Triggering Receptor Expressed on Myeloid cells-1: a new player in platelet aggregation

Lucie Jolly[1]; Jérémie Lemarié[1,2]; Kevin Carrasco[1]; Batric Popovic[1,3]; Marc Derive[4]; Amir Boufenzer[4]; Sébastien Gibot[1,2]

[1]INSERM UMRS-1116, Faculté de Médecine Nancy, Université de Lorraine, Nancy, France ; [2]CHU Nancy, Hôpital Central, Service de Réanimation Médicale, Nancy, France ; [3]CHU Nancy, Hôpital Brabois, Service de Cardiologie, Vandoeuvre-lès-Nancy, France ; [4]INOTREM SA, Nancy, France

Summary

Triggering Receptor Expressed on Myeloid cells-1 (TREM-1) is an immunoreceptor initially known to be expressed on neutrophils and monocytes/macrophages. TREM-1 acts as an amplifier of the inflammatory response during both infectious and aseptic inflammatory diseases. Another member of the TREM family, The Triggering receptor expressed on myeloid cells Like Transcript-1 (TLT-1) is exclusively expressed in platelets and promotes platelet aggregation. As the gene that encodes for TLT-1 is located in the TREM-1 gene cluster, this prompted us to investigate the expression of TREM-1 on platelets. Here we show that TREM-1 is constitutively expressed in α-granules and mobilised at the membrane upon platelet activation. Pharmacologic inhibition of TREM-1 reduces platelet activation as well as platelet aggregation induced by collagen, ADP, and thrombin in human platelets. Aggregation is similarly impaired in platelets from Trem-1$^{-/-}$ mice. *In vivo*, TREM-1 inhibition decreases thrombus formation in a carotid artery model of thrombosis and protects mice during pulmonary embolism without excessive bleeding. These findings suggest that TREM-1 inhibition could be useful adducts in antiplatelet therapies.

Keywords

Platelets, TREM-1

Correspondence to:
Prof. Sébastien Gibot
Service de Réanimation Médicale, Hôpital Central
29 avenue de Lattre de Tassigny
54035 Nancy Cedex, France
Tel.: +33 383852970, Fax: +33 383858511
E-mail: sgibot@yahoo.fr

Received: March 7, 2017
Accepted after minor revision: May 11, 2017
Epub ahead of print: July 27, 2017

https://doi.org/10.1160/TH17-03-0156
Thromb Haemost 2017; 117: 1772–1781

Supplementary Material to this article is available online at www.thrombosis-online.com.

Introduction

The main role of blood platelets is to ensure vascular integrity and haemostasis in case of vascular damage. Platelet functions involved in these physiological processes are also at work in arterial thrombosis, which is a dramatic complication of atherosclerosis that may lead to vascular occlusion. Despite the established benefits of currently approved antiplatelet drugs, a large number of patients continue to display adverse thrombotic events, highlighting the need for targeting other platelets activation pathways (1–3).

Over the past years, it has become clear that platelets are important, not only in haemostasis and thrombosis but also in inflammation and in distinct aspects of atherosclerosis (4). Platelets are able to interact with a large variety of cell types, such as leukocytes (5), endothelial cells (6, 7), and smooth muscle cells (SMCs) (8), and these interactions have been implicated in the pathophysiology of vascular inflammation. The question remains, however, to which extent these proinflammatory and prothrombotic functions can be separated and targeted (9).

The Triggering Receptor Expressed on Myeloid cells-1 (TREM-1) is an immune-receptor expressed by human and murine neutrophils, mature monocytes and macrophages that acts as an amplifier of the innate immune response (10, 11). Activation of TREM-1 signalling is initiated upon binding of its ligand, which triggers the association and phosphorylation of an immunoreceptor tyrosine-based activation motif (ITAM) of the adaptor protein DAP12 (12). TREM-1 engagement phosphorylates spleen tyrosine kinase (Syk) and leads to NF-κB activation, expression of inflammatory genes in a cell-specific manner, calcium influx (13), neutrophil degranulation and production of reactive oxygen species in addition to cytokines and chemokines (14, 15).

Pharmacological inhibition of TREM-1 by the use of synthetic peptides (such as LR12) or fusion protein repeatedly prevented hyper-responsiveness and death in various models of severe infections (16–20) or inflammation, including myocardial infarction (21, 22) and atherosclerosis (23, 24). LR12 is a 12-aa peptide that mimics a well conserved extra-cellular domain of TREM-1 involved in the binding of its putative ligand. LR12 thus acts as a decoy receptor for the TREM-1 ligand and modulates its activation (19, 21–24).

In this study, we examined the role of TREM-1 in platelets. We first showed that TREM-1 is constitutively expressed by platelets. Then we found that *Trem-1* genetic invalidation or pharmacological inhibition with LR12 decreased platelet secretion, spreading and aggregation. This reduction of platelet activation proved to be protective during arterial thrombosis and pulmonary embolism in mice. These findings provide new insights on the TREM-1 biology, with a potential impact on the pathophysiology and treatment of thrombotic disorders.

Methods

All methods were performed in accordance with the relevant guidelines and regulations and approved by the French Ministry of Research (under the no. 66).

Animals

Trem-1$^{-/-}$ adult male C57BL/6 (6–8 weeks) and wild-type (WT) littermates were used in the experiments. Trem-1$^{-/-}$ mice have recently been described in detail by Weber et al 18). Experiments were approved by our institutional Animal Care and Use Committee.

LR12 peptide

LR12 peptide (LQEEDAGEYGCM) or a scramble inactive peptide (EDGQYECLMEGA) were chemically synthesised (Pepscan Presto BV, Lelystad, The Netherlands) as a COOH terminally amidated peptides. The correct peptides were obtained with >99% yields and were homogeneous after preparative purification, as confirmed by mass spectrometry and analytic reversed-phase high-performance liquid chromatography. These peptides were free of endotoxin.

Preparation of mouse and human washed platelets

Mice were anesthetised with 2% isoflurane in 0.5 l/minute (min) oxygen, and whole blood was collected by cardiac puncture into syringes containing one-sixth the volume of an acid citrate dextrose. For human, after informed consent fresh blood was taken from free of medication healthy volunteers with citrate like anticoagulant. Platelet-rich plasma was obtained by centrifuging whole blood 190g for 4 min followed by 60 seconds at 1900g at room temperature (RT). Platelets were obtained from platelet-rich plasma by centrifugation (4 min at 6000g) and resuspended at a final concentration of 3×10^8/ml in Tyrode-BSA buffer (Sigma-Aldrich, Saint-Quentin Fallavier, France). Purity was constantly >99.99%.

Platelet aggregation assay

Platelet aggregation was monitored by measuring light transmission through the stirred suspension of washed platelets (3×10^8/ml) at 37°C using a TA-8V aggregometer (SD Innovation, Frouard, France). When required, platelets were first incubated with LR12 (50 µg/ml) for 5 min at 37°C. Platelet aggregation was triggered by adding 5 µg/ml collagen (SD Innovation), 5 µM ADP (SD Innovation) and 0.1 or 1 U/ml thrombin (HYPHEN Biomed, Neuville sur Oise, France). Aggregation was monitored for 10 min and results are expressed as the percentage change in light transmission with respect to the blank (buffer without platelets), set at 100%.

Platelet adhesion under flow

Vena8Fluoro+ Biochips (Cellix, Dublin, Ireland) were coated with fibrinogen (125 µg/ml) at 4°C overnight. On the next day, the chips were blocked with BSA (10 mg/ml) for 30 min at RT and then washed once more with PBS containing 0.9 mM $Ca2^+$ and 0.5 mM Mg^{2+}. Washed platelets were pretreated with vehicle or LR12 (20 µg/ml or 50 µg/ml). ADP (10 µM) was added directly prior to perfusion start. Platelets were then mixed with 1 mM $CaCl_2$ and immediately perfused over the fibrinogen-coated channels at constant shear stress of 2.5 dynes·cm^{-2} for 6 min using the Mirus nanopump (Cellix). Platelet adhesion was recorded on a ZEISS Axiovert 40 CFL fluorescence microscope, using a Q-IMAGING ROLERA-XR digital camera. Cell images of three microscopic fields from each channel were captured, and images were analysed using DucoCell software (Cellix).

In vitro thrombogenesis

Vena8Fluoro+ Biochips were coated with collagen, blocked and washed as described earlier. Whole blood collected in 3.8% sodium citrate was incubated with 3,3'-dihexyloxacarbocyanine iodide (1 µM) in the dark for 10 min. LR12 (20 µg/ml or 50 µg/ml) or PGE2 (10 µM) were added 5 min before the perfusion was started. Perfusion was performed at a shear rate of 30 dynes·cm^{-2}, and microscopy was carried out as described earlier for platelet adhesion.

Platelet dense granule secretion

Dense granule secretion was quantified by measuring adenosine triphosphate (ATP) release after platelet aggregation with the ATP Determination Kit (Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions.

Measurement of TXA_2 and cAMP generation

When required, LR12 (50 µg/ml) was pre-incubated for 5 min at 37°C. TXA_2, and cAMP generation were determined in conditions of platelet aggregation induced by 10 nM convulxin (Pentapharm, Basel, Switzerland) or 0.1 U/ml thrombin. Platelet aggregation was stopped after 5 min of stimulation and supernatant was obtained by centrifuging sample for 4 min at 6000g at RT. Levels of TXB2, the stable metabolite of TXA2, were determined with the Thromboxane B2 assay Kit following the manufacturer's instructions (R&D Systems, Abingdon, UK). Accumulation of cAMP was determined in platelets using cAMP Parameter assay Kit (R&D Systems), according to the manufacturer's instruction.

Measurement of intracellular free calcium concentration

Human platelets (2×10^8 platelets/ml) were loaded with a probe of the acetoxymethyl ester of the fluorescent Ca^{2+} indicator Fluo-3 (1 µM, Sigma) and pluronic acid (1 µM) for 30 min at RT. After pretreatment of platelets with LR12 (50 µg/ml), Ca^{2+} mobilisation induced by agonists (collagen 25 µg/ml, TRAP 25 µM, ADP 5 µM) was analysed in Tyrode-BSA buffer using an Accuri C6 (Becton Dickinson, Franklin Lakes, NJ, USA) flow cytometer. Individual fluorescence values were then analysed with FlowJo and Graphpad

Prism to normalize the fluorescence with the first value according to the equation (F/F0)–1, where 'F' is the fluorescence at specific time point and 'F0' is the fluorescence at time 0.

Immunoblotting

Platelets were lysed in RIPA buffer (Sigma Aldrich), kept for 5 min on ice and centrifuged for 10 min at 8000g at 4°C to collect the supernatant. Protein concentration was determined (BCA Protein Assay Kit, Pierce; ThermoScientific). Thirty micrograms of each sample were migrated on a Criterion XT Bis-Tris Gel 4–12% (Bio-Rad, Hercules, CA, USA) and transferred to a polyvinylidene difluoride membrane (Millipore, Saint-Quentin en Yvelines, France). The membrane was blocked with 5% w/v skim milk powder in TBST (0.1 M Tris-HCl pH 8,1.5 M NaCl and 1% Tween-20) for 2 hours (h) at RT, and subsequently incubated with anti-(p)ERK1/2, anti-(p)PLCγ2, anti-tubulin (Cell Signaling) or anti-TREM-1 (AbD Serotec, or R&D Systems) antibodies overnight at 4°C. After vigorous washing in TBST, the membrane was incubated with secondary antibody conjugated to horseradish peroxidase for 1 h at RT. Immunocomplexes were detected with the SuperSignal West Femto Substrate (Pierce; ThermoScientific). Acquisition and quantitative signal density analyses were performed by a LAS-4000 imager (FSVT) and Multi-Gauge software (LifeScience Fujifilm, Tokyo, Japan).

Real-time PCR

Total RNAs were extracted from platelets using RNeasy Plus Mini Kit (Qiagen, Venlo, The Netherlands) and quantified with NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) before being retrotranscripted using the iScript cDNA synthesis kit (Bio-Rad) and quantified by quantitative polymerase chain reaction (PCR) using Qiagen available probes (Quantitect Primers) for human Trem-1, Dap12, and ActB. ActB serves as housekeeping gene.

Flow cytometry analysis

Washed platelets (10^8/ml) were stimulated with ADP 5 µM or TRAP 25 µM. After incubation for 5 min at 37°C without stirring, platelets were incubated with fluorescein isothiocyanate (FITC)-fibrinogen, phycoerythrin (PE)-labeled anti-human PAC1, allophycocyanin (APC)-labeled anti-human CD62P (P-selectin) mAb (Miltenyi Biotech) and directly analysed on an Accuri C6 flow cytometer (Becton Dickinson).

For TREM-1 staining, platelets were fixed with 4% (wt/vol) paraformaldehyde (PFA) and permeabilised or not with 0.5% wt/vol Triton X-100. After washing, they were resuspended in Tyrode-BSA buffer and incubated for 15 min at 4°C in the dark with FITC-labeled anti-human TREM-1 mAb (BIOSS). Isotype controls were used for all antibodies (Miltenyi Biotech and BIOSS).

Confocal microscopy

Sterile glass coverslips were coated with 50 µg/ml human fibrinogen for 24 h at 4°C. Washed platelets were placed onto fibrinogen-coated coverslips for 1 h at 37°C. Platelets were fixed with 4% paraformaldehyde for 15 min, washed with PBS, permeabilised with 0.2% triton X-100, and blocked 2 h with 2% BSA. Cells were then incubated with the indicated primary antibodies (CD62p, DAP12, GPIIb, TLT-1, TREM-1) (BIOSS, MA, USA) overnight at 4°C. After washing with PBS coverslips were mounted on Vectashield (Vector Laboratories, Burlingame, CA, USA) and examined with a TCS SP5 X confocal microscope (Leica, Wetzlar, Germany), and images were processed using LAS AF Lite blue software (Leica). Alternatively, in order to prevent platelets activation, fixation, permeabilisation and blockade were performed before adding platelets to untreated coverslips. Co-localisation scores were calculated by ImageJ software based on 3 independent experiments. Human coronary artery thrombi were obtained using a thrombectomy catheter at the time of percutaneous coronary intervention for ST-segment elevation myocardial infarction. Thrombus fragments were placed in formalin, embedded in paraffin, sectioned (5 µm), and stained as above.

Platelets adhesion under static conditions

Glass coverslips were coated with fibrinogen (40 µg/ml) or bovine type I collagen (100 µg/ml) overnight, and then blocked with 2% BSA. Washed human platelets in Tyrode's buffer (3×10^7 platelets/ml) preincubated with LR12 for 5 min at 37°C were allowed to spread on the fibrinogen/collagen-coated surfaces. After 1 h, non-adherent platelets were discarded, and surface-bound platelets were washed with phosphate buffer saline (PBS). Coverslips were fixed in 4% paraformaldehyde, and labelled with CytoPainter Phalloidin iFluor 555 (Abcam, Cambridge, UK) for 1 h in the dark. Then platelets were visualised by TCS SP5 X confocal microscope (Leica), and images were processed using LAS AF Lite blue software (Leica).

$FeCl_3$-induced carotid artery thrombosis model

Mice were anesthetised with 2% isoflurane in 0.5 l/min oxygen and body temperature was kept constant using a heating plate. The right common carotid artery was isolated through a midline cervical incision and blood flow was monitored with an ultrasound transit-time flow probe (0.5 PSB Nanoprobe; Transonic, Ithaca, NY, USA). Mice were randomly assigned to receive i. p. injection of 100 µl PBS or 100 µl (=100 µg) LR12 just before thrombus formation was induced by applying a 1 mm^2 piece of filter paper soaked in 10% aqueous $FeCl_3$ solution. After 2 min, filter paper was removed and the vessel was washed with warm PBS. Time to complete carotid occlusion was monitored.

Pulmonary thromboembolism model

Collagen-induced pulmonary thromboembolism was carried out as follows. Mice were challenged with 100 µl of a mixture containing 100 µg/ml of collagen and 15 µg/ml of epinephrine (both from ABP, Epsom, UK) in PBS injected into right jugular vein, 2 min after IV injection of 100 µl of 1:1 LR12 solution or PBS. Except for

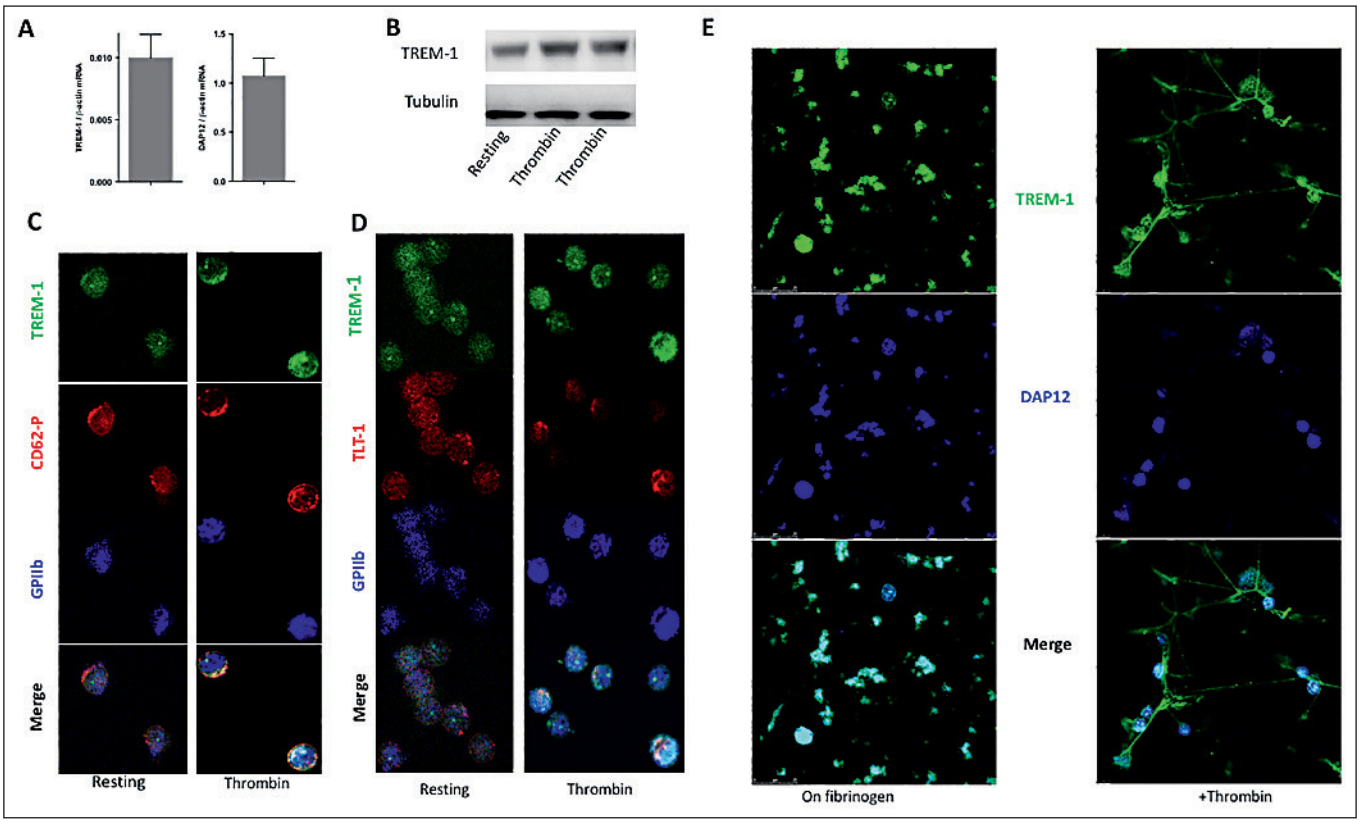

Figure 1: TREM-1 is expressed in platelets. A) Quantification of Trem-1 and Dap12 gene expression in human platelets by qRT-PCR; n=4–5 per condition; * P<0.05. B) Analysis of TREM-1 (anti-TREM-1 Ab Serotec) and tubulin (Cell Signalling) expression in resting or thrombin (1 U/ml, 5 min) activated-platelets by immunoblotting. C) Confocal fluorescent images of resting platelets (left panels) or thrombin (1 U/ml, 5 min) activated platelets (right panels). Platelets were stained with anti-human TREM-1 (green), CD62-P (red) and GPIIb (blue) mAb; or D) TREM-1 (green), TLT-1 (red) and GPIIb (blue), Scale bars: 10 µm; E) Confocal fluorescent images of fibrinogen-adherent platelets without (right panels) or with thrombin (1 U/ml, 5 min) (right panels). Platelets were stained with anti-humanTREM-1 (green) and DAP-12 (blue) mAb, Scale bar=25 µm.

survival analysis, mice were killed 3 min after collagen-epinephrine injection and blood and lungs were collected for platelet count and histological studies. Right-lower lobe was fixed in 4% paraformaldehyde and then paraffin-embedded. Lung sections stained with hematoxylin and eosin were digitally imaged. Five random X 20 fields were photographed for each specimen and thrombus number for each mouse was analysed. Lung single-cell suspension was obtained by manual grinding followed by a 45-min enzymatic digestion (2 mg of collagenase type-I and 30 µg of DNAse per ml of DMEM with 10% FCS, all from Sigma-Aldrich) before incubation in a cocktail of mAbs against CD45, CD11b, Ly6G, Ly6C and TREM-1 (all antibodies from Miltenyi Biotech). FACS data were acquired on Gallios (Beckman Coulter, Indianapolis, IN, USA) and expressed as total cell number per lobe.

Statistical analysis

All of the reported figures are representative of at least three different experiments and then are presented as mean ± SD. Statistical analysis was performed using Prism Version 6 software (GraphPad) and the data were compared by unpaired t-test. A p-value <0.05 was deemed significant.

Results

Trem-1 is constitutively expressed in platelets

The Triggering receptor expressed on myeloid cells Like Transcript-1 (TLT-1) (26) is a membrane-bound protein, abundant in the α-granules of resting platelets and at the surface of activated platelets (27). The gene that encodes for TLT-1 is located in the TREM gene cluster. This prompted us to investigate the expression of TREM-1 on human platelets.

To verify the presence of TREM-1 in platelets its gene expression was evaluated (▶Figure 1A). TREM-1 signalling requires its association with the adaptor protein DAP12. We also observed the presence of DAP12 mRNA in platelets (▶Figure 1A). The presence of the TREM-1 protein was confirmed by Western blot using 2 different antibodies (▶Figure 1B, Suppl. Figure 1, available online at www.thrombosis-online.com). Flow cytometry analysis revealed that TREM-1 was detectable at low level in resting non-permeabilised platelets, while staining was evident after activation and permeabilisation (Suppl. Figure 2, available online at www.thrombosis-online.com).

To get insights on the localisation of TREM-1 in platelets, we stained these cells for TREM-1, CD62p and GPIIb. In resting and

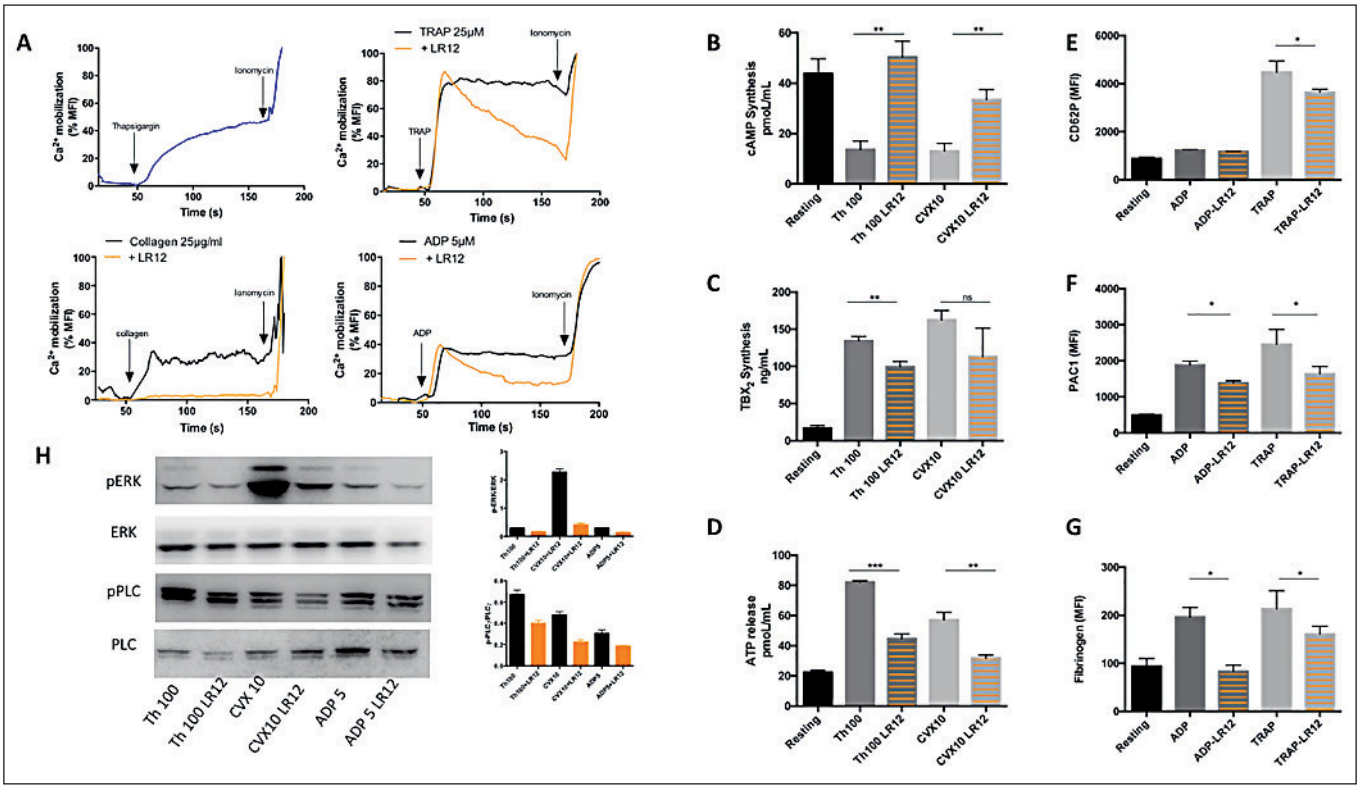

Figure 2: Effect of LR12 on Ca^{2+} mobilisation, cAMP and thromboxane B2 synthesis, platelet secretion and integrin αIIbβ3 inside-out activation. A) Effect of LR12 (50 µg/ml) on intracellular Ca^{2+} mobilisation in human platelets activated by TRAP (25 µM), collagen (25 µg/ml) and ADP (5 µM). Results are representative of three experiments and expressed as percentage of MFI normalised by difference between baseline and ionomycin (100 %). Accumulation of cAMP (B) and Thromboxane B2 (TBX2) (C) was determined in resting and thrombin (Th, 100 mU/ml) or convulxin (CVX, 10 nM) activated platelets (5 min) in the presence of LR12 (50 µg/ml) or control peptide (LR12 scramble, 50 µg/ml). Results represent the mean ± SD of at least three separate experiments, each performed in triplicate. Statistical significance was determined by unpaired Student t-test (*P<0.05; **P<0.01). C) Effect of LR12 (50 µg/ml) on dense granule secretion assessed by measuring ATP release induced by thrombin (Th, 100 mU/ml) or convulxin (CVX, 10 nM). Results were expressed as mean ± SD of at least three separate experiments, each performed in triplicate, and statistical significance was determined by unpaired Student t-test (**P<0.01; ***P<0.005). Flow cytometric analysis of APC-labelled CD62-P exposure (E), PE-labelled PAC-1 binding (F) or FITC-labelled fibrinogen binding (G) to washed human platelets treated with LR12 (50 µg/ml) or control peptide, stimulated with ADP (5 µM) or TRAP (50 µM). Data are expressed as mean ± SD of eight different donors. Statistical significance was determined by unpaired Student t-test (*P<0.05). H) Western blot of (p)ERK) and (p)PLCγ2) in resting and thrombin (Th, 100 mU/ml), convulxin (CVX, 1 nM), or ADP (ADP, 5 µM) activated platelets (5 min) in the presence of LR12 (50 µg/ml) or control peptide. Results are representative of three experiments.

thrombin activated platelets, TREM-1 seems to co-localise with CD62p and DAP12 but not GPIIb (colocalisation scores 0.619, 0.708, and 0.102, respectively) (▶Figure 1C, Suppl. Figure 3, available online at www.thrombosis-online.com). Upon platelet activation TREM-1 clusters at the membrane (▶Figure 1C). Interestingly, although TREM-1 and TLT-1 appear to co-localise in resting platelets, this is no more observed in activated cells (colocalisation score 0.171) (▶Figure 1D).

In fibrinogen-adherent platelets, TREM-1 and DAP12 are largely co-localised. Once activated with thrombin, TREM-1 clusters and platelets generates in-between strands containing large amounts of TREM-1, while DAP12 expression remains almost unchanged (▶Figure 1E, Suppl. Figure 4, available online at www.thrombosis-online.com).

Therefore, TREM-1 seems constitutively expressed in α-granules and clusters at the membrane upon platelet activation.

Coronary thrombi obtained from acute myocardial infarction patients also displayed an intense TREM-1 staining (Suppl. Figure 5, available online at www.thrombosis-online.com).

TREM-1 modulation reduces platelet secretion

Elevation in cellular calcium concentration is an immediate consequence of platelets stimulation and is a crucial event in their activation. To inhibit TREM-1, we used the synthetic peptide LR12 (19), the scramble LR12 peptide served as control. Pre-incubation of platelets with LR12 strongly reduced TRAP-, ADP- and collagen-induced calcium rising (▶Figure 2A). In addition to intracellular calcium mobilisation, platelet activation involves other signalling mechanisms, including cAMP, and TXA2 synthesis. We therefore assessed the effect of LR12 on these signalling pathways in resting and thrombin or convulxin activated platelets. Although

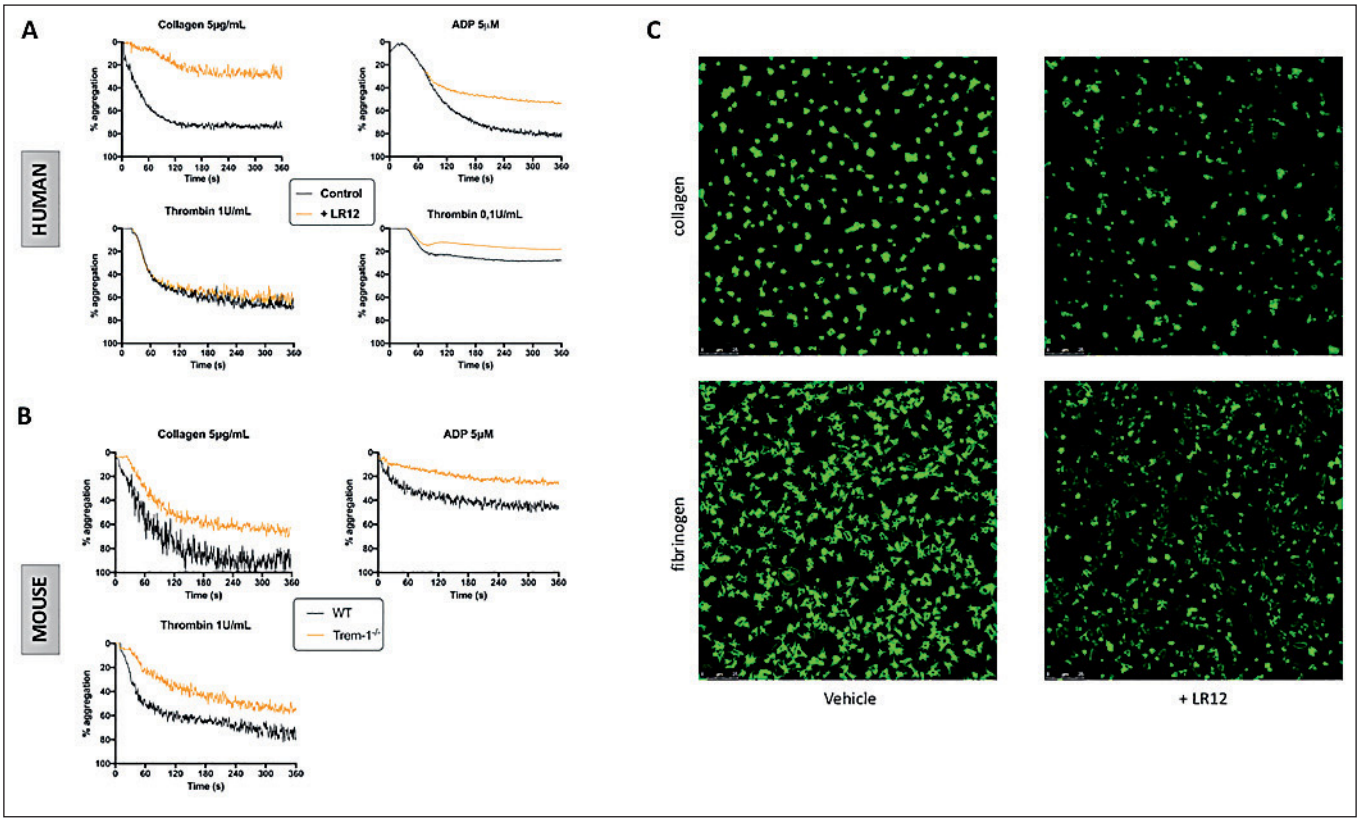

Figure 3: Effect of LR12 on platelet aggregation and adhesion. A) Aggregation of washed human platelets induced by indicated concentrations of collagen, thrombin or ADP with or without incubation with LR12 (50 µg/ml). Experiments were performed on 6–8 healthy blood donors. B) Aggregation of washed platelets from WT and Trem-1$^{-/-}$ mice induced by indicated concentrations of collagen, thrombin or ADP. Traces are representative of 5–6 animals per condition. Results are expressed as the percentage change in light transmission with respect to the blank (buffer without platelets), set at 100 %. C) Washed human platelets were preincubated with or without LR12 (50 µg/ml) for 5 min at 37 °C. Platelets were allowed to spread on fibrinogen or collagen-coated glasses for 1 h at 37 °C. Then platelets were fixed, labelled with phalloidin and photographed under a fluorescence microscope. Representative images from three different blood donors with similar results.

thrombin and convulxin inhibited cAMP accumulation in platelets, this phenomenon was prevented by LR12 (▶Figure 2B). As shown in ▶Figure 2C, thrombin-induced TXB2 (the stable metabolite of TxA2) release by platelets was reduced by LR12 and a trend was observed with convulxin. LR12 also dampened thromboxane production triggered by arachidonic acid (Suppl. Figure 6, available online at www.thrombosis-online.com). We next assessed the effect of TREM-1 inhibition on the secretion of dense granules by measuring the release of ATP upon stimulation by thrombin and convulxin. The presence of LR12 decreased ATP release after platelet activation by thrombin and convulxin (▶Figure 2D).

P-selectin (CD62P) is a major α-granule protein that is highly expressed on the platelet surface during activation. To examine the effects of LR12 on platelet activation, P-selectin expression was monitored by flow cytometry. Results showed that LR12 reduced TRAP-induced P-selectin expression (▶Figure 2E).

We next analysed whether the reduced platelet secretion observed in the presence of LR12 was associated with an impaired activation of integrin αIIbβ3 by measuring binding of the conformational dependent antibody PAC-1. As illustrated in ▶Figure 2F, TRAP and ADP induced PAC-1 binding was decreased by TREM-1.

Platelet inside-out signalling to integrin αIIbβ3 plays an essential role in the modulation of its conformation to increase affinity for plasma fibrinogen and von Willebrand factor (vWF) binding and subsequent platelet aggregation (24). As a consequence of a reduction of αIIbβ3 activation, LR12 decreased the binding of fibrinogen to activated platelets (▶Figure 2G).

TREM-1 inhibition was also associated with a reduction of thrombin, convulxin and ADP ERK1/2 and PLCγ2 phosphorylation (▶Figure 2H).

TREM-1 modulation therefore reduces platelet activation and secretion.

TREM-1 modulation impairs platelet aggregation and adhesion

To investigate the role of TREM-1 in the regulation of platelet activation, we evaluated the effect of LR12 on platelet aggregation in response to various platelet agonists, including collagen, ADP, and thrombin. Platelet aggregation induced by collagen, ADP, and low

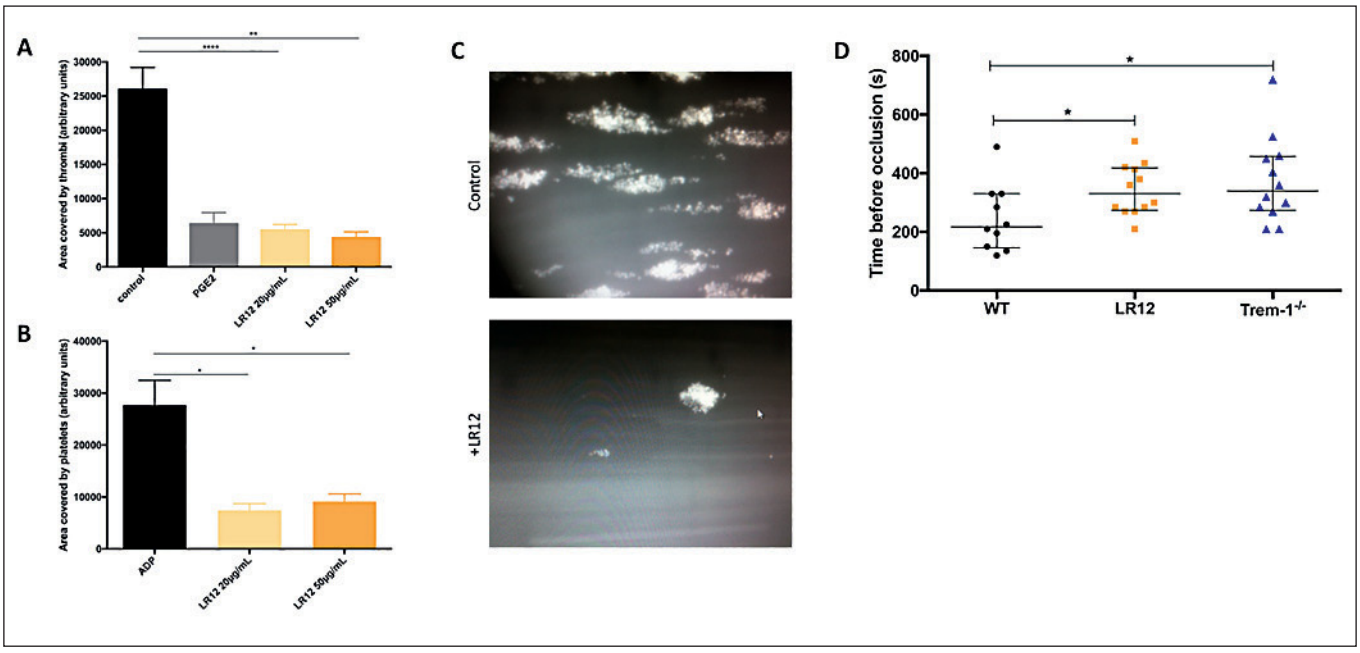

Figure 4: TREM-1 inhibition prevents thrombosis *in vitro* and *in vivo*. Effect of LR12 (20–50 µg/ml) pretreatment on whole blood (A) or washed platelets (B) perfusion into laminar flow chambers coated, respectively, with collagen and fibrinogen under a flow of 30 and 2.5 dynes.cm^{-2}, respectively. PGE2 (10 µM) was used as control of inhibition of thrombus formation. During perfusion, the formation of thrombi was observed under an epifluorescence microscope. The area covered by thrombi (A) or platelets (B) were calculated in three independent experiments. Results are mean ± SD. Statistical significance was determined by unpaired Student t-test (*P<0.05; **P<0.01; *** P<0.005). C) Representative image of thrombi formation in the capillary in absence or presence of LR12 (50 µg/ml). D) Carotid artery occlusion times induced by $FeCl_3$ in WT, WT treated by i.p. LR12 (100 µg), and Trem-1$^{-/-}$ mice. Results are mean ± SD (n=10–12 mice per group). Statistical significance was determined by unpaired Student t-test (*P<0.05).

dose of thrombin was impaired by LR12 in humans. The inhibitory effect of LR12 disappeared in the presence of highest concentration of thrombin (►Figure 3A) or lower doses of peptide (Suppl. Figure 7, available online at www.thrombosis-online.com). The scramble LR12 peptide showed no effect on platelet aggregation (Suppl. Figure 8, available online at www.thrombosis-online.com).

To definitely confirm the role of TREM-1 on aggregation we used washed platelets from Trem-1$^{-/-}$ mice. Platelet counts were similar and expression of CD31, CD36, CD41, P-Selectin, αIIbβ3, and TLT-1 did not differ between WT and Trem-1$^{-/-}$ mice (Suppl. Figure 9, available online at www.thrombosis-online.com). Gene expression of Treml1 (TLT-1) was also similar between WT and Trem-1 deficient platelets (Suppl. Figure 10, available online at www.thrombosis-online.com). ►Figure 3B shows that aggregation induced by collagen, ADP, and thrombin was strongly impaired in Trem-1-deficient platelets. While LR12 inhibited aggregation of WT platelets, it had no effect on Trem-1$^{-/-}$ platelets (Suppl. Figure 11, available online at www.thrombosis-online.com), ruling out an action of LR12 on TLT-1. TREM-1 inhibition also reduced platelets aggregation induced by arachidonic acid (Suppl. Figure 6, available online at www.thrombosis-online.com).

To further investigate whether LR12 interferes with integrin outside-in signalling, we performed platelet spreading assays using washed human platelets pretreated with LR12. As shown in ►Figure 3C, LR12 treatment decreased platelet spreading and platelet-platelet interactions on collagen or fibrinogen.

These data support a role of TREM-1 during *in vitro* platelet aggregation.

TREM-1 inhibition prevents thrombosis *in vitro* and *in vivo*

Inhibition of thrombus formation by LR12 was assessed in a whole-blood perfusion assay over a collagen matrix under a shear rate of 30 dynes·cm^{-2} (►Figure 4A). Platelets adhered to collagen matrix and rapidly built stable aggregates. By contrast, LR12 decreased the size and number of thrombi. We next investigated the adhesion of washed platelets to fibrinogen under flow conditions. Platelets were activated with ADP (10 µM) and perfused under constant low shear stress of 2.5 dynes·cm^{-2} over fibrinogen-coated biochips. Pretreatment of platelets with LR12 caused a significant decrease of platelet adhesion to fibrinogen ►Figure 4B and C).

Using a $FeCl_3$-induced carotid artery thrombosis model (►Figure 4D), we observed that genetic invalidation or pharmacological inhibition of TREM-1 delayed the time to arterial occlusion, and sometimes largely prevented the complete vessel occlusion, due to the presence of unstable thrombi.

Tail bleeding times, measured by transecting the tails of isoflurane anesthetised mice 5 mm from the tip that were then placed in a plastic cup filled with 37°C saline, were similar between the three groups of animals (WT: 295 ± 25 sec, WT+LR12: 306 ± 34 sec, Trem-1 Ko mice: 302 ± 24 sec).

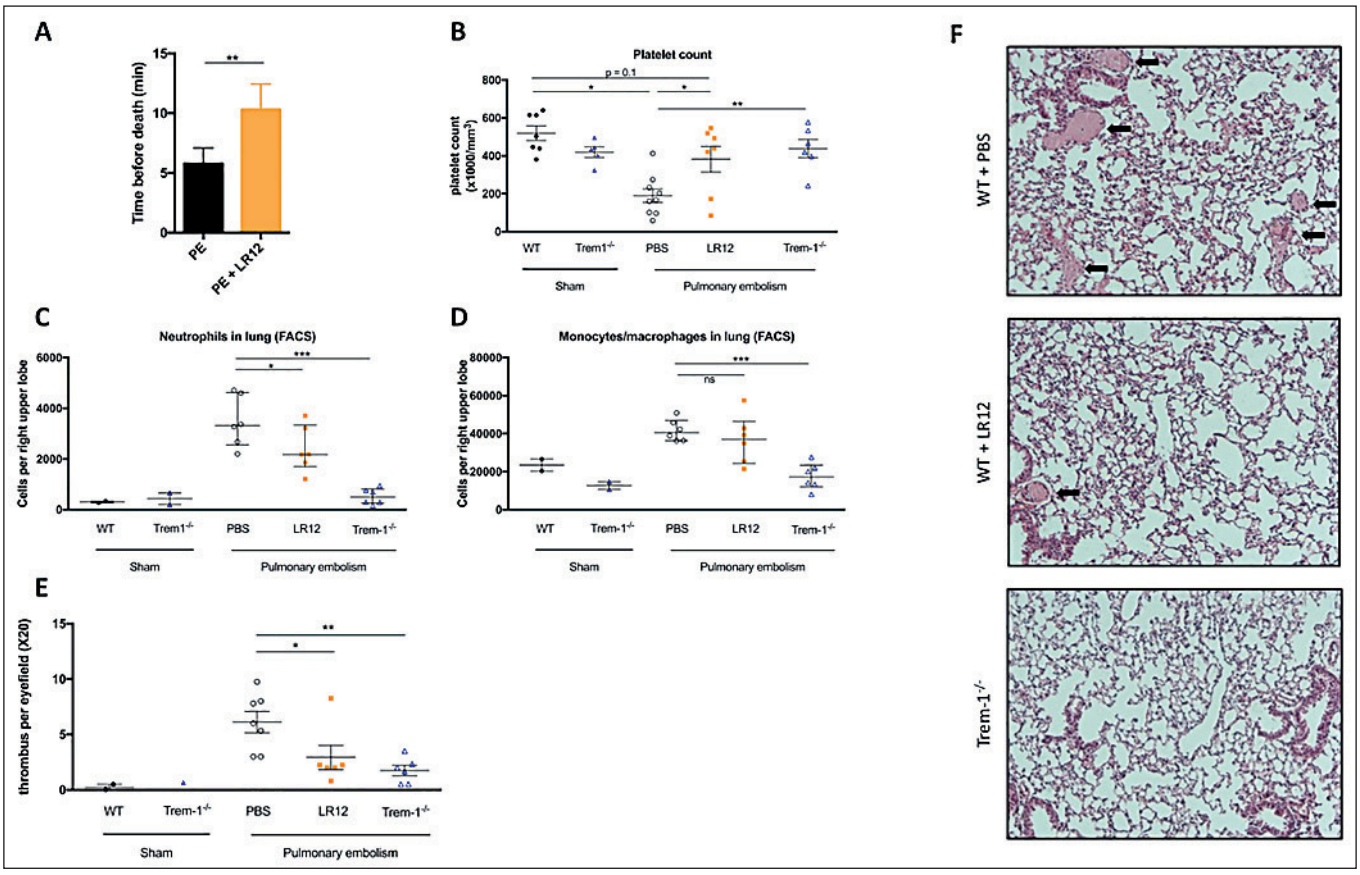

Figure 5: TREM-1 inhibition protects mice from pulmonary embolism in reducing lung leukocytes recruitment and thrombi formation. A) Pulmonary embolism (PE) associated mortality caused by injection of epinephrine plus collagen in control (100 µl PBS) (black bars) and LR12 (100 µl=100 µg) treated (orange bars) mice. Data are reported as the mean ± SD. Statistical significance was determined by unpaired Student t-test (**P<0.01). B) Platelet count in WT, WT+LR12 and Trem-1$^{-/-}$ mice before (Sham) and 3 min after pulmonary embolism (PE). Flow-cytometric quantification of neutrophils (C) and monocytes/macrophages (D) in lung from WT, WT+LR12 and Trem-1$^{-/-}$ mice before (Sham) and 3 min after pulmonary embolism. Data are reported as the mean ± SD. Statistical significance was determined by unpaired Student t-test (*P<0.5; **P<0.01; *** P<0.005). E) Number of thrombus per eye field in the lungs of WT+PBS, WT+LR12 and Trem-1$^{-/-}$ mice by count in 10 microscopic fields for each lung section. Data are reported as the mean ± SD. Statistical significance was determined by unpaired Student t-test (*P<0.05; **P<0.01) (F) Representative histological section of lungs of WT, WT+LR12 and Trem-1$^{-/-}$ mice 3 min after induction of pulmonary embolism. Black arrows: Occlusive intravascular thrombi.

TREM-1 inhibition thus decreases thrombus formation both *in vitro* and in a relevant model of arterial thrombosis.

TREM-1 inhibition protects mice from pulmonary embolism

We next assessed the role of TREM-1 in a pulmonary thromboembolism model. Time to death was delayed in Trem-1$^{-/-}$ or LR12 treated mice (▶Figure 5A). Pulmonary embolism (PE) was associated with a rapid thrombocytopenia. This phenomenon was prevented when TREM-1 was inhibited (▶Figure 5B).

Using flow cytometry, we analysed PE-induced leukocytes recruitment to the lungs. Very early (3 min) after the induction of PE, neutrophils and monocytes/macrophages infiltrated the lungs. TREM-1 inhibition largely reduced this inflammatory recruitment (▶Figure 5C and D).

Histologic analysis of isolated lungs collected 3 min after PE induction showed that the percentage of vessels occluded by thrombi was significantly lower in Trem-1$^{-/-}$ and LR12 treated mice (▶Figure 5E and F). These results indicate that TREM-1 plays a relevant role in thrombus formation *in vivo*.

TREM-1 inhibition protects mice during pulmonary embolism in reducing lung leukocytes recruitment and thrombi formation.

Discussion

The TREM family encompasses at least five different receptors sharing low sequence homology with each other. Among them, TREM-1 is expressed at the surface of neutrophils, mature monocytes, macrophages and hypoxic dendritic cells, and plays a major role in the pathophysiology of inflammation-associated disorders (11). During various models of sepsis, we observed that the

TREM-1 inhibition, in addition to reducing inflammation and improving survival, prevented from thrombocytopenia and disseminated intra-vascular coagulation (17, 19). Moreover, another member of the TREM family, the TREM-like transcript 1 (TLT-1) receptor, has been found in α-granules of megakaryocytes and platelets (26). Upon platelet activation, TLT-1 transports to the membrane where it enhances Ca2+ influx and promotes platelet aggregation (27, 28). Giomarelli et al. showed anti-TLT-1 scFvs inhibited low-dose thrombin-induced human platelets aggregation (29). Moreover, platelets isolated from Tlt-1$^{-/-}$ mice displayed poor aggregation *in vitro* (28). These different findings prompted us to investigate whether TREM-1 is also present in platelets.

Indeed, we observed that TREM-1 is constitutively expressed in resting platelets and clusters at the membrane upon activation. Interestingly, although TREM-1 and TLT-1 seems to co-localise in resting platelets, this is no more observed in activated platelets.

Platelet aggregation plays a key role in thrombosis, leading to many disorders. Platelet activation is achieved through various surface receptors that include G protein–coupled receptors, integrins, and glycoproteins receptors. Activation of these receptors is mainly mediated by the strong agonists thrombin and collagen, whereas other mediators released by activated platelets, such ADP and TxA2, potentiate the activation cascade by recruiting other platelets. We found that genetic invalidation or pharmacologic inhibition (with the use of the inhibitory peptide LR12) of TREM-1 inhibited ADP, collagen and thrombin induced platelet aggregation. Activation of the glycoprotein (GP)IIb/IIIa receptor is the most common pathway for agonists-induced platelet aggregation. As we observed in presence of LR12 a reduced expression of the conformational dependent anti-GPIIb/IIIa antibody PAC-1, a decreased fibrinogen binding and platelet spreading, this effect was likely the consequence of an impaired integrin αIIbβ3 inside-out activation.

In myeloid cells, TREM-1 engagement leads to the phosphorylation of several kinase including PLCγ2 and ERK1/2 (12). These kinases are also known to be involved in collagen, thrombin and ADP-dependent signalling in platelets (30). TREM-1 inhibition decreased PLCγ2 and ERK1/2 activation in stimulated platelets.

The role of activated platelets during acute inflammatory disorders is well established. For example, the adhesion molecule P-selectin (CD62P), a component of the α-granules membranes, plays a dominant role in modulating interactions between platelets and the endothelium, and may be involved in acute cardiovascular events (31, 32). Furthermore, P-selectin can lead activated platelets to adhere to monocytes and neutrophils: these three cell types cooperate to initiate and propagate thrombosis (33). This may be especially true during sepsis (34). Recent studies have indicated that antiplatelet medications may reduce mortality from infections and sepsis, which suggests a possible clinical relevance of modifying platelet responses to inflammation (35). As LR12 reduces platelet activation (including P-selectin expression) its beneficial effects during sepsis and myocardial infarction (19, 21) may not be restricted to an action on myeloid cells but also involves a modulation of platelet activation.

The reduced *ex vivo* aggregation of platelets from Trem-1$^{-/-}$ mice in response to collagen, ADP, and thrombin suggests that TREM-1 may also represent a novel molecular target for antithrombotic agents. This possibility is strengthened by our findings demonstrating an important role for TREM-1 in thrombosis *in vivo*. Using a $FeCl_3$-induced carotid artery thrombosis model, we showed a significant increase of time-to-occlusion in Trem-1 deficient or LR12 treated mice. Moreover, we also found that genetic invalidation or pharmacologic modulation of TREM-1 conferred protection against pulmonary thromboembolism with a reduced lung inflammation, number of occlusive thrombi and delayed death. These findings point to an important role of TREM-1 in regulating *in vivo* platelet function.

Our study has several limitations: the mouse model of Trem-1$^{-/-}$ is a full knockout that does not allow us to definitely implicate platelets in our carotid artery and pulmonary embolism experiments. However, aggregation defect was clearly observed in TREM-1 Ko isolated platelets. Another member of the TREM family, TLT-1, has been shown to be involved in platelet aggregation. Although we cannot completely exclude off-target effects of LR12, for example on TLT-1, this peptide has no action in TREM-1 Ko animals (who display a similar TLT-1 expression compared to WT controls), rendering this hypothesis less probable. Finally, the precise mechanism by which TREM-1 deletion decreases platelets activation remains elusive as many important steps are reduced at once (calcium mobilisation, integrin expression…). However, Haselmayer et al. observed the presence of a TREM-1 ligand in both resting and stimulated platelets (15). It is therefore tempting to speculate that blocking the interaction of platelet TREM-1 (overexpressed after stimulation) with its constitutively expressed platelet ligand decreases activation, at least in the presence of weak platelets agonists. But as the nature of this ligand remains elusive definite conclusions cannot be drawn.

In this study, we showed that TREM-1 is expressed by platelets where it plays an important role in mediating their activation. Pharmacological modulation or genetic invalidation of TREM-1 impairs platelet activation and prevents from arterial or venous thrombus formation. These findings suggest that TREM-1 modulating agents such as LR12 could potentially be useful adducts in antiplatelet therapies in the setting of thrombotic disorders. They also provide a new insight on the TREM-1 biology and may ex-

What is known about this topic?

- The Triggering Receptor Expressed on Myeloid cells-1 (TREM-1) is an immunoreceptor expressed by neutrophils and monocytes.
- TREM-1 activation on myeloid cells amplifies the inflammatory response triggered by Toll Like Receptor engagement.

What does this paper add?

- Here we show that TREM-1 is also expressed by platelets.
- TREM-1 plays an important role in mediating platelets activation.
- Modulation of TREM-1 impairs platelet activation and prevents from thrombus formation.

plain the protective action of the TREM-1 modulation during acute inflammatory diseases, beyond their effects on myeloid cells.

Acknowledgements
This work was supported by INSERM and INOTREM SA.

Author contributions
LJ and SG designed the research and wrote the paper. LJ, JL, KC, BP, MD, AB, and SG performed experiments and analysed the data.

Conflicts of interest
Marc Derive and Sébastien Gibot are co-founders of INOTREM SA, a company developing TREM-1 inhibitors.

References

1. American College of Emergency Physicians. 2013 ACCF/AHA guideline for the management of ST-elevation myocardial infarction: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. J Am Coll Cardiol 2013; 61: e78–140.
2. Members, 2012 Writing Committee. 2012 ACCF/AHA Focused Update of the Guideline for the Management of Patients With Unstable Angina/Non–ST-Elevation Myocardial Infarction (Updating the 2007 Guideline and Replacing the 2011 Focused Update) A Report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. Circulation 2012; 126: 875–910.
3. Franchi F, Angiolillo D Novel antiplatelet agents in acute coronary syndrome. Nat Rev Cardiol 2015; 12: 30–47.
4. Borissoff JI, Spronk HMH, ten Cate H. The hemostatic system as a modulator of atherosclerosis. N Engl J Med 2011; 364: 1746–1760.
5. van Gils JM, Zwaginga JJ, Hordijk PL. Molecular and functional interactions among monocytes, platelets, and endothelial cells and their relevance for cardiovascular diseases. J Leukoc Biol 2009; 85: 195–204.
6. Frenette PS, Johnson RC, Hynes RO, Wagner DD. Platelets roll on stimulated endothelium in vivo: an interaction mediated by endothelial P-selectin. Proc Natl Acad Sci USA 1995; 92: 7450–7454.
7. Frenette PS, Denis CV, Weiss L, et al. P-Selectin glycoprotein ligand 1 (PSGL-1) is expressed on platelets and can mediate platelet-endothelial interactions in vivo. Exp Med 2000; 191: 1413–1422.
8. Weber AA, Köppen HO, Schrör K. Platelet-Derived Microparticles Stimulate Coronary Artery Smooth Muscle Cell Mitogenesis by a PDGF-Independent Mechanism. Thromb Res 2000; 98: 461–466.
9. Projahn D, Koenen RR. Platelets: key players in vascular inflammation. Leukoc Biol 2012; 92: 1167–1175.
10. Bouchon A, Dietrich J, Colonna M. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. Immunol 2000; 164: 4991–4995.
11. Bouchon A, Facchetti F, Weigand MA, Colonna M. TREM-1 amplifies inflammation and is a crucial mediator of septic shock. Nature 2001; 410: 1103–1107.
12. Tessarz AS, Cerwenka A. The TREM-1/DAP12 pathway. Immunol Lett 2008; 116: 111–116.
13. Arts RJW, Joosten LAB, van der Meer JWM, Netea MG. TREM-1: intracellular signaling pathways and interaction with pattern recognition receptors. Leukoc Biol 2013; 93: 209–215.
14. Radsak MP, Salih HR, Rammensee HG, Schild H. Triggering receptor expressed on myeloid cells-1 in neutrophil inflammatory responses: differential regulation of activation and survival. Immunol 2004; 172: 4956–4963.
15. Haselmayer P, Grosse-Hovest L, Landenberg P, et al. TREM-1 ligand expression on platelets enhances neutrophil activation. Blood 2007; 110: 1029–1035.
16. Gibot S, Kolopp-Sarda MN, Béné MC, et al. A soluble form of the triggering receptor expressed on myeloid cells-1 modulates the inflammatory response in murine sepsis. Exp Med 2004; 200: 1419–1426.
17. Gibot S, Alauzet C, Massin F, et al. Modulation of the triggering receptor expressed on myeloid cells-1 pathway during pneumonia in rats. Infect Dis 2006; 194: 975–983.
18. Weber B, Schuster S, Zysset D, et al. TREM-1 deficiency can attenuate disease severity without affecting pathogen clearance. PLoS Pathog 2014; 10: e1003900.
19. Derive M, Boufenzer A, Gibot S. Attenuation of responses to endotoxin by the triggering receptor expressed on myeloid cells-1 inhibitor LR12 in nonhuman primate. Anesthesiology 2014; 120: 935–942.
20. Liu T, Zhou Y, Li P, et al. Blocking triggering receptor expressed on myloid cells-1 attenuates lipopolysaccharide-induced acute lung injury via inhibiting NLRP3 inflammasome activation. Sci Rep 2016; 6: 39473.
21. Boufenzer A, Lemarié J, Simon T, et al. TREM-1 Mediates Inflammatory Injury and Cardiac Remodeling Following Myocardial Infarction. Circ Res 2015; 116: 1772–1782.
22. Lemarié J, Boufenzer A, Popovic B, et al. Pharmacological inhibition of the triggering receptor expressed on myeloid cells-1 limits reperfusion injury in a porcine model of myocardial infarction. ESC Heart Fail 2015; 2: 90–99.
23. Zysset D, Weber B, Rihs S, et al. TREM-1 links dyslipidemia to inflammation and lipid deposition in atherosclerosis. Nat Commun 2016; 7: 13151.
24. Joffre J, Potteaux S, Zeboudj L, et al. Genetic and pharmacological inhibition of TREM-1 limits the development of experimental atherosclerosis. Am Coll Cardiol 2016; 68: 2776–2793.
25. Allcock RJN, Barrow AD, Forbes S, et al. The human TREM gene cluster at 6p21.1 encodes both activating and inhibitory single IgV domain receptors and includes NKp44. Eur Immunol 2003; 33: 567–577.
26. Washington AV, Gibot S, Acevedo I, et al. A TREM family member, TLT-1, is found exclusively in the alpha-granules of megakaryocytes and platelets. Blood 2004; 104: 1042–1047.
27. Washington AV, et al. TREM-like transcript-1 protects against inflammation-associated hemorrhage by facilitating platelet aggregation in mice and humans. Clin Invest 2009; 119: 1489–1501.
28. Barrow AD, Astoul E, Floto A, et al. 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. Immunol 2004; 172: 5838–5842.
29. Giomarelli B, Washington VA, Chisholm MM, et al. Inhibition of thrombin-induced platelet aggregation using human single-chain Fv antibodies specific for TREM-like transcript-1. Thromb Haemost 2007; 97: 955–963.
30. Ragab A, Séverin S, Gratacap MP, et al. Roles of the C-terminal tyrosine residues of LAT in GPVI-induced platelet activation: insights into the mechanism of PLCγ2 activation. Blood 2007; 110: 2466–2474.
31. Furie B, Furie BC, Flaumenhaft R. A journey with platelet P-selectin: the molecular basis of granule secretion, signalling and cell adhesion. Thromb Haemost 2001; 86: 214–221.
32. Blann AD, Nadar SK, Lip GYH. The adhesion molecule P-selectin and cardiovascular disease. Eur Heart J 2003; 24: 2166–2179.
33. von Brühl ML, Stark K, Stenhart A, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. Exp Med 2012; 209: 819–835.
34. Thomas MR, Storey RF. The role of platelets in inflammation. Thromb Haemost 2015; 114: 449–458.
35. Toner P, McAuley DF, Shyamsundar M. Aspirin as a potential treatment in sepsis or acute respiratory distress syndrome. Crit Care 2015; 19: 274.

