al., 2008. Inflammation induces hemorrhage in thrombocytopenia. *Blood* 111:4958-4964.

Klesney-Tait, et al., 2006. The TREM receptor family and signal integration. *Nat. Immunol.* 7:1266-

1273.

Bouchon, et al., 2000. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J. Immunol.* 164:4991-4995.

Bouchon, et al., 2001. A DAP12-mediated pathway regulates expression of CC chemokine receptor 7  
5 and maturation of human dendritic cells. *J. Exp. Med.* 194:1111-1122.

Washington, et al., 2002. Initial characterization of TREM-like transcript (TLT)-1: a putative inhibitory receptor within the TREM cluster. *Blood* 100:3822-3824.

Washington, et al., 2004. A TREM family member, TLT-1, is found exclusively in the alpha-granules of megakaryocytes and platelets. *Blood* 104:1042-1047.

10 Lu, et al., 2006. Preparation and characterization of monoclonal antibody against protein TREM-like transcript-1 (TLT-1). *Hybridoma (Larchmt.)* 25:20-26.

Gattis et al., 2006. The structure of the extracellular domain of triggering receptor expressed on myeloid cells like transcript-1 and evidence for a naturally occurring soluble fragment. *J. Biol. Chem.* 281:13396-13403.

15 Giomarelli, et al., 2007. Inhibition of thrombin-induced platelet aggregation using human single-chain Fv antibodies specific for TREM-like transcript-1. *Thromb. Haemost.* 97:955-963.

Levi and Ten, 1999. Disseminated intravascular coagulation. *N. Engl. J. Med.* 341:586-592.

Voves, et al., 2006. International Society on Thrombosis and Haemostasis score for overt disseminated intravascular coagulation predicts organ dysfunction and fatality in sepsis patients.  
20 *Blood Coagul. Fibrinolysis* 17:445-451.

Gibot, et al., 2004. A soluble form of the triggering receptor expressed on myeloid cells-1 modulates the inflammatory response in murine sepsis. *J. Exp. Med.* 200:1419-1426.

Nakamura, F et al., 1995. Phosphorylation of threonine 558 in the carboxyl-terminal actin-binding domain of moesin by thrombin activation of human platelets. *J. Biol. Chem.* 270:31377-31385.

25 Doi, et al., 1999. Normal development of mice and unimpaired cell adhesion/cell motility/actin-based cytoskeleton without compensatory up-regulation of ezrin or radixin in moesin gene knockout. *J. Biol. Chem.* 274:2315-2321.

Lankes and Furthmayr. 1991. Moesin: a member of the protein 4.1- talin-ezrin family of proteins. *Proc. Natl. Acad. Sci. U. S. A* 88:8297-8301.

30 Brozna, J.P. 1990. Shwartzman reaction. *Semin. Thromb. Hemost.* 16:326-332.

- Barrow, et al 2004. Cutting edge: TREM-like transcript-1, a platelet immunoreceptor tyrosine-based inhibition motif encoding costimulatory immunoreceptor that enhances, rather than inhibits, calcium signaling via SHP-2. *J. Immunol.* 172:5838-5842.
- Lehman, et al., 2004. Analytic validation and clinical evaluation of the STA LIATEST  
5 immunoturbidimetric D-dimer assay for the diagnosis of disseminated intravascular coagulation. *Am. J. Clin. Pathol.* 122:178-184.
- Nurden, et al., 2008. Phenotypic heterogeneity in the Gray platelet syndrome extends to the expression of TREM family member, TLT-1. *Thromb. Haemost.* 100:45-51.
- Gamulescu, et al., 2003. Platelet moesin interacts with PECAM-1 (CD31). *Platelets.* 14:211-217.
- 10 Fukata, et al., 1998. Association of the myosin-binding subunit of myosin phosphatase and moesin: dual regulation of moesin phosphorylation by Rho-associated kinase and myosin phosphatase. *J. Cell Biol.* 141:409-418.
- Retzer and Essler 2000. Lysophosphatidic acid-induced platelet shape change proceeds via Rho/Rho kinase-mediated myosin light-chain and moesin phosphorylation. *Cell Signal.* 12:645-648.
- 15 Nakamura, et al., 1999. Regulation of F-actin binding to platelet moesin in vitro by both phosphorylation of threonine 558 and polyphosphatidylinositides. *Mol. Biol. Cell* 10:2669-2685.
- Andre, et al., 2002. CD40L stabilizes arterial thrombi by a beta3 integrin--dependent mechanism. *Nat. Med.* 8:247-252.
- Angelillo-Scherrer, et al 2001. Deficiency or inhibition of Gas6 causes platelet dysfunction and  
20 protects mice against thrombosis. *Nat. Med.* 7:215-221.
- Podrez, et al 2007. Platelet CD36 links hyperlipidemia, oxidant stress and a prothrombotic phenotype. *Nat. Med.* 13:1086-1095.
- Prevost, et al., 2005. Eph kinases and ephrins support thrombus growth and stability by regulating integrin outside-in signaling in platelets. *Proc. Natl. Acad. Sci. U. S. A* 102:9820-9825.
- 25 Haselmayer, et al., 2007. TREM-1 ligand expression on platelets enhances neutrophil activation. *Blood* 110:1029-1035.
- Camerer, et al., 2006. Roles of protease-activated receptors in a mouse model of endotoxemia. *Blood* 107:3912-3921.
- Subramaniam, et al., 1996. Defects in hemostasis in P-selectin-deficient mice. *Blood* 87:1238-1242.
- 30 Knaus, et al 1991. The APACHE III prognostic system. Risk prediction of hospital mortality for critically ill hospitalized adults. *Chest* 100:1619-1636.

Vincent, et al., 1996. The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. Intensive Care Med 22:707-710.

5 Le, et al., 1993. A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study. JAMA 270:2957-2963.

Liu, et al., 2003. A highly efficient recombineering-based method for generating conditional knockout mutations. Genome Res. 13:476-484.

Yang and Seed 2003. Site-specific gene targeting in mouse embryonic stem cells with intact bacterial artificial chromosomes. Nat. Biotechnol. 21:447-451.

10 Offermanns, et al., 1997. Defective platelet activation in G alpha(q)-deficient mice. Nature 389:183-186.

We claim:

1. A use of an active sTLT-1 peptide for the preparation of a medicament for the treatment of inflammation.
2. The use of claim 1, wherein the inflammation is related to a coagulation disorder.
- 5 3. The use of claim 2, wherein active sTLT-1 family peptide promotes coagulation.
4. The use of claim 1, 2, or 3, wherein the inflammation is related to sepsis or hypovolemic shock.
5. The use of any of claims 1 to 4, wherein the inflammation is related to infection.
6. The use of claim 1, wherein the inflammation is related to a wound.
- 10 7. The use of claim 1, wherein the inflammation is related to trauma.
8. The use of claim 1, wherein inflammation is related to vascular damage.
9. The use of any of claims 1 to 8, wherein the active sTLT-1 peptide reduces inflammation.
10. A use of an active sTLT-1 peptide for the preparation of a medicament for the treatment of a coagulation disorder.
- 15 11. The use of claim 10, wherein the coagulation disorder is selected from the group consisting of sepsis, hypovolemic shock, stroke, vascular occlusion, and thrombosis
12. The use of any of claims 1 to 11, wherein the active sTLT-1 peptide comprises an amino acid sequence at least 80% identical to: CHYRLQDVKAQKQVWCRFLPEGCQPLVSSAVDRRAPAGRR TFLTDLGGGLLQVEMVTLQEEDAGEYGC.
- 20 13. A kit to detect a sTLT-1 peptide in a subject sample, wherein presence of the sTLT-1 is indicative of the subject suffering from sepsis or hypovolemic shock.
14. The kit of claim 13, wherein the presence of the sTLT-1 peptide in the sample comprises a concentration of at least 15 µg/ml, 20 µg/ml, 25 µg/ml, 30 µg/ml, 35 µg/ml, 40µg/ml, 45 µg/ml, or 50 µg/ml.
- 25 15. The kit of claim 13 or 14, wherein the test is performed at a first time and at a later second time, wherein an increase in the amount of sTLT-1 peptide in the sample from the first time to the later second time is indicative of the subject suffering from sepsis or hypovolemic shock.
16. The kit of claim 13 or 14, wherein the test is performed at a first time and at a later second time, wherein a decrease in the amount of sTLT-1 family peptide in the sample from the first time to
- 30 the later second time is indicative of the subject not suffering from sepsis or hypovolemic shock.

17. The kit of any of claims 13 to 16, wherein the kit comprises an antibody that binds specifically to an sTLT-1 peptide.

18. A method of diagnosing sepsis or hypovolemic shock in a subject comprising:

a) providing a serum sample from a subject;

5 b) detecting a sTLT-1 family peptide in the serum,

wherein a sTLT-1 family peptide in the serum is indicative of sepsis or hypovolemic shock is diagnosed.

19. The method of claim 18, wherein the amount of sTLT-1 peptide in the serum comprises at least 50 µg/ml of sTLT-1 polypeptide.

10 20. The method of claim 18, further comprising:

c) providing a serum sample from a subject obtained after the serum sample provided in step (a) from the subject;

d) comparing the amount of sTLT-1 peptide in the serum from step (c) to the serum from step (a),

15 wherein an increase in the amount of the sTLT-1 peptide in the sample from step (c) is indicative of sepsis or hypovolemic shock.

21. A method of treatment of inflammation comprising administration of an active sTLT-1 peptide.

22. The method of claim 21, wherein the inflammation is related to a coagulation disorder.

20 23. The method of claim 22, wherein active sTLT-1 family peptide promotes coagulation.

24. The method of claim 21, 22, or 23, wherein the inflammation is related to sepsis or hypovolemic shock.

25. The method of any of claims 21 to 24, wherein the inflammation is related to infection.

26. The method of claim 21, wherein the inflammation is related to a wound.

25 27. The method of claim 21, wherein the inflammation is related to trauma.

28. The method of claim 21, wherein inflammation is related to vascular damage.

29. The method of any of claims 21 to 28, wherein the active sTLT-1 peptide reduces inflammation.

30. A method of treatment of a coagulation disorder comprising administration of an active

sTLT-1 peptide.

31. The method of claim 30, wherein the coagulation disorder is selected from the group consisting of sepsis, hypovolemic shock, stroke, vascular occlusion, and thrombosis

32. The method of any of claims 21 to 31, wherein the active sTLT-1 peptide comprises an amino acid sequence at least 80% identical to: CHYRLQDVKAQKVWCRFLPEGCQPLVSSAVDR  
5 RAPAGRRRTFLTDLGGGLLQVEMVTLQEEDAGEYGC.

33. A pharmaceutical composition comprising an amino acid sequence at least 80% identical to: CHYRLQDVKAQKVW CRFLPEGCQPLVSSAVDRRAPAGRRRTFLTDLGGGLLQVEMVTLQE  
EDAGEYGC in a pharmaceutically acceptable carrier.

10 34. The composition of claim 33, wherein the amino acid sequence consists essentially of CHYRLQDVKAQKVWCRFLPEGCQPLVSSAVDRRAPAGRRRTFLTDLGGGLLQVEMVTLQ  
EEDAGEYGC.

35. A use of an active sTREM-1 peptide for the preparation of an adjuvant for administration in conjunction with an antigen to stimulate an immune response.

15 36. The use of claim 35, wherein the sTREM-1 polypeptide comprises an amino acid sequence at least 80% identical to: CDYTLEKFASSQKAWQIIRDGEMPKTLACTERPSKNSHPVQVGRILED  
YHDHGLLRVRM VNLQVEDSGLYQC.

37. A kit comprising a polypeptide comprising an amino acid sequence at least 80% identical to: CDYTLEKFASSQKAWQIIRDGEMPKTLACTERPSKNSHPVQVGRILED YHDHGLLRVRM  
20 VNLQVEDSGLYQC.

38. A pharmaceutical composition comprising a polypeptide comprising an active sTREM-1 peptide comprising an amino acid sequence at least 80% identical to: CDYTLEKFASSQKAWQIIRDGEMPKTLACTERPSKNSHPV QVGRILEDYHDHGLLRVRM  
VNLQVEDSGLYQC.

25 39. The pharmaceutical composition of claim 38, comprising a polypeptide consisting essentially of an amino acid sequence of: CDYTLEKFASSQKAWQIIRDGEMPKTLACTERPSKNSHPVQVGRILE DYHDHGLLRVRM  
VNLQVEDSGLYQC.

40. A method of immunization of a subject comprising co-administration of an active sTREM-1  
30 polypeptide and an adjuvant.

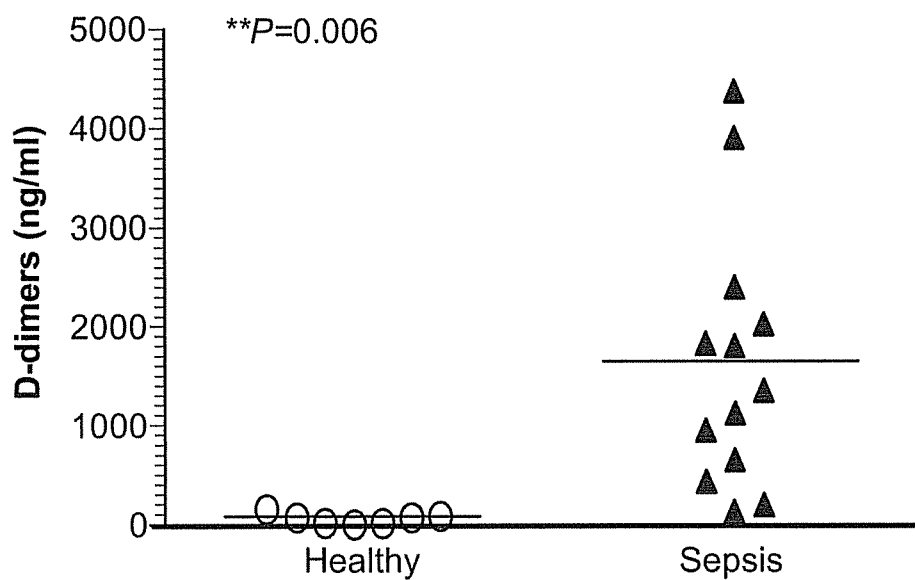


FIG. 1A

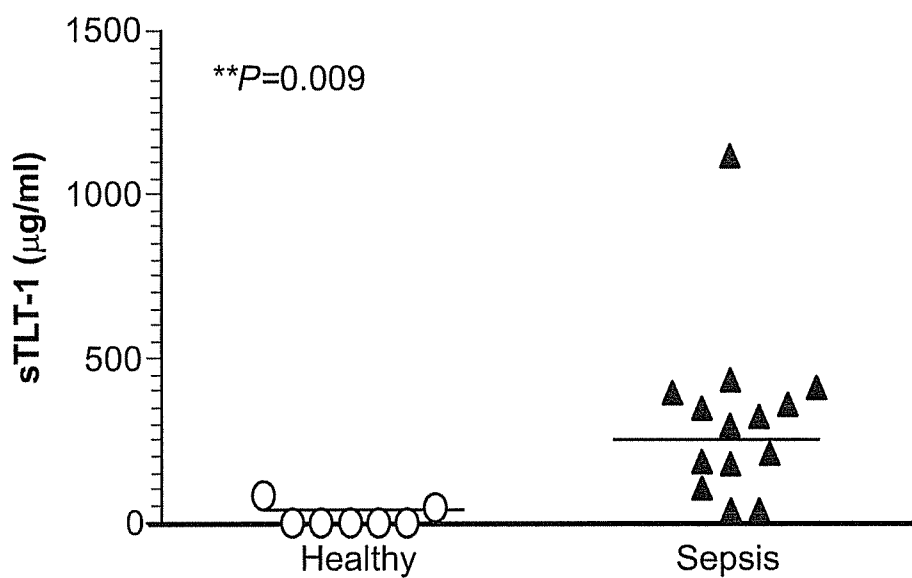


FIG. 1B

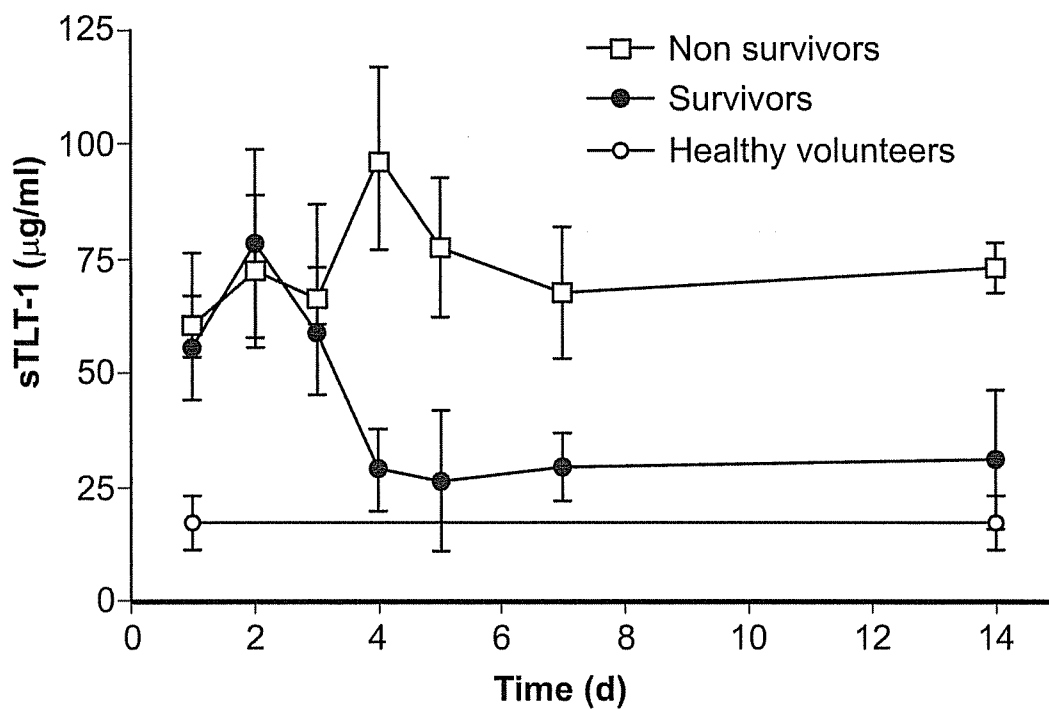


FIG. 2A

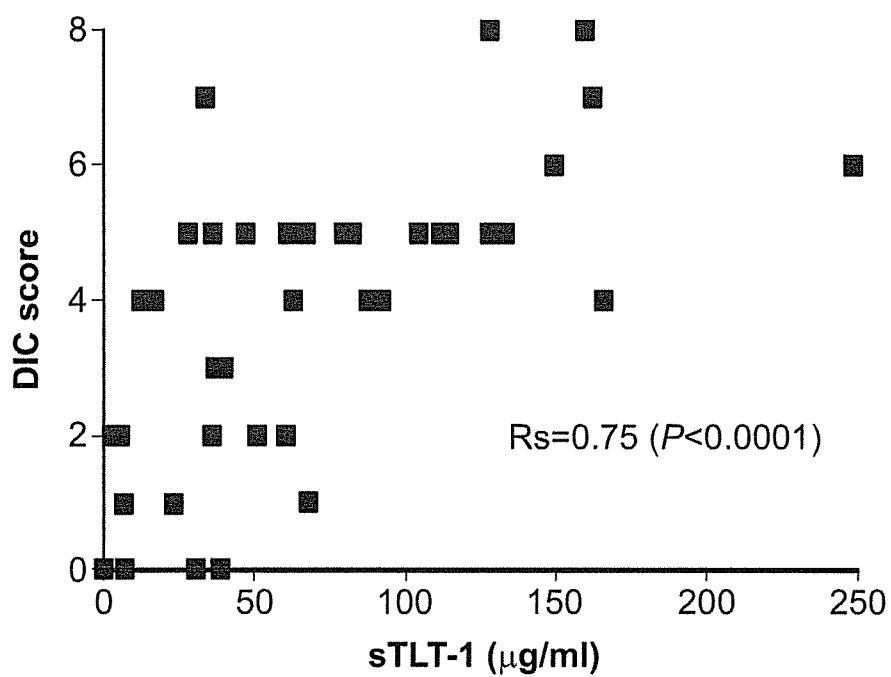


FIG. 2B

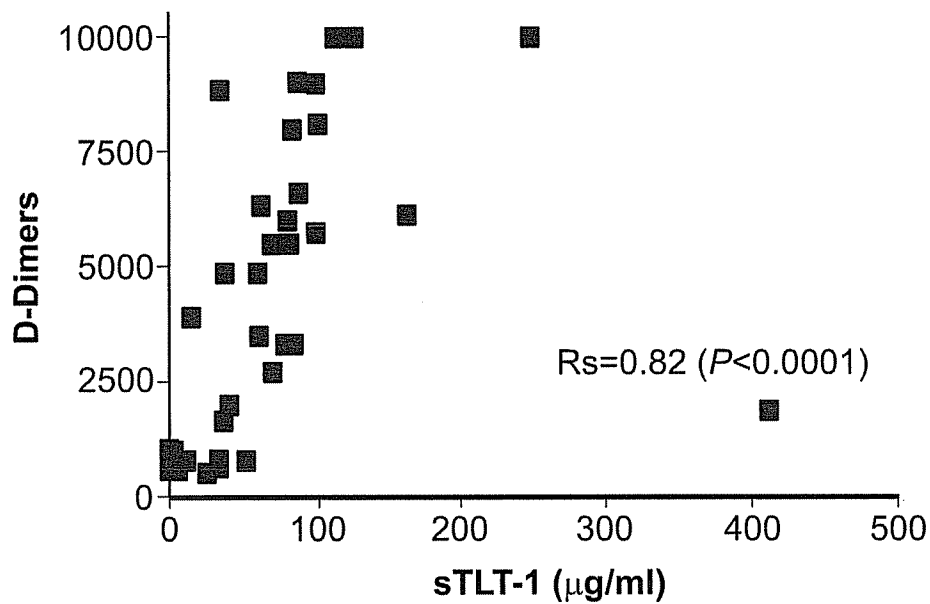


FIG. 2C

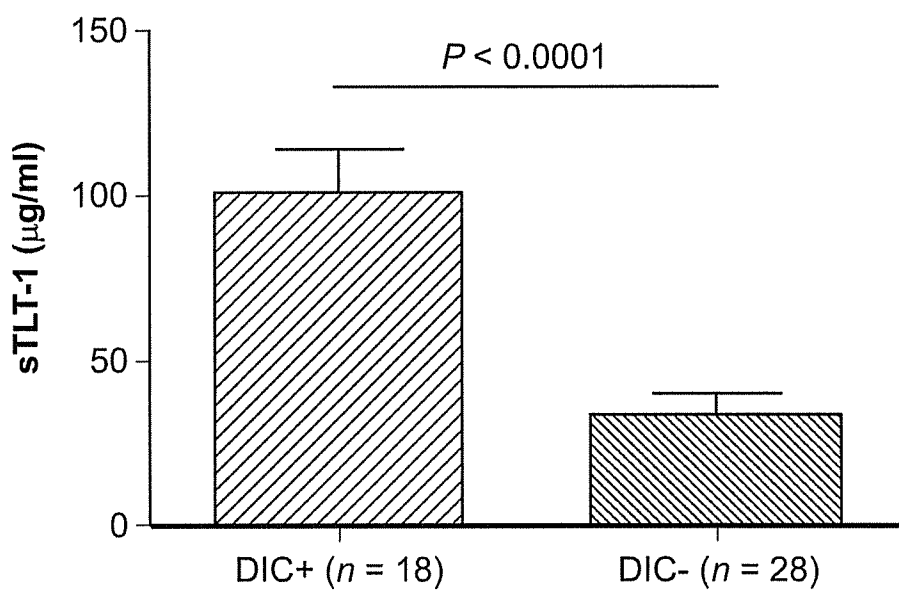


FIG. 2D

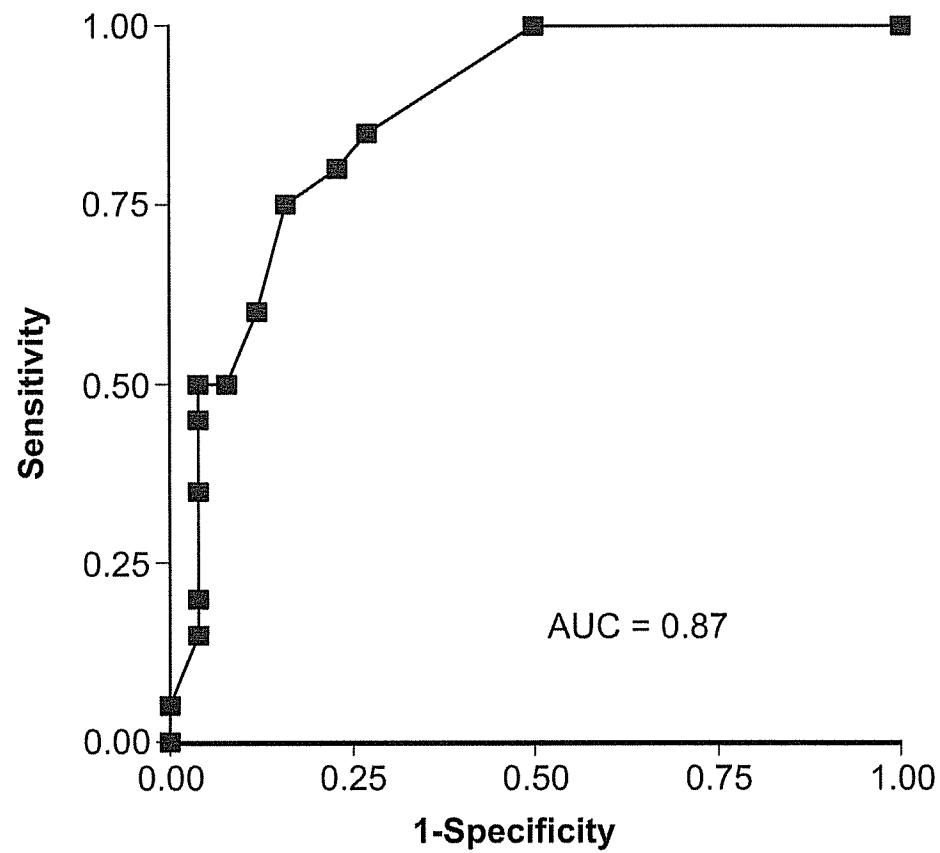


FIG. 2E

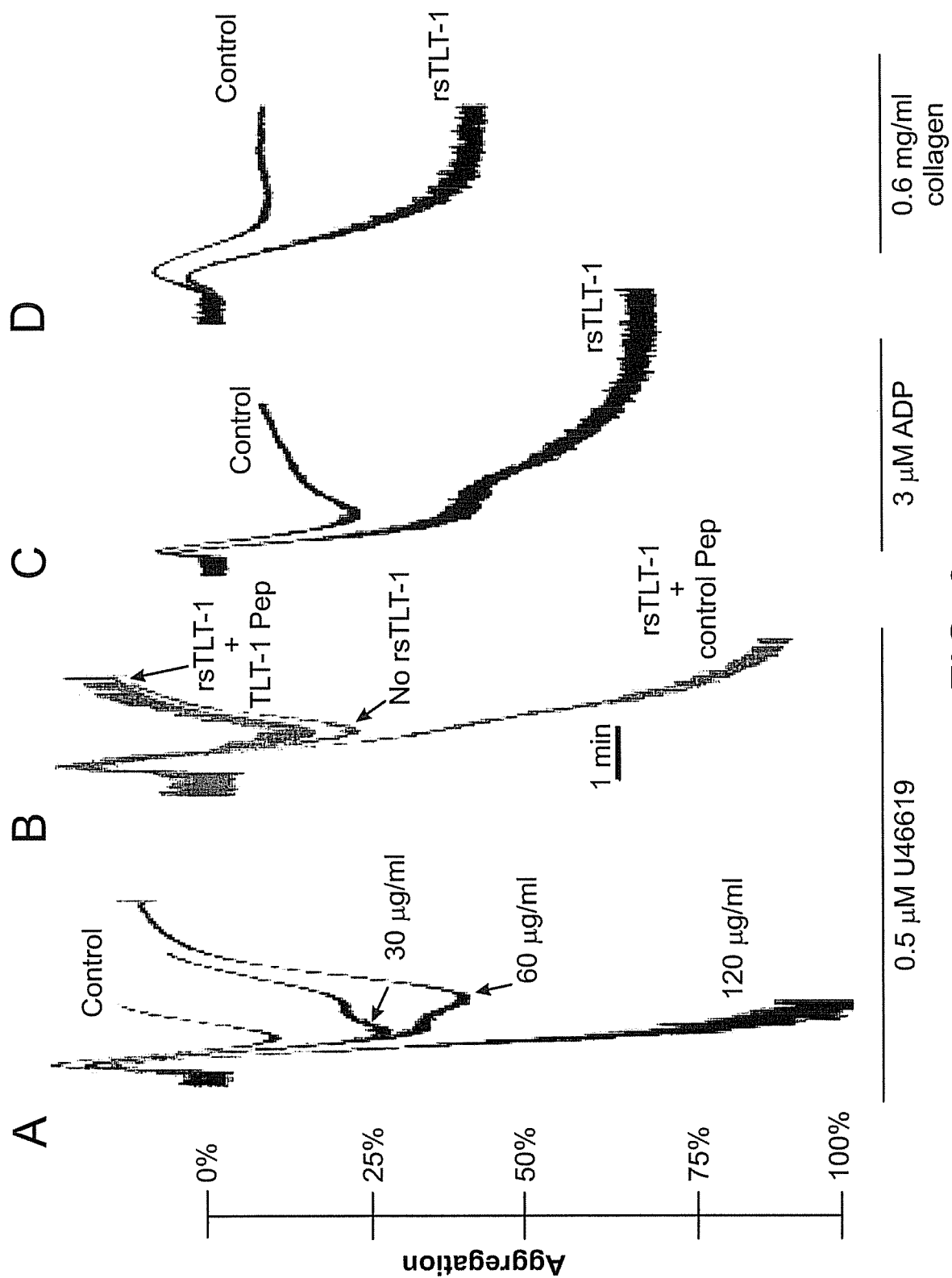


FIG. 3

6/35

FIG. 4A

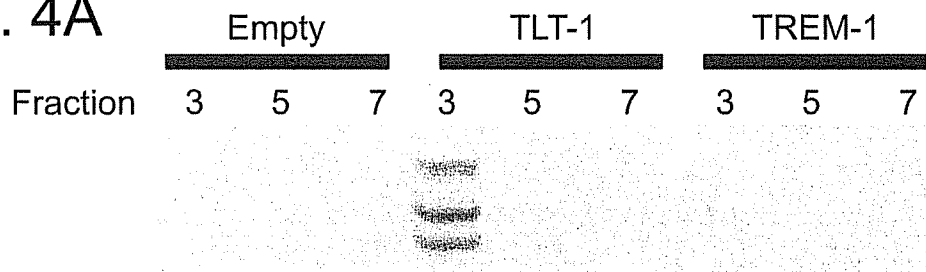


FIG. 4B

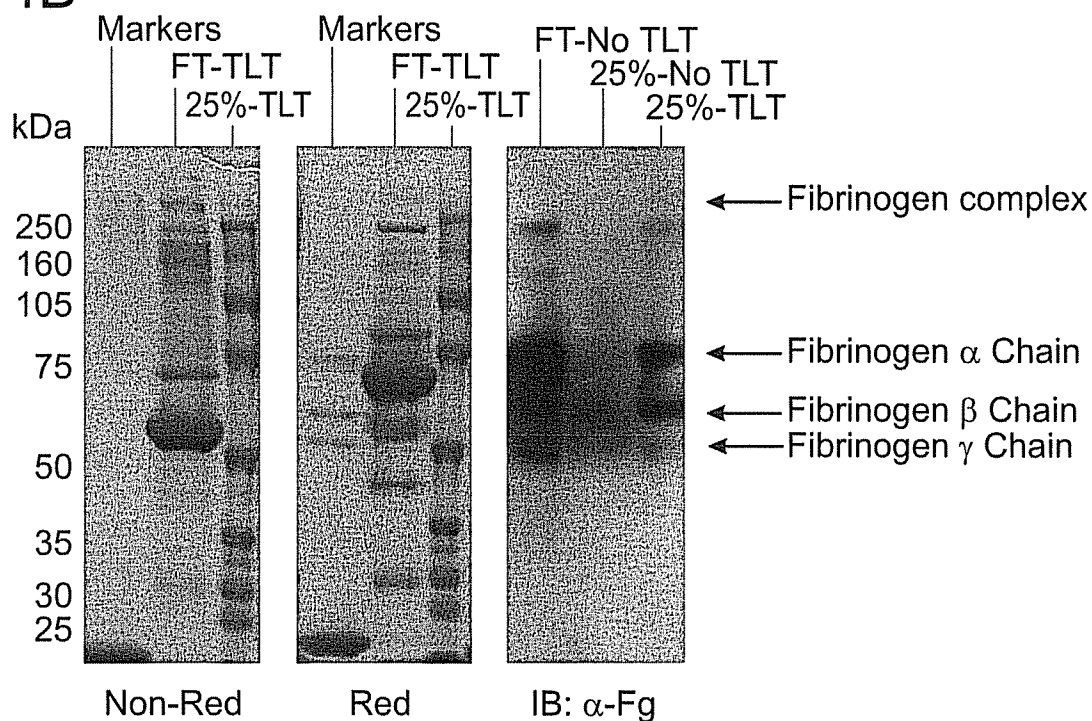


FIG. 4C

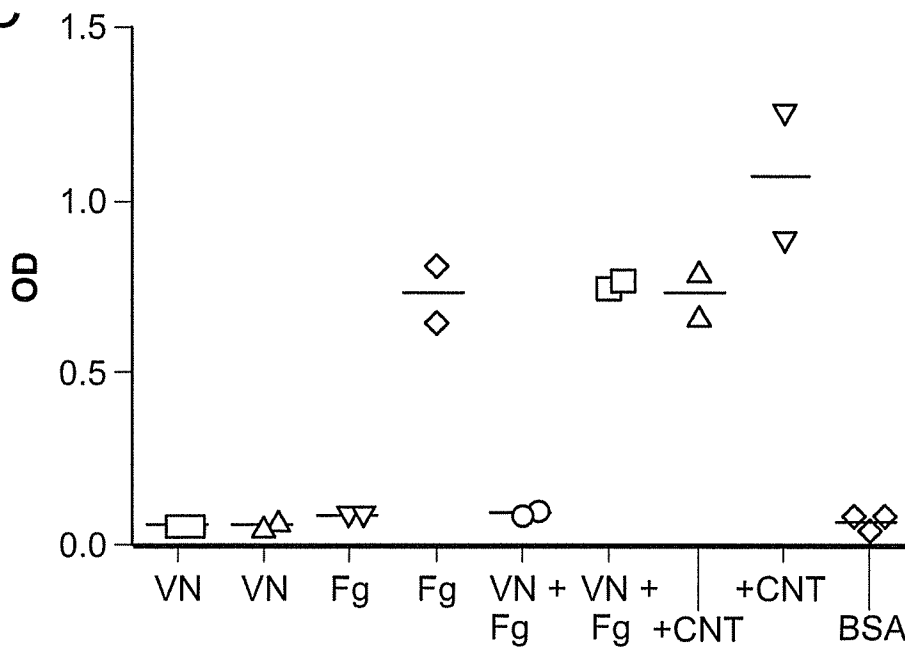


FIG. 5A

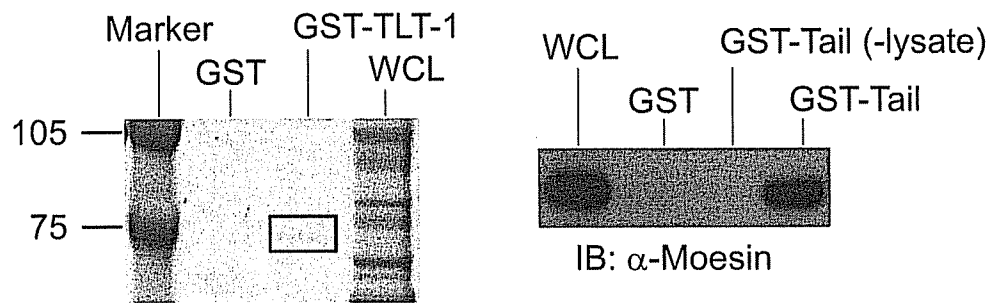


FIG. 5C

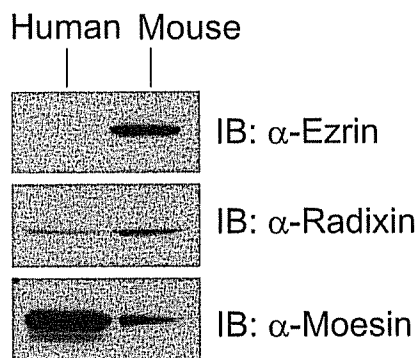


FIG. 5E

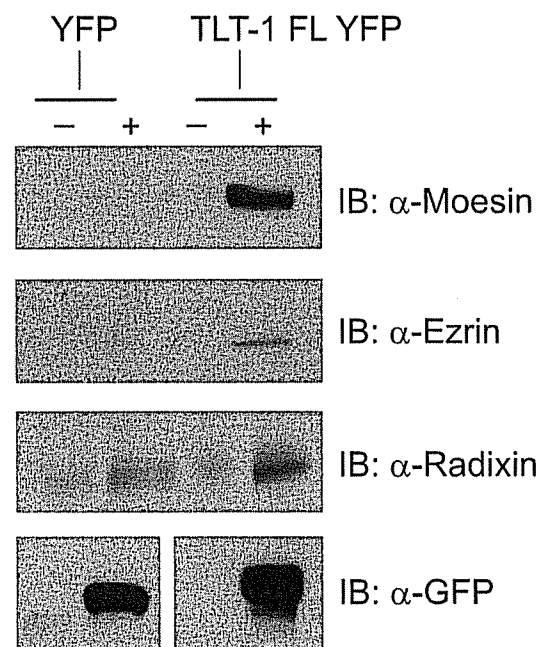


FIG. 5D

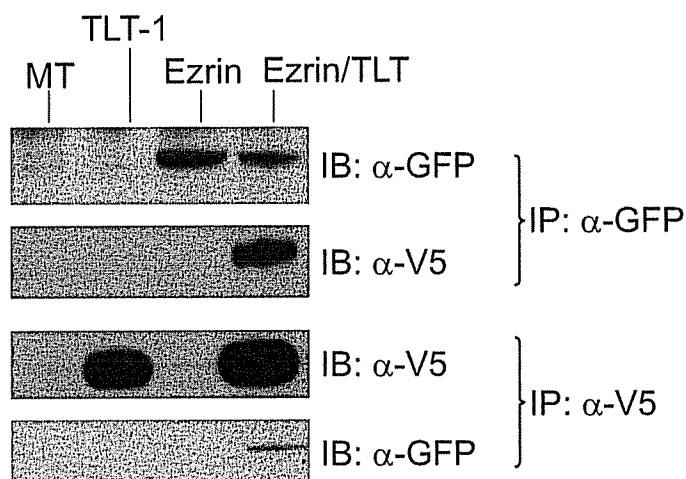


FIG. 5B

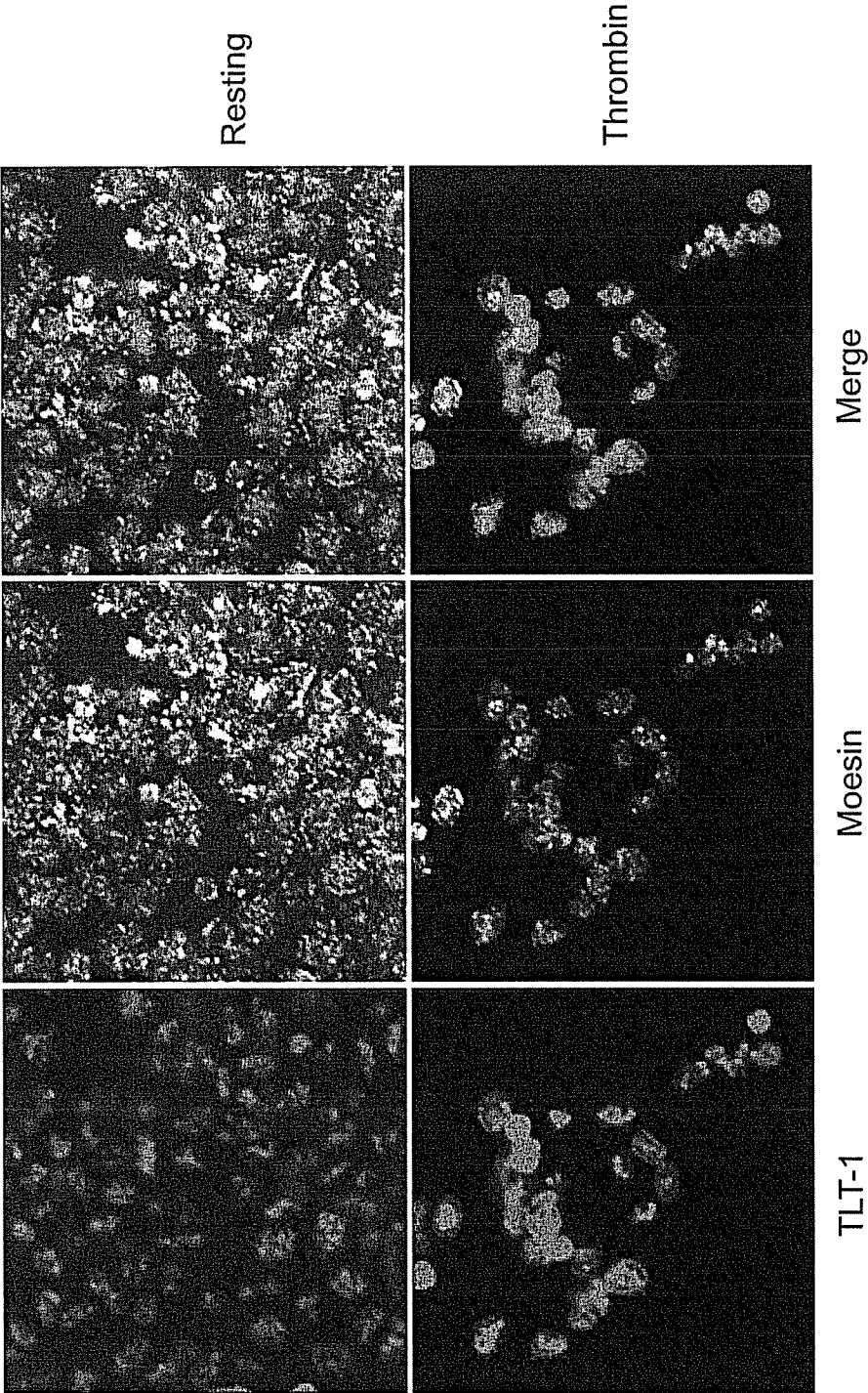


FIG. 5F

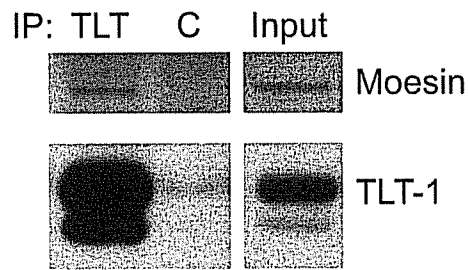


FIG. 6A

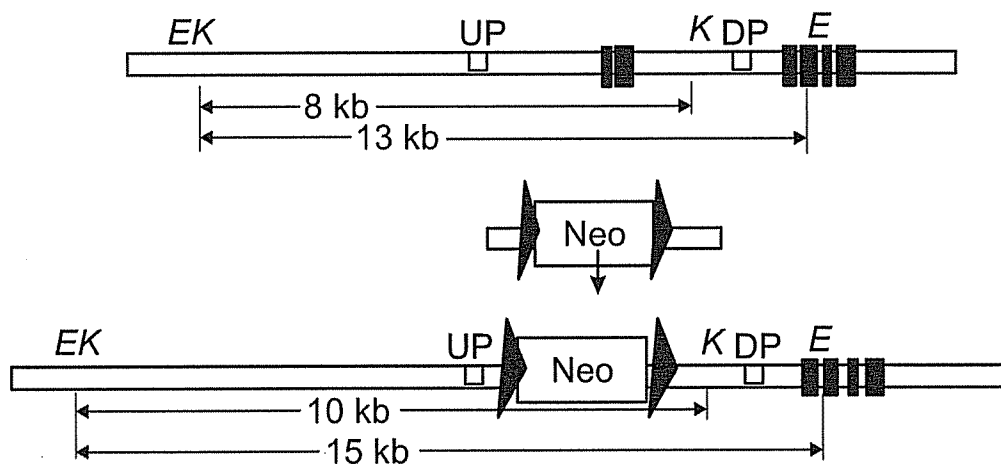


FIG. 6B

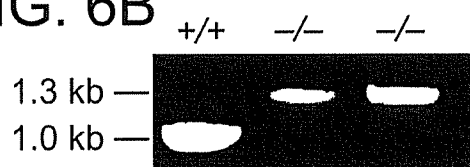


FIG. 6C

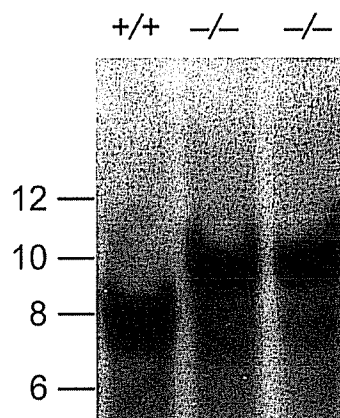


FIG. 6D

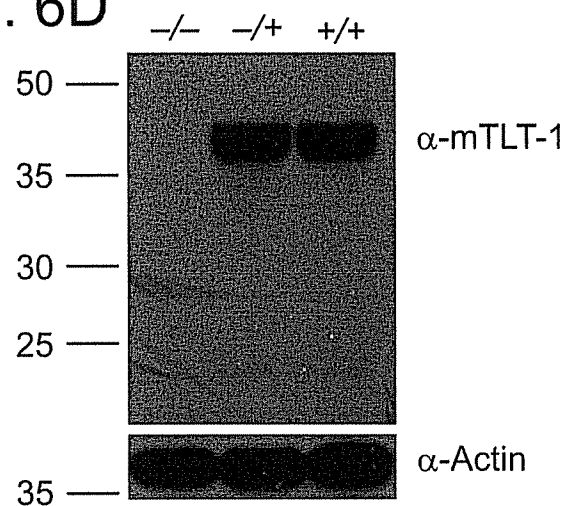
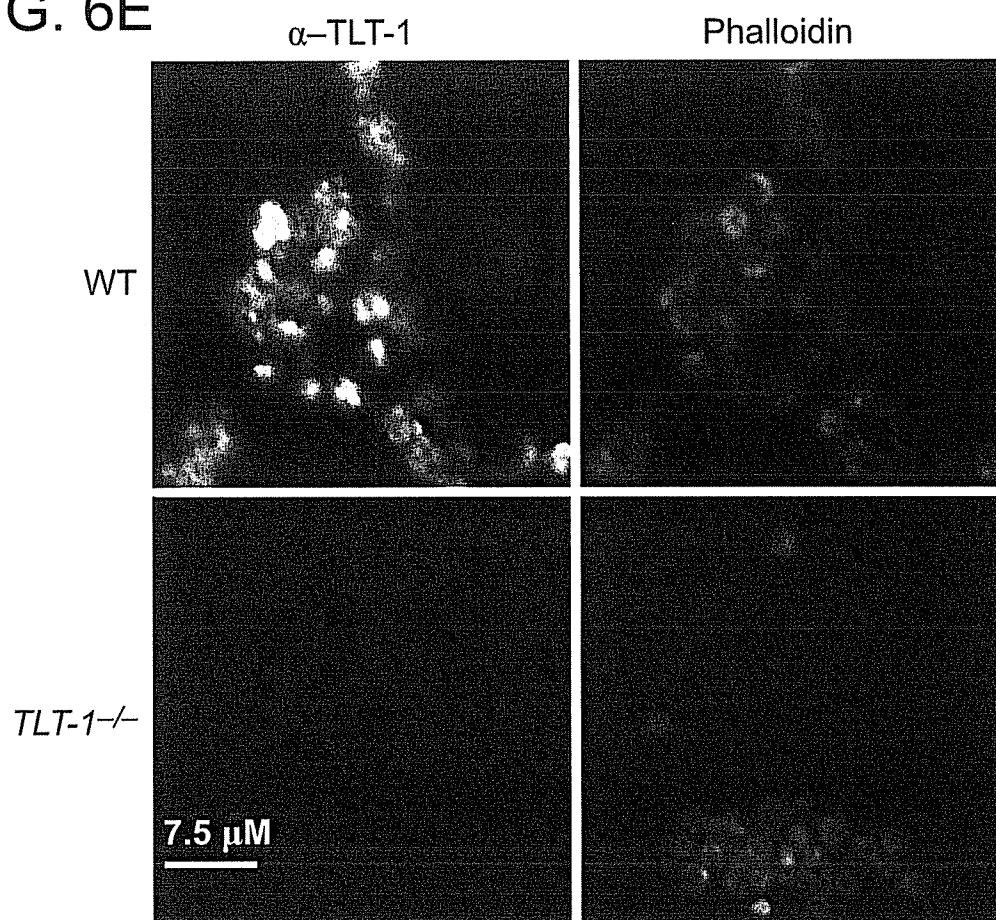


FIG. 6E



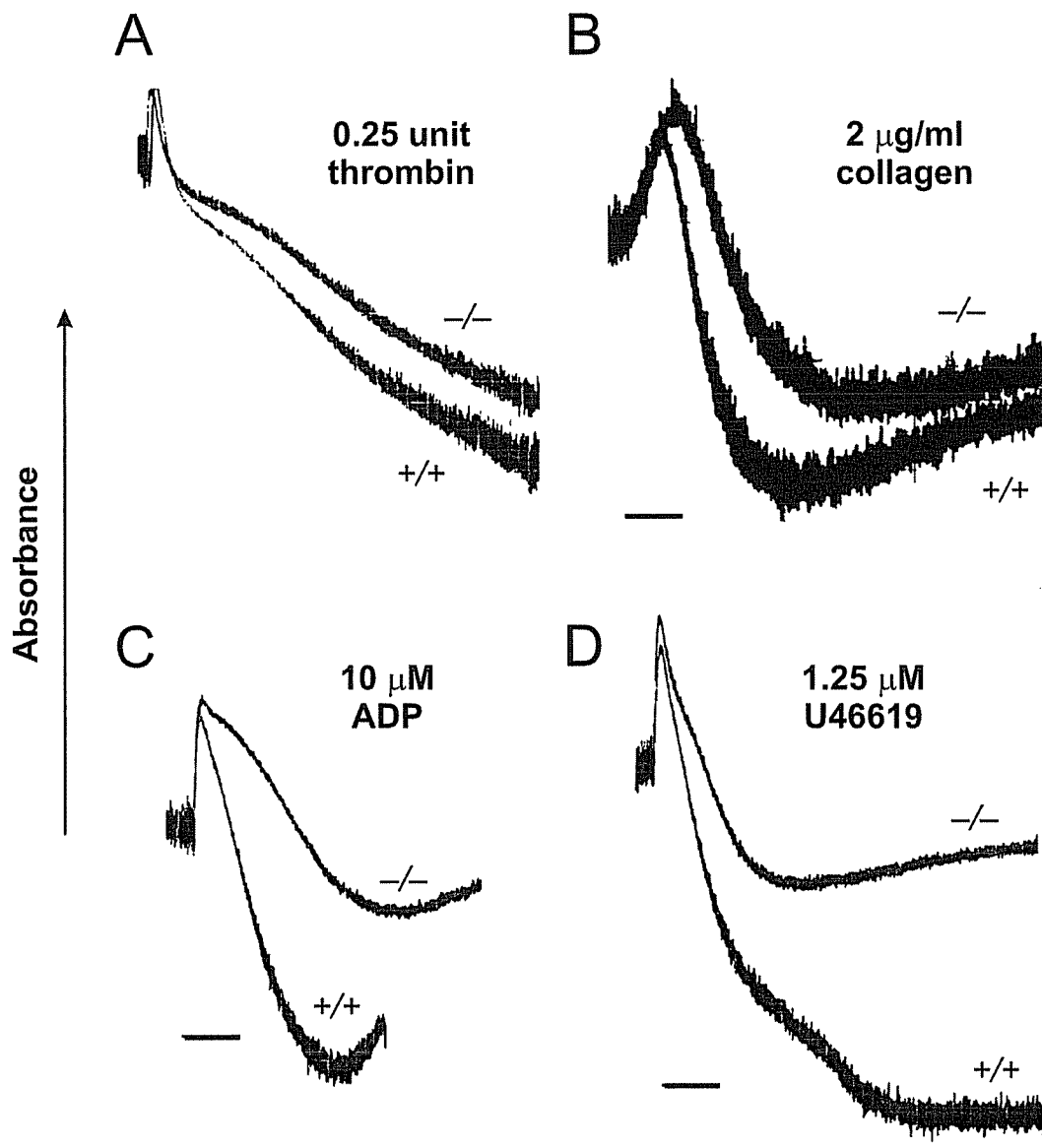


FIG. 7

FIG. 7E

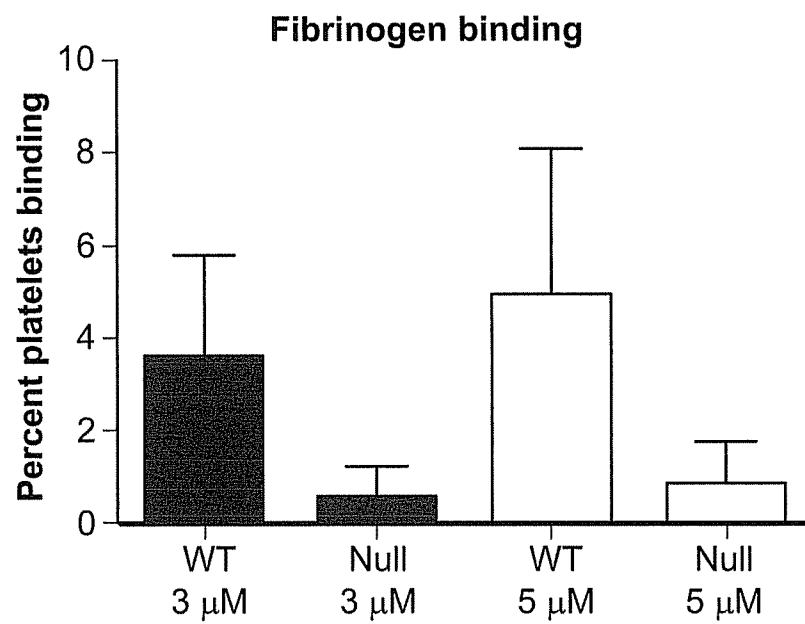


FIG. 7F

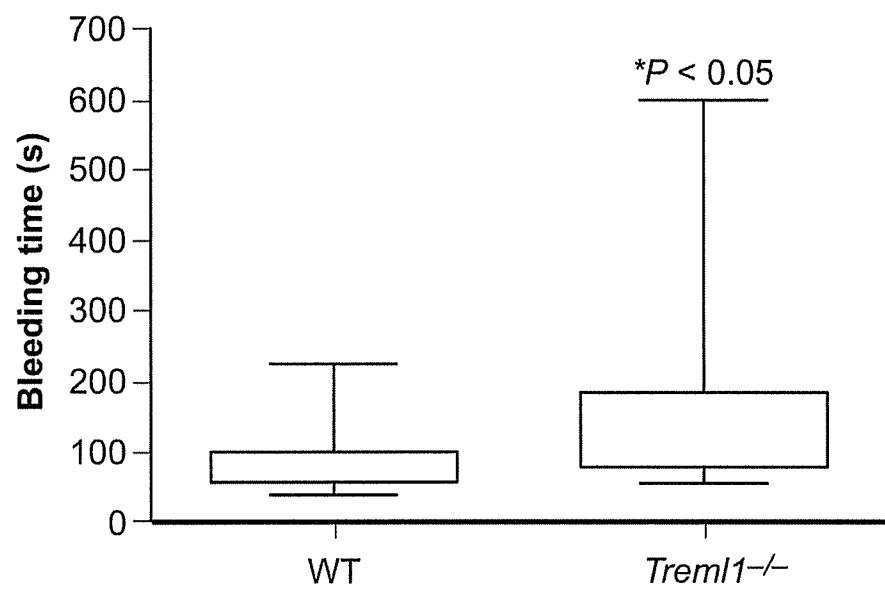


FIG. 8A

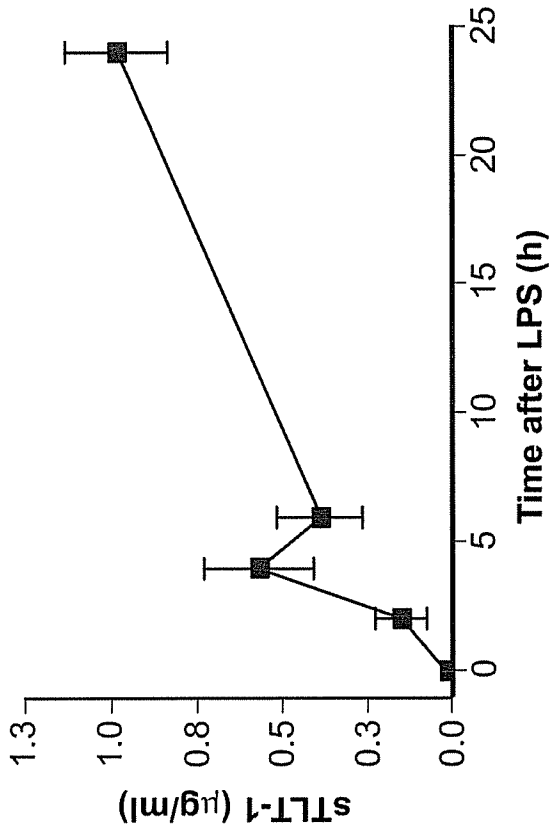


FIG. 8B

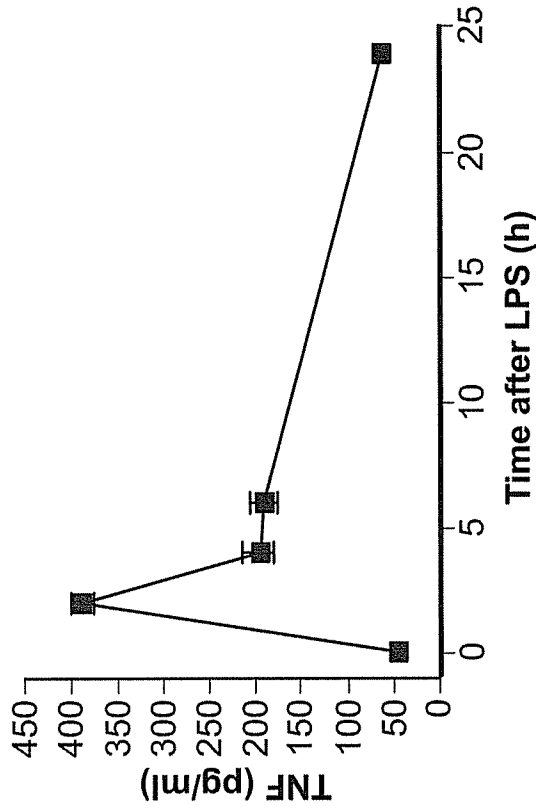


FIG. 8C

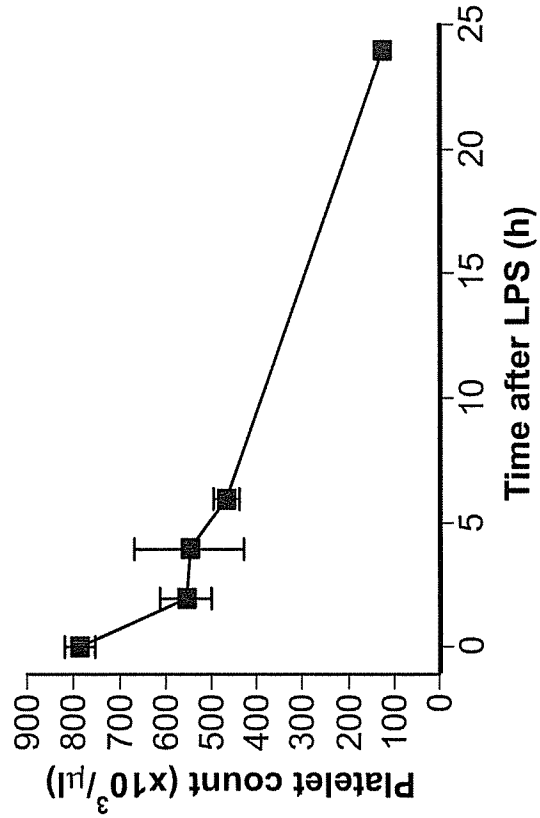


FIG. 8D

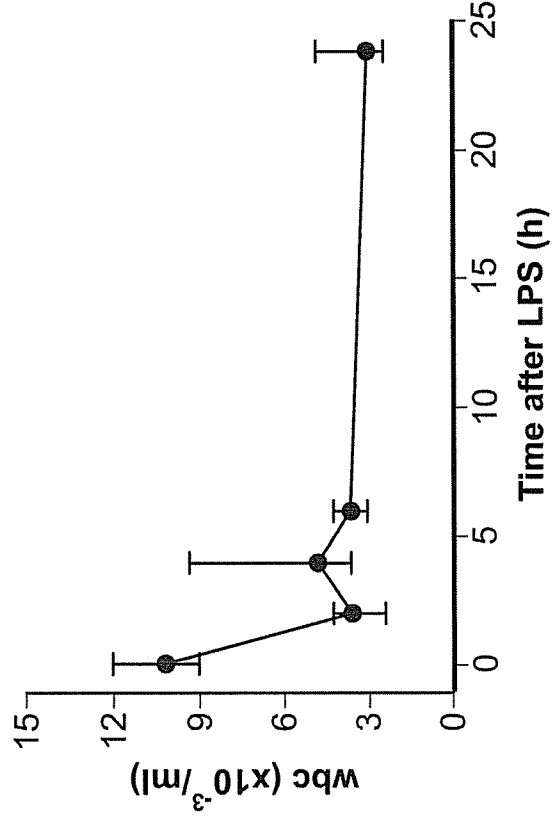


FIG. 9A

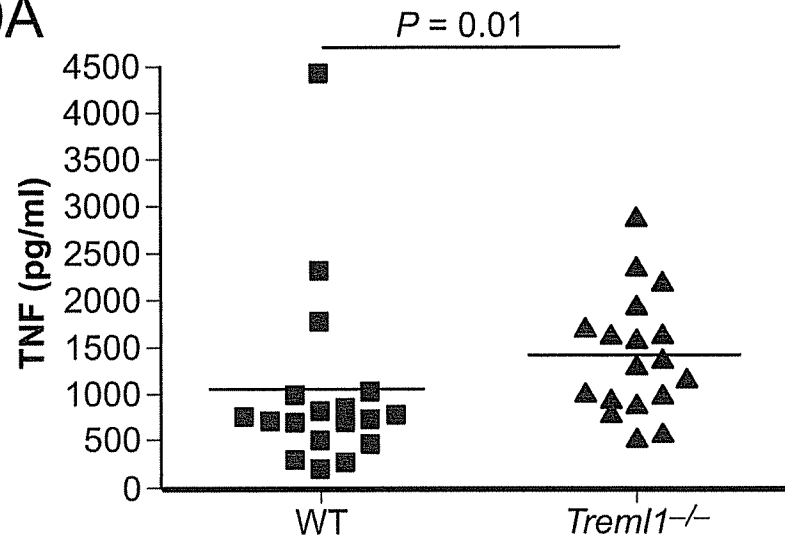


FIG. 9B

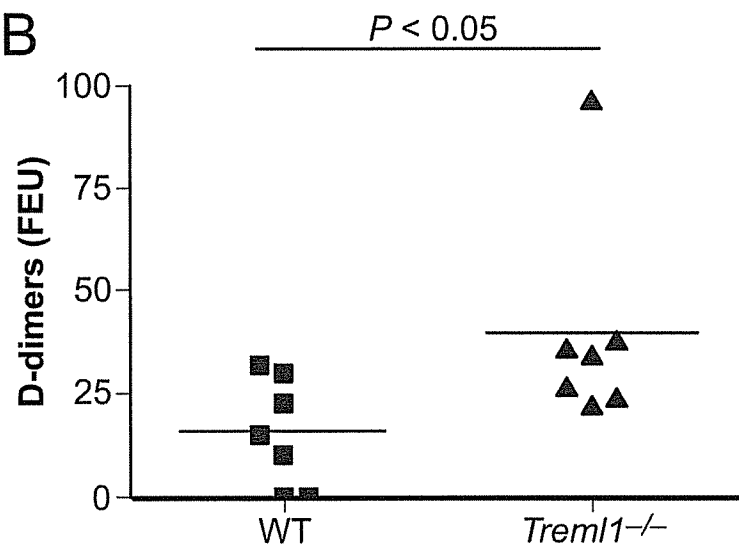
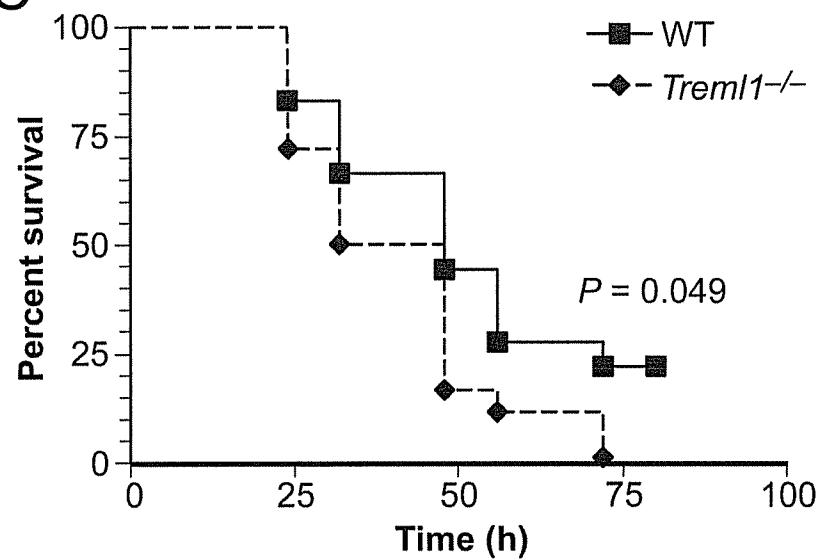


FIG. 9C



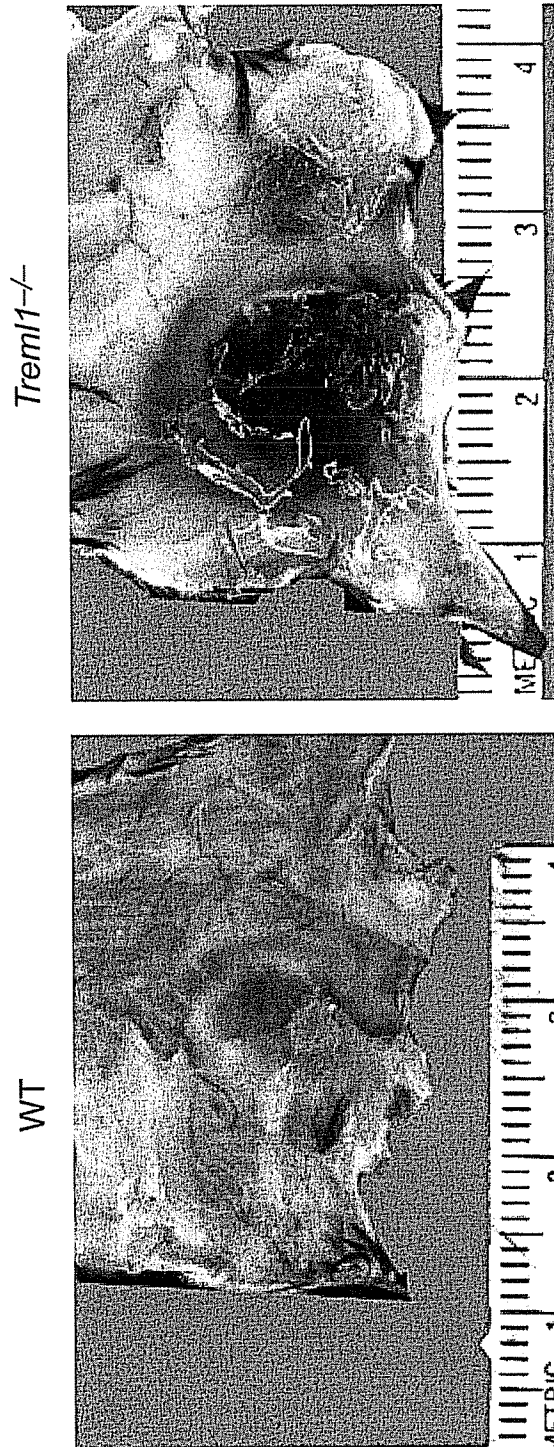


FIG. 10A

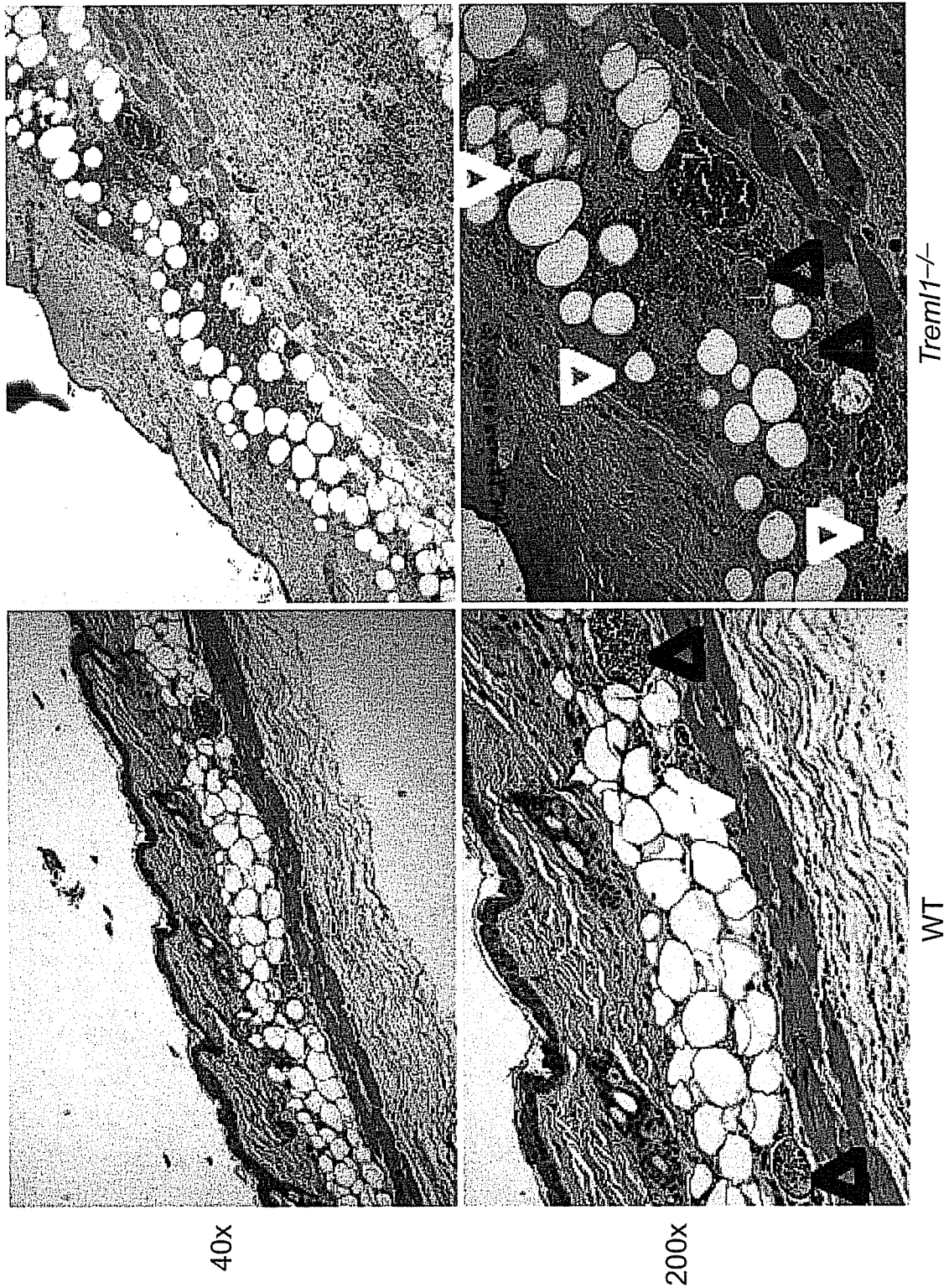


FIG. 10B

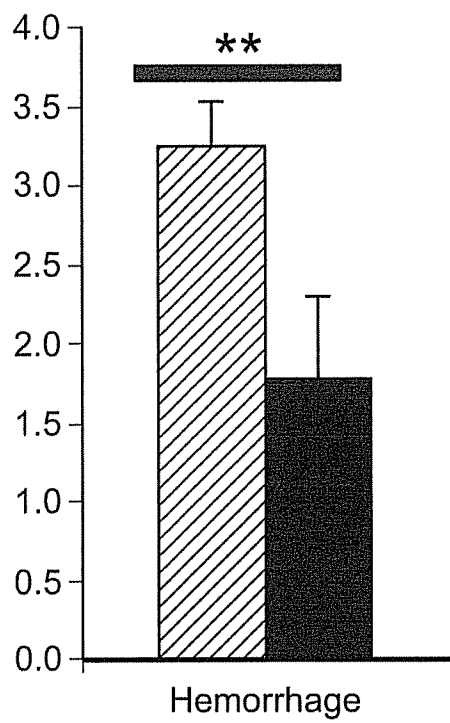


FIG. 10C

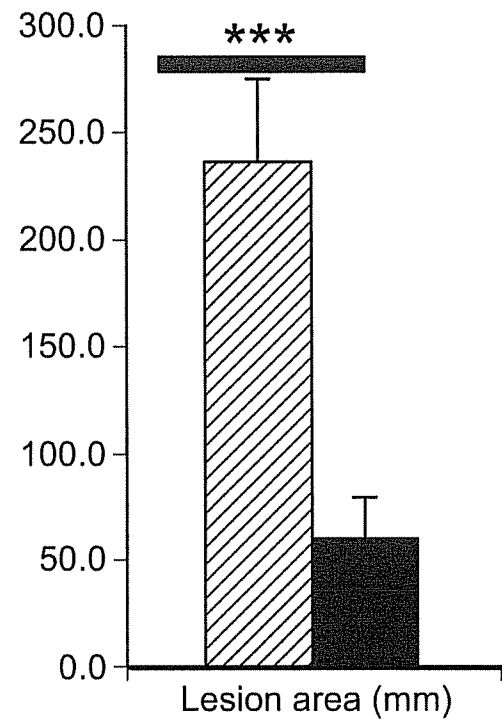
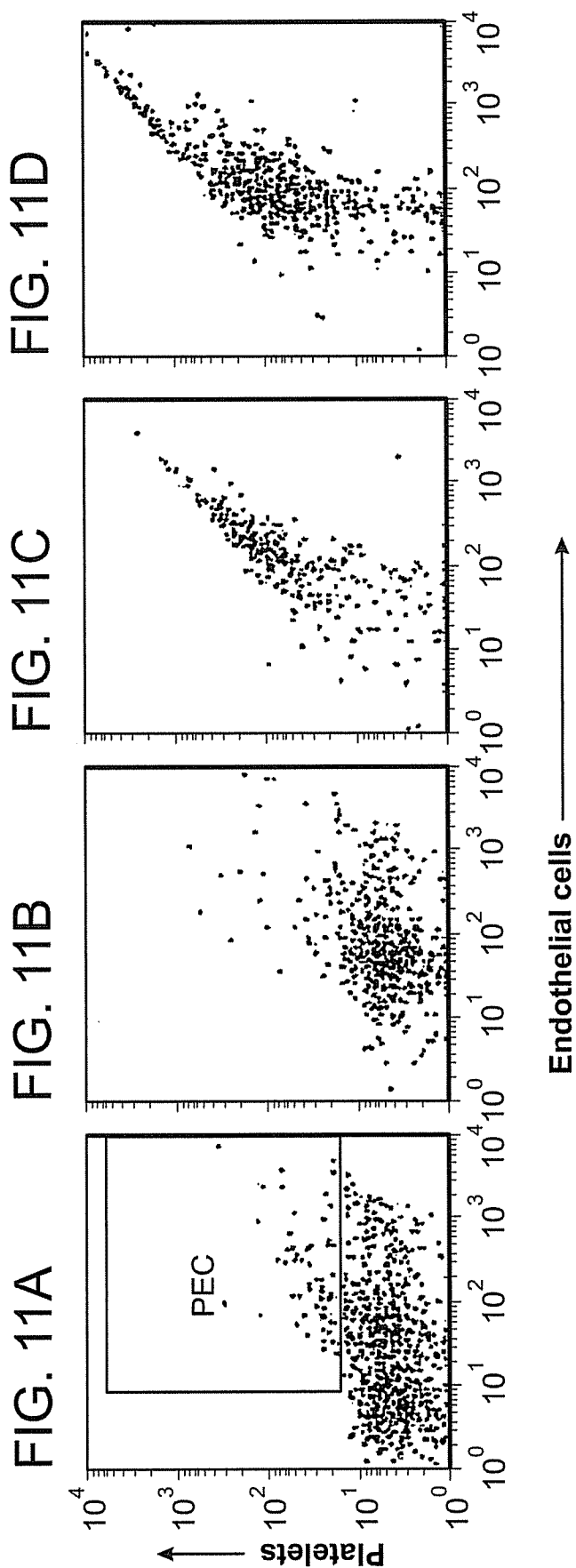


FIG. 10D



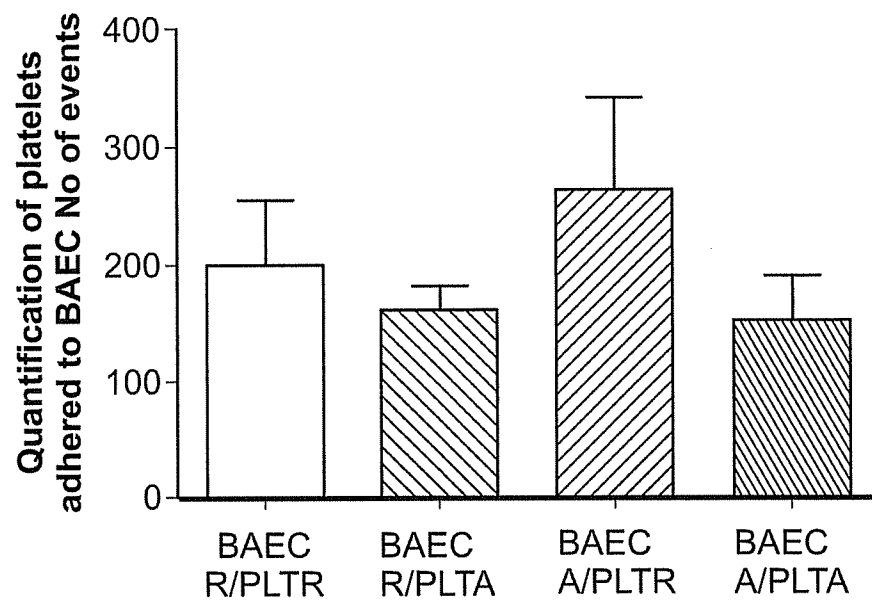


FIG. 11E

FIG. 12A

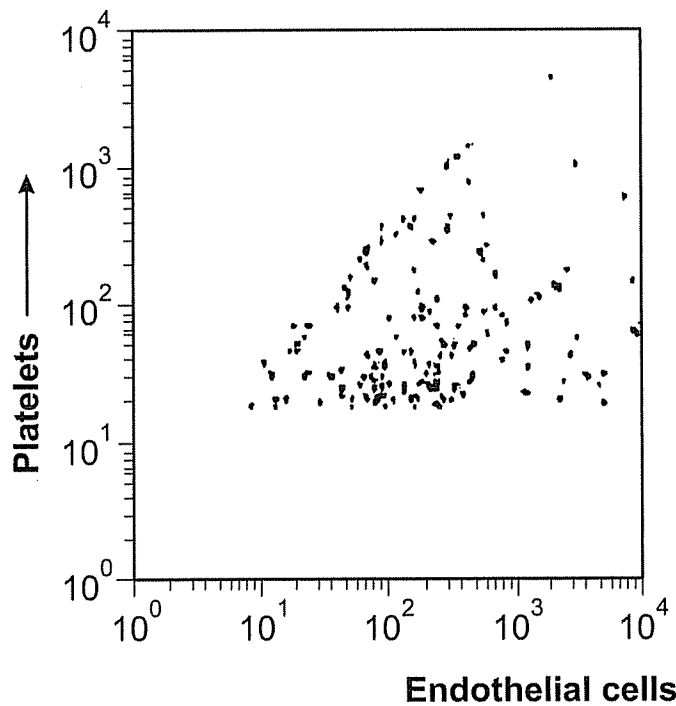


FIG. 12B

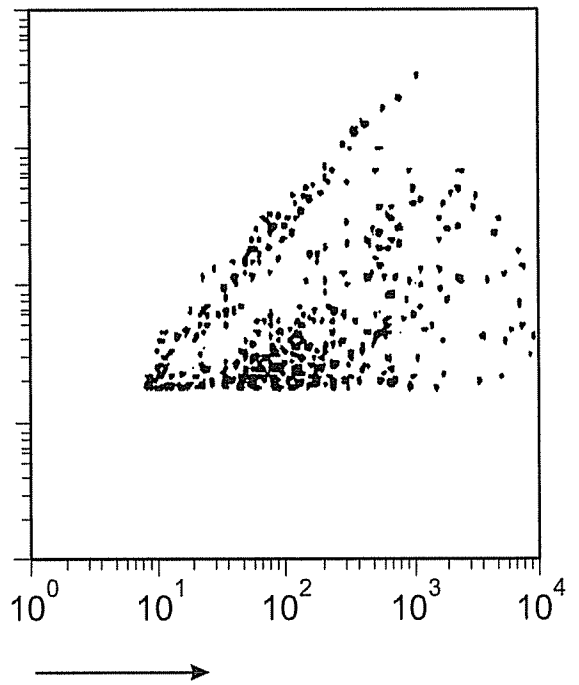


FIG. 12C

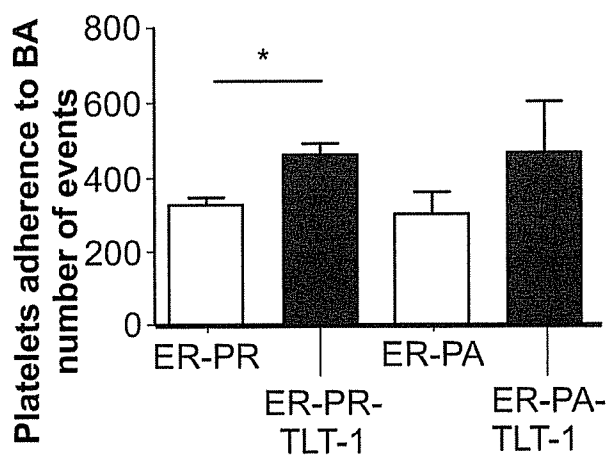
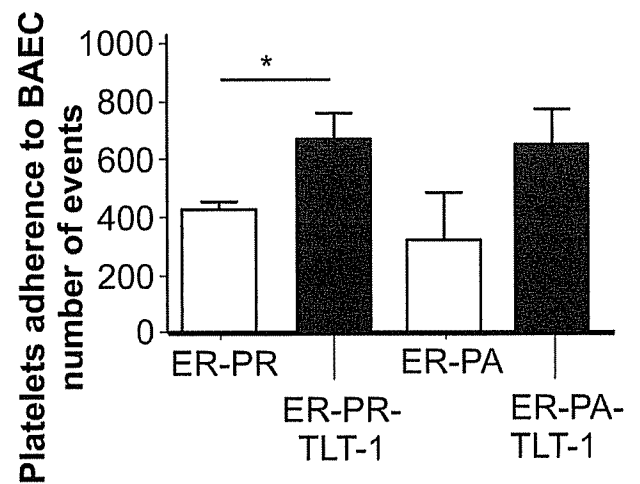


FIG. 12D



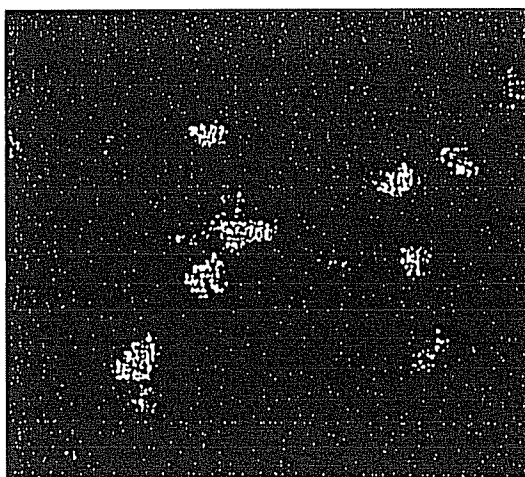


FIG. 13A

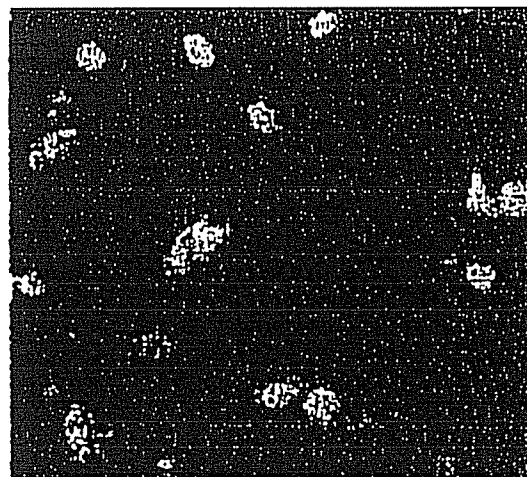


FIG. 13B

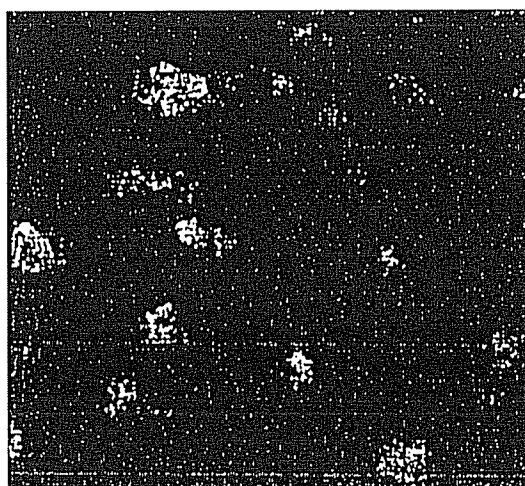


FIG. 13C

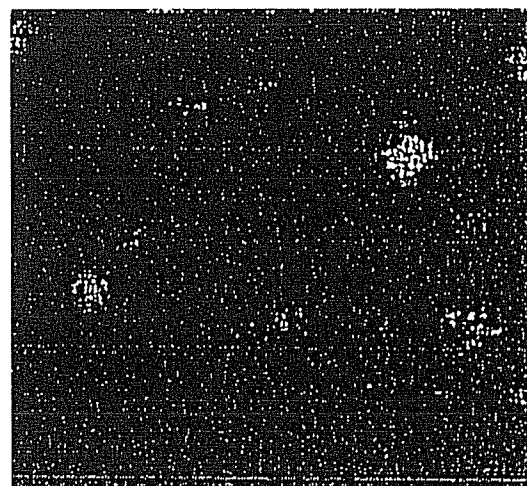


FIG. 13D

FIG. 13E

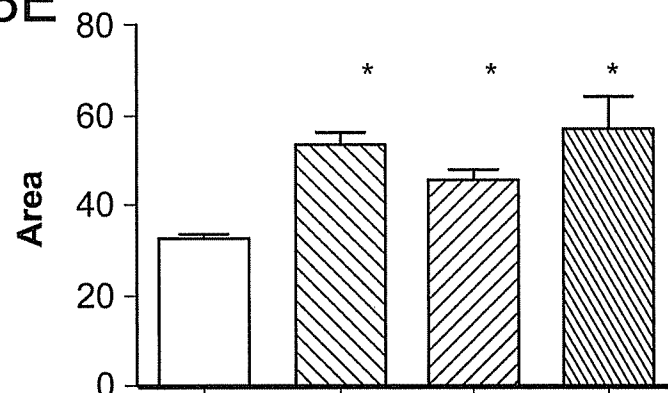
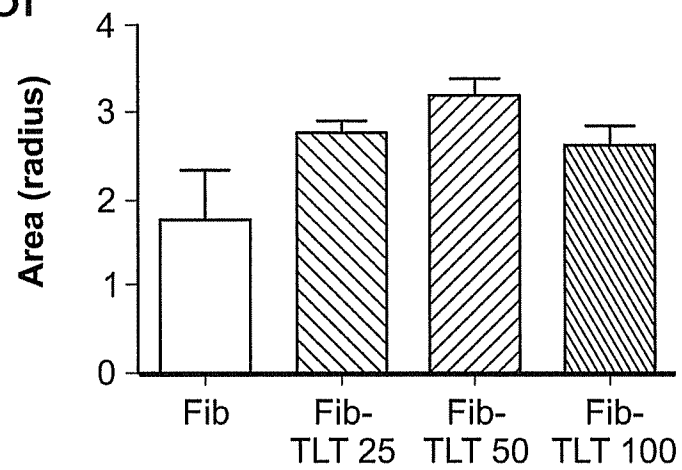


FIG. 13F



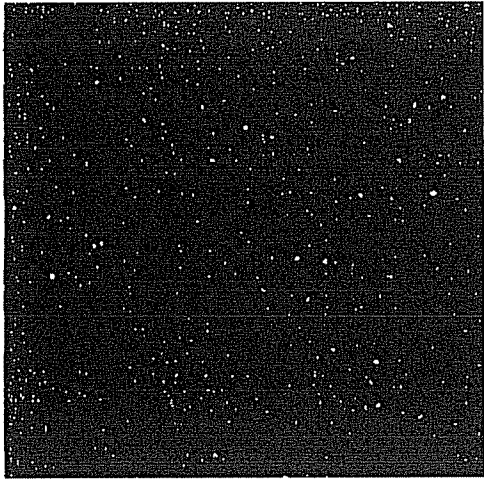


FIG. 14A

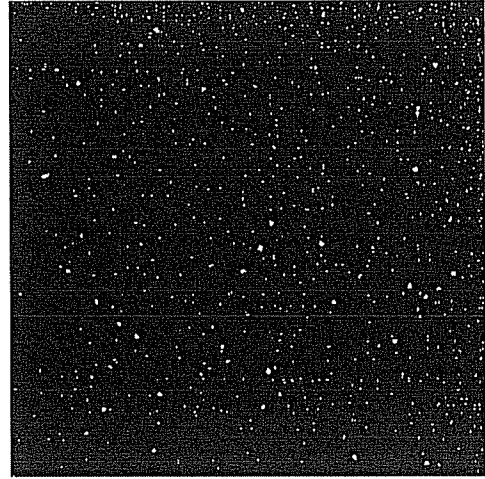


FIG. 14B

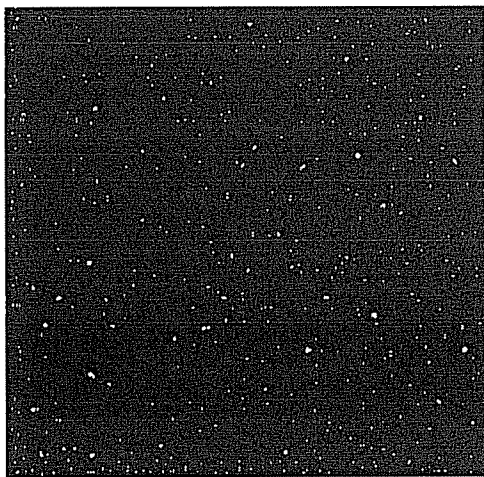


FIG. 14C

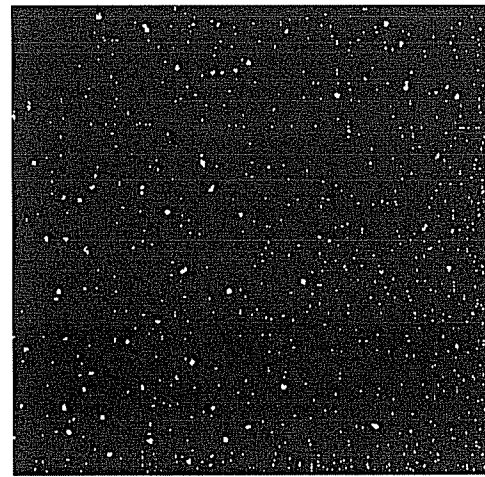


FIG. 14D

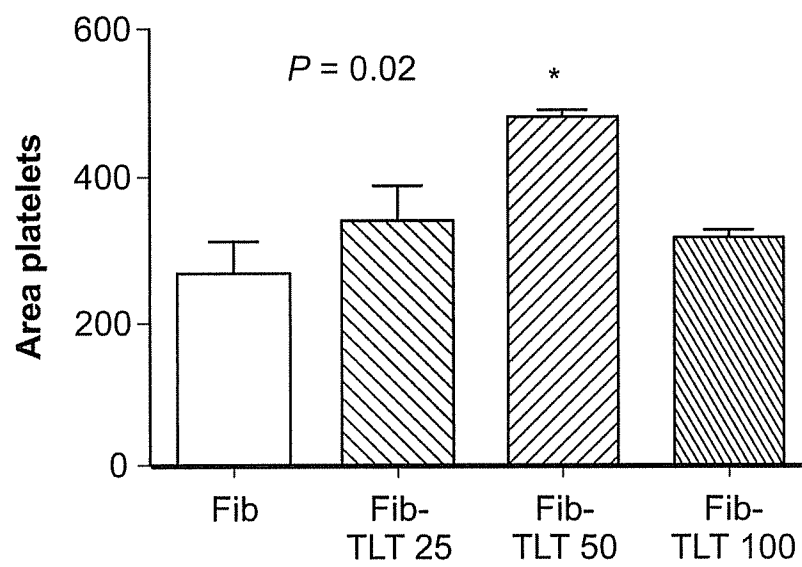
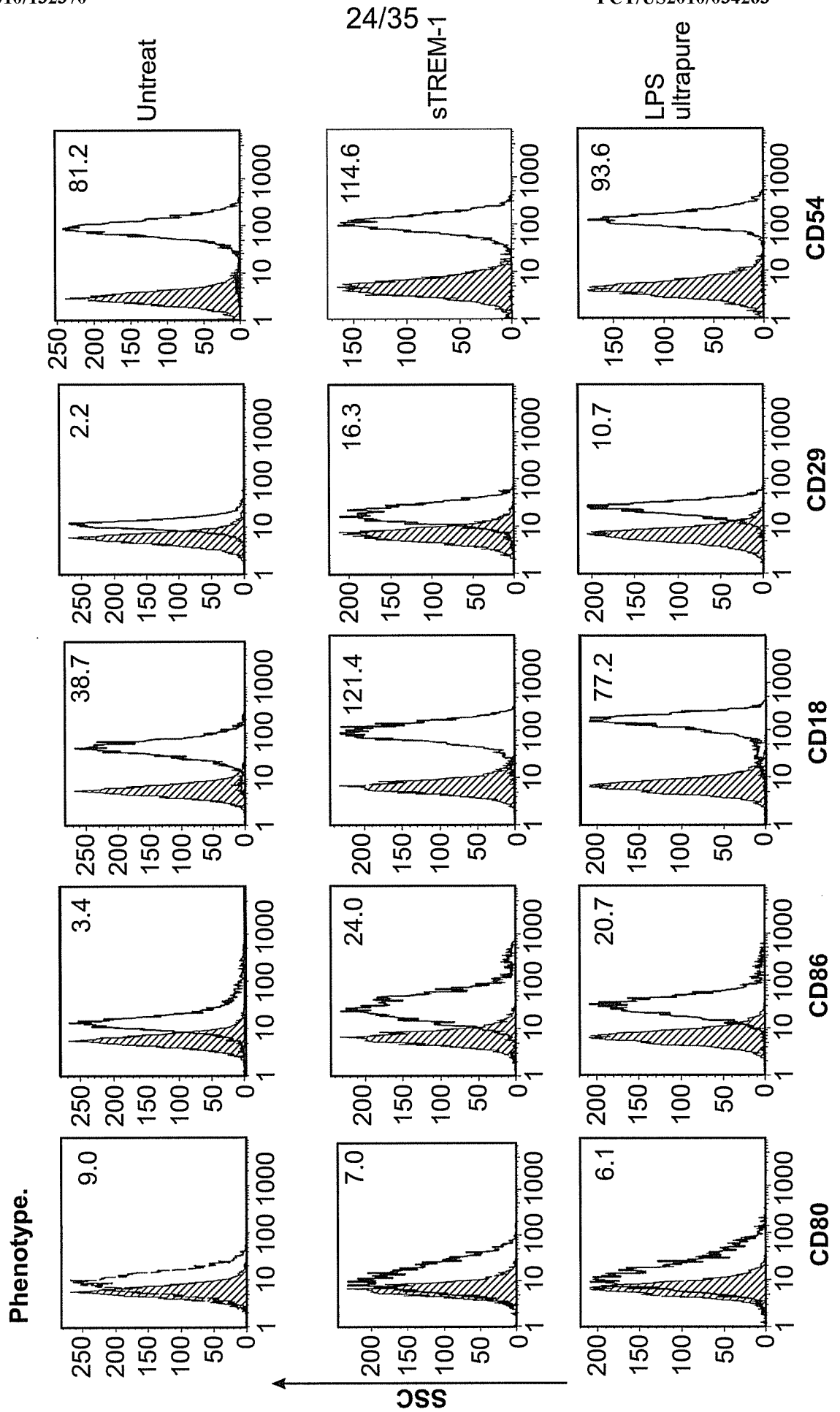


FIG. 14E

FIG. 15A



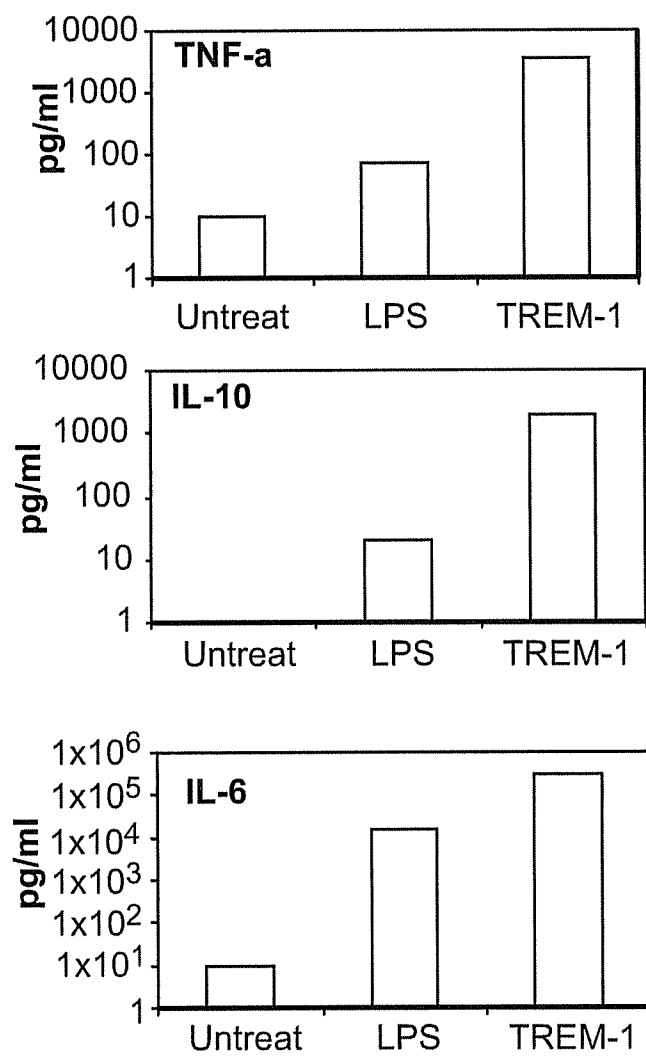
**Cytokine production.****FIG. 15B**

FIG. 16

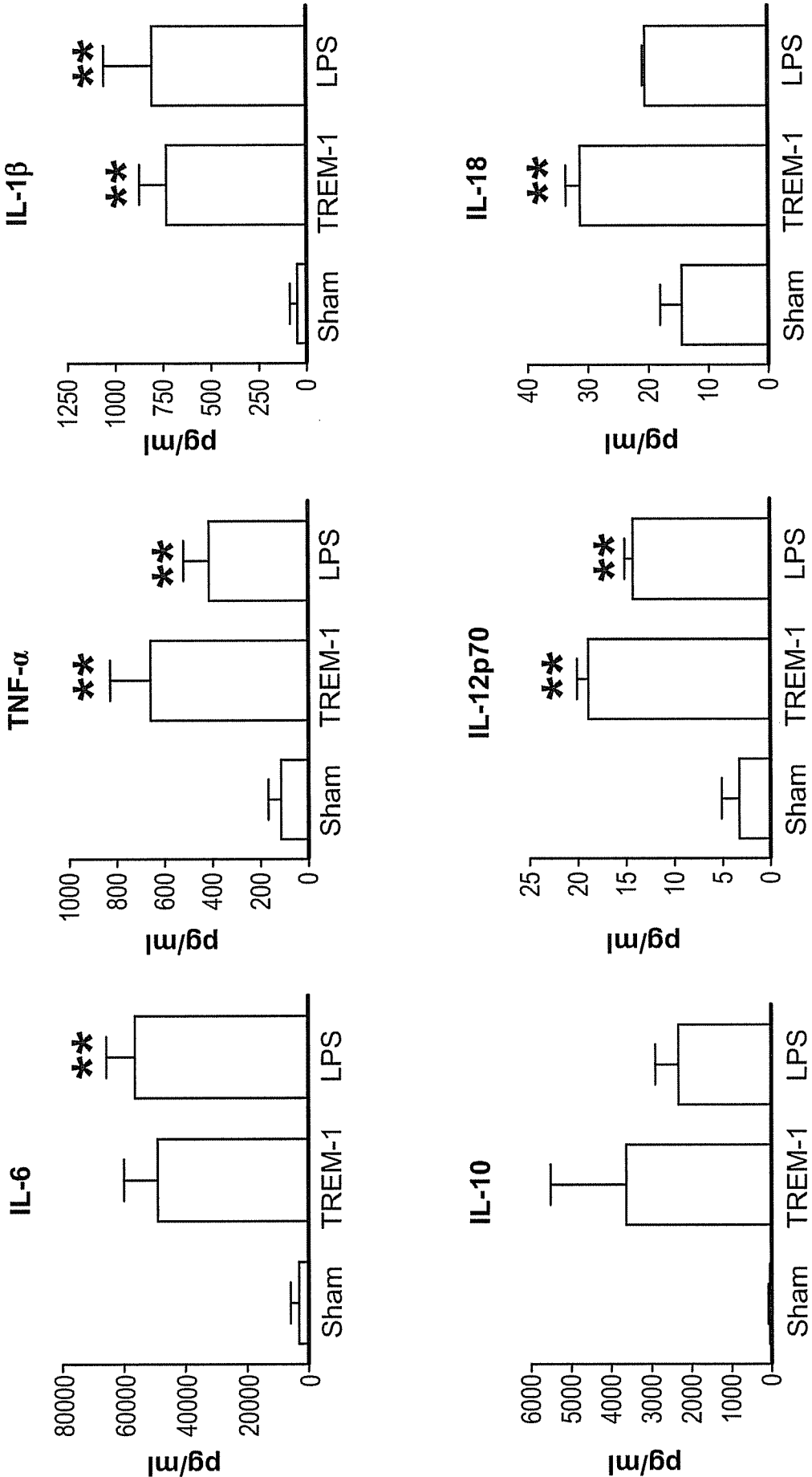


FIG. 16 (continued)

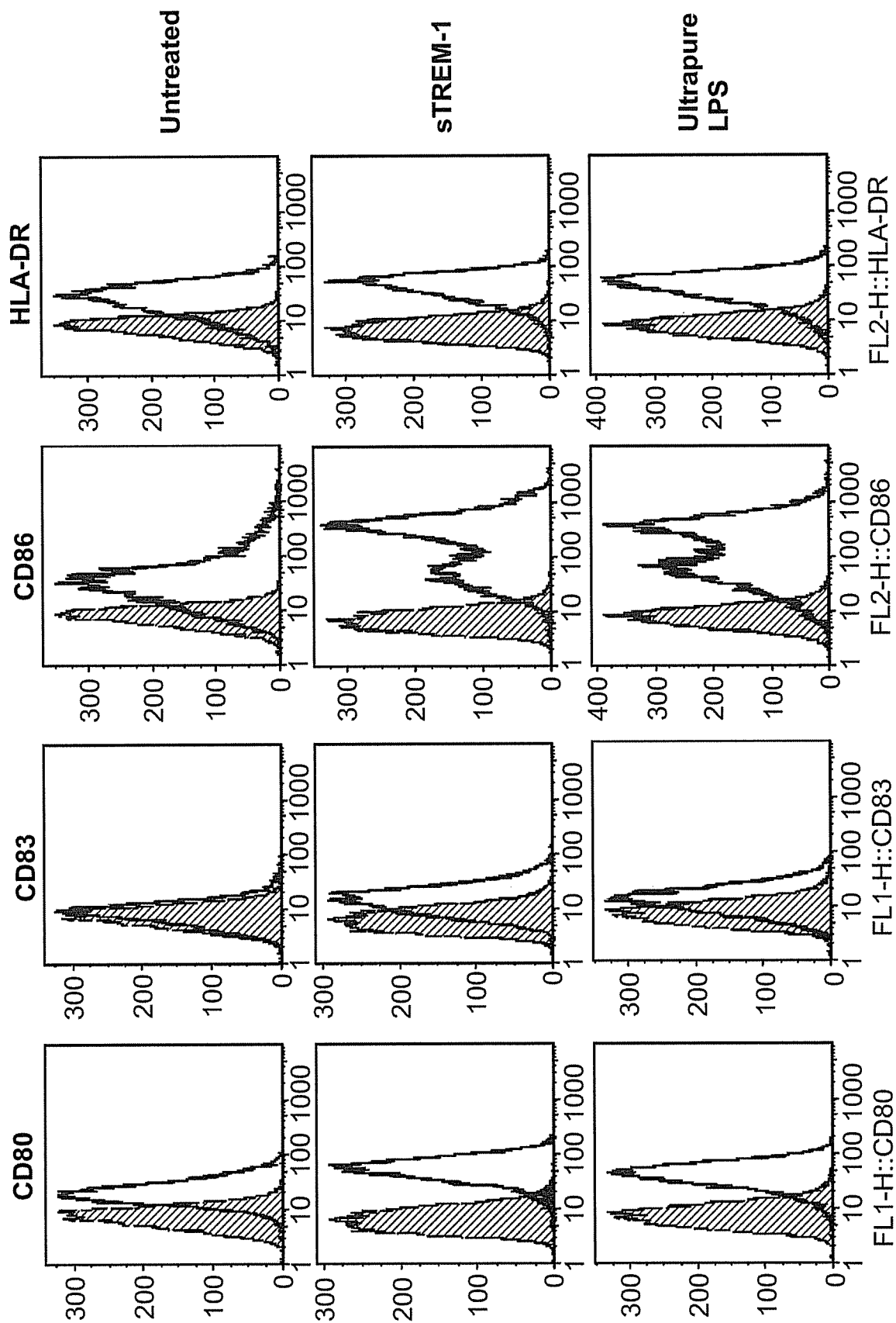


FIG. 16 (continued)

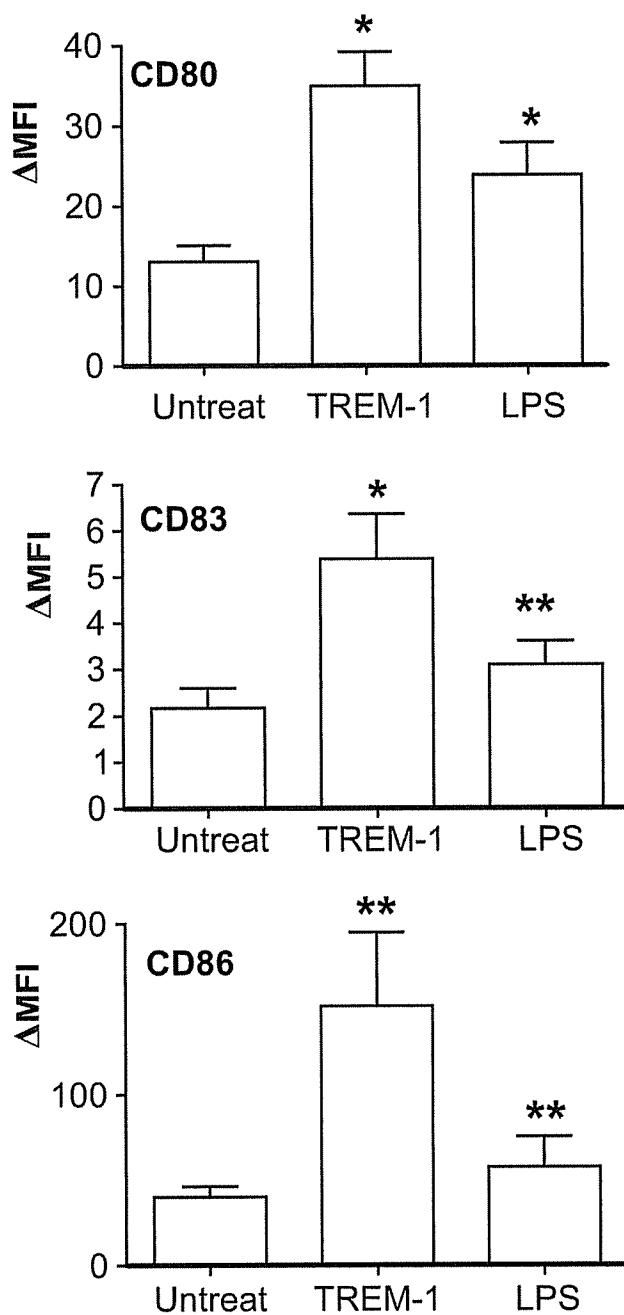
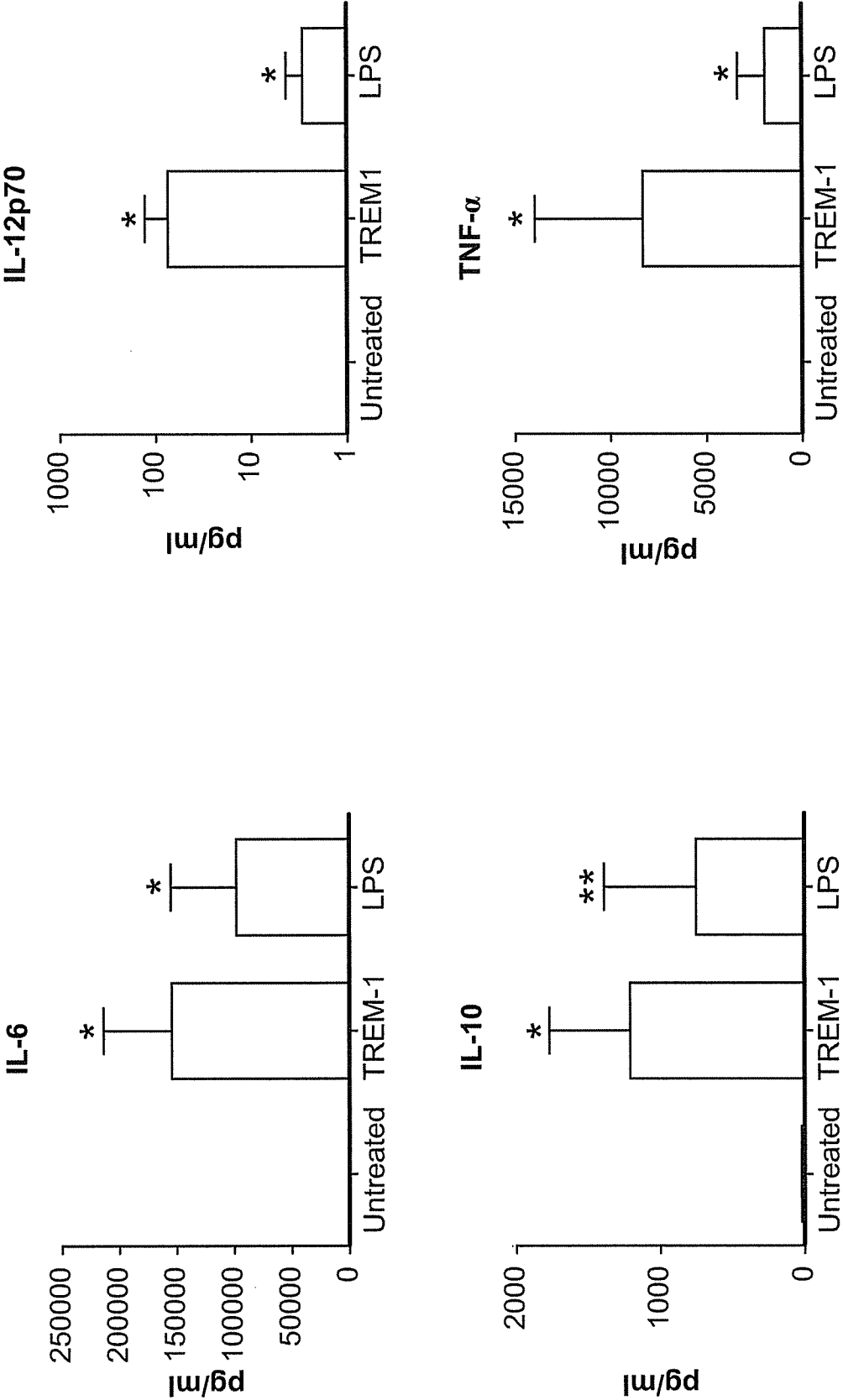


FIG. 16 (continued)



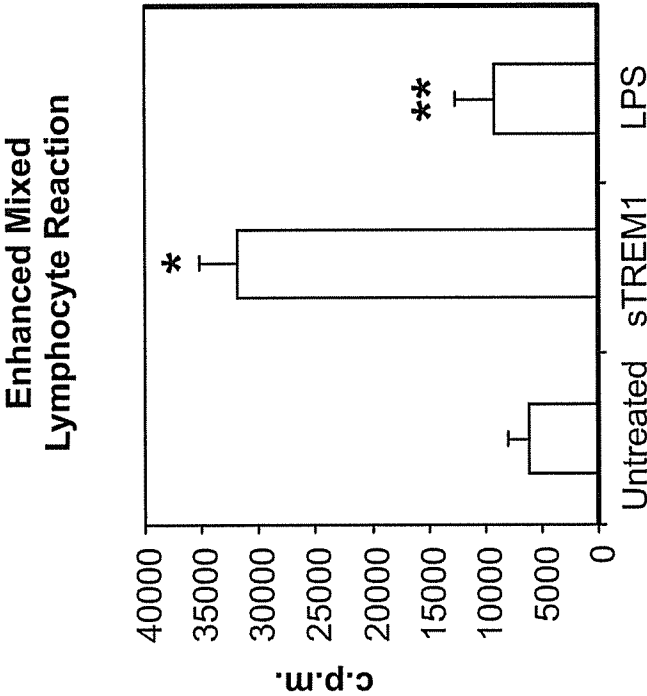


FIG. 17A

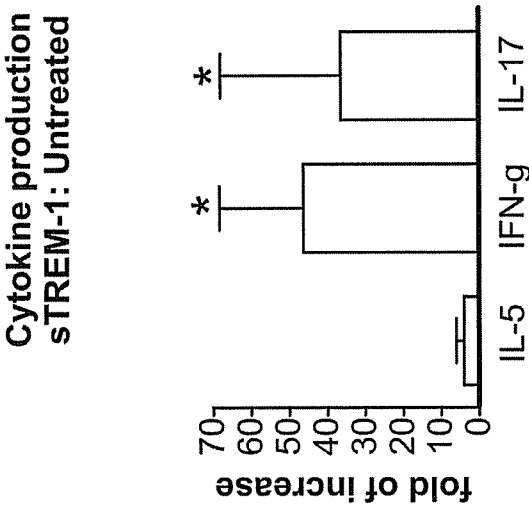


FIG. 17B

FIG. 18

BMDCs from HeN (wt)

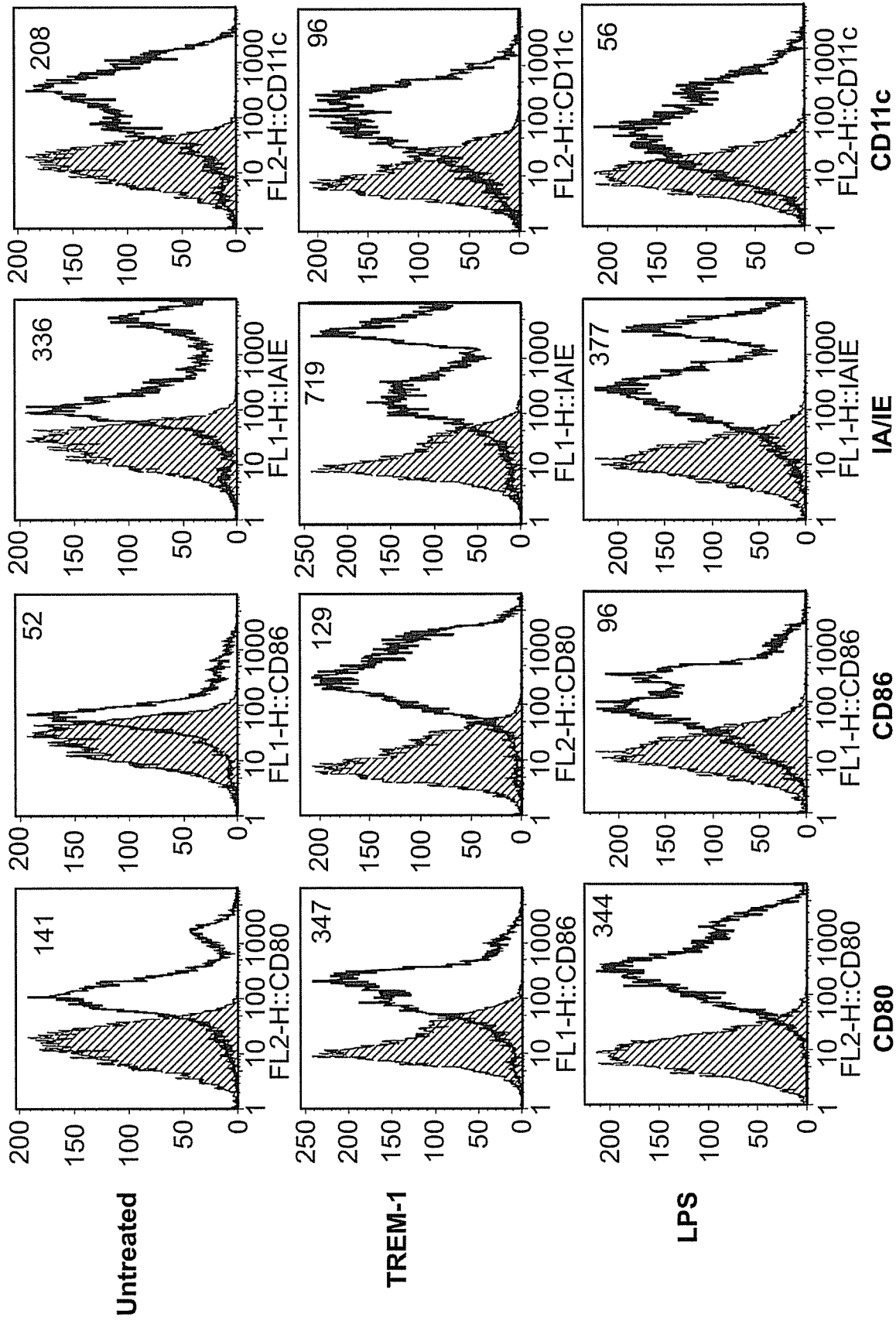


FIG. 18 (continued)

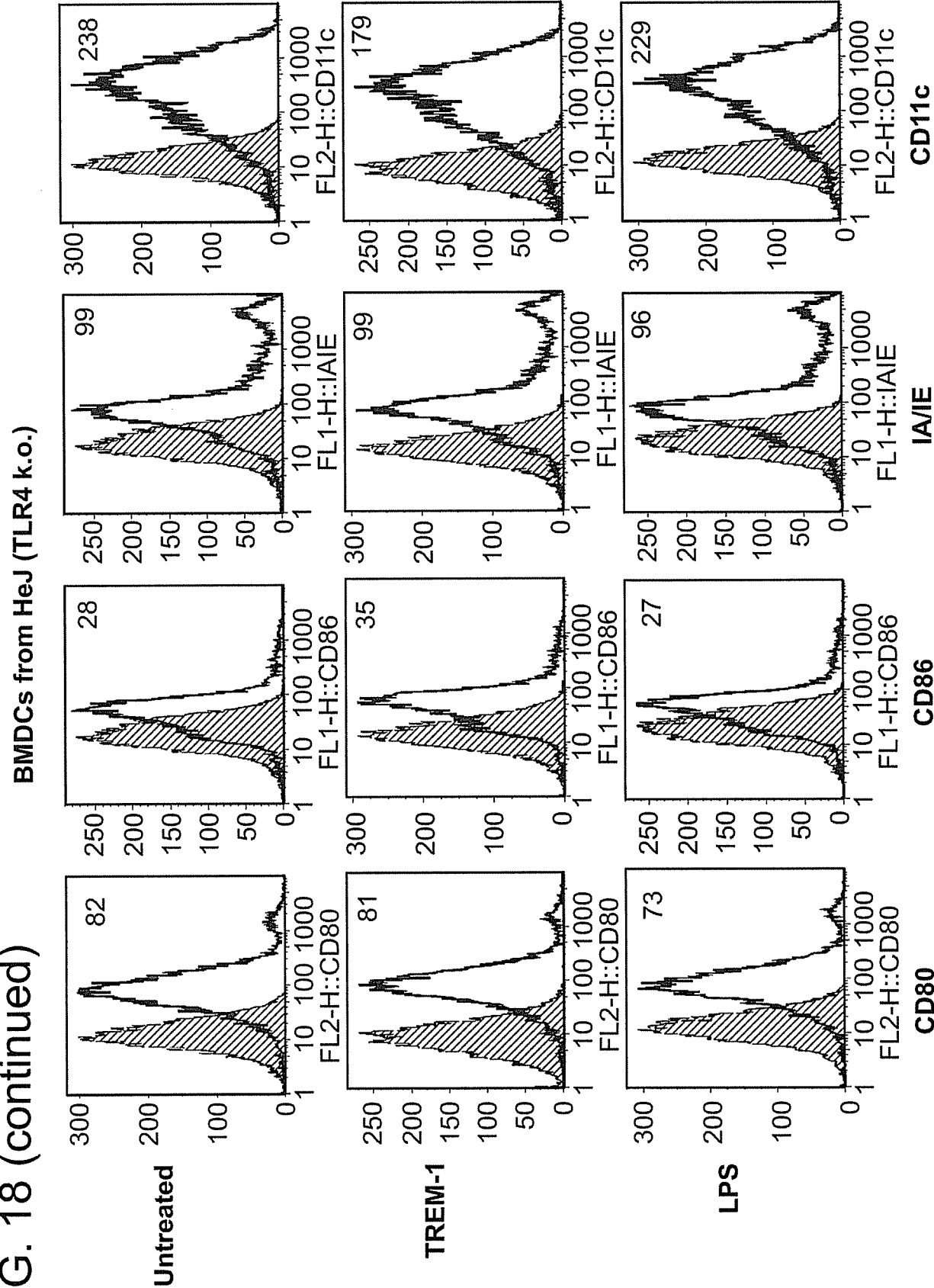


Figure 18 (2 of 2)

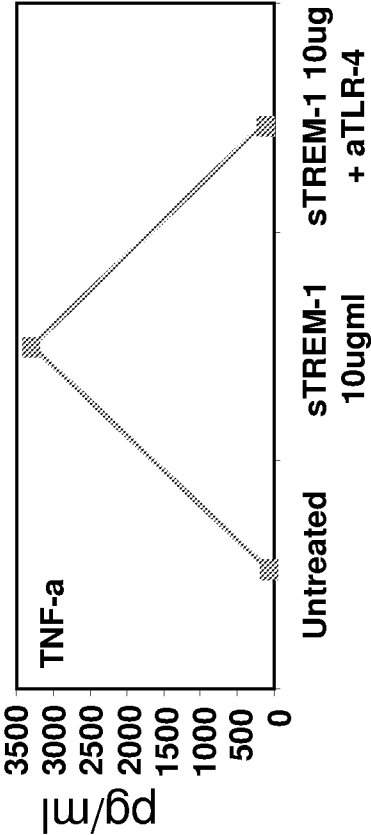


Fig 4.B

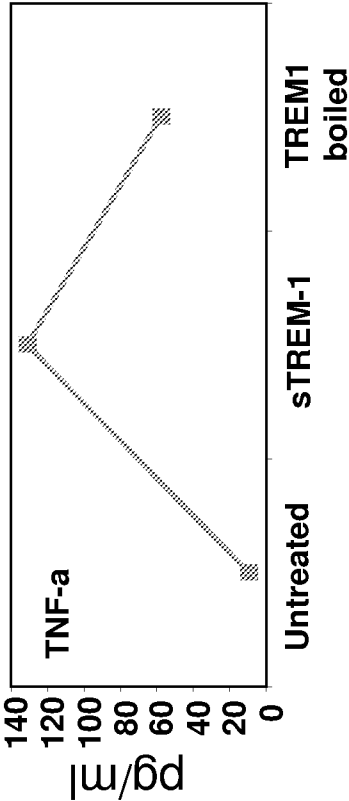


Fig 4.C

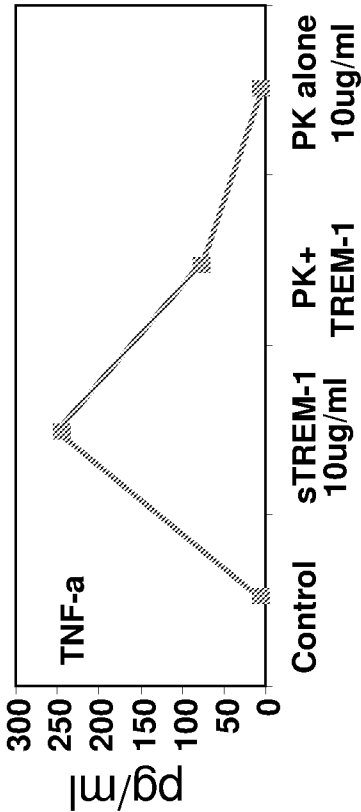


Fig 4.D

<sup>1</sup>MRKAGLWGLLCVFFVSEVKAIVLEEERYDLVEGQTLTVKCPFNIMKYANSQKAWQRLPDGKEPL  
TLVVTQRPFTRPSEVHMGKFTLKHDPSEAMLQVQMTDLQVTDGSLYRCVIYHPPNDPVVLFHPVR  
LVVTKGSSDVFTPIPIRTERPILITTKYSPSDTTTTRSLPKPTAVVSSPGLGVTIINGTDADSVSTSS<sup>2</sup>

FIG. 19A

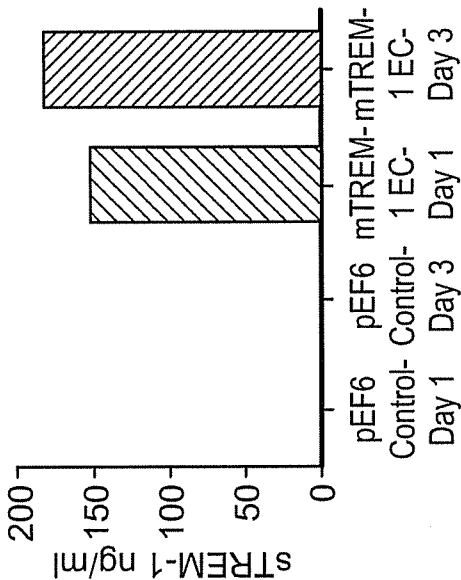


FIG. 19B

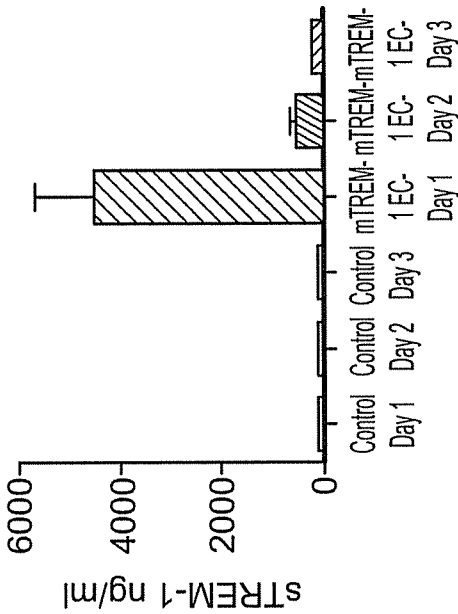


FIG. 19C

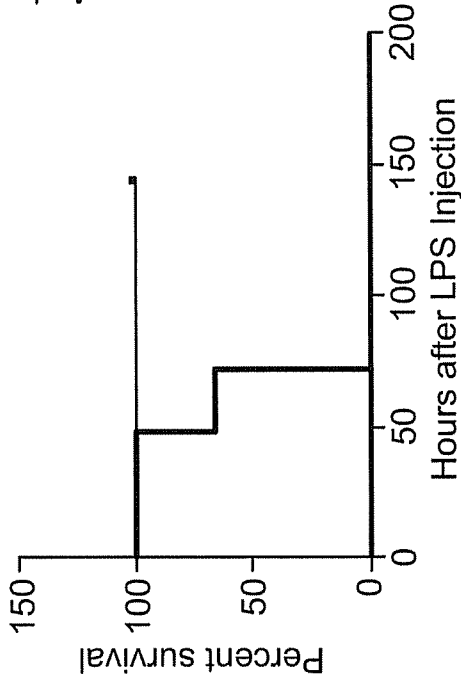


FIG. 19D

**FIG. 20****Human TLT-1 (GenBank AA037827.1)**

MGLTLLLLLL LGLEGQGIVG SLPEVLQAPV GSSILVQCHY RLQDVKAQKV WCRFLPEGCO  
PLVSSAVDRR APAGRRTFLT DLGGGLLQVE MVTLQEEDAG EYGCMVDGAR GPQILHRVSL  
NILPPEEEEE THKIGSLAEN AFSDPAGSAN PLEPSQDEKS IPLIWGAVLL VGLLVAAVVL  
FAVMAKRKQG NRLGVCGRFL SSRVSGMNPS SVVHHVSDSG PAAELPLDVP HIRLDSPPSF  
DNTTYTSLPL DSPSGKPSLP APSSLPLPLP KVLVCSKPVT YATVIFPGGN KGGGTSCGPA  
QNPPNNQTPS SKG

**Mouse TLT-1 (GenBank NP\_082039.1)**

MDCYLLLLLL LLGLAGQGSA DSHPEVLQAP VGSSILVQCH YRLQDVRALK VWCQFLQEGC  
HPLVTSVDR RAPGNRIFL TDLGGGLLQV EMVTLQEEDT GEYGCVVEGA AGPQTLHRVS  
LLVLPPVPGP REGEEAEDEK ETYRIGTGSL LEDPSLDPSA SAGPHEFRRR ENRCQKQCIP  
LIWGAVLLLA LVVVAVVIFA VMARKKGNRL VVCGPSQSTG VPGMDPPSAA HRSSDSGLPS  
DIPHVRLDSP PSFDSIYTGS SLDPPSSEPP APPSQPPLPP KVLMSKSVT YATVVFPGGD  
KGKIASCEPV QDPPNSQTPP SK

**Human TREM-1**

MRKTRLWGLL WMLFVSELRA ATKLTEEKYE LKEGQTLQDVK CDYTLEKFAS SQKAWQIIRD  
GEMPKTLACT ERPSKNSHPV QVGRIILEDY HDHGLLRVRM VNLOVEDSGL YQCVIYQPPK  
EPHMLFDRIR LVVTKGFSGT PGSNENSTQN VYKIPPTTTK ALCPLYTSR TVTQAPPKST  
ADVSTPDSEI NLTNVTDIIR VPVFNIVILL AGGFLSKSLV FSVLFAVTLR SFVP

**Mouse TREM-1**

MRKAGLWGLL CVFFVSEVKA AIVLEEERYD LVEGQTLTVK CPFNIMKYAN SQKAWQRLPD  
GKEPLTLVVT QRPFTRPSEV HMGKFTLKHD PSEAMLQVQM TDLQVTDSSL YRCVIYHPPN  
DPVVLFHPVR LVVTKGSSDV FTPVIIPITR LTERPILITT KYSPSDTTTT RSLPKPTAVV  
SSPGLGVTII NGTDADSVST SSVTISVICG LLSKSLVFII LFIVTKRTFG