TRAIL/Apo2L Mediates the Release of Procoagulant Endothelial Microparticles Induced by Thrombin In Vitro
A Potential Mechanism Linking Inflammation and Coagulation

Stéphanie Simoncini,* Makon-Sébastien Njock,* Stéphane Robert, Laurence Camoin-Jau, José Sampol, Jean-Robert Harlé, Catherine Nguyen, Françoise Dignat-George, Francine Anfosso

Abstract—Microparticles are small vesicles playing a crucial role in cell communication by promoting prothrombotic and proinflammatory responses. However, the molecular mechanisms underlying their release are still elusive. We previously established that thrombin promoted the generation of endothelial microparticles (EMPs). In the present study, gene profiling identified TRAIL/Apo2L, a cytokine belonging to the tumor necrosis factor-α superfamily, as a target of thrombin. Thrombin increased the expression of cell-associated and soluble forms of TRAIL (sTRAIL) in HMEC-1 cells and human umbilical vein endothelial cells (HUVECs). Blocking TRAIL by specific antibodies or by small interfering RNA reduced both the number and the procoagulant activity of EMPs released by thrombin. Consistent with an involvement of sTRAIL in thrombin-induced EMP release, we showed that (1) exogenously added sTRAIL generated procoagulant EMPs; (2) supernatants from thrombin-stimulated endothelial cells induced EMP release by HMEC-1 cells and HUVECs, whereas those recovered from TRAIL knockdown endothelial cells displayed no effect. TRAIL/TRAIL-R2 complex mediated EMP release by initiating the recruitment of adaptor proteins and the activation of nuclear factor κB. Moreover, sTRAIL modulated intercellular adhesion molecule-1 and interleukin-8 expression induced by thrombin by a downstream pathway involving nuclear factor κB activation. Our data reveal a novel mechanism controlling EMP release and identify TRAIL as a key partner in the pathway linking coagulation and inflammation elicited by thrombin. (Circ Res. 2009;104:943-951.)

Key Words: endothelium ■ vesiculation ■ cell signaling ■ inflammation ■ coagulation ■ thrombosis

Thrombin is a serine protease that plays a key role in the pathogenesis of vascular diseases. It is generated at sites of vessel damage and controls the formation of a hemostatic plug. Thrombin plays a central role in the crosstalk between inflammation and coagulation by inducing the synthesis of proinflammatory cytokines that activates the coagulation cascade and by impairing anticoagulant mechanisms. It also exerts pleiotropic effects on endothelium. Recent data from our laboratory indicated that thrombin induces the release of microparticles by endothelial cells (endothelial microparticles [EMPs]), an effect mediated by the receptor PAR-1.

Microparticles (MPs) are a heterogeneous population of small membrane–coated vesicles resulting from disruption of the membrane phospholipid asymmetry, leading to phosphatidylinerse exposure in response to cell activation or apoptosis. Their capacity to carry surface antigens, cytoplasmic proteins, or nucleic acids from their parent cells confers to MPs a major role in cell communication by binding to their targets or facilitating cell–cell interactions. In theory, all cells have the capacity to form MPs and endothelial cells generate EMPs that provide procoagulant surfaces able to trigger coagulation activation. Indeed, once available at the surface of the MP, phosphatidylinerse exposure promotes the assembly of the enzymes of the clotting system and provides a catalytic surface for tissue factor (TF) activity. Moreover, binding of EMPs to THP1 cells induces the synthesis of TF contributing to amplify a procoagulant pathway. In addition, EMPs also provide catalytic surfaces for the conversion of plasminogen into plasmin. The expression of other proteolytic systems such as matrix metalloproteinases confers to MPs a putative role in vascular remodeling and angiogenesis in vitro.

In human diseases, elevated levels of EMPs are found in disorders associated with thrombotic and/or inflammatory events such as diabetes, antiphospholipid syndrome, acute renal failure, sickle cell anemia, coronary syndrome, or preeclampsia. It is now obvious that elevated EMP levels are...
associated with most of the cardiovascular risk factors, are often correlated with endothelial dysfunction, and are associated with a poor clinical outcome. Although EMPs raised considerable interest as markers of vascular damage, the mechanisms leading to their generation are poorly understood.

We previously showed that the release of EMPs by HMEC-1 cells required the recruitment of caspase-2 in the absence of an apoptotic cell death. Caspase-2, in turn, activated the Rho-kinase II by its proteolytic activity. In the present study, using gene expression profiling, we identified TRAIL, a cytokine belonging to the tumor necrosis factor (TNF)-α superfamily, as a target of thrombin in HMEC-1 cells. The upregulation of the soluble form of TRAIL (sTRAIL) increases the release of EMPs carrying a procoagulant activity by a signaling network involving the interaction with the receptor TRAIL-R2, the recruitment of the adaptor proteins TRADD, TRAF2 and the kinase RIP1, and the activation of the transcription factor nuclear factor (NF)-κB in HMEC-1 cells and in the human umbilical vein endothelial cells (HUVECs). Moreover, sTRAIL participates to the thrombin-mediated upregulation of the inflammatory mediators intercellular adhesion molecule (ICAM)-1 and interleukin (IL)-8 by a pathway requiring NF-κB. Our data indicate a novel mechanism linking TRAIL/TRAIL-R2 system to the amplification of the endothelial vesiculation induced by thrombin and identify TRAIL as a key partner in the crosstalk between inflammation and coagulation.

Materials and Methods
Details of endothelial cultures, transfections, flow cytometry, and ELISA are described in the online data supplement at http://circres.ahajournals.org.

Results
Thrombin Generates Procoagulant EMPs
Thrombin induced the release of EMPs by HMEC-1 cells. We investigated whether these EMPs carried a procoagulant activity in a clotting assay measuring their capacity to convert FX into FXa. Thrombin induced a dose-dependent increase in FXa generation by HMEC-1 and HUVEC-derived EMPs (Figure 1A). Preincubation of EMPs with blocking monoclonal antibody against TF inhibited the procoagulant activity induced by thrombin (Figure 1B, lanes f versus d). These data indicate that thrombin generated EMPs carrying a TF-dependent procoagulant activity.

TRAIL Is Modulated by Thrombin
Thrombin modulates genes linked to inflammation and coagulation. By cDNA microarray, we identified a cluster belonging to the TNF-α superfamily (Figure I, A, in the online data supplement) and including TNFSF10 (TRAIL), a cytokine that displays multiple effects on endothelial cells. Relative quantification of changes in gene expression indicated a long-lasting upregulation of TRAIL in response to thrombin stimulation (Online Figure I, B).

Validation of microarray data by real-time PCR confirmed the upregulation of TRAIL expression that peaked at 6 hours in HMEC-1 and HUVECs (Figure 2A). Western blot indicated an increased protein expression peaking at 10 hours (Figure 2B). TRAIL is a type II transmembrane protein.

Thrombin induced a moderate increase in its transmembrane form in HMEC-1 and HUVECs (Online Figure II).

TRAIL interacts with 5 receptors, including 2 decoy receptors, a soluble receptor (osteoprotegerin), and 2 transmembrane receptors (DR4 [TRAIL-R1] and DR5 [TRAIL-R2]). Because these latter mediate signaling pathways, we determined whether thrombin modulated their expression. Thrombin upregulated TRAIL-R2 mRNA in HMEC-1 and HUVECs (Figure 2A) and also increased TRAIL-R2 protein (Figure 2B) and its membrane-bound form (Online Figure II). In contrast, TRAIL-R1 expression was unaffected by thrombin (Figure 2A and 2B and Online Figure II). TRAIL also exists as a full-active soluble protein (sTRAIL) and thrombin increased its release in cell-free supernatants (Figure 2C).

We previously showed that ROCK-II mediated EMP generation by thrombin. Stimulation of HMEC-1 or HUVECs by thrombin in the presence of the selective inhibitor of the Rho-kinase Y27632 (1 μmol/L) did not inhibit the release of sTRAIL (Online Table I) and did not modify TRAIL-R2 and TRAIL protein expression (Online Figure II, B). TRAP, the agonist peptide mimicking the effects of thrombin, induced EMP generation by HMEC-1 and HUVECs but did not modify sTRAIL levels (Online Table I). These findings showed that
thrombin upregulated the TRAIL/TRAIL-R2 system at the gene and protein levels and triggered the release of sTRAIL.

**TRAIL and TRAIL-R2 Are Involved in the Generation of Procoagulant EMPs by Thrombin**

The involvement of TRAIL/TRAIL-R2 complex in the generation of EMPs by thrombin was investigated by antibody blockade or specific cell silencing. Stimulation with thrombin in the presence of neutralizing anti-TRAIL or anti–TRAIL-R2 antibodies (Abs) resulted in an inhibition of EMP release (Online Figure III, A and B) that reached 40% and 32% inhibition in HMEC-1 and HUVECs, respectively (Figure 3A, lanes f and g versus e). Addition of neutralizing anti-TRAIL Abs at different times after the onset of thrombin stimulation, showed a significant inhibition of thrombin-induced EMP release beginning at 4 hours (Online Figure IV, A). When anti-TRAIL Abs were added at early times (2 or 3 hours), the inhibition only began after 4 hours of stimulation whereas anti-TRAIL Abs rapidly prevented the rise in EMP release between 4 and 10 hours. The data indicated that TRAIL did not control the early phases of EMP release.

The involvement of TRAIL and TRAIL-R2 in thrombin-induced EMP generation was confirmed by gene knockdown experiments. Three different small interfering (si)RNAs for each gene were tested for their capacity to reduce EMP release. Among them, TRAIL siRNA1 and TRAIL-R2

---

**Figure 2.** Thrombin-induced changes in TRAIL and TRAIL receptors in ECs. A, Real-time quantitative PCR analysis of the expression of TRAIL (ΔΔ), TRAIL-R1 (●●), and TRAIL-R2 (■■) mRNA in HMEC-1 or HUVECs. B, Time-course analysis of TRAIL and TRAIL receptors by Western blot in HMEC-1 and HUVECs: 40 μg of cell lysates were analyzed on 12% SDS-PAGE. The blots were probed with human anti-TRAIL, anti–TRAIL-R1, or anti–TRAIL-R2 monoclonal Abs and anti–β-tubulin as loading control. C, sTRAIL was assayed by ELISA in cell-free supernatants of HMEC-1 or HUVECs kept untreated (ΔΔΔΔ) or stimulated with thrombin (■■■■). Curves represent means±SEM (n=9). **P<0.03, ***P<0.001.
siRNA3 gave the more pronounced reduction of EMPs (Online Figure V, A), protein of interest, and mRNA expression (Online Figure V, B and C). They were used for the subsequent experiments. Knockdown of TRAIL or TRAIL-R2 mRNA decreased the level of EMPs released by thrombin by HMEC-1 and HUVECs (Online Figure VI, A; Figure 3B, lanes h and i versus f) in a time-dependent manner (Online Figure VI, B). Specific silencing of TRAIL or TRAIL-R2 siRNA also reduced the procoagulant activity of EMPs mediated by thrombin (5 IU/mL), and the FXa generation was quantified. Bars represent means±SEM (n=6). *P<0.01 vs thrombin (lane b).

**sTRAIL Mediated the Generation of Procoagulant EMPs by Thrombin**

To ascertain the role of sTRAIL in EMP generation, we investigated the capacity of supernatants from thrombin-stimulated HMEC-1 or HUVECs to induce EMP release by naive cells.

Cell media from HMEC-1 or HUVECs stimulated with thrombin were ultracentrifuged to eliminate cell debris or small vesicles carrying TRAIL molecules. sTRAIL levels determined in the remaining supernatants were not modified by the ultracentrifugation (Figure 4A, lanes d versus c) but were greatly reduced in supernatants from TRAIL knockdown HMEC-1 and HUVECs (lanes e versus c) compared to TRAIL-R2 knockdown cells (lanes g versus c).

The transfer of these ultracentrifuged supernatants to untreated monolayers increased both the number (Figure 4B, upper pattern) and the procoagulant activity (Figure 4B, lower pattern) of EMPs released by untreated HMEC-1 (Figure 4B, lanes c versus a) or HUVECs (Figure 4B, lanes k versus i). This increase was not modified by addition of hirudin (20 mg/mL), an inhibitor of thrombin (Figure 4B, lanes d versus c and l versus k). EMP and FXa levels were significantly reduced with supernatants from TRAIL-silenced HMEC-1 (lanes e versus c) or HUVECs (Figure 4B, lanes m versus k), whereas supernatants from TRAIL-R2 knockdown cells had no effect (Figure 4B, lanes g versus c and o versus k).

Recombinant human TRAIL (rhTRAIL) was tested for its capacity to generate EMPs. rhTRAIL dose-dependently increased EMP numbers (Online Figure VII, A). At a dose of 20 ng/mL, a concentration of sTRAIL found in thrombin-stimulated supernatants, rhTRAIL significantly increased EMPs carrying a procoagulant activity dependent of TF (Online Figure VII, B). Both the increase in EMP levels and procoagulant activity were inhibited in TRAIL-R2 knockdown HMEC-1 or HUVECs. These data showed that sTRAIL contributed to the generation of procoagulant EMPs induced by thrombin and that the selective interaction between TRAIL and TRAIL-R2 controlled this release.

**Signaling Pathways Involved in TRAIL-Mediated EMP Release**

Following engagement by TRAIL, TRAIL receptors form a complex with TRADD or FADD, 2 adaptors, respectively, involved in survival or death signaling pathway. We investigated which of TRADD or FADD adaptors mediated the signaling events that controlled TRAIL-induced EMP release.

Coimmunoprecipitations with anti–TRAIL-R2 indicated that on thrombin stimulation, TRADD, but not FADD, was recovered in the immunoprecipitates of HMEC-1 and HUVECs (Figure 5A, lanes d and e versus b). These findings showed that TRAIL/TRAIL-R2 complex mediated the release of procoagulant EMPs initiated by thrombin.
precipitation showed the recruitment of both TRAF2 and RIP1 to TRAIL-R2 (Online Figure VIII, A) that was inhibited when TRAIL or TRAIL-R2 were silenced in HMEC-1 cells. These data indicated that TRAIL/TRAIL-R2 interaction induced a signaling complex at the plasma membrane.

Translocation to the nucleus of the transcription factor NF-κB occurs downstream the engagement of TRADD.19 We investigated the involvement of NF-κB in the TRAIL-mediated EMP release by thrombin. Thrombin induced a time-dependent increase in p65 nuclear translocation that was reduced in HMEC-1 or HUVECs stimulated with thrombin in the presence of hirudin (20 μg/mL), with ultracentrifuged supernatants obtained from TRAIL or TRAIL-R2 knockdown HMEC-1 or HUVECs stimulated with thrombin. EMPs were numbered in the cell media after an 18-hour incubation with the supernatants (upper graph), and their procoagulant activity was determined (lower graph). Bars represent means ± SEM (n = 4). NS indicates not significant, *P < 0.05, **P < 0.001 vs thrombin (lane c). Addition of anti-TRAIL inhibited the p65 increase only for times greater than 4 hours of stimulation (Online Figure IX, B). Nevertheless, the addition of BAY1170082 at different times of the thrombin stimulation, inhibited early and late phases of EMP release (Online Figure X). All of these data indicated that TRAIL controlled the late phases of NF-κB activation mediated by thrombin, whereas EMP release depended on NF-κB activation for all the thrombin stimulation.

The role of the signaling complex in EMP generation was then investigated. Silencing of TRADD, TRAF2, and RIP1 (Online Figure VIII, C) resulted in a reduction of EMP numbers in HMEC-1 cells and HUVECs. The extent of the inhibition was similar to that observed when TRAIL or TRAIL-R2 were knocked down (Figure 5C, lanes g, h). These results indicated that TRAIL/TRAIL-R2 interaction triggered a signaling complex that participated to the thrombin-induced EMP release.

Figure 4. Involvement of sTRAIL in the release of procoagulant EMPs. A, Effect of ultracentrifugation on the release of sTRAIL. HMEC-1 or HUVECs were stimulated with thrombin and culture media were ultracentrifuged at 75 000g for 90 minutes. sTRAIL was assayed in the ultracentrifuged supernatants by ELISA. Bars represent means ± SEM (n = 4). NS indicates not significant, **P < 0.05, ***P < 0.001 vs thrombin (lane c). B, Generation of EMPs by thrombin-induced sTRAIL. Untreated HMEC-1 or HUVECs were incubated, in the presence or absence of hirudin (20 μg/mL), with ultracentrifuged supernatants obtained from TRAIL or TRAIL-R2 knockdown HMEC-1 or HUVECs stimulated with thrombin. EMPs were enumerated in the cell media after an 18-hour incubation with the supernatants (upper graph), and their procoagulant activity was determined (lower graph). Bars represent means ± SEM (n = 4). NS indicates not significant, *P < 0.01, **P < 0.05 vs thrombin (lanes c and k, respectively).

Participation of sTRAIL to the Inflammation Induced by Thrombin

We investigated whether TRAIL and its downstream pathway participated to the thrombin-induced inflammation. Thrombin...
indicated that TRAIL/TRAIL-R2 interaction modulated the inflammatory phenotype by a downstream pathway involving the activation of NF-κB.

**Discussion**

The present study defines a novel mechanism controlling the in vitro release of procoagulant EMP generation in response to thrombin. For the first time, to our knowledge, we identify TRAIL as a target of thrombin. We demonstrate that the soluble form of TRAIL contributes to the release of procoagulant EMPs by thrombin. The interaction between sTRAIL and its receptor TRAIL-R2 initiates the recruitment of downstream adaptor proteins TRADD, TRAF2, RIP1, and NF-κB. Moreover, the engagement of this signaling pathway controlled the thrombin-mediated upregulation of the inflammatory mediators ICAM-1 and IL-8. Thus, the present study provides insight into the mechanisms of EMP generation and unravels a new function of TRAIL as a mediator between coagulation and inflammation in response to thrombin.

Thrombin induced EMP generation in HMEC-1 and HUVECs. The higher capacity of HUVECs than HMEC-1 to form EMPs in response to thrombin would represent either an increased responsiveness of their plasma membrane to thrombin or structural differences with respect to the localization of these cells on the vascular tree, respectively, on macro- and microvessels.

TRAIL secretion in the cell culture medium was increased in HMEC-1 and HUVECs and participated to the thrombin-induced EMP release both in the transformed endothelial cell line or cells directly extracted from the vessel. Inhibition of TRAIL by blocking Abs or by silencing TRAIL mRNA reduced by ∼40% EMP release by thrombin in HMEC-1 cells and ∼32% in HUVECs. The higher inhibition in HMEC-1 cells than in HUVECs would reflect the higher TRAIL secretion by HMEC-1 cells compared to HUVECs. Because the inhibition was not complete, other pathways may be additionally involved in EMP generation by thrombin.

We postulate that the soluble form of TRAIL mediates EMP release. In accordance with a role of sTRAIL, we demonstrated that (1) thrombin increased TRAIL levels in cell-conditioned medium. (2) Exogenous rhTRAIL induced EMP release at a concentration found for sTRAIL in culture medium of cells stimulated with thrombin. (3) EMP release by HMEC-1 and HUVECs incubated with culture media from thrombin-treated identical endothelial cells was abolished with supernatants from TRAIL-silenced endothelial cells. This latter experiment supported the importance of sTRAIL in EMP release by thrombin and excluded an effect of residual thrombin because hirudin21 prevented EMP generation and did not modify EMP numbers generated by the supernatants. Another important finding in this work is the hitherto undescribed role of sTRAIL in the mediation of procoagulant EMPs generated by thrombin. Indeed, thrombin elicited the expression of TF in endothelial cells,20,22 and EMPs displayed a procoagulant activity dependent on TF. When TRAIL was silenced, the EMP procoagulant activity was reduced. Moreover, the generation of TF-dependent procoagulant EMPs by exogenous rhTRAIL confirmed the ability of sTRAIL to generate procoagulant EMPs. Therefore,
we propose that sTRAIL mediates the amplification of procoagulant EMP release by thrombin.

Among the receptors interacting with TRAIL, TRAIL-R1 and TRAIL-R2 are involved in the signaling pathway of apoptotic cell death.37 Our study indicated a critical role of TRAIL-R2 in EMP release by thrombin. The upregulation of TRAIL-R2 by thrombin and the fact that the inhibition of TRAIL-R2 with blocking Abs or with its specific siRNA decreased EMP generation demonstrated that the interaction of TRAIL-R2 with TRAIL was a key element in EMP release. Moreover, TRAIL-R1 receptor displayed a low expression in HMEC-1 and HUVECs37 and an absence of modulation by thrombin. These data indicated an absence of redundancy between the two receptors or a different sensitivity toward TRAIL.23,24 in the control of thrombin-induced EMP release. The generation of EMPs and the modulation of TRAIL/TRAIL-R2 by thrombin depended on different pathways. Indeed, (1) Rho-kinases did not control their expression although modulating EMP release, and (2) TRAP did not increase TRAIL or TRAIL-R2, indicating that the proteolytic activity of thrombin was necessary for their modulation.

The interaction of TRAIL with TRAIL-R2 triggered the recruitment of the death domain–containing adapter protein TRADD. TRAIL-R2 has 2 overlapping signaling pathways. It associates with FADD or TRADD adapter molecules.18 FADD initiates a downstream pathway linked to apoptosis in FADD−/− mice25 and to caspase-8 activation.26 Nevertheless, FADD did not associated with TRAIL-R2 in response to thrombin. The recruitment of TRADD, TRAF2, and RIP1 promoted the formation of the membrane-bound complex 1 associated with the nuclear translocation of NF-κB and the promotion of antiapoptotic responses.27 In addition, TRADD engages the cells toward a survival pathway by the recruitment of molecular complexes linked to NF-κB activation and TRAIL-R2 mediates NF-κB activation when cell death pathways are inhibited.28,29 Moreover, thrombin induced the nuclear translocation of p65.30 In our work, the use of TRAIL and TRAIL-R2 knockdown cells and the stimulation with rhTRAIL clearly conferred a role of TRAIL in the activation of NF-κB by thrombin. The blockade of TRAIL/TRAIL-R2 interaction clearly showed that TRAIL mediated NF-κB activation during the late phases of thrombin stimulation. Taken together, the recruitment of complex 1 molecules and NF-κB activation controlled the generation of EMPs by thrombin. Thrombin and TRAIL increased the expression of ICAM-1 and the inflammatory cytokine IL-8 in endothelial cells.32 One important finding in this work is the role played by TRAIL in the upregulation of inflammatory mediators by thrombin. Indeed, silencing TRAIL or TRAIL-R2 reduced the thrombin-mediated increase in ICAM-1 and IL-8, and the stimulation with rhTRAIL confirmed the involvement of TRAIL in the elicitation of an endothelial proinflammatory phenotype by thrombin. NF-κB plays a key role in inflammatory processes, and TRAIL also induces NF-κB activation.28,33 In our work, NF-κB prevented the increase in ICAM-1 or IL-8 mediated by rhTRAIL, demonstrating an involvement of the NF-κB pathway in the control of the inflammatory phenotype mediated by TRAIL. The inhibition of NF-κB prevented the increase in IL-8 over the entire course of the stimulation, and TRAIL only controlled the late phases of IL-8 secretion. These data suggested that 2 phases controlled EMP release or IL-8 secretion during thrombin stimulation. One occurred early and involved NF-κB activation, and during the course of thrombin stimulation, a second occurred lately and was mediated by TRAIL and NF-κB.

Despite its apoptotic role of TRAIL in cancer cells,34 TRAIL also signals for nonapoptotic responses on multiple cells, including endothelial cells.35,36 Although there is controversial evidence regarding the potential protective17 or detrimental role of TRAIL on the endothelium,32,37,38 our data suggest a dual role of TRAIL on endothelial cells. The engagement of signaling molecules involved in survival could protect the cells from apoptosis, but the amplification of the thrombin-induced release of procoagulant EMPs and proinflammatory mediators could be deleterious for endothelial cells.

sTRAIL is present in the plasma,39 and increased levels are associated with inflammatory processes characterized by a dysregulation of the coagulation and the inflammation such as systemic lupus erythematosus,40 sepsis,41 and rheumatoid arthritis.42 In these pathologies, plasma EMP levels are also increased.6,43,44 These data suggest a relationship between increased TRAIL levels and EMP release that remains to be proven.

Data based on in vivo and in vitro experiments indicated that EMPs behave as pathogenic vectors modulating cellular processes that underlie inflammation and thrombosis.45 Procoagulant microparticles originating from endothelial cells and other cell types are sequestered within the atherosclerotic plaque46,47 and could contribute to the rupture of the plaque. TRAIL is also present in the atherosclerotic plaque,48 and its expression is increased in vulnerable plaques.49,50 Because inflammation plays a role in plaque destabilization, and extensive thrombin generation occurs within plaques,47 thrombin would upregulate the local expression of TRAIL, thereby triggering an amplification loop of vesiculation by an autocrine and/or paracrine mechanism. TRAIL also acts on lymphocytes or smooth muscle cells. It could potentiate apoptosis or the release of inflammatory mediators, thereby contributing to plaque rupture.

In conclusion, the present study identifies sTRAIL/TRAIL-R2 system as a critical pathway involved in the late phases of the generation of procoagulant EMPs induced by thrombin in vitro. It establishes that TRAIL could take part in an inflammatory process and emphasizes its role in the interplay between coagulation and inflammation. Elucidation of the mechanisms underlying microparticle formation may help to design therapeutic approaches for a better control of EMP release.

Acknowledgments

We thank Dr C. Sapet for help with analyzing microarray and Dr K. Fallague for help with cell culture. We acknowledge the companies Stago and Biocytex for their support in providing funding.
Sources of Funding
This work was supported by L’Agence nationale de la recherche grant AO5064AS MIPRAMET and Assistance Publique-Hôpitaux de Marseille Programme Hospitalier de Recherche Clinique grant 02/07.

Disclosures
None.

References


TRAIL/Apo2L Mediates the Release of Procoagulant Endothelial Microparticles Induced by Thrombin In Vitro: A Potential Mechanism Linking Inflammation and Coagulation

Stéphanie Simoncini, Makon-Sébastien Njock, Stéphane Robert, Laurence Camoin-Jau, José Sampol, Jean-Robert Harlé, Catherine Nguyen, Françoise Dignat-George and Francine Anfosso

*Circ Res.* 2009;104:943-951; originally published online March 5, 2009;
doi: 10.1161/CIRCRESAHA.108.183285

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/104/8/943

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2009/03/05/CIRCRESAHA.108.183285.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org/subscriptions/
Online Materials and Methods

Endothelial cell cultures

The human microvascular endothelial cell line-1 (HMEC-1) was grown at subconfluence in MCDB131 medium, supplemented with 10% heat-inactivated fetal calf serum, penicillin streptomycin 100UI/ml, hydrocortisone 500µg/ml, human recombinant EGF (10ng/ml) and used between passages 8 and 12. HUVEC were isolated from the cord vein as described and used at passage 4. When required, endothelial cell culture were serum-deprived by culturing HMEC-1 in MCDB131 cell culture medium 0.5%BSA and HUVEC in EBM2 medium 0.5% SVF (Clonetics, Lonza, Verviers Switzerland) overnight. The endothelial cells were stimulated with human thrombin 5IU/ml (Sigma, Saint Louis, MO) for different doses and times. Viability in all the experimental conditions was determined by the incorporation of the fluorescent dye Alamar Blue (Molecular Probes, Eugene, OR). Absence of apoptosis was verified by nuclear staining with DAPI.

To study the effect of neutralizing antibodies, HMEC-1 or HUVEC were first incubated 1 hour with 100µg/ml of neutralizing anti-human TRAIL, TRAIL-R1 or -R2 mAbs (Diaclone, Besançon, France) on ice, washed with cold PBS, and then incubated for different times with thrombin (5IU/ml) in presence of 100µg/ml neutralizing antibodies at 37°C. In separate experiments, HMEC-1 were stimulated with thrombin and neutralizing anti-TRAIL abs (100µg/ml) were added at different times. When required, endothelial cells were stimulated with different doses of human recombinant soluble TRAIL (Biosource International, Invitrogen, Paisley UK)

Flow cytometry (FC)

EMP detection

EMP were assayed as previously described. Briefly, 1ml of cell supernatants from unstimulated or thrombin-stimulated HMEC-1 (1.2x10⁶ cells on 6 well plates) were collected and cleared from cell debris by centrifugation at 5000g for 5 minutes. EMP were quantified by flow cytometry in cell supernatant. 30 µl of cell-free supernatants were labeled for phosphatidylserine probing using annexin V–FITC kit (Beckman Coulter, Fullerton, CA). For counting, a known amount of
fluorescent latex beads (Flowcount, Beckman Coulter) was added as internal standard to samples before FC analysis. Samples were analyzed on a Coulter FC500 according to their size and fluorescence. Using 0.5 and 1.0 µm latex beads as gating parameters, EMP were defined as particles ≤1 µm size. EMP were enumerated from the region corresponding to annexin V-FITC+ events. Number of EMP were calculated using the formula: EMP = Num x [FC] / Num FC where Num is number of EMP passed through the flow cytometer, [FC] is the total amount of flow count beads, and Num FC is number of acquired beads.

Identification of membrane-bound TRAIL and TRAIL receptors

HMEC-1 were rapidly detached by trypsin digestion, centrifuged and washed twice in PBS 1% BSA. Cells were incubated 1 hr with 50µg/ml mAbs raised against human TRAIL, TRAIL-R1, -R2 or isotype control mouse IgG1 (R&D systems, Minneapolis, MN). Immunolabeling was revealed by addition of FITC-conjugated goat anti-mouse IgG1 (Beckman Coulter, Fullerton Ca). Analysis was performed with a Coulter FC500 using the CPX software. For each sample, the mean fluorescence intensity (MFI) was calculated by subtracting the MFI of the immunolabeled isotype control.

TF cofactor activity of EMP

The procoagulant activity of EMP generated by TRAIL was assayed as previously described5. Briefly, EMP released in the supernatants were pelleted by ultracentrifugation at 20,000g at 4°C during 1h30. The pellet was resuspended washed once with PBS and centrifuged again as above. EMP were resuspended in 250µl PBS, enumerated by FACS, and adjusted at the concentration of 200EMP/µl in phosphate buffer saline (PBS). Procoagulant activity (PCA) was measured by a chronometric clotting test according to Aras et al6. Tissue factor (TF) activity associated with EMP was evaluated by the activation of FX into FXa by FVIIa. To do this, EMP (25µl) were incubated with FX (10nM) and FVII (2nmol/L) (Kordia, Leiden, the Netherlands) in Tris HCl 0.05mol/L pH=7.4 buffer, at 37°C for 15min in presence of 5mmol/L CaCl2. Then, 50µl of ½ diluted CS-1132™ chromogenic substrate for FXa in EDTA 0.25 mmol/L were added at 37°C (Hyphen Biomed, Neuville/Oise, France). The absorbance was measured in an ELISA plate reader at 405 nm every 5 seconds during 10 minutes. The concentration of Xa produced in
response to EMP was determined on a standard curve obtained by incubation of increasing concentrations (10ng/ml to 100ng/ml) of FXa (Kordia, Leiden, the Netherlands) with CS-1132™ substrate as above. Results were expressed as ng FXa/µl/min. Inhibition of TF activity by a murine monoclonal anti-human TF blocking antibody (American Diagnostica, Stamford, CT) was assessed. For this purpose, the EMP suspension was pre-incubated for 20min with 1/50th (20µg/ml) diluted mAb and the residual cofactor activity was measured as above.

**Transfer of supernatants**

Ultracentrifuged supernatants (1ml) were incubated for 18 hours with a sub-confluent monolayer of untreated HMEC-1 or HUVEC plated on a 12-well cell-culture plate and EMP were assayed by flow cytometry. When required, HMEC-1 or HUVEC were incubated 18 hours with supernatants in presence of hirudin (20mg/ml).

**Endothelial cell transfections**

HMEC-1 or HUVEC were seeded in 6-well plates and transfected at 60% confluence by magnetofection (OZ Biosciences, Marseille France) as described by the manufacturers.

For studies of RNA silencing, transient transfections were performed with TRAIL, TRAIL-R1 or TRAIL-R2 validated siRNA (50 nM) (Stealth™ validated siRNA duplex Invitrogen), or FADD or RIP1 or TRAF2 (siGENOME ON-TARGETplus SMARTpool, duplex, Dharmacon Lafayette, Co) using three different siRNAs each time, or a scrambled non silencing siRNA as negative control (Invitrogen) complexed with Silence MAG beads (OZ Biosciences) as described by the manufacturer’s. 24 and 48 hours after transfection, HUVEC and HMEC-1 were lysed respectively for determining mRNA expression and protein content. 48 hours after transfection, the endothelial cells were stimulated with thrombin (5IU/ml) for supplemental 18 hours.

**Total RNA extraction**

Total RNA was isolated using the RNeasy plus mini kit (Qiagen, Valencia, CA). The RNA concentration was spectrophotometrically estimated and RNA integrity was checked using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).
Quantitative real-time PCR

Two-step RT-PCR was performed. Total RNA from the same sample as those used for the microarray experiments was reversed-transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, Ca). cDNA product (200ng) was amplified in a 20 µl reaction on a MX3000 (Stratagene, NL) using the Taqman Universal PCR Master Mix. Primers pairs and Taqman probes were purchased as pre-designed and validated reagents (Assays-on-Demand Gene Expression Probes, Applied Biosystems). The PCR program consisted of an initial denaturation at 95°C for 10 minutes followed by amplification for 40 cycles (95°C for 15 seconds, 60°C for 1 minute, and 72°C for 15 seconds). A standard curve for serial dilutions of 18S rRNA used as calibrator was similarly generated. Data were generated from each reaction, analyzed using Mx3000P software and normalized to 18S RNA. Quantification was performed using the \( \Delta\Delta^C \)T calculation. The relative standard curve method was used to calculate the amplification difference between thrombin stimulated and unstimulated cells.

cDNA arrays and cDNA labeling

cDNA arrays were used as previously described using custom-made high density cDNA arrays containing 8074 spotted PCR products from 7874 selected IMAGE human cDNA clones (MRC Rosalind Franklin Centre for Genomics research, Geneservice, Cambridge, UK) and 200 control clones. The IMAGE clones were divided into 6664 cDNA and 1210 expressed sequence tags (EST). About 10% of the genes are represented by two or more different cDNA clones. Reverse transcription was performed as previously described, using 2 µg of total RNA in the presence of [\( \alpha ^{33}\)P]dCTP. Hybridizations were performed for 48 h at 68°C using three different extracts on three different cDNA arrays. After washing, arrays were exposed to phosphor imaging plates, which were scanned using a BAS 5000 (Fuji, Tokyo, Japan) at a 25-µm resolution. After image acquisition, the data were processed using the ArrayGauge software (Fuji, Tokyo, Japan). For each array, background intensity was calculated on the basis of the average of the 400 lowest values and subtracted. For each sample, the 6000 highest values were flagged. Data were filtered (100%), log2 transformed, and centered relative to the median for each gene and each
array. Hierarchical supervised clustering in the array axis was applied to the data set using Cluster software and results were visualized with Treeview software.8

**Differential analysis:** Distinct classes of samples, each of them containing the three replicates of the same time were defined. Only clones present in all the samples and exhibiting a SD ≤ to 0.35 in each class were kept. The mean of the normalized values of each gene from thrombin-stimulated HMEC-1 was divided by the mean of normalized values of the same gene in unstimulated cells. Genes with a ratio of 2.0 or above were considered positively regulated by thrombin, whereas those that had a ratio of 0.5 or below were considered negatively regulated. All data are MIAME compliant and have been submitted to Gene Expression Omnibus (GEO) database9 (accession N° E-MEXP-477).

Genes of interest were followed up by referring to web SOURCE (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch) and Gene Ontology (http://www.geneontology.org) databases for functional informations and subsequently categorized into functional classes.

**Immunoprecipitation and western blotting**

HMEC-1 or HUVEC were scraped and lysed in TRIS HCl 0.1M buffer pH=7.5 containing EDTA 0.001M, NP40 10% and the protease inhibitor Complete® mixture (Roche Diagnostic). For immunoblots, equal amounts of protein lysates (40µg) were separated on 12% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose C+ membrane (Amersham Bioscience, GE Healthcare, UK). They were probed with anti-TRAIL, anti-TRAIL-R1, -R2 mAbs (R&D systems). Bound antibodies were visualized using peroxidase-conjugated goat anti-mouse (Fab’)2 antibody (Jackson laboratories) and ECL reagent. Reblotting with anti-human α-tubulin ensured equal protein loading.

Immunoprecipitations were performed by mixing 300µg of cell lysates with anti-TRAIL-R2 (10µl) mAb and Protein G sepharose (30µl). After incubation overnight at 4°C the proteins were eluted from the beads by heating at 90°C and separated in 12% SDS-PAGE gel. Immunoblotting was
performed by probing with anti-TRADD, -RIP1, -TRAF2 pAbs (Santa-Cruz Biotechnology, Santa Cruz, Ca); and monoclonal anti-FADD (Zymed, Invitrogen Paisley UK).

**ELISA**

Cell supernatants were centrifuged at 2000g for 5 min to eliminate cell debris. Soluble TRAIL and IL-8 were assayed in the cell-free supernatants by commercially available ELISA kit (Diaclone, Besançon France). ICAM-1 was determined by a fluorescent-based cell assay as previously described. Briefly, after stimulation, the cell monolayers were washed and incubated on ice with an anti-ICAM-1 mAb (clone 84H10 Beckman Coulter, Marseille France) and then with an alkaline phosphatase labelled goat anti-mouse antibody. After washing, the cells were incubated with Attophos substrate (Roche Diagnostic) and the fluorescence was determined in a plaque-reader spectrofluorimeter using 450 and 580nm excitation and emission wavelengths.

**P65 NF-κB assay**

The activation of NF-κB was determined by the p65 ELISA-based kit using the TransAM assay according to the manufacturer’s instruction (Active Motif Carlsbad CA). HMEC-1 and HUVEC were stimulated by thrombin (5IU/ml) for different times. In brief, cytoplasmic and nuclear fractions were obtained by cell lysis. 10 µg of nuclear proteins were incubated for 1 h in a 96-well plate coated with an oligonucleotide that contains the NF-κB consensus site (5’-GGGACTTTCC-3’) to which activated NF-κB factors contained in nuclear extracts specifically bind. By using an antibody directed against an epitope on p65 accessible only when NF-κB is activated and bound to its target DNA, the NF-κB complex bound to the oligonucleotide is detected. After incubation for 1 h with a secondary HRP-conjugated antibody, specific binding was detected by colorimetric reaction on a spectrophotometer at 450 nm. The inhibition of p65 nuclear translocation was performed by pre-incubating HMEC-1 or HUVEC with 20µM BAY117082 and then stimulation with thrombin up to 18hrs in presence of the same amount of the inhibitor. When required, the inhibitor was added at different times after the onset of the stimulation of HMEC-1 by thrombin.
**Statistical analysis**

Values presented are means ± SEM for the indicated number of experiments. Data were compared with the use of a two-tailed unpaired Student’s *t* test to calculate statistical significance, with *P* values <0.5 considered statistically significant.

**Reference List**


Online Legends

Online Figure I: Genome-wide analysis of thrombin-stimulated HMEC-1.
The image shows a hierarchical supervised clustering of gene expression in HMEC-1 in response to thrombin (5.0 IU/ml). After data filtering, patterns of genes with at least a two-fold variation are represented.

A: *Left pattern:* total tree presenting up- and down regulated patterns; *right pattern:* Cluster of genes modulated by thrombin and belonging to the TNFα superfamily. The color image shows the numerical values encoded by color according to the method introduced by Eisen. Red represents positive values, and green the negative values.

B: cDNA microarray quantification of changes in the expression of genes belonging to selected cluster. The graph represented the mean of three individual replicates over a 18h-stimulation course.

Online Figure II: Modulation of TRAIL and TRAIL receptors in HMEC-1 and HUVEC:
A: Expression of membrane-bound TRAIL and TRAIL receptors by flow cytometry: HMEC-1 (left pattern) and HUVEC (right pattern) were recovered by dissociation after a 10 hr incubation in presence or absence of thrombin (5IU/ml). Cells were identified according to forward and side scatter parameters. Staining of TRAIL (upper profile), TRAIL-R1 (central profile) and TRAIL-R2 (lower profile) with their specific antibody was determined on cells cultured in absence (dark lines) or in presence (dotted lines) of thrombin. Grey histograms represented staining with the isotype-control IgG1 mAb. Representative graphs of three independent experiments.

B: Absence of TRAIL and TRAIL-R2 modulation by the inhibition of the Rho-Kinases. HMEC-1 or HUVEC were pre-incubated with Y27632 (1µM); kept untreated or stimulated with thrombin (5IU/ml) in presence of the inhibitor. Cells were lysed and lysates (40µg) were resolved by 12% SDS PAGE. Western blotting was performed with anti-TRAIL and anti-TRAIL-R2 respectively. Representative pattern of 2 independent experiments.

Online Figure III: Inhibition of thrombin-induced EMP release by neutralizing antibodies
A: Effects of neutralizing antibodies against TRAIL and TRAIL-R1 or TRAIL-R2 on EMP release by thrombin. HMEC-1 or HUVEC were pre-incubated 1hr at 4°C with neutralizing antibodies (100µg/ml) against TRAIL, TRAIL-R2 or TRAIL-R1, washed and incubated again with or without thrombin in absence or presence of the same neutralizing antibodies for additional 18 hours at 37°C as described in Materials and Methods. The plots were representative of 3 independent experiments.

B: Time-course inhibition of EMP release. HMEC-1 or HUVEC were incubated without thrombin in presence of isotype-matched mAbs (■—■) or with thrombin (▲—▲) in presence of TRAIL (●▪▪▪●) (upper profile) or TRAIL-R2 (○—○) (lower profile) neutralizing antibodies. Curves represented the mean values ± SEM (n=8). **p<0.05, ***p<0.001, (thrombin + abs vs thrombin alone)

Online Figure IV: Late involvement of TRAIL in EMP release by thrombin
HMEC-1 were stimulated with thrombin (5IU/ml) over a 18h-stimulation course. At different times (arrow) neutralizing anti-TRAIL antibodies (100µg/ml) were added to the medium and EMP were assayed by flow cytometry in the cell-free supernatants up to 18 hrs. The curves represented the mean of two individual experiments

Online Figure V: Transient transfections of HMEC-1 by siRNAs.
A: HMEC-1 were transiently transfected with commercially available 3 different siRNA for TRAIL or TRAIL-R2 as described in Materials and Methods. 48hr after transfection, the cells were stimulated with thrombin during 18 hrs. EMP were assayed in cell-free supernatants by flow cytometry. The graph represented the means of 2 independent experiments.

B: HMEC-1 were kept untreated or stimulated with thrombin (5IU/ml) 24hrs after transfection to determine changes in RNA expression by qRT-PCR as described in Materials and Methods. PCR products were then identified on a 2% agarose gel.

C: 48 hours after transfection, HMEC-1 were lysed and TRAIL or TRAIL-R2 protein expression was determined by immunoblot. 40 µg of cell lysates of untreated or thrombin-treated HMEC-1 transfected with scrambled siRNA (sc-siRNA) or specific siRNA were analyzed on 12% SDS-
PAGE under reducing conditions and the blots were probed with: anti-TRAIL, -TRAIL-R2 or -TRAIL-R1 Abs. These patterns were representative of 2 independent experiments.

**Online Figure VI: Inhibition of thrombin-induced EMP release by TRAIL and TRAIL-R2 specific siRNA**

A: Effect of TRAIL and TRAIL-R2 gene silencing on thrombin-induced EMP generation. HMEC-1 or HUVEC were transiently transfected with scrambled siRNA or siRNAs against TRAIL, TRAIL-R2 or TRAIL-R1 as described in Materials and Methods. They were kept untreated (control) or stimulated with thrombin (5IU/ml) during 18hrs. EMP were quantified in cell-free supernatants by flow cytometry. The plots were representative of 3 independent experiments.

B: Time-dependent inhibition of thrombin-induced EMP generation by HMEC-1 or HUVEC untreated (■—■), or transfected with sc-siRNA as control (□—□), or stimulated with thrombin in untransfected (▲—▲) or transfected with TRAIL - (●—●) or TRAIL-R2 (O—O) siRNA. The curves represented the means ± SEM (n=4). * P<0.5; **P<0.03, ***P<0.05 (thrombin + siRNA vs thrombin alone).

**Online Figure VII: Induction of procoagulant EMP by rhTRAIL**

A: Dose-dependent release of EMP by rhTRAIL. HMEC-1 or HUVEC (empty bars) or TRAIL-R2 knocked-down cells (filled bars) were kept untreated (-) or stimulated with increasing doses of rhTRAIL for 18 hours or with TNF-α (25ng/ml) as a positive control of vesiculation. EMP were numerated in the cell-free supernatants. The graphs represented the means ± SEM of 3 experiments performed in triplicate. *P<0.05, **P<0.01, ***P<0.001 vs untreated cells.

B: Tissue-factor-dependent pro-coagulant activity. EMPs were recovered in the supernatants of HMEC-1 or HUVEC kept untreated or stimulated with 20ng/ml rhTRAIL. Their pro-coagulant activity was determined as above without or with pre-incubation with TF-specific blocking mAb or IgG1 isotype control. The graphs represented the means ± SEM of 3 experiments performed in duplicate. *P<0.01, P<0.05 vs thrombin (lane d).

**Online Figure VIII: Involvement of TRAF2 and RIP1 in the signaling pathway.**
A: Recruitment of TRAF2 and RIP1 by TRAIL/TRAIL-R2 complex in HMEC-1. Cell lysates (200µg) were immunoprecipitated with anti-TRAIL-R2 and protein G-agarose beads. Immunoprecipitates were resolved by 12% SDS PAGE, and western blotting was performed with anti-TRAF2 and anti-RIP1 respectively. Representative pattern of 3 independent experiments.

B: Inhibition of TRAF2 and RIP1 proteins by their specific siRNAs
Representative Immunoblot determination of the expression of TRAF2 and RIP-1 in transfected HMEC-1. 40 µg of cell lysates of untreated or thrombin-treated HMEC-1 transfected with TRAIL or TRAIL-R2 siRNAs were analyzed on 12% SDS-PAGE under reducing conditions and the blots were probed with anti-TRAF2 or anti-RIP1 Abs. Control: untreated cells

C: HMEC-1 or HUVEC were knocked-down for TRAF2 or RIP1 or treated with a scrambled siRNA. They were stimulated with thrombin (5IU/ml) during 18 hrs or kept untreated (control). EMP were numerated in cell-free supernatants as described in Materials and Methods. The plots were representative of 2 independent experiments.

Online Figure IX: Activation of NF-κB.
A: Time-course of p65 nuclear localization. HMEC-1 or HUVEC were incubated with thrombin (5IU/ml) up to 18 hrs. Cells were lysed and p65 was assayed by TRANS-AM ELISA in the nuclear fraction at the different times (•—•). HMEC-1 or HUVEC were pre-incubated 30min with either neutralizing anti-TRAIL (100µg/ml) (▲----▲) or BAY117082 (20µM) (■……■) and then incubated with thrombin in presence of the same quantity of the inhibitors up to 18 hrs.

B: Involvement of TRAIL in p65 nuclear translocation. HMEC-1 were stimulated with thrombin up to 18hrs. At different times (arrows) neutralizing anti-TRAIL antibodies (100µg/ml) were added to the medium and p65 was assayed by TRANS-AM ELISA in the nuclear fractions of HMEC-1 incubated up to 18 hrs. The curves represented the mean of two individual experiments

Online Figure X: Time-dependent involvement of NF-κB in EMP release by thrombin
HMEC-1 were stimulated with thrombin (5IU/ml) over a 18h-stimulation course. At different times (arrow) BAY117082 (20µM) was added to the medium and EMP were assayed by flow cytometry
in the cell-free supernatants up to 18 hrs. The curves represented the mean of two individual experiments.

**Online Figure XI: Inhibition of IL-8 secretion in HMEC-1.**

HMEC-1 were stimulated with thrombin (5IU/ml) over a 18h-stimulation course. At different times (arrow) neutralizing anti-TRAIL antibodies (100µg/ml) (A) or BAY117085 (20µM) (B) were added to the medium and IL-8 was assayed by ELISA in the cell-free supernatants up to 18 hrs. The curves represented the mean of two individual experiments.
<table>
<thead>
<tr>
<th></th>
<th>EMP x10^3/10^6 cells</th>
<th>sTRAIL (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HMEC-1</td>
<td>*P</td>
</tr>
<tr>
<td>Control</td>
<td>237.21 ± 47.29</td>
<td></td>
</tr>
<tr>
<td>Thrombin</td>
<td>886.5 ± 97.3</td>
<td></td>
</tr>
<tr>
<td>Control + Y32632†</td>
<td>218.61 ± 39.71</td>
<td></td>
</tr>
<tr>
<td>Thrombin +Y32632†</td>
<td>273.60 ± 82.45</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TRAP‡ 10μM</td>
<td>648.62 ± 84.90</td>
<td>NS</td>
</tr>
<tr>
<td>TRAP‡ 20μM</td>
<td>814.26 ± 107.29</td>
<td>NS</td>
</tr>
<tr>
<td>TRAP‡ 50μM</td>
<td>767.31 ± 118.46</td>
<td>NS</td>
</tr>
<tr>
<td>Hirudin* 5 mg/ml</td>
<td>272.28 ± 67.31</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hirudin* 10mg/ml</td>
<td>236.54 ± 35.93</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hirudin* 20mg/ml</td>
<td>217.12 ± 23.57</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Online Table 1: Thrombin-induced EMP and sTRAIL release by HMEC-1 and HUVEC. Cells were pre-incubated with the different agonists and let untreated or stimulated with thrombin (5.IU/ml) in presence of the same content of agonists for 18 hours. EMP and sTRAIL were assayed in cell-free supernatants as described in Materials and Methods. Results represent means ± SEM of 3 experiments performed in triplicate.*P: determined vs thrombin.

Control: unstimulated cells; †Y27632: Rho-kinase inhibitor; ‡TRAP:SFLLRN agonist peptide; *Hirudin: thrombin inhibitor.
Online Figure I

(A) Heatmap of cDNA microarray levels for different time points (0.5H, 1H, 2H, 4H, 6H, 12H, 18H) with clusters of genes showing significant changes.

(B) Graph showing the fold increase in microarray levels over time for TNFSF10, TNFSF7, LTBR, and LTA.
Online Figure II

B

<table>
<thead>
<tr>
<th></th>
<th>Thrombin</th>
<th>Y27632</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HMEC-1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anti-TRAIL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-TRAIL-R2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HUVEC</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Anti-TRAIL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-TRAIL-R2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Online Figure II
Figure III

EMP/μl

HMEC-1

Control cells

THROMBIN +

- IgG1 Isotype control

- anti-TRAIL

anti-TRAIL R2

anti-TRAIL R1

HUVEC

125

187

1431

973

1012

1359

Online Figure III
Online Figure IV

HMEC-1

EMP x10^3 / 10^6 cells

TIME (hours)

W/O anti-TRAIL

Anti-TRAIL after:

- 2 hrs
- 3 hrs
- 4 hrs
- 6 hrs
- 10 hrs
A

EMP/μl

HMEC-1

HUVEC

Control cells
Thrombin +

- Sc siRNA siRNA TRAIL siRNA TRAIL-R2 siRNA TRAIL-R1

B

EMP x10^3/10^6 cells

HMEC-1

HUVEC

TIME (hr)

Online Figure VI
**Figure VII**

A

- **HMEC-1**
  - EMP x10^3 / 10^6 cells
  - hrTRAIL (ng/ml): 10, 20, 40, 100
  - Control: -
  - TNF-α: +,
  - Significance: * (p < 0.05), ** (p < 0.01), *** (p < 0.001)

- **HUVEC**
  - EMP x10^3 / 10^6 cells
  - hrTRAIL (ng/ml): 250, 500, 750, 1000
  - Control: -
  - Anti-TF mAb: +,
  - Significance: * (p < 0.05), ** (p < 0.01), *** (p < 0.001)

B

- rhTRAIL
  - Ig control
  - Anti-TF mAb

- **HMEC-1**
  - Fxa/min (ng/ml)
  - Significance: * (p < 0.05), ** (p < 0.01)

- **HUVEC**
  - Fxa/min (ng/ml)
  - Significance: * (p < 0.05), ** (p < 0.01)
### Online Figure VIII

**A**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Thrombin</th>
<th>Sc siRNA</th>
<th>siRNA TRAIL</th>
<th>siRNA TRAIL-R2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**IP:**
- anti-TRAIL-R2
- anti-TRAF2
- Anti-RIP-1

**B**

**WB:**
- Anti-TRAF2
- Anti-RIP-1

**C**

<table>
<thead>
<tr>
<th>EMP (μl)</th>
<th>HMEC-1</th>
<th>HUVEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>295</td>
<td></td>
<td></td>
</tr>
<tr>
<td>812</td>
<td></td>
<td></td>
</tr>
<tr>
<td>771</td>
<td></td>
<td></td>
</tr>
<tr>
<td>458</td>
<td></td>
<td></td>
</tr>
<tr>
<td>398</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Control**

<table>
<thead>
<tr>
<th></th>
<th>Thrombin</th>
<th>Sc siRNA</th>
<th>siRNA TRAF2</th>
<th>siRNA RIP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Online Figure VIII**
Online Figure X

HMEC-1

W/O BAY117082
BAY117082 after:
- - 2 hrs
- - 3 hrs
- - 4 hrs
- - 6 hrs
- - 10 hrs

TIME (hours)

EMP x10^3 / 10^6 cells
Online Figure XI

A

HMEC-1 anti-TRAIL Ab

IL-8 (pg/ml x10)

0 25 50 75 100

0 1 2 4 6 8 10 12 18

Anti-TRAIL after:

- 2 hrs
- 3 hrs
- 4 hrs
- 6 hrs
- 10 hrs

W/O anti-TRAIL

B

HMEC-1 BAY117082

IL-8 (pg/ml x10)

0 25 50 75 100

0 1 2 4 6 8 10 12 18

BAY117082 after:

- 2 hrs
- 3 hrs
- 4 hrs
- 6 hrs
- 10 hrs

W/O BAY117082

TIME (hours)

Online Figure XI