CEACAM1 negatively regulates platelet-collagen interactions and thrombus growth in vitro and in vivo.

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6DEJ is a recipient of an NHMRC Senior Research Fellowship.

Running Title: CEACAM1 modulates platelet-collagen interactions.

Scientific Heading: Hemostasis, Thrombosis and Vascular Biology.

This work was supported by grants from the National Heart Foundation of Australia and National Health and Medical Research Council of Australia (to DEJ) and the Canadian Institutes of Health Research (to NB).


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Abstract

Carcinoembryonic antigen cell adhesion molecule-1 (CEACAM1) is a surface glycoprotein expressed on various blood cells, epithelial cells and vascular cells. CEACAM1 possesses adhesive and signaling properties mediated by its intrinsic immunoreceptor tyrosine-based inhibitory motifs that recruits SHP-1 protein-tyrosine phosphatase. In this study, we demonstrate that CEACAM1 is expressed on the surface and in intracellular pools of platelets. In addition, CEACAM1 serves to negatively regulate platelet collagen GPVI-FcR gamma chain signaling. *Ceacam1*^−/−^ platelets displayed enhanced Type I collagen and GPVI-selective ligand, collagen-related peptide, CRP-mediated platelet aggregation, enhanced platelet adhesion on Type I collagen and elevated CRP-mediated alpha and dense granule secretion. Platelets derived from *Ceacam1*^−/−^ mice form larger thrombi when perfused over a collagen matrix under arterial flow compared to wild-type mice. Furthermore, using intravital microscopy to ferric chloride-injured mesenteric arterioles, we show that thrombi formed in *Ceacam1*^−/−^ mice were larger and were more stable than wild-type mice *in vivo*. GPVI depletion using monoclonal antibody JAQ1 treatment of *Ceacam1*^−/−^ mice showed a reversal in the more stable thrombus growth phenotype. *Ceacam1*^−/−^ mice were more susceptible Type I collagen induced pulmonary thromboembolism than wild-type mice. Thus, CEACAM1 acts as a negative regulator of platelet-collagen interactions and thrombus growth involving collagen GPVI receptor *in vitro* and *in vivo*. 
Introduction

*In vivo*, several negative regulatory mechanisms have been described that down modulate platelet-collagen interactions. These include prostacylin and nitric oxide released from endothelium and the immunoglobulin (Ig)-immunoreceptor tyrosine-based inhibitory motif (ITIM) superfamily member, PECAM-1 that serves an autoregulatory role when platelets come into contact with each other after exposure to collagen.\(^1\)\(^-\)\(^4\) Given the importance of collagen in promoting platelet adhesion and thrombus formation particularly involving GPVI/FcR gamma chain signaling, it is essential that natural inhibitors that contribute to autoregulatory mechanisms that modulate collagen GPVI/FcR gamma chain-mediated signaling are characterized in platelets.

While the ITIM-bearing receptor regulation of ITAM-associated pathways has been well described in the leukocyte world, they are poorly defined in platelets. This concept is further complicated by the observation that Ig-ITIM-bearing receptors have the capacity to bind multiple phosphatases including SHP-1, SHP-2 and SHIP to initiate signaling attenuation.\(^5\) However, there is also evidence that Ig-ITIM bearing receptors may differentially associate with distinct phosphatases depending upon the cellular context and activational status of cells.\(^6\),\(^7\)

These inhibitory mechanisms are further complicated by the fact that ITAM-bearing receptors can mediate inhibitory ITAM signaling under defined conditions using either DAP12 adaptor or FcR gamma chain.\(^8\),\(^9\) This switch between activating and inhibitory ITAM signaling is controlled by the avidity of the ligand cross-linking the receptor. It
appears that low-avidity ligand interactions result in inhibitory signaling possibly involving a phosphatase and high-avidity ligand interactions produce activating ITAM-mediated signaling.\textsuperscript{8,9} Therefore, it is important to dissect out the respective mechanisms of activation and inhibitory mechanisms in platelets.

As platelets lack the prototypic inhibitory Ig-ITIM superfamily member, FcγRIIb, there has been a prevailing view that PECAM-1 represents the sole surrogate Ig-ITIM superfamily member that serves a negative regulatory role.\textsuperscript{1,2} However, based upon several studies it would appear that platelets contain multiple Ig-ITIM superfamily members that are likely to have a modulatory role in regulating platelet-collagen interactions. Several reports suggest that platelets contain other Ig-ITIM superfamily members including G6B and TREM (TLT-1) that may attenuate platelet function.\textsuperscript{10,11} In immunological systems, SHP-1 protein-tyrosine phosphatase is classified as a negative regulator, while in collagen GPVI platelet responses, it has been proposed to act as a positive regulator.\textsuperscript{12} Given this controversy, it is important that we study respective Ig-ITIM superfamily members that are linked to distinct protein-tyrosine phosphatases such as SHP-1 in platelets to determine their relative importance in regulating platelet thrombus formation.

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1/CD66a/Bgp) is a member of the CEACAM family of genes that is a type I-transmembrane receptor that is broadly expressed on a wide range of cells including immune, haematological, epithelial and endothelial cells.\textsuperscript{13} CEACAM1 is the only CEACAM superfamily member
expressed in rodents and humans. It shares some similarities to PECAM-1 in its capacity to mediate homophilic and heterophilic ligands (CEA and CEACAM6) but generally has a preference towards homophilic CEACAM1 interactions. CEACAM1 is composed of an N-terminal IgV-like domain that is responsible for homophilic adhesion, followed by one to three IgC-like domains in its extracellular structure. In addition, alternative mRNA splicing gives rise to different isoforms that differ in length of their cytoplasmic domain including the long ITIM-containing isoform (CEACAM1-L) (70-73 amino acids) or a short ITIM-less isoform (CEACAM1-S) (10-12 amino acids). The long form of CEACAM1 protein has two ITIMs that preferentially recruit SHP-1 protein-tyrosine phosphatase and to a lesser extent SHP-2 protein-tyrosine phosphatase. Based upon recent studies in T-cells, CEACAM1 functions as a negative regulator via its recruitment and activation of SHP-1 protein-tyrosine phosphatase. However, there is little known about the presence and functional role of CEACAM1 in regulating platelet-collagen interactions and thrombus formation in vitro and in vivo. The only exception was a report by Hansson et al., 1990 which suggested that C-CAM (old terminology) was present in the intracellular compartment of rat platelets.

In this current study, we provide the first report that CEACAM1 serves as a negative regulator of platelet-collagen interactions, thrombus growth in vitro and in vivo and susceptibility to Type I collagen-induced pulmonary thromboembolism. Based upon these studies, we propose CEACAM1 as a new negative regulator in platelets that serves an autoregulatory role when platelets come into contact with each other after exposure to collagen.
Materials and Methods

Materials

Antibodies
Rabbit anti-mouse CEACAM1 has been previously described.\(^{18}\) Anti-rabbit FITC was purchased from Dako Australia (Glostrup, Denmark). Anti-mouse GPVI\(^{19}\), anti-mouse GPI\(\beta\)α/IX/V complex\(^{20}\), anti-mouse integrin α2β1\(^{21}\), anti-mouse CD9\(^{22}\) anti-mouse GPVI (JAQ1)\(^{23}\) were purchased or obtained from Emfret analytics (Wurzburg, Germany). Anti-mouse integrin αIIbβ3\(^{24}\), anti-mouse CD44\(^ {25}\) and HRP-conjugated anti-phosphotyrosine RC20 antibody were obtained from BD Biosciences Pharmingen (Franklin Lakes, NJ). Anti-mouse ‘390’ PECAM-1 antibody was provided by Dr. Steve Albelda (Philadelphia, PA).\(^{26}\) PLC\(\gamma\)2 and ERK-2 antibodies were purchased from Santa Cruz (Santa Cruz, CA).

Mice
The generation of Ceacam1\(^{+/−}\) mice and breeding on C57BL/6 background has been described.\(^{18}\) Wild-type and Ceacam1\(^{+/−}\) mice were age- and sex-matched C57BL/6. These mice were housed in the Burnet Institute at Austin Animal House and the St. Michael’s Hospital Research Vivarium. All procedures were approved by the Austin Health Animal Ethics committee #2005:2098 and #2005:02354, and Animal Care Committee of St. Michael’s Hospital.

Preparation of mouse platelets
Platelet-rich plasma (PRP) or washed mouse platelets were prepared as previously described.  

**Static platelet adhesion assays**

Static platelet adhesion assays were performed according to Yuan et al., 1999. Washed platelets derived from wild-type, Ceacam1+/− and Ceacam1−/− mice were examined for binding to immobilised Type I fibrillar collagen and fibrinogen in the absence of Mg2+ over time, fixed and quantitated by differential interference contrast (DIC) microscopy (x63 oil objective).

**Platelet adhesion and thrombus formation under flow**

Platelet adhesion and thrombus formation under flow was performed as previously described.  

**Platelet aggregation assays**

Platelet aggregation assays were monitored by measuring light transmission using a 4-channel platelet aggregometer (Chronolog, Havertown, PA). Platelet rich plasma (PRP) was diluted in RCD buffer, pH 7.4, (108 mM NaCl, 38 mM KCl, 1.7 mM NaHCO3, 21.2 mM sodium citrate, 27.8 mM glucose and 1.1 mM MgCl2.6H2O) to generate a platelet count of 100x10⁹/L. Reactions were performed in glass cuvettes in a 250 μL volume in the presence of 100 μg/ml fibrinogen and 1 mM CaCl2 at 37°C with constant stirring (1000 rpm).
Flow cytometry

The surface and intracellular expression of CEACAM1 was performed essentially as previously described. The only exception was that platelets were labelled with either preimmune rabbit serum (1:500), rabbit anti-mouse CEACAM1 2457 serum (1:500) or FITC-conjugated antibody to P-selectin (10 μg/mL) and where appropriate then labelled with swine anti-rabbit F(ab’)2 FITC-conjugated antibody (1:200)(Dako Australia, Glostrup, Denmark).

5-Hydroxytryptamine dense granule secretion

Dense granule secretion was performed essentially as described with the inclusion of 2 mM Imipramine hydrochloride (Sigma Chemical Co., St. Louis, MO).

Ferric chloride-induced model of thrombosis and intravital microscopy

Wild-type, Ceacam1+/− or Ceacam1−/− C57BL/6 mice (4-5 weeks old) were anaesthetized with a ketamine/xylazine (200:10 mg/kg) mixture and the mesentery was exteriorized through a midline abdominal incision. A catheter was inserted into the jugular vein to deliver rhodamine dye and anaesthetic as required. Mesenteric arterioles (60-100 μm in diameter) were visualized with a Zeiss Axiovert 135 M1 microscope (Carl Zeiss, Gottingen, Germany), and z-stack slices of the vessel captured with a AxioCam MRm camera. A strip of filter paper (1 x 4 mm) was dipped in a 462 mM (7.5% w/v) FeCl₃ solution for 3 seconds, applied to a 2 – 5 mm length of arteriole for 4 minutes and then removed. Z-stack images of the vessel were taken over five 2 minute cycles (determined as the time the vessel begins to occlude). The rendered z-stack images were deconvolved with AxioVision Rel 4.6 software, to produce a three-dimensional image, and the volume of the thrombi were determined from the area and height of the clot. Assessment of platelet thrombi was blinded to detect quantitative differences between wild-type, Ceacam1+/− and Ceacam1−/− mice. Stability was determined by calculating the
percentage of the vessel that is occupied by the thrombus over a period of 2 minutes, and scored from 1 – 10, with 1 being 0 – 10% occupancy and 10 being 91 – 100% occupancy (i.e. complete vessel occlusion).

**Pulmonary Thromboembolism mouse model**

Mouse models of pulmonary thromboembolism were essentially performed as previously described. The only exceptions include that 350 µg/kg of type I fibrillar collagen and 0.200 µl/g tissue thromboplastin (Thromborel S) were used in respective models. The endpoint of the pulmonary thromboembolism was defined as the time to restrained breathing within 10 second intervals.

**Statistical analysis**

Data was checked for normal distribution using the Shapiro-Wilk normality test and statistical significance was determined using unpaired Student’s t test. P values of 0.05 or less were taken to indicate significant differences.
Results

CEACAM1-deficient mice have normal haematopoiesis

Unlike other CEACAM1 family members, CEACAM1 is broadly expressed on a wide range of cells including immune, epithelial cells, endothelial cells and platelets. In order to test if deletion of CEACAM1 influenced haematopoiesis and particularly platelet production, we determined peripheral blood haematological parameters on age- and sex matched wild-type versus CEACAM1-deficient mice. As shown in Table 1, Ceacam1−/− mice displayed normal white blood cell (WBC) count, red blood cell (RBC) count and parameters including haemoglobin and platelet count compared to wild-type C57BL/6 mice (P>0.05, n=18). Differential counting of white blood cells revealed that Ceacam1−/− mice had an increased percentage of neutrophils compared to wild-type mice (*P<0.05, n=18).

CEACAM1 is expressed on the surface and in intracellular pools in murine and human platelets

The only evidence in the literature in relation to CEACAM1 in platelets was the demonstration that C-CAM (old terminology) was present in the intracellular compartment of rat platelets. In order to determine the presence of CEACAM1 protein expressed on the surface and intracellular pools of resting murine and human platelets, and if this expression is upregulated by intracellular granule release, we examined the binding of specific polyclonal anti-murine CEACAM1 antibody to either resting and thrombin-stimulated wild-type murine and human platelets, monitored by flow cytometry. In order to characterize the surface expression of CEACAM1 in murine
platelets, washed platelets were incubated with either normal rabbit pre-immune serum (1/500 dilution) or polyclonal anti-mouse CEACAM1 antibody (1/500 dilution) followed by FITC-labelled anti-rabbit conjugate (1/200 dilution) and then analyzed by flow cytometry. In addition, characterization of the total distribution of CEACAM1 in murine platelets was determined by permeabilization of the platelet membrane with 0.1% (w/v) saponin treatment. As shown in Figure 1A and C, a two-fold increase in the mean fluorescence intensity was observed in resting murine platelets using a specific polyclonal anti-CEACAM1 antibody compared to normal rabbit pre-immune serum (1/500 dilution) (15.24±1.61 □; vs 7.74±1.71; ■; n=3, **P<0.005), indicating the presence of CEACAM1 on the surface of murine platelets. In addition, a four-fold increase in the mean fluorescence intensity (MFI) was observed in saponin-treated murine platelets using a specific polyclonal anti-CEACAM1 antibody compared to normal rabbit pre-immune serum (1/500 dilution) (43.64±8.84 □; vs 11.99±2.75, ■, n=3, *P<0.05), indicating the presence of CEACAM1 in an intracellular distribution in murine platelets. As shown in Figure 1B and D, a seven-fold increase in the MFI was observed in resting human platelets using a specific monoclonal anti-CEACAM1 antibody compared to an isotype control antibody (35.3±1.3 □, vs 4.0±1.2; ■, **P<0.005, n=3). In addition, a four-fold increase in the MFI was observed in saponin-treated human platelets using a specific monoclonal anti-CEACAM1 antibody compared to an isotype control antibody (50.25±17.4 □, vs 12.05±0.35 ■, *P<0.05, n=3). Cell surface expression of CEACAM1 was increased upon agonist stimulation with different doses of thrombin compared to resting murine platelets and normal pre-immune rabbit serum (24.08±2.3 □, vs 15.25±0.93 □, *P<0.05, n=3; 8.41±0.37 ■)(Figure 1E). A similar trend was observed
with cell surface expression of CEACAM1 following agonist stimulation with different doses of thrombin compared to resting human platelets and negative isotype antibody (58.93±7.11 □, vs 31.63±2.59 □, *P<0.05, n=3; 10.23±1.15 ■)(Figure 1F). At higher concentrations of thrombin, a modest reduction in CEACAM1 surface expression is observed. *Ceacam1*+/− platelets had 60% ceacam1 expression compared to wild-type murine platelets (Figure 1G-H). These results indicate that CEACAM1 is expressed on the surface and in intracellular pools of resting murine and human platelets.

**CEACAM1 serves as a negative regulator of collagen GPVI-mediated platelet responses**

Recent studies in T-cells have demonstrated that CEACAM1 acts as an inhibitory co-receptor via the interface of its intrinsic ITIMs with recruitment and activation of SHP-1 protein-tyrosine phosphatase. This finding supports the concept in immunological systems that SHP-1 protein-tyrosine phosphatase acts as a negative regulator. However, in collagen GPVI-mediated platelet responses, SHP-1 has been proposed to act as a positive regulator. Given this controversy, it is important to define the functional role of CEACAM1 negative regulatory signaling in platelets. In order to test the hypothesis that CEACAM1, like PECAM-1, normally functions to modulate collagen GPVI-mediated platelet responses, we first examined the ability of platelets derived from wild-type versus CEACAM1-deficient mice to bind to immobilised Type I fibrillar collagen and fibrinogen in the absence of Mg2+ over time. As shown in Figure 2A, *Ceacam1*+/− platelets bound significantly better at all time points on Type I fibrillar collagen compared to wild-type and *Ceacam1*+/− platelets (***P<0.005, n=3). This effect was
specific as adhesion of bovine serum albumin over the same time points was insignificant for both wild-type, $Ceacam1^{+/+}$ and $Ceacam1^{-/-}$ platelets. This hyper-responsive platelet adhesion observed with $Ceacam1^{-/-}$ platelets was selective for Type I fibrillar collagen as platelet adhesion on fibrinogen did not show increased adhesion (Fig. 2B). In fact, a mild reduction in platelet adhesion on fibrinogen is observed in $Ceacam1^{-/-}$ platelets but not wild-type or $Ceacam1^{+/+}$ platelets (*P<0.05, n=3).

In order to examine the functional role of CEACAM1 negative regulatory signaling in platelets, we performed a time course (0-3 min) of CRP stimulation of wild-type and $Ceacam1^{-/-}$ platelets and determined global tyrosine phosphorylation profiles of 30 μg of platelet lysates. As shown in Figure 3A, CRP stimulation of $Ceacam1^{-/-}$ platelets showed an increased level of tyrosine phosphorylation of a number of proteins including Syk over time with both quantitative and kinetic differences than wild-type platelets. In addition, Erk-2 blot was included as a loading control (lower panel). Measurement of tyrosine phosphorylation of PLCγ2 was performed by immunoprecipitation of resting versus CRP stimulated wild-type and $Ceacam1^{-/-}$ platelets (T=90 secs) followed by immunoblotting with an anti-phosphotyrosine antibody (Fig. 3B). PLCγ2 antigen loading was confirmed by reprobing with a polyclonal anti-PLCγ2 antibody. As shown in Fig. 3B, resting and CRP stimulated $Ceacam1^{-/-}$ platelets showed increased tyrosine phosphorylation of PLCγ2 compared to wild-type platelets. These results support the concept that CEACAM1 acts as a negative regulator to modulate ITAM-coupled signaling pathways.
As CEACAM1 has been demonstrated to function as an regulator in T cells upon TCR ligation, we wanted to test the hypothesis that CEACAM1 serves to negatively regulate ITAM-bearing collagen GPVI-mediated platelet responses. In order to test the possibility in platelets, a comparison of the ability of platelets derived from wild-type and CEACAM1-deficient mice in platelet aggregation responses over a dose-dependent range of GPVI selective agonist, CRP (0.62-2.5 μg/mL)(j-l) and acid soluble Type I collagen (1.5-4 μg/mL)(m-o) with G-protein coupled agonists, PAR-4 (100-250 μM)(a-c), ADP (5-20 μM)(d-f) and calcium ionophore (CI)(0.3-2.5 μg/mL)(g-i). As shown in Figure 4, wild-type and Ceacam1+/− platelets display equivalent platelet aggregation profiles over a dose-dependent range of PAR-4, ADP and calcium ionophore. In contrast, Ceacam1−/− platelets show enhanced amplitude and slope of platelet aggregation responses to CRP and collagen particularly at subthreshold doses. This hyper-responsive effect is independent of GPVI expression and other platelet glycoprotein expression (integrin α2β1, GPIb-IX-V complex, integrin αIIbβ3, CD9, CD44, PECAM-1) as Ceacam1−/− platelets are comparable to wild-type platelets (Table 2). These results indicate that the absence of the Ig-ITIM superfamily member, CEACAM1 leads to hyper-responsive GPVI-mediated platelet responses to collagen and CRP.

As Ceacam1+/− platelets displayed enhanced adhesion of Type I fibrillar collagen and hyper-responsive GPVI-mediated platelet aggregation responses, we wanted to determine if the absence of CEACAM1 influenced the platelet release reaction stimulated with GPVI-specific agonists is restricted to GPVI. In order to test this possibility, we compared the ability of wild-type, Ceacam1+/− and Ceacam1−/− platelets to secrete alpha
(P-selectin) and dense granule serotonin upon stimulation of GPVI. As shown in Figure 5A-B, Ceacam1<sup>−/−</sup> platelets displayed increased release of their alpha and dense granules at subthreshold doses of fibrillar collagen and CRP compared to low or high doses of thrombin treated wild-type and Ceacam1<sup>+/−</sup> platelets (*P<0.05, ***P<0.001, n=3). These data are consistent with the concept that CEACAM1 normally acts as a negative regulator of platelet GPVI-mediated alpha and dense granule release.

CEACAM1-deficient mice display increased thrombus growth <i>in vitro, in vivo</i> and enhanced susceptibility to Type I collagen induced pulmonary thromboembolism

While platelets lack the prototypic Ig-ITIM superfamily member, FcγRIIib, other surrogate Ig-ITIM superfamily members, like PECAM-1, serve as potent negative regulators of platelet thrombus formation. It is however, not clearly defined which Ig-ITIM superfamily members are functionally important in regulating <i>in vivo</i> platelet thrombus formation and this issue warrants investigation. In order to explore the possibility that CEACAM1 is important in modulation of platelet thrombus formation <i>in vivo</i>, we compared age- and sex-matched wild-type versus Ceacam1<sup>−/−</sup> mice in platelet adhesion and thrombus growth on exposed extracellular matrix of the injured mesenteric arterioles (60-100 μm sized vessels) as a vascular injury model by ferric chloride-mediated denuding of endothelium monitored by fluorescence intravital microscopy. The ferric chloride (FeCl<sub>3</sub>) oxidative injury model was used to induce formation of free radicals to disrupt the vascular endothelium in mesenteric arterioles. The ferric chloride is used to initiate the formation of free radicals, which disrupt the vascular endothelium. Following application of FeCl<sub>3</sub> to the mesentery, platelets in both
wild-type and Ceacam1+/− mice rapidly commenced interacting with the injured vessel wall and within 10-15 mins after the injury, vessel occlusion occurred. Thrombus characteristics were assessed in real time measuring the area (µm²), height (µm) and volume (µm³) occupied by the thrombus together with a stability score for each thrombus over a 2 min time frame. Typically, Ceacam1+/− mice showed increased thrombus growth and more stable thrombi over time compared to wild-type control mice (Fig. 6A). Typically, Ceacam1−/− arterioles showed an increase in thrombus area (reflecting surface coverage of platelets) over a 2 minute time interval compared to wild-type and Ceacam1+/− arterioles (6212±268.7 vs 3294±223.4 and 2859±293.9µm²; ***P<0.0001, n=15)(Fig. 6B). In addition, Ceacam1−/− mice developed thrombi that were significantly larger in volume compared to wild-type and Ceacam1+/− mice (122400±6794 vs 49430±4602 and 63680±5478µm³; ***P<0.0001, n=15) (Fig. 6C). Moreover, the thrombi formed in Ceacam1−/− arterioles were typically more stable than those in wild-type and Ceacam1+/− arterioles (4.067±0.3712 vs 2.400±0.2138 and 1.05±0.12; ***P<0.001, n=15)(Fig. 6D). In contrast, Ceacam1−/− mice showed no abnormality in time to first thrombi > 20 µm², number of thrombi > 20 µm², the increase in thrombus diameter over 1 min, thrombus height and time to vessel occlusion (P>0.05, n=15) (data not shown). In order to directly test the contribution of collagen GPVI receptor in this thrombus growth phenotype in ceacam1−/− arterioles, we deleted GPVI in vivo using JAQ1 treatment (100 µg/mouse) and left for 5 days before ferric chloride injury and intravital studies23. A different cohort of ceacam1−/− were similarly treated with control IgG (100 µg/mouse). As shown in Figure 6E-F, JAQ1 treated Ceacam1−/− mice showed a 3-fold reduction in thrombus volume and 2-fold reduction in stability score compared to control
IgG treated and untreated Ceacam1+/+ and Ceacam1−/− arterioles (n=10 arterioles from 3 mice/group; ***P<0.001). Importantly, Ceacam1−/− arterioles showed reversal of the more stable thrombus phenotype upon JAQ1 treatment and in thrombus growth phenotype compared to Ceacam1+/+ arterioles. We confirmed successful clearance of GPVI after JAQ1 treatment by western blotting of platelet lysates from Ceacam1−/− platelets (not shown). This data supports the concept that the in vivo thrombus growth phenotype in ceacam1−/− arterioles is dependent on presence of collagen GPVI receptor.

To further investigate the role of CEACAM1 in modulating platelet thrombus formation on immobilized collagen, in vitro flow studies were performed with blood obtained from wild-type, Ceacam1+/− and Ceacam1−/− mice. DiOC₆-labelled whole blood was perfused through Type I collagen-coated microcapillary tubes at a wall shear rate of 1800 seconds⁻¹, and thrombi were imaged at 4 minutes of blood perfusion by confocal microscopy. Ceacam1−/− platelets (136200±18640 μm³) displayed increased thrombus volume than wild-type (83870±8065 μm³) or Ceacam1+/− platelets (86640±15330 μm³) (P<0.05, n=9)(Fig. 6F). Collectively, these data demonstrate that CEACAM1 is required for modulation of platelet-collagen interactions in platelet thrombus growth in vitro and in vivo.

To further determine the consequences of CEACAM1 deficiency in vivo, we tested wild-type, Ceacam1+/− and Ceacam1−/− mice in a model of pulmonary thromboembolism induced by infusion of type I fibrillar collagen or tissue thromboplastin. Typically, Ceacam1−/− mice displayed enhanced susceptibility to Type I collagen induced pulmonary
thromboembolism than wild-type and Ceacam1+/− mice with >70% reduction in circulating platelet counts within 3 minutes of challenge (Figure 7A-B). Following challenge with Type I fibrillar collagen, Ceacam1+/− mice reached maximal clinical scoring of restrained breathing earlier than wild-type and Ceacam1+/− mice (252.1±15.39 versus 343.5±23.73 and 327.0±28.29 secs; **P<0.005; n=20). In this system, both wild-type, Ceacam1+/− and Ceacam1+/− mice had comparable reductions in circulating platelet numbers over a three minute time frame tested following challenge with type I fibrillar collagen (73.49±3.15 versus 77.24±2.25 and 74.21±3.50%; P>0.05, n=20). It should be noted that early time points could not be tested for detection of kinetic differences in platelet consumption due to animal welfare considerations. Consistent with these observations, histologic sections of lung tissue and analysis of occlusive thrombi in the lungs at the end of the experiment revealed similar numbers of occluded vessels between wild-type, Ceacam1+/− and Ceacam1+/− mice (data not shown). In contrast, wild-type and Ceacam1+/− mice were equally susceptible and had comparable reductions in circulating platelets over time following tissue thromboplastin-induced pulmonary thromboembolism (Figure 7C-D)(P>0.05, n=10). Collectively, these data highlight that CEACAM1 regulates platelet-collagen interactions in controlling susceptibility to Type I collagen-induced pulmonary thromboembolism in vivo.
Discussion

Using several approaches, we have defined a novel role of CEACAM1 as a new inhibitory co-receptor in murine platelets that serves to negatively modulate platelet-collagen interactions and regulate platelet thrombus formation \textit{in vitro} and \textit{in vivo}. This study suggests that CEACAM1 serves an autoregulatory role when platelets come into contact with each other when exposed to Type I collagen. Firstly, CEACAM1 is expressed on the surface and in intracellular pools of murine and human platelets. Secondly, CEACAM1 serves as a negative regulator of collagen GPVI-mediated platelet responses \textit{in vitro}. Thirdly, CEACAM1 serves to negatively regulate platelet thrombus formation \textit{in vitro} and \textit{in vivo} under conditions where Type I collagen is exposed in the injured arteriole and in the presence of collagen GPVI receptor on platelets. Finally, CEACAM1 serves as a negative regulator of thrombosis following Type I collagen-induced pulmonary thromboembolism \textit{in vivo}.

There is little evidence in the literature in relation to the presence and functional role of CEACAM1 in platelets. The only exception is a report by Hansson \textit{et al.}, 1990 which suggested that C-CAM (old terminology) was present in the intracellular compartment of rat platelets. In these studies, these workers only found significant amounts of C-CAM when $^{125}$I-labelled rat platelets were solubilised with detergent and immunoprecipitated with C-CAM antibodies. These workers suggested that almost no $^{125}$I-labelled C-CAM could be immunoprecipitated from intact rat platelets under resting or ADP-activated conditions. This study suggested that intracellular expressed C-CAM may play a functional role under conditions of platelet activation. This concept is supported by studies of CEACAM1 in leukocytes. Traditionally, CEACAM1 (formerly known as
CD66a) was defined as a neutrophil-specific “activation antigen” in that it is detected in low density on resting cells but its surface expression is upregulated upon stimulation with the chemotactic peptide FMLP, calcium ionophore A23187 and 12-O-tetradecanoyl-phorbol-13-acetate.\textsuperscript{35} Subcellular localization studies revealed that CEACAM1 was present in the secondary granules of neutrophils suggesting that antigens could be recruited to the cell surface with activation.\textsuperscript{36} In contrast, in mouse spleen T lymphocytes, CEACAM1 was not present on the surface of resting cells but was rapidly upregulated on CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells after activation with either Con A or anti-CD3 stimulation.\textsuperscript{37} Overall, these studies in leukocytes are somewhat different from the CEACAM1 expression profile that we have observed in murine and human platelets with moderate basal expression on the surface of resting platelets with an intracellular pool evident (Fig.1A-D). This was further supported by agonist stimulation using thrombin that resulted in upregulation of cell surface expression of CEACAM1 on murine and human platelets (Fig.1E-F).

There is now a consensus view that platelet thrombus formation under physiological conditions involves platelet adhesion to subendothelial matrix component, Type I collagen which becomes exposed by tissue trauma, injury or disease such as atherosclerotic plaque formation. A ‘two-site, two-step’ model for platelet-collagen interactions has been proposed that requires the initial engagement of collagen to be mediated by the high-affinity receptor integrin α2β1, followed by activation generated through the low-affinity collagen receptor, GPVI.\textsuperscript{38,39} In this model, integrin α2β1 is now thought to have a supportive, rather than a primary role as the adhesive collagen receptor, while GPVI is a major platelet-collagen activating receptor that interacts with collagen
fibrils via a triplicate GPO sequence (glycine, proline, hydroxyproline) and integrin α2β1 interacts with collagen via GFOFER (glycine, phenylalanine, hydroxyproline, glutamic acid and arginine). For in vitro studies, we used collagen-related peptide (CRP) as the GPVI-selective agonist as this peptide contains ten GPO repeat sequences that serves as a potent activator of GPVI-mediated signaling and does not recognize integrin α2β1. In this way, we can differentiate GPVI-mediated platelet responses from integrin α2β1-mediated platelet responses. Signaling through GPVI/FcR gamma chain ITAM-dependent pathway is thought to be counterbalanced by proteins bearing ITIM motifs such as those contained in members of the Ig-ITIM superfamily. As mouse platelets lack the low-affinity ITAM containing FcγRIIa, but contain GPVI/FcR gamma chain ITAM-dependent pathway, we wanted to test the hypothesis that CEACAM1 may serve as an inhibitory co-receptor in platelets to negatively regulate collagen GPVI-mediated platelet responses. Consistent with this hypothesis, Ceacam1−/− platelets displayed hyper-tyrosine phosphorylated proteins including PLC gamma 2 following GPVI stimulation compared to wild-type platelets indicating modulation of ITAM-associated signaling events in murine platelets (Fig. 3).

In order to test this hypothesis, platelet aggregation studies were conducted to examine the slope and amplitude of platelet responses of wild-type versus CEACAM1-deficient platelets using a range of agonists and dose-dependent ranges including collagen and CRP. In addition, to test specificity of platelet responses, different agonists were selected to stimulate different receptor signaling pathways including G-protein-coupled receptors using PAR-4 and ADP, calcium ion channels using calcium ionophore and collagen
coupled receptors such as acid soluble collagen and CRP. \textit{Ceacam1}\textsuperscript{−/−} platelets displayed normal platelet aggregation responses over a dose-dependent range of PAR-4, ADP and calcium ionophore. In contrast, \textit{Ceacam1}\textsuperscript{−/−} platelets displayed enhanced platelet aggregation responses over a dose-dependent range of collagen and CRP (Fig.4). This hyper-responsive feature was most evident at subthreshold concentrations of either collagen or CRP indicating that CEACAM1 serves to negatively regulate collagen GPVI-mediated ITAM platelet responses. Importantly, the fact that the platelet aggregation responses to PAR-4, ADP and calcium ionophore were normal reveals that \textit{Ceacam1}\textsuperscript{−/−} platelets do not have a global platelet defect and that the defect is restricted to GPVI-mediated platelet-collagen interactions.

This hyper-responsive feature of \textit{Ceacam1}\textsuperscript{−/−} platelets with either subthreshold doses of collagen or CRP is consistent with the phenotype observed by us and others with \textit{Pecam1}\textsuperscript{−/−} platelets.\textsuperscript{1,2} The subtle phenotype was most evident at concentrations of collagen at 1 μg/mL but was not so evident at higher concentrations of collagen 10-30 μg/mL. This was also the case using CRP as the GPVI-selective agonist. At 0.5 μg/mL CRP, wild-type platelets were not responsive while \textit{Pecam-1}\textsuperscript{−/−} platelets yielded approx. 70% CRP-mediated platelet aggregation response. At 5-10 μg/mL CRP, wild-type and \textit{Pecam-1}\textsuperscript{−/−} platelets yielded less hyper-responsive platelet responses.\textsuperscript{1,2}

Platelets from \textit{Ceacam1}\textsuperscript{−/−} mice exhibited increased adhesion on Type I fibrillar collagen but not fibrinogen. This hyper-responsive adhesion phenotype of \textit{Ceacam1}\textsuperscript{−/−} platelets on Type I fibrillar collagen was evident at all time points. In addition, \textit{Ceacam1}\textsuperscript{−/−} platelets
displayed a lowered threshold for alpha and dense granule release relative to wild-type and Ceacam1+/− platelets following stimulation with GPVI-selective ligand, CRP particularly at 0.1-0.5 μg/mL and type I collagen at 1 μg/mL but not G-protein-coupled receptor agonist, thrombin. Overall, these features suggest that in the absence of an inhibitory co-receptor, platelets are more responsive to stimulation by collagen agonists particularly involving collagen GPVI receptor-mediated signaling. This trend is consistent with CEACAM1 serving as a negative regulator of platelet-collagen interactions.

In order to investigate the role of CEACAM1 in thrombus formation in vitro and in vivo, we employed intravital microscopy to study thrombus formation in real time and ferric chloride-induced vascular injury of mesenteric arterioles and in vitro flow studies on immobilised Type I collagen. Using this ferric chloride-induced vascular injury model results in exposure of subendothelial collagens including Type I collagen. This enables collagen GPVI platelet interactions to occur with exposed Type I collagen. Under these conditions, we demonstrated that thrombi formed in mesenteric arterioles following ferric chloride injury in CEACAM1 knockout mice were significantly larger in area and volume reflecting increased thrombus growth than those formed in wild-type and Ceacam1+/− mice (Fig.6B-C). These differences in thrombus size were consistent over time. In addition, thrombi formed in CEACAM1 knockout mice appear to have greater stability than wild-type and Ceacam1+/− mice (Fig.6D). In addition, this in vivo thrombus growth phenotype observed in Ceacam1−/− mice was dependent on the presence of collagen GPVI receptor on platelets (Fig. 6E-F). These data are consistent with
CEACAM1 playing a role in negative regulation of thrombus growth in vivo in mesenteric arterioles. In addition, our studies have shown an important role for CEACAM1 in regulating thrombus formation on immobilised collagen under in vitro flow conditions (Fig.6F). As CEACAM1 is expressed on platelets, leukocytes and endothelium further studies will be required to dissect out the relative importance of platelet CEACAM1 versus endothelial CEACAM1 in regulating thrombus growth in vivo. Importantly, CEACAM1 appears to represent a second example of an Ig-ITIM superfamily member that plays an important functional role in negatively regulating stationary adhesion of platelet-collagen interactions and thrombus growth in vivo, but not tethering and rolling of platelets on the thrombogenic collagen surface.

Previous studies with PECAM-1-deficient mice in the ferric chloride induced vascular injury model of carotid arteries revealed a subtle defect in the time to 75% vessel occlusion. The mean occlusion rate of Pecam-1−/− mice was shorter than wild-type control mice. However, the magnitude of difference was only very modest. A more noticeable difference was observed in the kinetics of platelet thrombus formation using the laser-induced injury model that requires tissue factor generated platelet/fibrin thrombus and not Type I collagen exposure. Therefore, it appears that PECAM-1 and CEACAM1 both play a role in negatively regulating platelet-collagen interactions in vivo.

In order to examine the consequences of CEACAM1 deficiency in vivo in platelet-collagen interactions, we utilised a second model of Type I collagen-induced pulmonary
thromboembolism. In this model, type I fibrillar collagen is infused intravenously and platelet thrombi are deposited in the lung architecture leading to development of pulmonary thromboembolism and restrained breathing that in wild-type mice ultimately leads to morbidity. Using this model, monitoring of the restrained breathing rate of the mice revealed that Ceacam1−/− mice have enhanced susceptibility to Type I collagen induced pulmonary thromboembolism but not tissue thromboplastin induced pulmonary thromboembolism compared to wild-type and Ceacam1+/− mice (Fig.7B and 7D). Ceacam1−/− mice showed a reduction in time for restrained breathing following challenge with Type I fibrillar collagen compared to wild-type and Ceacam1+/− mice (Fig.7B) despite having comparable consumption of platelets (Fig.7A). These data further implicate CEACAM1 as a physiological regulator of platelet-collagen interactions in vivo.

In conclusion, this study has defined a new inhibitory co-receptor, CEACAM1 present in murine and human platelets that is expressed on the surface and in intracellular compartments. CEACAM1 serves as a negative physiological regulator of platelet-collagen interactions as shown by its hyper-responsive features in collagen and CRP-mediated platelet aggregation, increased adhesion on Type I collagen and enhanced alpha and dense granule release using GPVI-selective ligand. In vitro and in vivo, CEACAM1 serves to negatively regulate arteriolar thrombus growth and thrombosis associated with Type I collagen induced pulmonary thromboembolism. These negative regulatory properties of CEACAM1 may prove to be of physiological benefit in diseased vessels such as rupture of atherosclerotic plaques where Type I collagen is exposed.
Acknowledgements

C.Wong, Y.Liu, J. Yip, R. Chand, J. Wee, A. Reheman and L. Oates performed research and analysed data; N. Beauchemin supplied the CEACAM1-deficient mice, B. Nieswandt supplied the JAQ1 antibody and advice for the GPVI depletion experiment, H. Ni directed the in vitro flow experiments and DE Jackson directed the research and wrote the paper.
References


6. Chemnitz JM, Parry RV, Nichols KE, June CH and Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon
primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J. Immunol.* 2004;173:945-954.


Figure Legends

Figure 1. CEACAM1 is expressed on the surface and in intracellular pools in murine and human platelets.

A. Flow cytometric analysis of CEACAM1 surface and total expression on resting murine platelets. Platelets were stained with a polyclonal anti-murine CEACAM1 2457 antibody followed by a secondary FITC-conjugated anti-rabbit antibody. Normal rabbit serum and Ceacam1<sup>−/−</sup> platelets were included as negative controls. Data was collected through a live platelet gate based on forward versus side scatter profiles on a FACS Canto flow cytometer. Results are cumulative data derived from three independent experiments and represented as mean fluorescence intensity (MFI) ± SEM.

B. Flow cytometric analysis of CEACAM1 surface and total expression on resting human platelets. Platelets were stained with a monoclonal anti-human CEACAM1 4D1C2 antibody followed by a secondary FITC-conjugated anti-mouse antibody. Normal mouse IgG and Ceacam1<sup>−/−</sup> platelets were included as negative controls. Data was collected through a live platelet gate based on forward versus side scatter profiles on a FACS Canto flow cytometer. Results are cumulative data derived from three independent experiments and represented as mean fluorescence intensity (MFI) ± SEM.

C-D. Representative histogram profiles of CEACAM1 surface and total expression on resting murine and human platelets. CEACAM1 surface and total expression was determined as described in A and B.

E. Agonist stimulation of murine platelets using thrombin (0.25-1.00 U/mL) over a dose-dependent range. CEACAM1 surface expression was determined as described in A.
F. Agonist stimulation of human platelets using thrombin (0.25-1.00 U/mL) over a dose-dependent range. CEACAM1 surface expression was determined as described in B.

G. Flow cytometric analysis of CEACAM1 surface expression on resting murine platelets from wild-type versus Ceacam1<sup>+/−</sup> and Ceacam1<sup>−/−</sup> mice. CEACAM1 surface expression was determined as described in A.

H. Representative histogram profiles of CEACAM1 surface expression on resting murine platelets from wild-type versus Ceacam1<sup>+/−</sup> and Ceacam1<sup>−/−</sup> mice.

**Figure 2. Ceacam1<sup>−/−</sup> platelets demonstrate increased adhesion on immobilised Type I fibrillar collagen.**

A. Wild-type, Ceacam1<sup>+/−</sup> and Ceacam1<sup>−/−</sup> platelets were allowed to adhere to type I fibrillar collagen (50 μg/mL) in the absence of magnesium, or to plates coated with bovine serum albumin (50 μg/mL) for 15, 30, 45 or 60 minutes at 37°C. After removal of non-adherent platelets, adherent platelets were measured as described in Materials and Methods. Each assay was performed in triplicate and is representative of three independent experiments. Each data point represents platelet adhesion per high powered field and was expressed as the mean ± SEM. Note that wild-type, Ceacam1<sup>+/−</sup> and Ceacam1<sup>−/−</sup> platelets bound to a similar extent on BSA-coated platelets, where at all time points, Ceacam1<sup>−/−</sup> platelets showed higher levels of adhesion to type I fibrillar collagen compared to wild-type and Ceacam1<sup>+/−</sup> platelets (***P<0.005, n=3).

B. As described in A, but for platelet adhesion on fibrinogen (50 μg/mL) for wild-type, Ceacam1<sup>+/−</sup> and Ceacam1<sup>−/−</sup> platelets for 15, 30, 45 or 60 minutes at 37°C.
C. Representative micrographs of platelet adhesion on Type I fibrillar collagen (50 μg/mL) for wild-type and Ceacam1−/− platelets over time. Adherent platelets were fixed and imaged for DIC microscopy (x 63 oil objective).

Figure 3. Ceacam1−/− platelets display hyper-phosphorylated proteins including PLC-gamma2 following CRP stimulation over time.

A. Tyrosine phosphorylation in whole-cell platelet lysates from wild-type and Ceacam1−/− mice were stimulated with CRP (10 μg/mL) over a 3 min time frame. 30 μg of each platelet lysate was loaded onto a 10% SDS-PAGE gel and tyrosine phosphorylation detected on the western blot using a HRP-conjugated anti-phosphotyrosine RC20 antibody. Erk-2 blot (lower panel) was included as a protein loading control. This is a representative blot of three experiments performed. B. PLCγ2 was immunoprecipitated from lysates of wild-type or Ceacam1−/− platelets stimulated by CRP (10 μg/mL) at T=0 and T=90 seconds and immunoblotted for phosphotyrosine using a HRP-conjugated anti-phosphotyrosine RC20 antibody. PLCγ2 antigen is included as a loading control of the immunoprecipitates (lower panel). Relative intensity of tyrosine phosphorylated PLCγ2 to total PLCγ2 antigen was quantitated using Image J software version 1.40g from three replicate experiments.

Figure 4. Ceacam1−/− platelets show hyper-responsive aggregation in response to stimulation with GPVI-selective agonists.

Aggregation responses of PRP (platelet count adjusted to 100 x10^9/l) for wild-type and Ceacam1−/− mice were determined following activation with different concentrations of
various agonists: PAR-4 agonist peptide (100-250 μM), ADP (5-20 μM), calcium ionophore (0.3-2.5 μg/mL), collagen-related peptide (0.62-2.5 μg/mL) and collagen (1.5-4 μg/mL) respectively. Note that Ceacam1⁺⁻ platelets are hyper-responsive in collagen and CRP-induced platelet aggregation. These experiments are representative of at least three independent experiments performed.

Figure 5. Ceacam1⁺⁻ platelets display a lower threshold in alpha and dense granule release in response to stimulation with GPVI-selective agonists.

A. Wild-type (black bars), Ceacam1⁺⁻ (gray bars) and Ceacam1⁺⁻ platelets (white bars) were stimulated with varying concentrations of GPVI-selective agonists including type I fibrillar collagen in the absence of magnesium and CRP, or with thrombin. The amount of 5-hydroxytryptamine released was measured as described in Materials and Methods. The assays were performed in triplicate, and the results expressed as the mean ± SEM. These results are representative of three independent experiments. Note that the release of dense granular contents in response to stimulation at subthreshold levels of GPVI-selective agonists was elevated in Ceacam1⁺⁻ platelets compared to the wild-type and Ceacam1⁺⁻ platelets.

B. Surface expression of P-selectin (alpha granule release) was determined for washed platelets stimulated by thrombin, PAR-4 agonist peptide or collagen-related peptide (CRP) at different concentrations and then stained with either a buffer control and FITC-P-selectin mAb for both wild-type and Ceacam1⁺⁻ platelets. FITC-labelled samples were analyzed on a FACS Canto analyzer. Results are representative of three independent experiments.
Figure 6. Thrombi are larger and more stable in Ceacam1−/− mice both in vitro and in vivo.

A. Images of thrombus formation in response to ferric chloride induced vascular injury was visualized in arterioles of wild-type versus Ceacam1−/− mice over time. The different lengths of time after ferric chloride application are indicated. Note that Ceacam1−/− arterioles formed larger thrombi over time compared to wild-type control arterioles (n=15).

B-D. Quantitative analysis of arterial thrombogenesis of wild-type, Ceacam1+/− and Ceacam1−/− arterioles. Compared to wild-type arterioles, Ceacam1−/− arterioles exhibited a significant increase in thrombus area over two minutes (6212±268.7 versus 3294±223.4 and 2859±294 μm²; ***P<0.0001, n=15), showed greater stability in thrombi formed (4.07±0.37 versus 2.40±0.21 and 1.05±0.12; ***P<0.001, n=15) and increased thrombus volume (122400±6794 versus 49430±4602 and 63680±5478 μm³; ***P<0.0001, n=15).

The primary stability of the thrombi was scored from 1 to 10, with 1 being 0-10% occupancy and 10 being 91-100% occupancy (ie. complete vessel occlusion) monitored over time.

E-F. Platelet thrombus formation following inhibition of GPVI using monoclonal antibody JAQ1 administration to Ceacam1+/− and Ceacam1−/− mice compared to control IgG treated Ceacam1+/− and Ceacam1−/− or untreated Ceacam1+/− and Ceacam1−/− mice. Mice received either 100 μg Control IgG or JAQ1 antibody and were left for 5 days before ferric chloride injury and intravital microscopy. Compared to untreated Ceacam1−/− or control IgG treated Ceacam1−/− arterioles, JAQ1 treated Ceacam1−/− arterioles
exhibited a 3-fold reduction in thrombus volume over two minutes (135500±2137 versus 134000±1837 compared to 40400±1127 μm³; ***P<0.001, n=10 arterioles from 3 mice/group) and a 2-fold reduction in stability score over two minutes (6.150±0.2115 versus 5.700±0.3000 compared to 3.350±0.2115; ***P<0.001, n=10 arterioles from 3 mice/group). In contrast, compared to untreated Ceacam1+/+ or control IgG treated Ceacam1+/+ arterioles, JAQ1 treated Ceacam1+/+ arterioles exhibited a 3-fold reduction in thrombus volume over two minutes (107800±3526 versus 102100±2607 compared to 32050±599 μm³; ***P<0.001, n=10 arterioles from 3 mice/group) and a modest reduction in stability score over two minutes (2.750±0.2609 versus 3.000±0.2357 compared to 2.150±0.0764; ***P<0.001, n=10 arterioles from 3 mice/group).

G. DiOC₆-labelled whole blood of wild-type, Ceacam1+/− and Ceacam1−/− mice was perfused over 100 μg/mL Type I fibrillar collagen-coated microslides at a wall shear rate of 1800 seconds⁻¹. Thrombi were imaged at 4 minutes of blood flow by confocal microscopy (x60, 1-μm sections) and thrombus was quantified using Slidebook software (Intelligent Imaging Innovations Inc., Denver, CO, USA). Each data point showed on the graph represents the thrombus volume for each individual mouse performed independently.

Figure 7. Ceacam1−/− mice are more susceptible to Type I collagen-induced pulmonary thromboembolism.

A. The percentage reduction in platelet count over three minutes for wild-type, Ceacam1+/− and Ceacam1−/− mice after intravenous injection of 350 μg/kg type I fibrillar
collagen was determined. Note that wild-type, *Ceacam1*+/− and *Ceacam1*+/+ mice showed a similar reduction in platelet count over time (77.24±2.246 versus 73.49±3.157 and 74.21±3.507%; P>0.05, n=20).

B. The time until restrained breathing, quantified by a breathing interval of 10 secs, for wild-type and *Ceacam1*−/− mice following intravenous injection of 350 μg/kg of type I fibrillar collagen. Note that *Ceacam1*−/− mice showed enhanced susceptibility to type I collagen-induced pulmonary thromboembolism compared to wild-type control mice (343.5±23.73 versus 327.0±28.29 and 252.1±15.39 secs; **P<0.001, n=20).

C. The percentage reduction in platelet count over three minutes for wild-type and *Ceacam1*+/− mice after intravenous injection of 0.200 μl/g tissue thromboplastin Thromborel S was determined. Note that wild-type and *Ceacam1*+/− mice showed a similar reduction in platelet count over time (94.30±2.162 versus 93.22±1.755%; P>0.05, n=10).

D. The time until restrained breathing, quantified by a breathing interval of 10 secs, for wild-type and *Ceacam1*+/− mice following intravenous injection of 0.200 μl/g tissue thromboplastin Thromborel S was determined. Note that *Ceacam1*+/− mice showed equal susceptibility to tissue thromboplastin induced pulmonary thromboembolism compared to wild-type control mice (214.2±4.052 versus 223.8±7.979 secs; P>0.05, n=10).
Table 1. Summary of peripheral blood haematological parameters for Ceacam1⁻⁻ mice (n=18).

<table>
<thead>
<tr>
<th>Haematological parameter</th>
<th>Ceacam1⁻⁻</th>
<th>Ceacam1⁺⁺</th>
<th>P value</th>
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<tr>
<td>WCC (x 10⁹/l)</td>
<td>1.63±0.17</td>
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<tr>
<td>Hb (g/l)</td>
<td>107.8±2.7</td>
<td>108.3±3.0</td>
<td>&gt;0.05</td>
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<tr>
<td>RBC (x 10¹²/l)</td>
<td>8.34±0.148</td>
<td>8.66±0.230</td>
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<tr>
<td>PCV (l/l)</td>
<td>0.396±0.007</td>
<td>0.412±0.009</td>
<td>&gt;0.05</td>
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<tr>
<td>MCV (fl)</td>
<td>474.8±1.35</td>
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<td>&gt;0.05</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>129.6±3.37</td>
<td>125.5±1.53</td>
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<td>MCHC (x 10³ g/l)</td>
<td>2.73±0.07</td>
<td>2.62±0.03</td>
<td>&gt;0.05</td>
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<td>Plat (x 10⁹/l)</td>
<td>776±39</td>
<td>680±65</td>
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<td>% Neut./100 WBC</td>
<td>9.71±0.84</td>
<td>13.78±1.88</td>
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<td>% Lymph./100 WBC</td>
<td>83.8±1.7</td>
<td>78.2±2.7</td>
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<tr>
<td>% Mono./100 WBC</td>
<td>6.29±1.21</td>
<td>7.56±1.14</td>
<td>&gt;0.05</td>
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WCC  White cell count  
Hb  Haemoglobin  
RBC  Red blood cell count  
PCV  Packed cell volume  
MCV  Mean cell volume  
MCH  Mean cell haemoglobin  
MCHC  Mean cell haemoglobin concentration  
Plat.  Platelet count
Figure 1.

A. Control Antibody
Rabbit anti-CEACAM1

B. Control Antibody
Mouse anti-CEACAM1

C. Mouse Extracellular
Rabbit anti-CEACAM1
Control Antibody

D. Human Extracellular
Mouse anti-CEACAM1
Control Antibody

E. Murine
Rabbit anti-CEACAM1

F. Human
Control
ADV13

G. Ceacam1 Expression

H. Ceacam1 Expression

MFI

Platelet only
NRS
Ceacam1

No of cells

FITC
Table 2. Glycoprotein expression in Ceacam1<sup>−/−</sup> mice.

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>Wild-type mice (MFI±SEM)(n=3)</th>
<th>Ceacam1&lt;sup&gt;−/−&lt;/sup&gt; mice (MFI±SEM)(n=3)</th>
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<tr>
<td>GPVI</td>
<td>87.9 ± 5.9</td>
<td>78.3 ± 9.8</td>
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<tr>
<td>Integrin αIIbβ3</td>
<td>254.7 ± 15.6</td>
<td>245.0 ± 16.5</td>
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<tr>
<td>GPIbα/IIXV complex</td>
<td>242.6 ± 23.2</td>
<td>231.9 ± 29.4</td>
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<tr>
<td>Integrin α2β1</td>
<td>60.7 ± 1.9</td>
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<tr>
<td>PECAM-1</td>
<td>39.9 ± 12.7</td>
<td>32.4 ± 10.1</td>
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<tr>
<td>CD9</td>
<td>258.2 ± 1.0</td>
<td>256.3 ± 43.7</td>
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<tr>
<td>CD44</td>
<td>134.7 ± 2.5</td>
<td>113.3 ± 10.8</td>
</tr>
<tr>
<td>CEACAM1</td>
<td>39.7 ± 1.4</td>
<td>3.8 ± 0.04</td>
</tr>
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Cell surface expression of platelet glycoprotein expression was monitored by flow cytometry using specific monoclonal antibodies for wild-type and Ceacam1<sup>−/−</sup> mice. Mean fluorescence intensity (MFI) were reported with a standard error of mean for three independent experiments.
Figure 3.

A.

![Image of a gel blot showing time-course analysis of phosphorylated tyrosine (Phos-tyrosine) and ERK-2 levels in wild-type and cecam1^-/- mice.](image)

B.

![Image of Western blots showing phosphorylated PLC2 (pTyr PLC2) and total PLC2 levels in +/+ and +/- mice with and without CRP stimulation.](image)

Relative increase in intensity of pTyr PLC2 to total PLC2:
- +/+: 1.1 ± 0.2%
- +/-: 8.6 ± 0.4%
- +/+ Rest.: 35.7 ± 3.2%
- +/- CRP: 100 ± 5.4%
Figure 4.
Figure 5.

A. 5-Hydroxy-3[H]-tryptamine release (%)

<table>
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<tr>
<th>Condition</th>
<th>Resting</th>
<th>Thrombin 0.1U/ml</th>
<th>Thrombin 1.0 U/ml</th>
<th>Fibrillar Collagen 1.0 μg/mL</th>
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B. P-selectin exposure (MFI)

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<th>Agonist</th>
<th>Platelet only</th>
<th>Resting</th>
<th>THR 0.5 U/ml</th>
<th>THR 1.0 U/ml</th>
<th>PAR4 100 μM</th>
<th>PAR4 300 μM</th>
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Figure 6.

A. Wild-type C57BL/6

T=2 mins  
T=4 mins  
T=6 mins

T=2 mins  
T=4 mins  
T=6 mins

Ceacam1<sup>−/−</sup>

B. Thrombus Area

C. Thrombus Volume

D. Stability Score

E. Thrombus Volume

F. Stability Score

G. In vitro Thrombus Volume

---

*P<0.0001

*P<0.001

*P<0.0001

*P<0.05
Figure 7.

A. % Platelet reduction over 3 mins

B. Time until restrained breathing

C. % Platelet reduction

D. Time until restrained breathing