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Sara Martínez de Lizarrondo, Carmen Roncal, Olivier Calvayrac, Cristina Rodríguez, Nerea Varo, et al.. Synergistic Effect of Thrombin and CD40 Ligand on Endothelial Matrix Metalloproteinase-10 Expression and Microparticle Generation In Vitro and In Vivo. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 2012, 32 (6), pp.1477-1487. 10.1161/ATVBAHA.112.248773 . hal-03026076

**HAL Id: hal-03026076**

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Submitted on 26 Nov 2020

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# Arteriosclerosis, Thrombosis, and Vascular Biology



JOURNAL OF THE AMERICAN HEART ASSOCIATION

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*Arterioscler Thromb Vasc Biol.* 2012;32:1477-1487; originally published online April 5, 2012;  
doi: 10.1161/ATVBAHA.112.248773

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://atvb.ahajournals.org/content/32/6/1477>

Data Supplement (unedited) at:

<http://atvb.ahajournals.org/content/suppl/2012/04/05/ATVBAHA.112.248773.DC1.html>

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# Synergistic Effect of Thrombin and CD40 Ligand on Endothelial Matrix Metalloproteinase-10 Expression and Microparticle Generation In Vitro and In Vivo

Sara Martínez de Lizarrondo, Carmen Roncal, Olivier Calvayrac, Cristina Rodríguez, Nerea Varo, Ana Purroy, Leonardo Lorente, José A. Rodríguez, Loïc Dœuvre, Sandra Hervás-Stubbs, Eduardo Angles-Cano, José A. Páramo, José Martínez-González, Josune Orbe

**Objective**—Thrombin induces CD40 ligand (CD40L) and matrix metalloproteinases (MMPs) under inflammatory/prothrombotic conditions. Thrombin and CD40L could modulate endothelial MMP-10 expression in vitro and in vivo.

**Methods and Results**—Human endothelial cells were stimulated with thrombin (0.1–10 U/mL), CD40L (0.25–1 µg/mL), or their combination (thrombin/CD40L) to assess MMP-10 expression and microparticle generation. Thrombin/CD40L elicited higher MMP-10 mRNA (5-fold;  $P < 0.001$ ) and protein levels (4.5-fold;  $P < 0.001$ ) than either stimulus alone. This effect was mimicked by a protease-activated receptor-1 agonist and antagonized by hirudin,  $\alpha$ -protease-activated receptor-1,  $\alpha$ -CD40L, and  $\alpha$ -CD40 antibodies. The synergistic effect was dependent on p38 mitogen-activated protein kinase and c-Jun N-terminal kinase-1 pathways. Thrombin also upregulated the expression of CD40 in endothelial cell surface increasing its availability, thereby favoring its synergistic effects with CD40L. In mice, thrombin/CD40L further increased the aortic MMP-10 expression. Septic patients with systemic inflammation and enhanced thrombin generation ( $n = 60$ ) exhibited increased MMP-10 and soluble CD40L levels associated with adverse clinical outcome. Endothelial and systemic activation by thrombin/CD40L and lipopolysaccharide also increased microparticles harboring MMP-10 and CD40L.

**Conclusion**—Thrombin/CD40L elicited a strong synergistic effect on endothelial MMP-10 expression and microparticles containing MMP-10 in vitro and in vivo, which may represent a new link between inflammation/thrombosis with prognostic implications. (*Arterioscler Thromb Vasc Biol.* 2012;32:1477-1487.)

**Key Words:** matrix metalloproteinase-10 ■ endothelium ■ thrombin ■ CD40 ligand ■ microparticles

Growing evidence supports a relationship between inflammation and coagulation in vascular diseases, and a bidirectional cooperation between both mechanisms has been proposed.<sup>1,2</sup> Thrombin is a critical mediator in the coagulation/inflammation vessel wall crosstalk and can amplify inflammation induced by other stimuli, directly via protease-activated receptors (PARs) or indirectly through generation of downstream mediators.<sup>3,4</sup> In addition, CD40, a type I transmembrane protein receptor of the tumor necrosis factor- $\alpha$  superfamily, and its immunomodulating ligand (CD40L/CD154) elicit a series of inflammatory and prothrombotic responses involved in atherogenic and thrombotic mechanisms (eg, overexpression of adhesive proteins, chemokines, metalloproteinases [MMPs], and tissue factor).<sup>5–7</sup> In fact, we and others have reported elevated levels of CD40L in patients with both stable

and unstable angina, acute myocardial infarction, and diabetes mellitus with coronary artery disease, as well as the presence of CD40L in microparticles (MPs) from human atherosclerotic plaques that trigger an inflammatory/proliferative response on endothelial cells (ECs).<sup>8,9</sup>

MPs are submicron vesicles released from cell membranes after cell activation or apoptosis in response to different molecules, such as thrombin.<sup>10</sup> MPs expose and harbor a broad spectrum of bioactive substances (cytokines, proteins, mRNA, and microRNA) and receptors, promoting the dissemination and exchange of information among vascular cells.<sup>11</sup>

We have recently shown that different inflammatory/prothrombotic stimuli, such as C-reactive protein and thrombin, significantly induce the expression of MMP-10 (stromelysin-2) in human ECs in vitro and in vivo.<sup>12,13</sup> Moreover, MMP-10

Received on: May 6, 2011; final version accepted on: March 23, 2012.

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The online-only Data Supplement is available with this article at <http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.112.248773/-/DC1>.

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*Arterioscler Thromb Vasc Biol* is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.112.248773

is enhanced in patients with clinical and subclinical atherosclerosis,<sup>12,14</sup> as well as in pathological conditions with increased thrombin generation,<sup>13</sup> suggesting a potential role of MMP-10 in these processes.

In spite of the role of thrombin and CD40L in the cross-talk of inflammation and coagulation, it still remains unclear whether these 2 proteins cooperate to regulate proteolytic activity in the vessel wall. Here, we investigated the effect of thrombin, CD40L, and thrombin/CD40L on endothelial MMP-10 expression and MP generation in vitro and in vivo. These results were correlated with pre-clinical and clinical studies of sepsis as a model of systemic inflammation.<sup>15</sup>

## Methods

A detailed description is presented in the online-only Data Supplement.

### Cell Cultures

Human umbilical vein endothelial cells (HUVEC) and human aortic endothelial cells were grown and stimulated with thrombin (0.1–10 U/mL), soluble CD40L (0.25–1 µg/mL), purified platelet-derived MPs (PMPs) (0.6 mg/mL), or different PAR agonist peptides, in the presence or absence of specific inhibitors.

When needed, cells were pretreated for 30 minutes with different inhibitors of intracellular signaling pathways.

### Phosphorylation Pattern of the Signaling Pathways

Cells were stimulated with thrombin, CD40L, or their combination, and protein extracts analyzed by Western blot as described.<sup>16</sup>

### Gene Expression Analysis

RNA was extracted from cells (6100 Nucleid Acid PrepStation; Applied Biosystems). Real-time polymerase chain reaction was performed on an ABI PRISM 7900 sequence detector (Applied Biosystems) using TaqMan gene expression assays-on-demand (Applied Biosystems) for MMP-10, CD40, and tissue inhibitor of metalloproteinases-1.

### MMP-10 Protein Secretion

MMP-10 levels were measured in conditioned medium by ELISA (Quantikine, R&D Systems) and Western blot. Inter- and intra-coefficients of variation were <8%.

### Analysis of Thrombin-Induced CD40 by EC

HUVEC were stimulated with thrombin (0.1–1 U/mL) and cell lysates analyzed by Western blot under nonreducing conditions. CD40 expression on the EC surface was assessed by flow cytometry (FACSCalibur; BD Biosciences) after thrombin stimulation using anti-human CD40 antibody and its isotype control. Immunofluorescence assays with HUVEC grown on gelatin-coated slides were performed after thrombin (0.25 U/mL) stimulation.

### Generation and Characterization of Thrombin/CD40L MPs

Endothelial-derived MPs (EMPs) were purified from conditioned medium of HUVEC stimulated for 48 hours with thrombin (1–10 U/mL) with or without CD40L (0.25–1 mg/mL) by serial centrifugations at 20 000g.<sup>17</sup> PMPs were obtained after platelet activation with thrombin (1 U/mL=9 nM for 30 minutes) and harvested as described for EMPs.

Standardization of MPs was achieved by flow cytometry using a mix of fluorescent beads of varied diameters (Megamix, BioCytex)

and annexin V labeling. The total amount was assessed by protein quantification in lysed MPs. The presence of MMP-10 and CD40L was analyzed by Western blot in EMPs and PMPs, respectively.

### Induction of MMP-10 in Mouse Aorta In Vivo

Male C57Bl/6 wild-type (WT) and knockout mice for MMP-10 (*Mmp10*<sup>-/-</sup>) (6 months old, n=30) were procured at the CIMA animal facility, and fed a normal chow diet. Anesthetized mice were injected with thrombin (2.5 U/mouse), CD40L (2.5 µg/mouse), thrombin/CD40L, or saline via the ocular plexus. Animals were euthanized 24 hours after treatment and aortic arteries dissected. MMP-10 protein in aorta was analyzed by immunohistochemistry and Western blot using polyclonal MMP-10 and β-actin antibodies.

A necropsy was performed to determine the cause of death during the procedure. All experimental procedures were approved by the local animal ethics committee.

### Endotoxemia Model in WT and *Mmp10*<sup>-/-</sup> Mice

Six-month-old WT and *Mmp10*<sup>-/-</sup> male mice were intraperitoneally injected with lipopolysaccharide (LPS) (5–15 mg/kg, *Escherichia coli*). Citrated blood was obtained from the ocular plexus before LPS injection when died or euthanized (7 days after injection) to measure platelet number (Hemavet). Platelet-poor plasma was used to measure thrombin/antithrombin complexes and for MP isolation as described in the online-only Data Supplement. Cellular origin of circulating MPs was determined by flow cytometry using specific markers for platelets (CD41<sup>+</sup>), leukocytes (CD45<sup>+</sup>) and endothelium, platelets and leukocytes (CD31<sup>+</sup>). MMP-10 and CD40L in MPs were detected by double immunostaining.

### MMP-10, CD40L, and Thrombin Generation in Patients With Sepsis

Serum and plasma samples were obtained from 60 patients (64% men, mean age 63 years) with sepsis from abdominal origin, hospitalized in Spanish intensive care units, and 50 age- and sex-matched controls. Sepsis was diagnosed according to standard criteria.<sup>18</sup> The study was approved by the institutional review boards of the corresponding hospitals, and informed consent from patients or family members was obtained. MMP-10 (R&D Systems), CD40L (Bender MedSystems), and D-dimer (Innovance D-dimer; Siemens) were assayed with specific ELISAs.

### Statistical Analysis

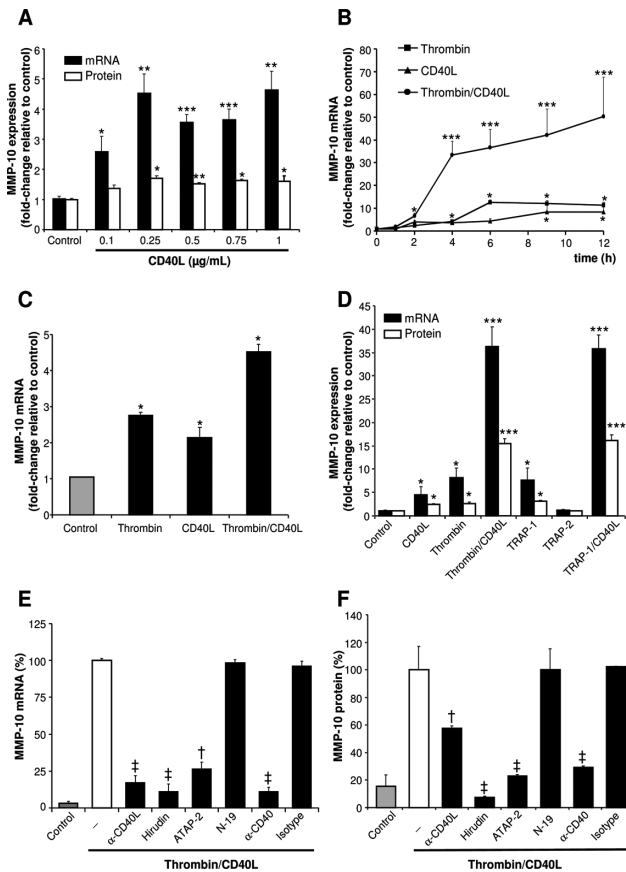
Continuous variables were expressed as mean±SD (unless otherwise stated). Differences between experimental groups were assessed by using appropriate statistical tests (SPSS version 15.0). Statistical significance was established at *P*<0.05.

## Results

### Synergistic Expression and Secretion of MMP-10 by Thrombin/CD40L on Human ECs

We had previously shown that thrombin induces endothelial MMP-10 in a dose-dependent manner.<sup>13</sup> Here, we report that MMP-10 expression in human ECs also increased upon stimulation with increasing concentrations of CD40L (0.1–1 µg/mL). As shown in Figure 1A, endothelial MMP-10 expression and secretion of the medium increased by 4.5-fold and 1.7-fold, respectively, 12 hours after CD40L stimulation (0.25 µg/mL), with no further elevation upon higher concentrations of the ligand. Therefore, 0.25 U/ml of thrombin and 0.25 µg/mL





**Figure 1.** Synergistic effect of thrombin/CD40 ligand (CD40L) on human endothelial cells (ECs). **A**, Matrix metalloproteinase-10 (MMP-10) mRNA (black) and protein levels (white) in human umbilical vein endothelial cells upon increasing concentration of CD40L (0.1–1  $\mu\text{g/mL}$ ). **B**, Time course of endothelial MMP-10 expression (mean  $\pm$  SD,  $n=4$ ) after stimulation with thrombin (0.25 U/mL; squares), CD40L (0.25  $\mu\text{g/mL}$ ; triangles), or thrombin/CD40L (circles). **C**, MMP-10 mRNA levels in human aortic endothelial cells 12 hours after stimulation (as in **B**). **D**, Thrombin (0.25 U/mL) and CD40L (0.25  $\mu\text{g/mL}$ ) increased MMP-10 mRNA (black) and protein (white) levels in ECs 12 hours after stimulation (mean  $\pm$  SEM,  $n=6$ ). A protease-activated receptor-1 (PAR-1) agonist peptide (thrombin receptor agonist peptide [TRAP]-1; 50  $\mu\text{M}$ ) mimicked the effect of thrombin on MMP-10 expression, whereas no effect was observed for a PAR-2 agonist (TRAP-2; 100  $\mu\text{M}$ ) (**E** and **F**). Thrombin/CD40L-induced (12 hours) endothelial MMP-10 upregulation (mRNA [**E**] and protein [**F**]) was prevented by blocking CD40L ( $\alpha$ -CD40L; 7.5  $\mu\text{g/mL}$ ), thrombin (hirudin; 10  $\mu\text{M}$ ),  $\alpha$ -PAR-1 (ATAP-2; 1.2  $\mu\text{g/mL}$ ), or CD40 ( $\alpha$ -CD40; 7.5  $\mu\text{g/mL}$ ). No effect was observed for  $\alpha$ -PAR-1 (N-19; 1.2  $\mu\text{g/mL}$ ) or isotype control antibodies (7.5  $\mu\text{g/mL}$ ) ( $*P<0.05$ ;  $**P<0.01$ ;  $***P<0.001$  vs control, and  $\dagger P<0.05$  and  $\ddagger P<0.01$  vs thrombin/CD40L).

of CD40L were chosen for further experiments. Simultaneous addition of thrombin and CD40L to HUVEC induced stronger MMP-10 expression than each stimulus alone. As shown in Figure 1B, treatment of HUVEC with thrombin or CD40L significantly increased MMP-10 mRNA from 6 hours ( $12.5 \pm 1.12$  and  $4.38 \pm 1.13$ ;  $P<0.05$ ). The combination of both stimuli, however, drove an earlier (2 hours) and higher ( $\approx 5$ -fold) MMP-10 induction compared with single stimulation. Our results were confirmed in human aortic endothelial cells (Figure 1C).

MMP-10 protein could not be detected in HUVEC lysates by Western blot (data not shown), likely due to its

fast secretion of the medium. Therefore, MMP-10 protein expression was measured in cell culture supernatants by ELISA. Thrombin and CD40L increased MMP-10 secretion 2-fold after 12 hours ( $2.7 \pm 0.3$  and  $2.5 \pm 1.7$ ;  $P<0.05$ ), whereas a 7-fold increase was observed for the combination ( $n=4$ ,  $15.5 \pm 1.1$ ;  $P<0.001$ ) (Figure 1D). Costimulation with thrombin/CD40L, however, did not modify the mRNA levels of the protease inhibitor tissue inhibitor of metalloproteinases-1 in HUVEC (fold change relative to control:  $1 \pm 0.01$  control versus  $0.7 \pm 0.03$  thrombin/CD40L,  $n=4$ ).

In addition, thrombin receptor agonist peptide-1, a peptide agonist for PAR-1, alone or in combination with CD40L mimicked the effect of thrombin on thrombin receptor agonist peptide-10 expression, while an agonist for human PAR-2 (thrombin receptor agonist peptide-2) had no effect (Figure 1D).

To study the mechanisms of this synergy, HUVEC were treated with hirudin, a specific thrombin inhibitor, or a blocking antibody against CD40L ( $\alpha$ -CD40L) before stimulation. As shown in Figure 1E and F, both hirudin and  $\alpha$ -CD40L prevented the synergistic increase in MMP-10 expression induced by thrombin/CD40L, indicating that catalytically active thrombin and biologically functional CD40L are required. To assess the role of PAR-1 and CD40 in this process, HUVEC were preincubated with a PAR-1 blocking antibody (ATAP-2) or a control antibody (N-19), and with CD40 blocking antibody ( $\alpha$ -CD40) or an isotype-matched control IgG before stimulation. Both ATAP-2 and  $\alpha$ -CD40 prevented the synergistic increase in MMP-10 expression, whereas N-19 and the control antibody exerted no significant effect (Figure 1E and F).

### p38 MAPK and JNK-1 Mediated the Synergistic Induction of MMP-10 by Thrombin/CD40L

We assessed whether the synergistic induction was due to the stabilization of MMP-10 mRNA or the increase of its transcription rate. MMP-10 mRNA half-life was similar in HUVEC regardless of the treatment (Figure 1A in the online-only Data Supplement). Actinomycin D, however, completely blocked MMP-10 upregulation by CD40L or thrombin/CD40L stimulation (Figure 1B in the online-only Data Supplement), suggesting an increment of the transcriptional rate in both conditions.

When analyzing the kinases involved in MMP-10 induction, thrombin (0.25 U/mL) increased the phosphorylation of extracellular signaling-regulated kinases-1/2, p38 mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinase-1 (JNK-1) within 5 minutes, returning to baseline at 15 to 30 minutes (Figure 2A). CD40L (0.25  $\mu\text{g/mL}$ ) also enhanced phosphorylation of extracellular signaling-regulated kinases-1/2, p38 MAPK, and JNK-1, but showed a delayed effect (maximal between 15 and 30 minutes) compared with thrombin. Costimulation with thrombin/CD40L resulted in a higher and more sustained phosphorylation of these signaling kinases. This effect was further analyzed by using specific inhibitors for extracellular signaling-regulated kinases-1/2 (PD98059), p38 MAPK (SB203580), and JNK-1 (SP600125). All of these reduced the effect of CD40L on MMP-10 mRNA levels ( $n=4$ , 46.6%, 82.7%, and 69.9% inhibition, respectively,  $P<0.05$ , Figure 2B), whereas only SB203580 and SP600125 significantly diminished the upregulation of MMP-10 induced

by the combination of thrombin/CD40L (Figure 2C). In contrast, phosphoinositide 3-kinase inhibition did not show any effect on MMP-10 expression (data not shown). Therefore, both p38 MAPK and JNK-1 seem to be critical in the synergistic upregulation of MMP-10 by thrombin/CD40L. Similar results were obtained when the endothelial release of MMP-10 to the medium was measured by ELISA (Figure 2D and E).

### Thrombin-Mediated Induction of CD40 Expression in Human ECs

To understand the molecular basis for the observed synergy, we determined whether thrombin modulated the expression of CD40 in ECs. As shown in Figure 3A, thrombin (0.5 U/mL, 12 hours) induced a significant increase in CD40 mRNA, which was prevented with hirudin. This effect is dependent on PAR-1, as thrombin receptor agonist peptide-1 mimicked the effect of thrombin and ATAP-2 prevented it. Moreover, as determined by immunoblot analyses, CD40 levels increased in dose-dependent manner upon thrombin stimulation in HUVEC (Figure 3B). Analysis of endothelial CD40 by flow cytometry in HUVEC showed 1.7-fold increase in CD40<sup>+</sup> cells upon thrombin stimulation (0.5 U/mL) compared with controls (Figure 3C). Moreover, we determined CD40 surface availability to the ligand by incubating ECs with thrombin for 30 minutes, a time lapse too short to enable new protein synthesis. As shown in Figure 3D, thrombin rapidly promotes CD40 expression on EC surface, suggesting that it mediates CD40 clustering to the membrane, increasing its availability for the ligand.

As shown in Figure 3A, CD40L did not modify CD40 expression by itself and did not further increase the effect of thrombin.

### Effect of Thrombin/CD40L on MMP-10 Expression In Vivo

To further characterize this synergistic effect in vivo, WT mice were treated with saline (control), thrombin (2.5 U), CD40L (2.5 µg), or both stimuli, and 24 hours later levels of MMP-10 in aortic tissues were analyzed by Western blot and immunohistochemistry. Control animals presented low levels of MMP-10 in aortic tissue (Figure 4A, B), whereas higher levels of MMP-10 were detected after thrombin and CD40L injection. This effect was significantly increased when both proteins were infused simultaneously (fold change relative to control:  $3.02 \pm 0.41$  versus  $1.73 \pm 0.52$  and  $1.69 \pm 0.14$ ,  $P < 0.05$ ), indicating that thrombin and CD40L also potentiate vascular expression of MMP-10 in vivo (Figure 4A). Immunohistochemical analysis showed that thrombin/CD40L induced MMP-10 in ECs, smooth muscle cells, and infiltrated inflammatory cells (Figure 4B). Interestingly, the combination of thrombin/CD40L was associated with a higher acute mortality in WT mice compared with thrombin (8/16 versus 2/16,  $P < 0.05$ ) and CD40L (0/16,  $P < 0.01$ ). Histological analysis of dead mice injected with thrombin/CD40L showed widespread intravascular fibrin deposition, as well as fibrin deposits in extravascular sites, as the main cause of death (Figure II

in the online-only Data Supplement). Similar mortality in *Mmp10*<sup>-/-</sup> and WT mice after thrombin/CD40L treatment (6/16 versus 8/16) suggests that other mediators, unrelated to MMP-10, might trigger this response after thrombin/CD40L administration.

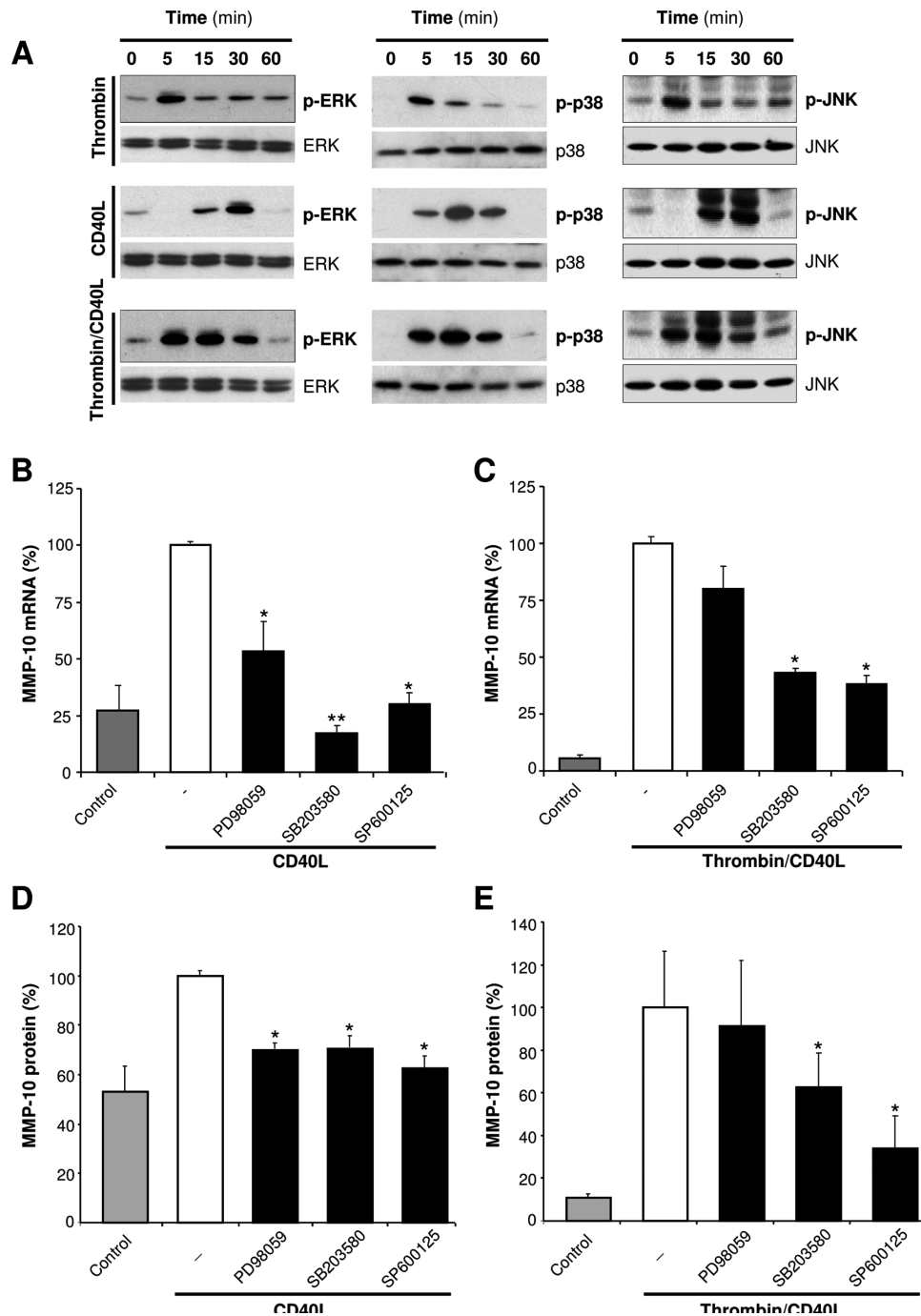
### Circulating Levels of MMP-10 and CD40L in Relation to Mortality in Patients With Sepsis

Sepsis is characterized by the systemic activation of coagulation and inflammation which triggers the activity of thrombin and CD40L among others.<sup>2</sup> To demonstrate whether increased thrombin generation and CD40L were associated with elevated MMP-10 levels in pathological situations, samples from patients with sepsis (n=60) and healthy subjects (n=50) were collected. Circulating levels of MMP-10 ( $2903 \pm 400$  pg/mL versus  $496 \pm 257$  pg/mL,  $P < 0.001$ ), d-Dimer ( $6.90 \pm 0.23$  mg/L versus  $0.30 \pm 0.19$  mg/L,  $P < 0.001$ ), and soluble CD40L ( $4.47 \pm 2.48$  ng/mL versus  $3.45 \pm 1.72$  ng/mL,  $P < 0.05$ ) were increased in patients compared with controls (Figure 4C). MMP-10 levels were also associated with lactic acid, a marker of sepsis severity ( $r = 0.33$ ,  $P < 0.01$ ), and with mortality. The main determinants of mortality in patients with sepsis were disseminated intravascular coagulation accompanied by septic shock. Interestingly, we observed that nonsurviving patients (n=23) presented higher circulating levels of CD40L ( $5.8 \pm 0.7$  ng/mL versus  $4.1 \pm 0.5$  ng/mL,  $P < 0.05$ ) and MMP-10 ( $5843 \pm 1048$  pg/mL versus  $2957 \pm 615$  pg/mL,  $P < 0.01$ ) than survivors. Multivariate analysis showed that increased levels of MMP-10 and CD40L remained statistically associated with mortality after adjustment for age and sex (Table).

### MMP-10 in Experimental Endotoxemia in Mice

To unravel the role of MMP-10 in sepsis in vivo, we performed a model of endotoxemia-induced sepsis by injecting LPS (IP 5–15 mg/kg, *E. coli*) in WT and *Mmp10*<sup>-/-</sup> mice. Analysis of blood samples from the group receiving the higher dose of LPS (15 mg/kg) showed similar platelet count in WT and *Mmp10*<sup>-/-</sup> mice ( $1223 \pm 28$  k/µL versus  $1120 \pm 23$  k/µL; n=20) that dropped by 35% 24 hours after LPS injection (platelets k/µL:  $831 \pm 29$  WT versus  $727 \pm 24$  *Mmp10*<sup>-/-</sup>, n=20;  $P < 0.05$ ). LPS-injected WT mice exhibited increased thrombin/antithrombin levels compared to *Mmp10*<sup>-/-</sup> endotoxemic mice ( $479 \pm 85$  µg/L versus  $310 \pm 36$  µg/L, n=20;  $P = 0.05$ ), suggesting reduced thrombin generation in the absence of MMP-10 activity. However, no differences in survival between WT and *Mmp10*<sup>-/-</sup> mice were observed with high (14/20 and 15/20, respectively) or low dose of LPS (5 mg/kg) (Figure 4D).

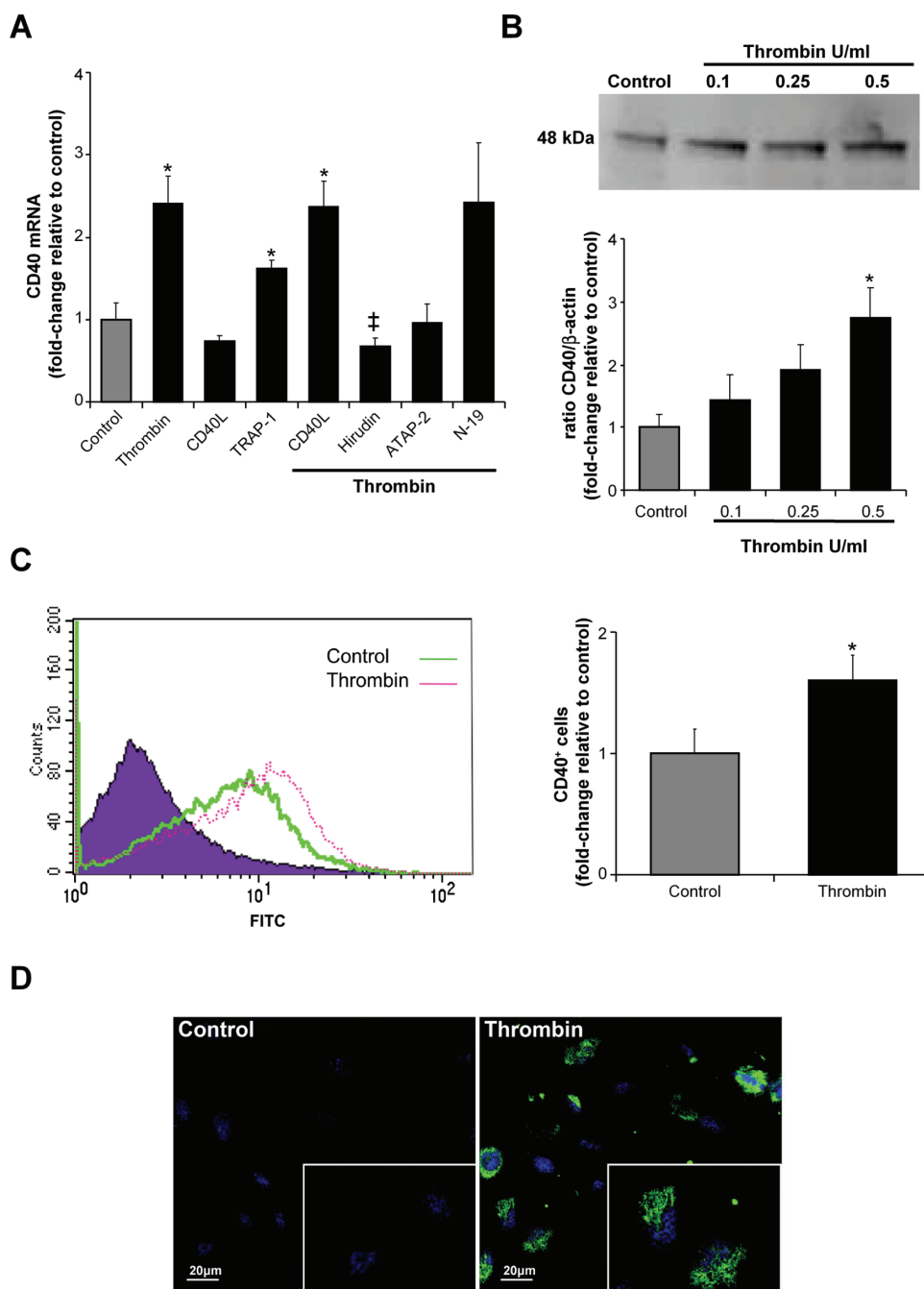
In sepsis, systemic inflammation is a major cause of cellular dysfunction and generation of MPs.<sup>19</sup> We assessed the levels of MPs in our experimental model (Figure III in the online-only Data Supplement). As shown in Figure 5A, the amount of MPs isolated from platelet-poor plasma of LPS-injected mice was higher in WT and *Mmp10*<sup>-/-</sup> animals compared with saline. In control conditions, CD41<sup>+</sup> MPs were the most abundant (32%) followed by CD31<sup>+</sup> MPs (18%) and CD45<sup>+</sup> MPs (6%). After LPS injection, the percentage



**Figure 2.** Induction of matrix metalloproteinase-10 (MMP-10) by CD40 ligand (CD40L) and thrombin/CD40L via mitogen-activated protein kinase (MAPK). **A**, Western blot analysis showing the time-dependent increase in the phosphorylation of extracellular signaling-regulated kinases (ERK)-1/2 (p-ERK), p38 MAPK (p-p38), and c-Jun N-terminal kinase-1 (JNK-1; p-JNK) in human umbilical vein endothelial cells (HUVEC) treated with thrombin (0.25 U/mL; upper panel), CD40L (0.25  $\mu$ M; middle panel), or thrombin/CD40L (lower panel). **B**, **C**, MMP-10 mRNA levels in CD40L (**B**) and thrombin/CD40L (**C**) stimulated HUVEC in presence of different inhibitors: 10  $\mu$ M PD98059 (ERK1/2 inhibitor), 10  $\mu$ M SB203580 (p38 MAPK inhibitor), and 10  $\mu$ M SP600125 (JNK-1 inhibitor). Inhibition of p38 MAPK and JNK-1 signaling pathways significantly prevented MMP-10 upregulation. **D** and **E**, MMP-10 protein levels (ELISA) in CD40L (**D**) and thrombin/CD40L (**E**) stimulated HUVEC in the presence of different inhibitors (as in **B** and **C**: (n=4, \*P<0.05, \*\*P<0.01 vs CD40L or CD40L/thrombin).

of CD45<sup>+</sup> MPs in WT mice increased whereas no differences were displayed in CD41<sup>+</sup> MPs and CD31<sup>+</sup> MPs in WT or *Mmp10*<sup>-/-</sup> mice compared with saline group (Figure 5B and Figure IV in the online-only Data Supplement). LPS increased the levels of CD40L in CD41<sup>+</sup> MPs in WT mice, but not in *Mmp10*<sup>-/-</sup> mice that exhibited similar CD40L

content in control and LPS-treated animals ( $\approx$ 50%) (Figure 5C). Finally, we determined whether WT MPs from different origin bore MMP-10. CD45<sup>+</sup> MPs, CD31<sup>+</sup> MPs, and CD41<sup>+</sup> MPs expressed MMP-10 on their surface in baseline conditions that were significantly increased after LPS administration in CD31<sup>+</sup> MPs and CD41<sup>+</sup> MPs (Figure 5D).



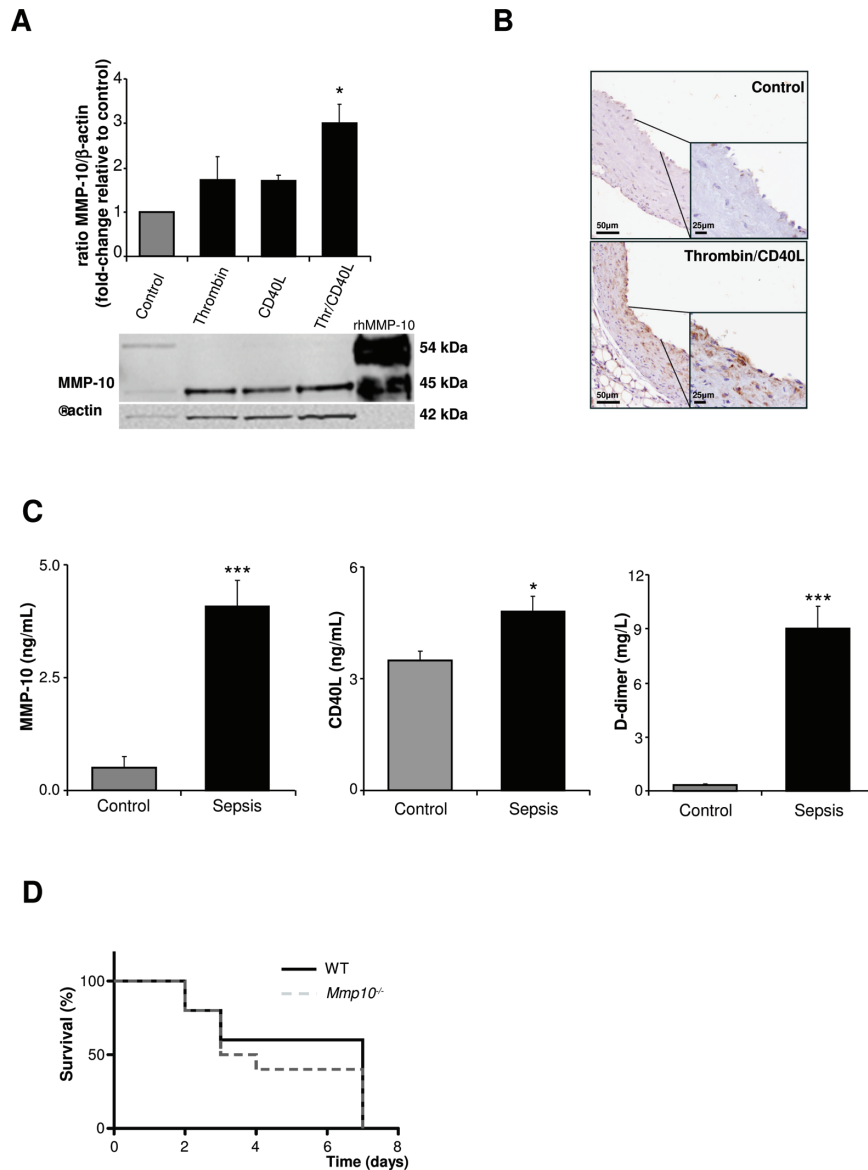
**Figure 3.** Thrombin increases CD40 expression in endothelial cells (ECs). **A**, CD40 mRNA levels in human umbilical vein endothelial cells (HUVEC) stimulated for 12 hours with thrombin (0.5 U/mL), CD40L (0.5 μg/mL), thrombin receptor agonist peptide (TRAP)-1 (50 μM), and thrombin coincubated with CD40 ligand (CD40L), hirudin (10 μM), ATAP-2, or N-19 (1.2 μg/mL) (mean±SD, n=4). **B**, Western blot analysis of CD40 receptor (48 kDa) in lysates from HUVEC stimulated with thrombin (24 hours). Histogram represents CD40/β-actin ratio. **C**, Flow cytometry analysis of CD40<sup>+</sup> ECs stimulated with thrombin for 24 hours (dashed pink line) compared with controls (solid green line). **D**, Immunofluorescence for CD40 (green) in HUVEC treated with thrombin (30 minutes, 0.25 U/mL). Insets show high-power magnification. Draq5 (blue) was used as nuclear staining (\**P*<0.05 vs controls and ‡*P*<0.01 vs thrombin).

### Endothelial and Platelet Activation Induce MP Generation Bearing MMP-10 and CD40L

MPs shedding in response to different stimuli result in the production of biologically active vesicles.<sup>20</sup> First, we determine whether thrombin, CD40L, or the combination induced the release of endothelial MPs harboring MMP-10 (MMP-10<sup>+</sup> EMPs), as well as the contribution of MMP-10<sup>+</sup> EMPs to the total amount of active

MMP-10 in cell culture supernatants. HUVECs were stimulated for 24 hours and supernatants collected. Samples were centrifuged to eliminate MPs (EMPs-free) or used as collected (EMPs+). Western blot analysis showed that MMP-10 levels were higher in EMP+ supernatants compared with EMPs-free (Figure 5E), representing ≈22 % of the total amount of the secreted MMP-10 after thrombin/CD40L stimulation.





**Figure 4.** Pathophysiological role of matrix metalloproteinase-10 (MMP-10) in vivo. **A**, MMP-10 protein levels (Western blot) were upregulated in aortic lysates from mice 24 hours after thrombin (2.5 U) or CD40 ligand (CD40L; 2.5  $\mu$ g) administration, although the strongest upregulation was achieved by the combination of both stimuli. rhMMP-10 (proenzyme: 55 kDa; active enzyme: 45 kDa) was used as positive control and  $\beta$ -actin (42 kDa) as loading control ( $n=14$ ,  $*P<0.05$  vs controls). Results are expressed as the ratio between MMP-10 and  $\beta$ -actin and expressed as fold increase relative to controls. **B**, Morphological analysis for MMP-10 in mouse aortas after thrombin/CD40L administration. **C**, Circulating levels of MMP-10, CD40L, and D-dimer (mean $\pm$ SEM) in patients with sepsis (black bars;  $n=60$ ) and controls (gray bars;  $n=50$ ). **D**, Survival curve in lipopolysaccharide-injected (5 mg/kg) wild-type and *Mmp10*<sup>-/-</sup> mice ( $n=20$ /genotype) ( $*P<0.05$ ,  $***P<0.001$ ).

We then characterized by flow cytometry the size and binding to annexin V (data not shown) of MPs shed by HUVEC stimulated with thrombin, CD40L, and thrombin/CD40L at high concentrations to increase the production of EMPs. As shown in Figure 5F, we observed that the number of EMPs increased upon thrombin stimulation, and was higher with thrombin/CD40L (mg/mL:  $0.53\pm 0.12$  versus  $1.21\pm 0.16$ ,  $n=3$ ;  $P<0.05$ ). CD40L alone did not increase the release of EMPs, suggesting a synergistic effect of these stimuli when added together. Western blot analyses showed that shed EMPs bear MMP-10 upon thrombin stimulation, and in greater extent if thrombin is combined with CD40L (Figure 5F, top).

Second, we assessed whether CD40L<sup>+</sup> PMPs could amplify thrombin-mediated endothelial MMP-10 upregulation. As shown in Figure 5G, PMPs alone were unable to induce MMP-10; however, they enhanced thrombin-induced endothelial MMP-10 upregulation (fold change relative to control:  $5.89\pm 0.37$  versus  $4.13\pm 0.27$ ,  $n=3$ ;  $P<0.01$ ). This effect was much lower than that of rhCD40L (0.25  $\mu$ g/mL), because the amount of available ligand in PMPs is lower than in the recombinant protein (Figure 5G, top). The effect was CD40L dependent, because the treatment of PMPs with an  $\alpha$ -CD40L antibody prevented thrombin-related synergistic induction (Figure 5G, bottom).

**Table. Multivariate Analysis of Soluble CD40L and MMP-10 in Relation to Survival in Patients With Sepsis**

	Survivors (n=37)	Nonsurvivors (n=23)	P
CD40L, ng/mL	4.1±0.5	5.9±0.6	0.03
MMP-10, pg/mL	2915.6±706.7	5911.0±897.3	0.01

CD40L indicates CD40 ligand; MMP-10, matrix metalloproteinase-10. Adjusted for age and sex, mean±SEM is shown.

## Discussion

Vascular and inflammatory diseases, such as atherosclerosis and sepsis, manifest thrombosis-driven augmentation of the endothelial response. This is associated with increased circulating levels of prothrombotic/inflammatory mediators, such as thrombin and CD40L,<sup>4,7</sup> and enhanced protease activity.<sup>13,21</sup> In this study, we describe for the first time that thrombin and CD40L act synergistically to induce MMP-10 both in vitro and in vivo, and to generate MPs carrying active MMP-10. The fact that thrombin mediates CD40 expression in ECs and that its inhibition significantly reduced MMP-10 expression strongly suggests that thrombin-induced CD40 upregulation could underlie this synergy.

Our study confirms previous reports showing that thrombin and CD40L induce the overexpression of MMP-10 through the activation of JNK-1, p38 MAPK, and extracellular signaling-regulated kinases.<sup>13,22</sup> Interestingly, costimulation resulted in higher and more sustained phosphorylation of these signaling kinases, highlighting a major role of p38 MAPK and JNK-1 in the synergistic upregulation of MMP-10. These results do not exclude that other endothelial inflammatory markers (eg, interleukin-6, interleukin-8, vascular cell adhesion molecule-1, intracellular adhesion molecule-1) activated by these signaling pathways could also be regulated in our experimental conditions.

Our data suggest that the mechanism of this synergy relies on the ability of thrombin to enhance CD40 expression in ECs via PAR-1,<sup>23,24</sup> a prototypic thrombin receptor ubiquitously distributed and most sensitive to thrombin stimulation. Previous reports have demonstrated that PAR-1 activation upregulates CD40 expression, resulting in the expression of other inflammatory cytokines such as tumor necrosis factor- $\alpha$ .<sup>24,25</sup> Our data indicate that PAR-1 activation not only increased CD40 expression in ECs,<sup>26</sup> but also induced its mobilization to the membrane.<sup>27</sup> This mechanism may favor CD40 interaction with its ligand, and contribute to increased MMP-10 expression and other proinflammatory and proatherogenic genes.<sup>6</sup> To translate these in vitro data, we analyzed MMP-10 expression in the vascular wall of mice after thrombin and CD40L injection. Both stimuli independently induced MMP-10, but the combination elicited a further increase in aortic MMP-10 expression, thus linking this protease with thrombin generation and inflammation in vivo.

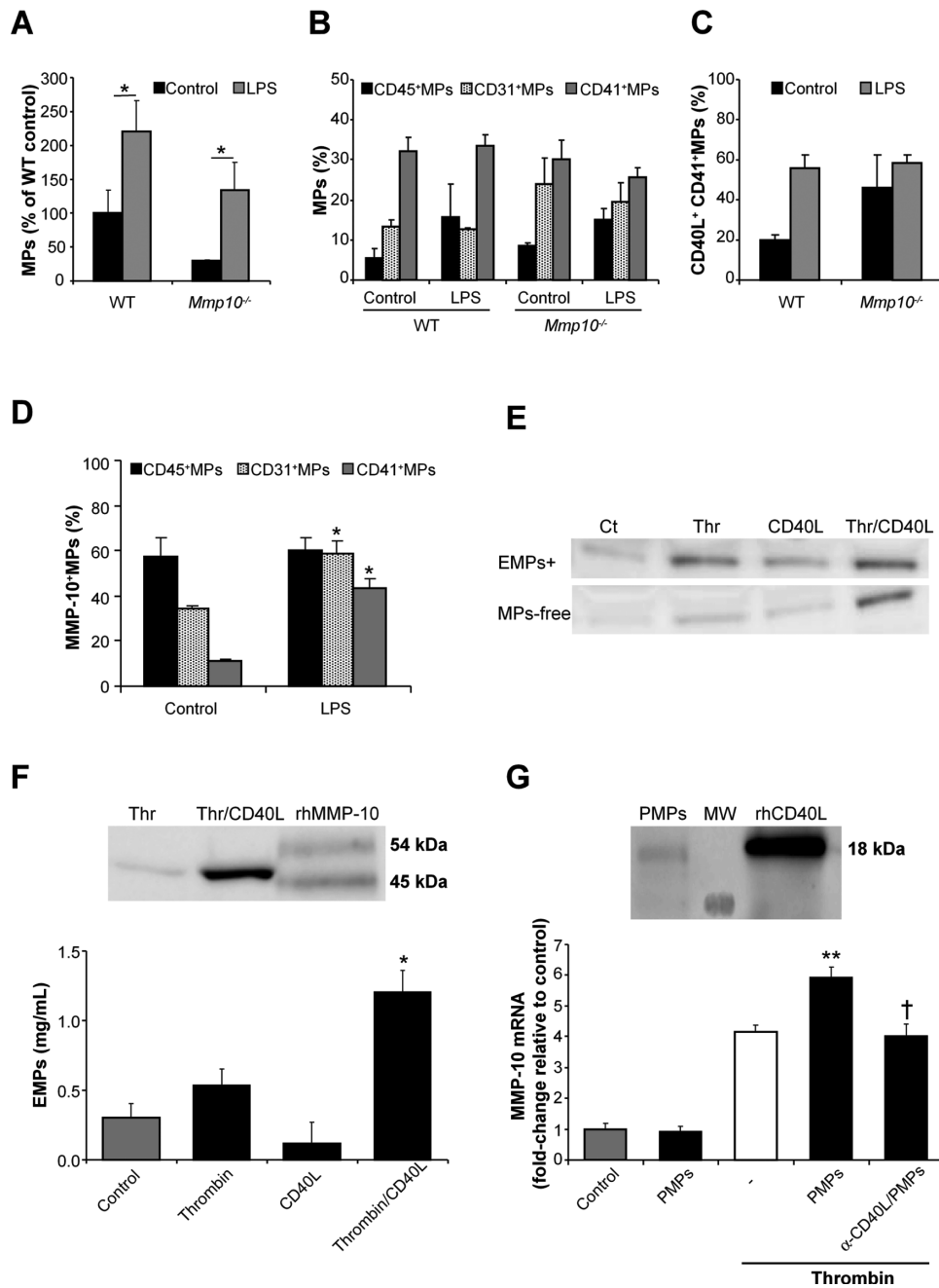
To assess the role of MMP-10 in a clinical setting, its circulating levels were measured in septic patients characterized by systemic inflammation and thrombin and fibrin generation, as assessed by increased D-dimer levels.<sup>21</sup> We found a significant increase of MMP-10 and CD40L in septic patients, particularly in the nonsurviving group. Thrombin, protein C pathway, activators and inhibitors of fibrinolysis, and PARs play vital roles in the crosstalk between inflammation and coagulation in sepsis,<sup>28</sup> but the role of CD40L in sepsis still remains unclear.

CD40/CD40L upregulation has been confirmed in experimental studies of endotoxemia in mice<sup>24,29</sup> and septic patients.<sup>30</sup> Once the soluble CD40L binds to its receptor, it promotes activation of monocytes and ECs with subsequent upregulation of proinflammatory and prothrombotic factors<sup>30</sup> that could facilitate tissue damage and death. In agreement with our previous study, MMP-10 levels were also associated with markers of sepsis severity, such as lactic acid. Overall, our results suggest that thrombin, via PAR-1, and CD40L potentiate MMP-10 production in sepsis, which is associated with worse outcome and mortality. These data support previous studies reporting a correlation between MMPs and sepsis-related mortality.<sup>31</sup>

We moved into experimental animals to define the role of MMP-10 in endotoxemia by using WT and *Mmp10*<sup>-/-</sup> mice on an LPS model. No differences were detected in hematological parameters between both genotypes in baseline, and they changed similarly after LPS injection. We observed a reduction in thrombin/antithrombin complexes in LPS-induced sepsis in *Mmp10*<sup>-/-</sup> mice suggesting a diminution in thrombin activation associated with the absence of MMP-10. Previous data showing impaired inflammation associated with a secondary reduction in coagulation<sup>32,33</sup> and the existing association between inflammation and MMP-10<sup>13,21</sup> could explain a reduced inflammatory/hemostatic status in *Mmp10*<sup>-/-</sup> mice. Interestingly, the mortality rate following LPS was similar in *Mmp10*<sup>-/-</sup> mice compared with WT, suggesting that the detrimental effects of LPS on survival were unrelated to the presence of MMP-10.

Taken together, differences in outcome among human sepsis and LPS experimental model could be related to the fact that the observed increase of MMP-10 in human sepsis may be the consequence of several inflammatory/prothrombotic stimuli operating in these patients rather than a causal mechanism.<sup>34</sup> Our results point to a role of MMP-10 as a biomarker of severity in human sepsis.<sup>21</sup>

Regarding possible sources of thrombin-induced MMP-10, we and others have found that the endothelium and other cell types, particularly leukocytes, could be responsible for its circulating levels.<sup>35,36</sup> MPs also convey a broad spectrum of bioactive molecules, and may serve as shuttles promoting cellular crosstalk in various pathological settings such as sepsis. We assessed the amount, origin, and composition of circulating MPs in the endotoxemic model of sepsis. We described reduced MP levels at baseline in *Mmp10*<sup>-/-</sup> that may suggest lower cell activation in the absence of this protease. After LPS injection we observed an increase of circulating MPs in both genotypes mainly in CD45<sup>+</sup> MPs as marker of leukocyte-derived MPs. During acute inflammatory events, such as those that occur during sepsis, the generation of procoagulant MPs from endothelial, platelet, erythrocyte, and leukocyte origins has been reported.<sup>37</sup>



**Figure 5.** Generation of microparticles (MPs) in vivo and in vitro. **A**, Detection (flow cytometry) of circulating MPs in saline or lipopolysaccharide (LPS)-injected (15 mg/kg, n=3/condition) wild-type (WT) and *Mmp10*<sup>-/-</sup> mice. **B**, Determination of cellular origin (flow cytometry) of circulating MPs in control and LPS-injected WT and *Mmp10*<sup>-/-</sup> mice; CD45<sup>+</sup> MPs are leukocyte-derived vesicles, CD31<sup>+</sup> MPs correspond to endothelial, platelet, or leukocyte origin, and CD41<sup>+</sup> MPs are platelet-derived vesicles (n=3/condition). **C**, Circulating CD41<sup>+</sup> MPs were immunostained for CD40 ligand (CD40L) in saline and LPS-injected WT and *Mmp10*<sup>-/-</sup> mice (n=3/condition). **D**, MPs from different cellular origins harbor MMP-10 in WT mice (n=3/condition). **E**, Western blot for matrix metalloproteinase-10 (MMP-10) in human umbilical vein endothelial cell (HUVEC) supernatants 24 hours after stimulation with thrombin (0.25 U/mL), CD40L (0.25 μg/mL), or the combination of both of them before and after MP removal. Active MMP-10 was detected (45 kDa). **F**, lower panel: quantification of endothelial-derived MPs (EMPs) originated from HUVEC stimulated with thrombin (1 U/mL), CD40L (0.25 μg/mL), or thrombin/CD40L for 48 hours. Upper panel: MMP-10 (Western blot) in EMPs originated from cells stimulated with thrombin (Thr, 10 U/mL) +/- CD40L (1 μg/mL). **G**, CD40L (18 kDa) was detected by Western blot in lysed platelet-derived MPs (PMPs; upper panel). rhCD40L was used as positive control (0.25 μg/mL). Lower panel: Endothelial MMP-10 expression 6 hours after stimulation with thrombin (0.25 U/mL), CD40L<sup>+</sup> PMPs (0.6 mg/mL), and CD40L<sup>+</sup> PMPs preincubated with α-CD40L antibody (7.5 μg/mL) before thrombin addition. Data (mean±SD, n=3) (\*P<0.05 vs controls, \*\*P<0.01 vs thrombin, †P<0.05 vs thrombin/PMPs).

Leukocyte-derived MPs have been specifically related to processes that converge in sepsis such as hemostasis and pathological thrombosis, inflammatory response, and endothelial function. In addition, we observed that most platelet-derived MPs (CD41<sup>+</sup> MPs) bear CD40L, mainly in endotoxemic mice. Finally, we show that MPs harboring MMP-10 are more abundant after LPS injection. Previous studies have documented the presence of other MMPs in MPs related to vascular remodeling and cell invasion.<sup>38</sup> Whether MMP-10<sup>+</sup> MPs could modulate the procoagulant/inflammatory states in sepsis should be further analyzed in other studies designed for this purpose.

Finally, we assessed the capacity of thrombin/CD40L on the generation of MPs harboring MMP-10. Herein, we describe that thrombin/CD40L synergistically increased the generation of EMPs containing MMP-10. This novel observation agrees with previous reports showing that shed vesicles act as protease carriers.<sup>17</sup> In this setting, MMP-10 harbored by EMPs could regulate the focalized proteolytic activity essential in clinically relevant processes. Because the ability of transferring membrane-associated molecules to other cell types is a common feature for MPs,<sup>11</sup> we further checked the capacity of PMPs to bear CD40L activity. Our results showed that PMPs harbored CD40L on their surface and were able to increase MMP-10 expression in the presence of thrombin in a CD40L-dependent manner. Taken together, these data suggest that the combination of thrombin with CD40L, either secreted by activated platelets or carried by PMPs, has functional consequences on ECs.

This study has some limitations. The series of sepsis analyzed is rather small, and plasma samples from these patients were not properly collected to determine MPs because serial centrifugation of platelet-poor plasma was not done before storage.

Overall, our observations suggest that MMP-10 upregulation can be the result of a positive feedback loop between inflammation and thrombosis, carried out by thrombin in combination with CD40L likely participating in circulating MPs. These effects may be of pathophysiological significance in patients with cardiovascular disease and prothrombotic disorders. Further studies are needed to establish whether MMP-10 is critical in mediating the effects of thrombin on inflammation to define its role in the pathophysiology of thrombin-related disorders and to gain more insights into MMP-10 as a new therapeutic target.

### Acknowledgments

We thank Dr W.C. Parks (University of Washington, Seattle) and Guillermo Martínez de Tejada (Microbiology Department, University of Navarra) for kindly providing *Mmp10*<sup>-/-</sup> mice and lipopolysaccharide from *Escherichia coli* respectively, and Lara Montori for her technical assistance.

### Sources of Funding

This work was funded through the "UTE project CIMA" (University of Navarra) and grants SAF2009-11949, SAF2009-12039 from Ministerio de Ciencia e Innovación (MICINN), and Red Temática de Investigación Cardiovascular RECAVA (RD06/0014/0008 and RD06/0014/0027) and FIS/PS09/00143, FIS/PI-10-01572, and I3SNS-INT-11-063 from Instituto de Salud Carlos III (Madrid, Spain). Sara Martínez de Lizarrondo and Ana Purroy were supported by a fellowship from the Education Department, Gobierno de Navarra. Carmen Roncal was supported by the Ministry of Science and Innovation MICINN-PTQ -09-02-01941.

### Disclosures

None.

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## SUPPLEMENT MATERIAL

### Cell cultures

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords by digestion with collagenase A (Invitrogen) and cultured as described<sup>1</sup>. Human artery endothelial cells (HAEC, Cambrex BioScience) were grown in endothelial growth medium (EGM-2, Cambrex) according to the manufacturer's instructions. Cells were seeded in multi-well plates and at confluence were arrested overnight and further stimulated with recombinant human thrombin (0.1-10 U/mL; Enzyme Research Laboratory), recombinant human soluble CD40L (rhCD40L, 0.25-1 µg/mL; Peprotech, US), purified platelet-derived microparticles (PMPs) (0.6 mg/mL) or different PAR agonist peptides, in the presence or absence of specific inhibitors.

When needed, cells were also pre-treated for 30 min (unless otherwise stated) with several blocking antibodies or inhibitors of intracellular signaling pathways before thrombin and/or CD40L stimulation: 10 µM PD98059 (mitogen extracellular kinase-1/2 [MEK1/2] inhibitor, Calbiochem), 10 µM SB203580 (p38 mitogen-activated protein kinase [MAPK] inhibitor, Calbiochem), 10 µM SP600125 (c-Jun N-terminal kinase [JNK] inhibitor, BioSource) and, 1 µM wortmannin (phosphatidylinositol-3 kinase [PIK3] inhibitor, Sigma). Blocking antibodies were: ATAP-2 and N-19 antibodies (2 µg/mL, Santa Cruz Biotechnology, Inc.), against the active and the N-terminal site of PAR-1 respectively<sup>2</sup>. Recombinant hirudin (2 µM, Refludan, Schering AG) and PAR-1 and PAR-2 agonist peptides, TRAP-1 (TFLLRNPNDK; 50 µM) and TRAP-2 (SLIGRL; 100 µM), generated as described<sup>3</sup>, were also assayed. The CD40/CD40L system was inhibited using antibodies against CD40 (α-CD40, 7.5 µg/mL, R&D Systems) and CD40L (α-CD40L, 7.5 µg/mL, BenderMed Systems), using an isotype control antibody (α-IgG2b, R&D Systems).

Additionally, actinomycin D (0.5 µM, Sigma) was assayed to determine changes at transcriptional rate.

## **Phosphorylation pattern of the signaling pathways**

To study the phosphorylation pattern of the different signaling pathways, cells were stimulated with thrombin, CD40L or their combination and protein extracts analyzed by Western blot as described<sup>4</sup>. Briefly, cell cultures were washed twice with buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 100 mM NaF, 10 mM NaPPi, 10 mM EDTA, 2 mM Na<sub>3</sub>VO<sub>4</sub>) and lysed with buffer (containing 1 mM PMSF, 5 μM leupeptin, 1% triton X- 100). Protein content of whole cell lysates was determined by bicinchoninic acid (BCA) protein assay TM (Pierce) using bovine serum albumin as standard. Proteins were separated by SDS-PAGE (12.5% acrylamide:bisacrylamide) and electrotransferred onto nitrocellulose membranes (Bio-Rad Laboratories) in Tris-Glycine transfer buffer with 20% methanol in a Bio-Rad Trans-Blot Cell (Bio-Rad Laboratories). Membranes were blocked with 5% skimmed milk in TBS-Tween for two hours at room temperature before incubation with antibodies to ERK1/2 (#9102, Cell Signaling Technology) and phosphorylated ERK1/2 (#9106, Cell Signaling Technology), p38 MAPK (sc-7194, Santa Cruz Biotechnology) and phosphorylated p38 MAPK (#9216, Cell Signaling Technology), JNK-1 (sc-474, Santa Cruz Biotechnology, Inc) and phosphorylated JNK-1 (#9251, Cell Signaling Technology) overnight at 4°C. Membranes were thoroughly washed and incubated with horseradish peroxidase-coupled anti-rabbit or anti-mouse immunoglobulin G antibodies (Dako) for 1 h at room temperature and finally washed four times in TBS-Tween 20. Bands were detected using a chemiluminescent detection system (Supersignal West Dura™, Pierce) after exposure to X-Ray AX film (Agfa).

## **Gene expression analysis**

RNA from cells was extracted using a semi-automated system for the isolation and purification of nucleic acids (Abi Prism 6100, Applied Biosystems) and reverse transcribed with 1 μg of total RNA, random primers and Moloney Murine Leukaemia Virus (MMLV) reverse

transcriptase (Invitrogen). Real-time PCR was performed on an ABI PRISM 7900 sequence detector (Applied Biosystems) using TaqMan™ gene expression Assays-on-demand (Applied Biosystems) for MMP-10 (Hs00233987\_m1), CD40 (Hs00374176\_m1) and TIMP-1 (Hs00171558\_m1). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 4326317E) was used as housekeeping gene.

### **MMP-10 protein levels**

MMP-10 levels were measured in conditioned medium by ELISA (Quantikine, R&D Systems), which recognizes the zymogen form, following the manufacturer's instructions. Inter- and intra-coefficients of variation were < 8%. Moreover, MMP-10 protein levels were also measured by Western blot in conditioned medium of thrombin, CD40L or thrombin/CD40L stimulated endothelial cells (HUVEC and HAEC, 24 h after stimulation). To determine whether thrombin, CD40L or the combination induced the release of endothelial MPs harbouring MMP-10 (MMP-10<sup>+</sup>EMPs), as well as the contribution of MMP-10<sup>+</sup>EMPs to the total amount of active MMP-10 in cell culture supernatants, HUVEC were stimulated for 24h and supernatants collected. Samples were centrifugated to eliminate MPs (EMPs-free) or used as collected (EMPs<sup>+</sup>). EMPs-free fraction was subjected to a centrifugation of 1,500 xg, 10 min, at 4°C to eliminate cellular debris followed by a centrifugation 20,000 xg, 90 min at 4°C to eliminate EMPs. Samples were separated by SDS-PAGE (4-12% Bis-Tris gel, Invitrogen) and transferred onto nitrocellulose membrane by iBLOT transfer stacks (Invitrogen). Blots were incubated overnight with an anti-MMP-10 antibody ( $\alpha$ -MMP-10, dilution 1:1000, R&D Systems) followed by incubation with a peroxidase-conjugated goat anti-mouse antibody (1:10000 Santa Cruz) and developed by a chemiluminescent detection system (ECL Advance, GE Healthcare).

### **Analysis of thrombin-induced CD40 by EC**

HUVEC were stimulated with thrombin (0.1-1 U/mL) (Sigma) for 24 h and protein extracts were analyzed by Western blot (non reducing conditions). Briefly, cells were scrapped and centrifuged for 10 min at 3,200 xg. Cell pellets were resuspended and lysed with a phosphate solution 50 mM, pH=7 (K<sub>2</sub>HPO<sub>4</sub> 50 mM, KH<sub>2</sub>PO<sub>4</sub>, EDTA 0.01 mM and a cocktail of protease inhibitors). Protein concentration was measured by the BioRad DC protein assay (BioRad labs). Proteins (6 µg total homogenate) were separated by SDS-PAGE (4-12% Bis-Tris gel, Invitrogen) and transferred onto nitrocellulose membrane by iBLOT transfer stacks (Invitrogen). Blots were incubated overnight with a monoclonal antibody α-CD40 (2 µg/mL, R&D Systems) followed by incubation with a peroxidase-conjugated rabbit anti-mouse antibody (GE Healthcare) and developed by a chemiluminescent detection system (ECL Advance, GE Healthcare).

In addition, CD40 expression on the EC surface was assessed by FACS after thrombin (0.1-1 U/mL, 24 h) stimulation. HUVEC were detached and then 1x10<sup>6</sup>/mL cells were incubated with anti-human CD40 antibody (10 µg/mL, R&D Systems) or its isotype control (10 µg/mL IgG2b, eBiosciences). After subsequent incubation with a FITC-labeled anti-mouse Ig antibody (1 µg/mL BD Biosciences), fluorescence was determined by flow cytometry (FACSCalibur, BD Biosciences). Nonviable cells were assessed by measuring cell-surviving rate after 7-AAD (7-amino-Actinomycin D) incubation.

In order to study whether thrombin could be able to rapidly expose membrane CD40 on the endothelial surface, we performed immunofluorescence in ECs grown on slides (Nalgene Nunc International) precoated with 0.1% gelatin. After reaching confluence, cells were stimulated with 0.25 U/mL thrombin for 30 min. Then cell nuclei were stained with Draq5 (10 µM, Invitrogen) for 5 min at 37°C. Once the nuclei were stained cells were fixed with 4% paraformaldehyde and immunofluorescence done incubating cells with 10 µg/mL mouse anti human CD40 (R&D Systems), followed by incubation with 10 µg/mL anti-mouse IgG Alexa

Fluor 488® (Molecular Probes). Finally, they were mounted in Glycerol Gelatin reagent (Sigma) and visualized under laser confocal microscope (Zeiss LSM-510 Meta; 40× objective). Forty image stacks (512×512 pixels each) were captured from representative areas. The specificity of the fluorescent signal was checked in samples prepared by omitting the first antibody.

### **Generation and characterization of thrombin/CD40L microparticles (MPs)**

Endothelial-derived MPs (EMPs) were purified from conditioned medium after stimulating HUVEC treated for 48 h with thrombin and/or CD40L (1-10 U/mL and 0.25-1 µg/mL) respectively, as previously described<sup>5</sup>. Briefly, culture supernatants were collected and cleared from detached cells or large cell fragments by centrifugation at 1,500 xg for 15 min. The supernatants were then centrifuged at 20,000 xg for 90 min at 4°C. Pelleted EMPs were washed twice and resuspended in HEPES-NaCl buffer pH=7.4.

To obtain platelet-derived MPs (PMPs), platelets were isolated from blood collected into acid-citrate-dextrose from healthy volunteers and platelet-rich plasma was obtained by centrifugation at 200 xg for 15 min at room temperature, followed by a second centrifugation at 1,200 xg for 12 min. Platelets were then washed twice in 36 mM citric acid buffer, pH 6.5, containing 100 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, and 5 mM glucose. Isolated platelets were activated with 9 nM =1 U/mL of human thrombin for 30 min at room temperature. PMPs were then harvested as described for EMPs.

Standardization of MPs was achieved by flow cytometry on a BD FACSCanto II cytometer (BD Biosciences), using a mix of monodisperse fluorescent beads of three diameters (0.5, 0.9 and 3 µm) (Megamix, BioCytex, Stago, France). Forward scatter (FS) and side scatter (SS) parameters were plotted on logarithmic scales to best cover a wide size range. Each individual bead subset was gated on the basis of their SS and FL1 properties; with discriminator on FS,



the MPs region was then set up between the 0.5  $\mu\text{m}$  bead and 0.9  $\mu\text{m}$  bead gates. For MPs labeling, 5  $\mu\text{l}$  of Annexin V–Alexa Fluor 568 conjugate (Beckman Coulter, Fullerton, CA) were added to 100  $\mu\text{l}$  of freshly isolated MPs. After 30 min of incubation at room temperature, samples were diluted in 300  $\mu\text{l}$  of binding buffer, using EDTA buffer as control (PBS, 1mM EDTA, 25mM HEPES pH 7, 0.5% Fetal Bovine Serum and 1% penicillin/streptomycin). The total amount was assessed in lysed MPs by protein quantification at 280 nm using NanoDrop spectrophotometer (NanoDrop 1000; Thermo Fisher Scientific).

The presence of MMP-10 and CD40L was analyzed by Western blot in previously lysed EMPs and PMPs respectively, using specific  $\alpha$ -MMP-10 (dilution 1:1000, R&D Systems) and  $\alpha$ -CD40L (10  $\mu\text{g}/\text{mL}$ , MedBender Systems) antibodies.

#### **Induction of MMP-10 in mouse aorta *in vivo***

Male C57Bl/6 (originating from breeders purchased from Charles River) wild-type (WT) and knockout mice for MMP-10 (*Mmp10*<sup>-/-</sup>, kindly provided by Dr. WC Parks, Washington University, USA) (6 months old, 25-30 g, n = 30) were procured at the CIMA animal facility and fed a normal chow diet. The research was performed in accordance with the European Community guidelines for ethical animal care and use of laboratory animals (Directive 86/609), and approved by the Animal Research Review Committee of the University of Navarra.

In order to assess the role of thrombin and CD40L on MMP-10 expression *in vivo*, anesthetized mice (2.5% isoflurane) were injected with human thrombin (2.5 U/mouse), rhCD40L (2.5  $\mu\text{g}/\text{mouse}$ ), their combination or saline (as control), via the ocular plexus. Animals were sacrificed by CO<sub>2</sub> inhalation 24 h after treatment and aortic arteries were obtained. MMP-10 in aorta was quantified by Western blot as indicated above using a polyclonal  $\alpha$ -MMP-10 (dilution 1:1000, Anaspec Inc). Immunoblots were quantified using a computer program (Quantity One) and MMP-10 expression was calculated using  $\beta$ -actin as endogenous control.

Briefly, after developing, the membrane was incubated with stripping buffer (Thermo Scientific) at 37°C during 15 min. The membrane was washed thoroughly and blocked with 5% Amersham™ ECL Blocking reagent in TBS-Tween for 1 h at room temperature. Membranes were then washed thoroughly and incubated overnight at 4°C with a monoclonal anti-β-actin antibody (1:20000 dilution, SIGMA), washed again and incubated with horseradish peroxidase coupled goat anti-mouse (1:30000, Santa Cruz) for 1 h at room temperature with 5% blocking reagent in TBS-Tween. The bands corresponding to β-actin were detected using a chemiluminescent detection system (Amersham™ ECL detection system).

### **Immunohistochemistry analysis in mice after thrombin/CD40L administration**

Male C57Bl/6 were intraocularly injected with the combination of human thrombin (2.5 U/mouse) and rhCD40L (2.5 μg/mouse) via the ocular plexus as previously described. Mice were sacrificed by CO<sub>2</sub> inhalation 24 h after injection and aortas dissected for histological analysis. Tissue sections were deparaffinized, treated with 5% H<sub>2</sub>O<sub>2</sub>, immersed in Target retrieval solution (DAKO) for 20 min, and incubated in 10% normal sheep serum for 30 min at room temperature. Samples were incubated with a polyclonal antibody against MMP-10 (10 μg/mL; Acris Antibodies), further incubated with biotinylated anti-goat antibodies (GE Healthcare), and then followed by the EnVision™ HRP. Rabbit (DAB+) kit (Dako). Histological analysis of different organs fixed in paraformaldehyde (4%) was performed in death mice.

### **Endotoxemia model in WT and *Mmp10*<sup>-/-</sup> mice**

6 month-old WT and *Mmp10*<sup>-/-</sup> male mice were intraperitoneal injected with LPS (5-15 mg/Kg). Blood was drawn from the ocular plexus before and after LPS injection to measure platelet number (Hemavet). Mice were sacrificed at 24 h and blood collected in 0.129 M, pH 7.4 citrate buffer for MPs isolation and determination of thrombin/antithrombin (TAT) complexes.

### **Isolation of circulating MPs in mice**

Blood was drawn from the heart of anaesthetized mice into citrate-anticoagulated syringes 24 h after LPS administration, and centrifuged at 650 xg, 10 min, at 4°C. Plasma was collected and centrifuged at 13,000 xg, 2 min, at 4°C to remove cellular debris. Resulting platelet poor plasma (PPP) was centrifuged at 16,000 xg for 25min, at 20 °C to pellet MPs. MPs containing pellet was washed in HEPES-NaCl buffer pH=7.4 and centrifuged at 16,000 xg for 25min, at 20°C. The resulting pellet was resuspended in HEPES-NaCl buffer pH=7.4 and the standardization of MPs achieved by flow cytometry analysis as previously described for EMPs (Supplemental Figure 3). The cellular origin of PPP-derived MPs was determined by flow cytometry using specific markers for; platelets (PE rat anti-mouse CD41 antibody, BD Pharmingen), endothelial cells, platelets and leukocytes (PE rat anti-mouse CD31 antibody, BD Pharmingen), leukocytes (APC rat anti-mouse CD45 antibody, BD Pharmingen) and corresponding isotype control antibodies. MMP-10 and CD40L were detected by using a rabbit anti-human MMP-10 antibody (Acris) and a rabbit anti-human CD40L antibody (Santa Cruz) respectively, that were labelled with Zenon® Alexa Fluor® 488 Mouse IgG2a labeling Kit (Life technologies) following the manufacturer's instructions before staining. Briefly, MPs were resuspended in HEPES-NaCl buffer pH=7.4, and incubated at room temperature for 20 min with 0.5 µg/mL PE rat anti mouse CD41, PE rat anti-mouse CD31, APC rat anti-mouse CD45, and labeled anti-MMP10 or anti-CD40L antibodies when needed. Stained MPs were centrifuged at 16000 xg for 25 min at 20°C to pellet them. The supernatant was discarded and MPs diluted in 400 µl of HEPES-NaCl buffer pH=7.4 to determine their cellular origin by flow cytometry (BD FACSCanto II, BD Biosciences).

The relative amount of MPs was determined in relation to a mix of monodisperse fluorescent beads (Megamix, BioCytex, Stago, France). 4 µL of MPs isolated from 100 µL of PPP were resuspended in 400 µL of HEPES-NaCl buffer pH=7.4, and mixed with 25 µL of beads. The

acquisition was stopped when the gate of 0.5  $\mu\text{m}$  beads reached 3000 events, and number of MPs calculated.

### **Determination of thrombin/antithrombin complexes**

PPP was obtained as previously described and TAT complexes determined by an enzyme immunoassay (Enzygnost<sup>®</sup> TAT micro, Siemens) following the manufacturer's instructions.

### **Circulating levels of MMP-10 and CD40L in patients with sepsis**

Samples were obtained from 60 patients with sepsis from abdominal origin hospitalized in six Spanish intensive care units (ICU). The clinical characteristics and the different variables are reported in Supplemental table 2. Data from the survivors (n=33) and non-survivors (n=27) during ICU hospitalization were compared.

Blood samples were collected in serum separator and citrated tubes (Vacutainer, Becton Dickinson). Serum and plasma were obtained by centrifugation within 30 min at 1,000 xg for 15 min, and then removed and frozen at -80°C until measurement. MMP-10 (R&D Systems), fibrin degradation products (Innovance D-Dimer, Siemens) and soluble CD40L (Bender MedSystems) were assayed with specific ELISAs. The inter and intra-assay coefficients of variation for ELISAs were < 8%. The study was approved by the Institutional Review Boards of the corresponding hospitals, and informed consent from patients or from family members was obtained.

### **Statistical analysis**

Results from *in vitro* studies are expressed as mean $\pm$ SD of 3 independent experiments in triplicate. Continuous variables were expressed as mean $\pm$ SD (unless otherwise stated). Statistical analysis was performed by Kruskal–Wallis followed by Mann–Whitney U-test for comparisons between groups. Fisher's exact test was used to calculate differences in mice

mortality rate. Spearman correlation test was used for unvaried associations of continuous variables. Estimated marginal means for MMP-10 and CD40L for survivors and non-survivors were calculated by factorial ANOVA with age and sex as covariables. The statistical analysis was performed with SPSS for Windows software package version 15.0.

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### Supplemental Table I

Mortality rate in wild-type (WT) and *Mmp10*<sup>-/-</sup> mice after the administration of CD40L (2.5 µg), thrombin (Thr, 2.5 U), thrombin/CD40L (2.5 U + 2.5 µg) or saline (control).

<b>Mortality</b>	<b>Control</b>	<b>CD40L</b>	<b>Thr</b>	<b>Thr/CD40L</b>
WT	0/16	0/16	2/16	8/16*
<i>Mmp10</i> <sup>-/-</sup>	0/16	0/16	3/16	6/16

\* p < 0.05 vs Thr.

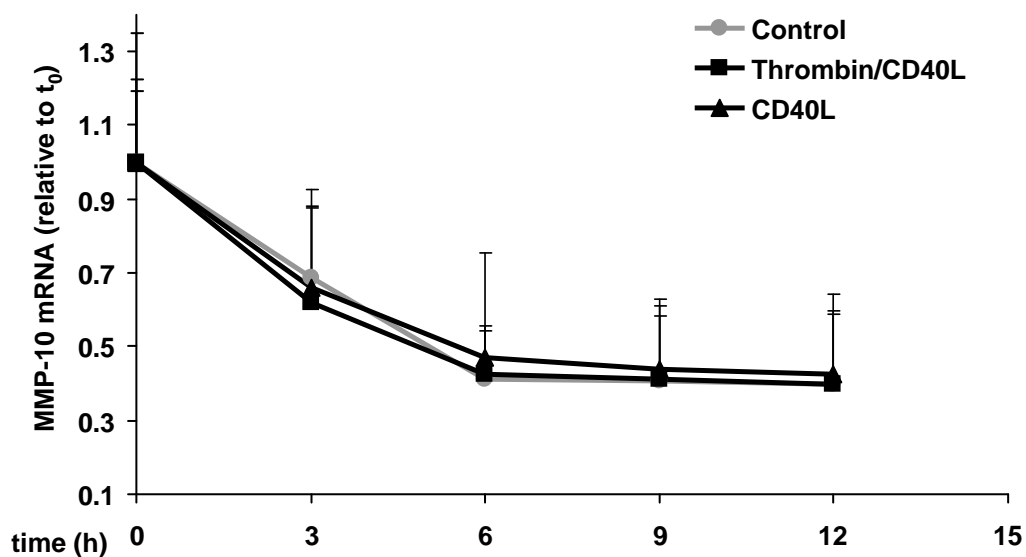
## Supplemental Table II

Demographic and clinical parameters of surviving and non-surviving septic patients. Median and interquartile range (IQR) or percentage, when indicated, are shown.

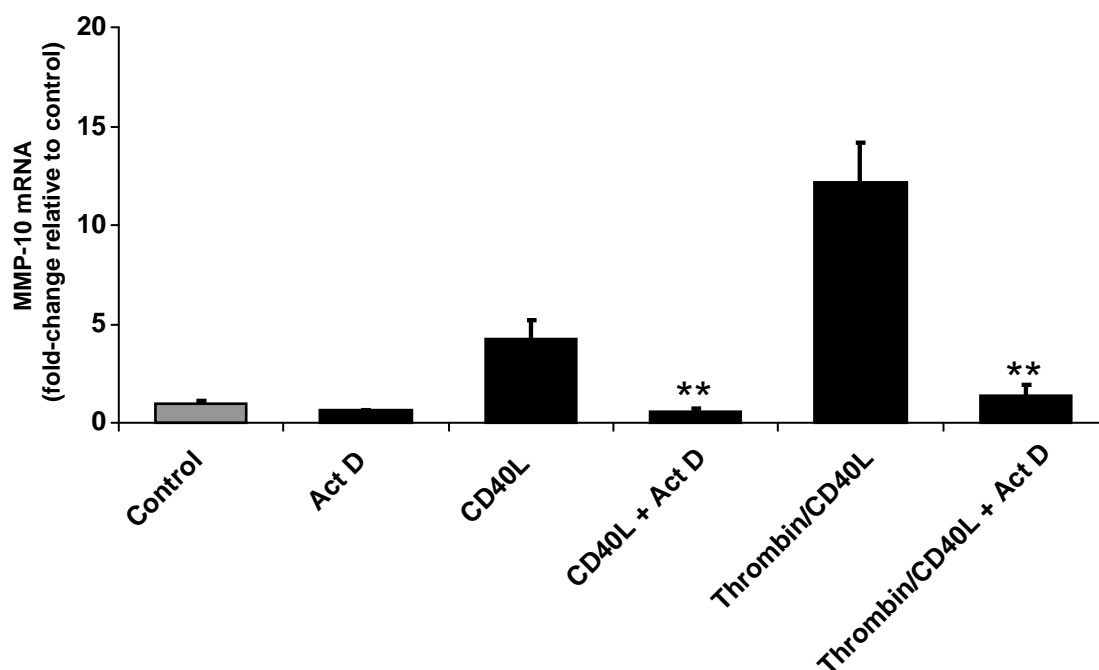
	Survivors (n=33)	Non-survivors (n=27)	p
Gender female: number (%)	19 (45.23)	10 (41.66)	0.460
Age: median years (IQR)	65.5 (29)	67.5 (26)	0.779
Diabetes mellitus: number (%)	11 (26.2)	4 (16.6)	0.374
COPD: number (%)	7 (16.66)	1 (4.16)	0.174
APACHE-II score: median (IQR)	20 (6)	23 (6)	<b>0.002</b>
Creatinine (mg/mL): median (IQR)	1.6 (1.1)	2.6 (2.9)	<b>0.023</b>
Leucocytes: median/mm <sup>3</sup> (IQR)	12,200 (16,450)	13,800 (16,300)	0.913
Lactic acid: median mmol/L (IQR)	2.0 (2.5)	4.6 (4.4)	<b>&lt;0.001</b>
Platelets: median/mm <sup>3</sup> (IQR)	158,000 (114,500)	137,000 (144,500)	0.218
INR: median (IQR)	1.5 (0.44)	1.8 (1.73)	<b>0.025</b>
aPPT: median seconds (IQR)	64 (27)	49 (35)	<b>0.028</b>
SOFA score: median (IQR)	9 (5)	10.5 (7)	0.083

APACHE-II = Acute Physiology and Chronic Health Evaluation; aPTT = activated partial thromboplastin time; COPD = chronic obstructive pulmonary disease; INR = international normalized ratio; SOFA = Sepsis-related Organ-failure Assessment score.

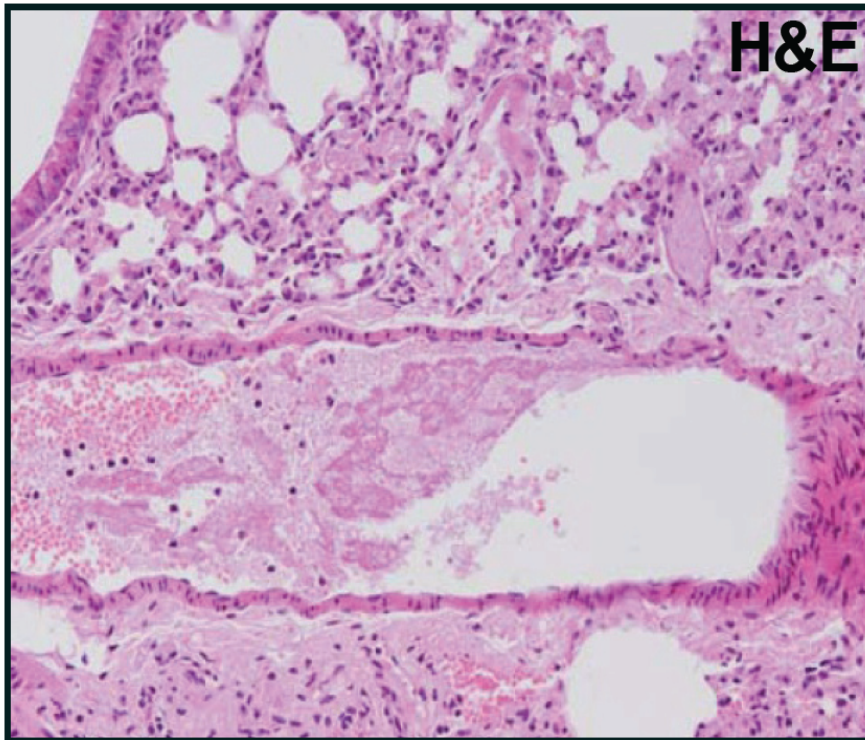
A



B

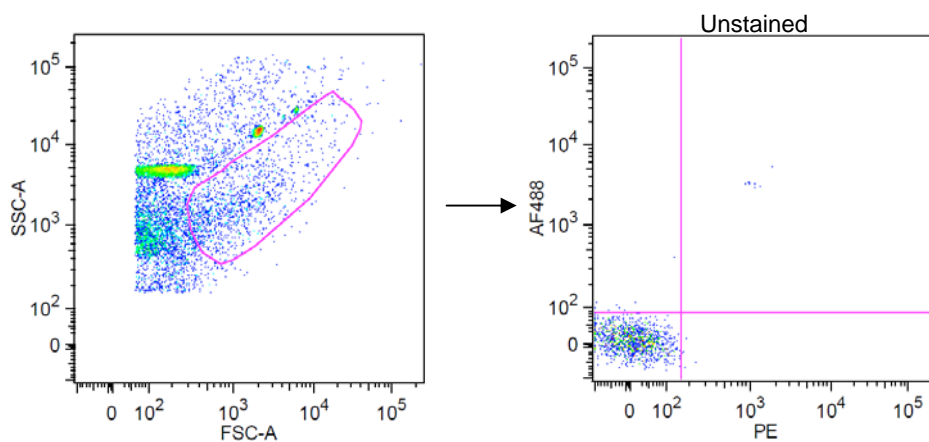


**Supplemental Figure I.** Induction of MMP-10 by CD40L and thrombin/CD40L occurs at transcriptional level. (A) MMP-10 mRNA levels of HUVEC stimulated with vehicle, CD40L or thrombin/CD40L for 12 h and then treated with actinomycin D for additional 12 h. Results were plotted semilogarithmically as a function of time (relative to time 0 [ $t_0$ ]). Neither CD40L nor thrombin/CD40L modified MMP-10 mRNA stability (half-life). (B) Preincubation with actinomycin D (Act D, 30 min) completely blocked CD40L or thrombin/CD40L upregulation of MMP-10, indicating that MMP-10 up-regulation occurs at transcriptional level. \*\* $p < 0.01$  vs treatment without Act D.

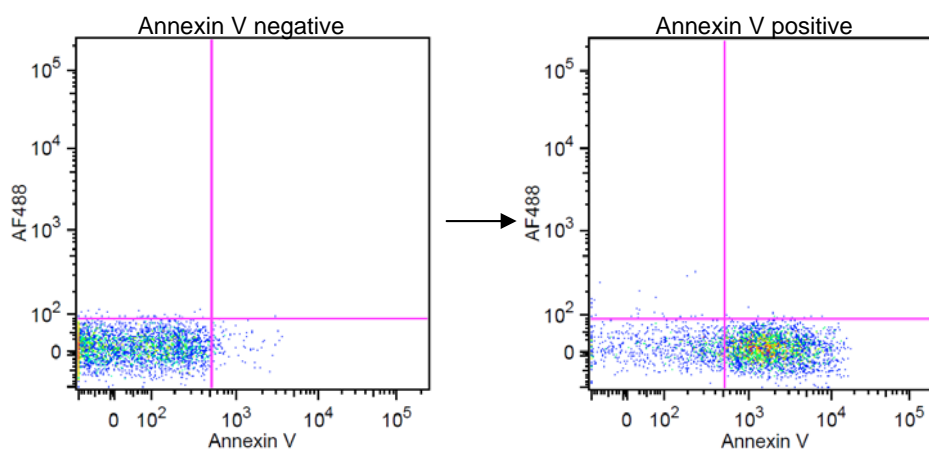


**Supplemental Figure II.** Lung section (hematoxylin & eosin staining) in thrombin/CD40L treated mice showing intense intravascular fibrin deposits (200x).

**A**



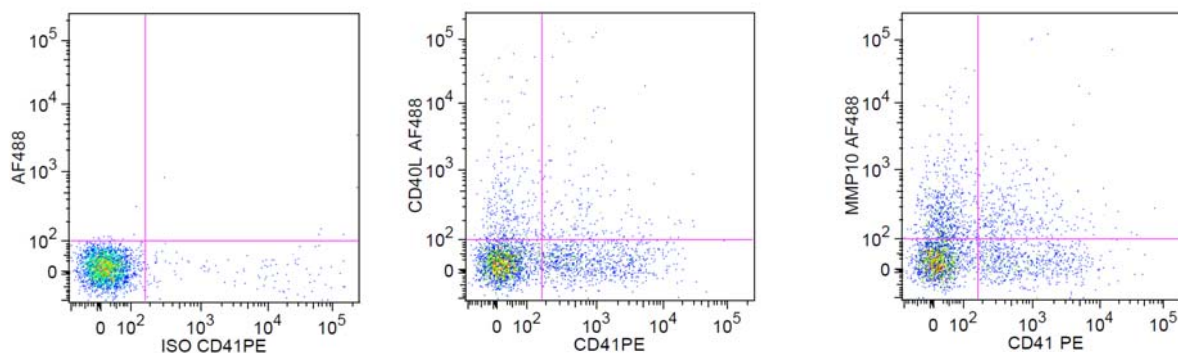
**B**



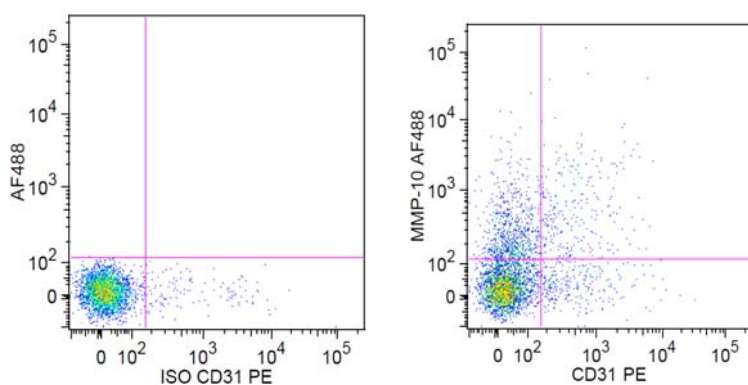
**Supplemental Figure III.** Identification of mouse MPs by flow cytometry. A) Representative dot plot showing the gating strategy for MPs based on beads of 0.5, 0.9 and 3  $\mu\text{m}$  size. B) 80% of the population gated in A is Annexin V positive (right panel, Annexin V–Alexa Fluor 568 conjugate). Annexin V negative MPs refers to MPs stained for Annexin V in presence of EDTA as a calcium chelator.

## Figure Suppl IV

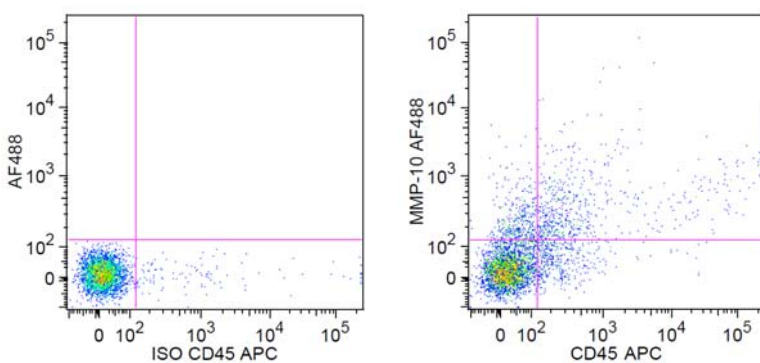
### A



### B



### C



**Supplemental Figure IV.** CD41<sup>+</sup>MPs, CD31<sup>+</sup>MPs and CD45<sup>+</sup>MPs were identified by flow cytometry using Isotype control antibodies to determine the specificity of specific antibodies. A) Left hand panel represent WT MPs stained with isotype control antibodies for CD41 and CD40L/MMP10. Middle panel shows MPs stained with CD41PE and CD40LAF488 antibodies. Right hand panel represent CD41PE and MMP10AF488 stained MPs. B) MPs stained for CD31 (right panel) and its corresponding isotype control (left panel). C) MPs stained for CD45 (right panel) and its corresponding isotype control (left panel).