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ORIGINAL ARTICLE

EMMPRIN (CD147/basigin) mediates platelet–monocyte interactions *in vivo* and augments monocyte recruitment to the vascular wall

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Summary. *Background:* Platelets play a central role in hemostasis, in inflammatory diseases such as atherosclerosis, and during thrombus formation following vascular injury. Thereby, platelets interact intensively with monocytes and enhance their recruitment to the vascular wall. *Objectives:* To investigate the role of the extracellular matrix metalloproteinase inducer (EMMPRIN) in platelet–monocyte interactions. *Methods and Results:* Isolated human monocytes were perfused in vitro over firmly adherent platelets to allow investigation of the role of EMMPRIN in platelet–monocyte interactions under flow conditions. Monocytes readily bound to surface-adherent platelets. Both antibody blockade and gene silencing of monocyte EMMPRIN substantially attenuated firm adhesion of monocytes to platelets at arterial and venous shear rates. *In vivo*, platelet interactions with the murine monocyte cell line ANA-1 were significantly decreased when ANA-1 cells were pretreated with EMMPRIN-silencing small interfering RNA prior to injection into wild-type mice. Using intravital microscopy, we showed that recruitment of EMMPRIN-silenced ANA-1 to the injured carotid artery was significantly reduced as compared with control cells. Further silencing of EMMPRIN resulted in significantly fewer ANA-1–platelet aggregates in the mouse circulation as determined by flow cytometry. Finally, we identified glycoprotein (GP)VI as a critical corresponding receptor on platelets that mediates interaction with monocyte EMMPRIN. Thus, blocking of GPVI inhibited the effect of EMMPRIN on firm monocyte adhesion to platelets under arterial flow conditions in vitro, and abrogated EMMPRIN-mediated platelet–monocyte aggregate formation *in vivo*. *Conclusions:* EMMPRIN supports platelet–monocyte interactions and promotes monocyte recruitment to the arterial wall. Therefore, EMMPRIN might represent a novel target to reduce vascular inflammation and atherosclerotic lesion development.

Keywords: adhesion, atherosclerosis, extracellular matrix metalloproteinase inducer, glycoprotein VI, monocytes, platelets.

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Introduction

Atherosclerosis is a progressive inflammatory disease of the vascular wall, and a common cause of morbidity and mortality worldwide [1]. Platelets and monocytes are critically involved in atherogenesis [2]. Notably, platelets are recruited to the vascular endothelium of atherosclerotic arteries at very early stages of lesion development, and even before vascular alterations become visible [3]. Adherent platelets thereby pave the way for subsequent leukocyte recruitment [3]. During atherogenesis, circulating monocytes are recruited to the vascular wall in a coordinated multistep process of tethering, rolling, and then firm adhesion to the activated endothelium [4]. This process is strongly supported by adherent platelets [4]. Furthermore, interaction of platelets with circulating monocytes induces inflammatory and procoagulatory pathways in both cell types [5–7]. In the clinical setting, increased formation of platelet–monocyte aggregates (PMAs) has been noted in

patients with acute myocardial infarction [8], and interactions between platelets and monocytes play a relevant role in the procoagulant state found in these patients [9].

Various ligand–receptor pairs have been identified that mediate PMA formation. So far, firm binding of monocytes and platelets has been mainly attributed to the interaction of P-selectin with P-selectin glycoprotein (GP) ligand-1 (PSGL-1), binding of the β_2-integrin MAC-1 to platelet GPIbα, and to fibrinogen-bridged binding of MAC-1 (and also cellular adhesion molecules, i.e. ICAM) to platelet GPIIb–IIIa [4]. However, the contribution of other adhesion molecules in this process remains largely unknown.

Adherent monocytes secrete matrix metalloproteinases (MMPs) and express membrane-type (MT) MMPs, which enable them to migrate into the developing plaque [10–12]. De novo synthesis of certain metalloproteinases, such as MT1-MMP, MMP-2, and MMP-9, is induced by the immunoglobulin-like GP extracellular matrix metalloproteinase inducer (EMMPRIN [CD147]; basigin in mice) [13]. The basic mechanisms of this process have been partially elucidated in tumor cells: EMMPRIN-expressing tumor cells induce the synthesis of MMPs such as MMP-1, MMP-2, MMP-3, and MMP-9, as well as MT1-MMP and MT2-MMP, in adjacent fibroblasts by means of a transcellular homophilic EMMPRIN–EMMPRIN interaction [14–16]. In consequence, EMMPRIN expression on various tumor cells correlates with metastasis development and patient mortality [17]. Recently, we determined that certain proatherogenic stimuli upregulate expression of EMMPRIN on monocytes in vitro [18,19]. We also showed enhanced surface expression of EMMPRIN on monocytes from patients with acute myocardial infarction. EMMPRIN stimulates expression of MMP-9 in monocytes and of MMP-2 in smooth muscle cells, indicating a key regulatory role for MMP activity at the vascular wall [20]. Interestingly, EMMPRIN ligation fosters platelet activation and degranulation in vitro [20]. In addition, platelets induce nuclear factor-κB-regulated activation pathways in monocytes in an EMMPRIN-dependent manner, triggering the subsequent release of proinflammatory cytokines and MMPs from monocytes [21]. In the present study, we demonstrate that EMMPRIN enhances platelet–monocyte interactions *in vivo* and promotes monocyte recruitment to the vascular wall. Furthermore, using genetic ablation or antibody blockade of common platelet adhesion receptors, we expand recent data by identifying platelet GPVI as a relevant counter-receptor for monocyte EMMPRIN.

Materials and methods

Animals

Specific pathogen-free wild-type C57BL6/J and B6.129s7-Selp/J ($CD62P^{-/-}$) mice were obtained from Charles River (Sulzfeld, Germany). Mice lacking the GPIIb integrin (*Itga2b*$^{-/-}$) were generated as previously described [22]. Mice lacking the transcription factor nuclear factor-erythroid 2 ($NF\text{-}E2^{-/-}$) were provided by B. Isermann (University of Heidelberg) [23,24]. All groups were age-matched (8–16 weeks) and sex-matched. NF-E2-deficient, CD62P-deficient and *Itga2b*-deficient mice were all on a C57BL6/J background. All experimental procedures performed on animals met the requirements of the German legislation on protection of animals and were approved by the Government of Bavaria/Germany.

Reagents

For fluorescence-activated cell sorting (FACS) experiments with human cells, mouse anti-human EMMPRIN/CD147 mAb (clone HIM6), anti-human PAC-1 (clone PAC-1) and anti-human CD62P (clone AK-4) were from BD Pharmingen (Heidelberg, Germany). Mouse anti-human CD11b (clone BEAR), CD18 (clone 7E4), CD29 (clone K20), CD162 PL1 (clone 3E2.25.2, blocking antibody) and CD162 PL2 (clone 5D8.8.12, non-blocking antibody) were from Immunotech (Hamburg, Germany). Mouse anti-human CD14 phycoerythrin (clone TUK4) was from Dako (Hamburg, Germany). For FACS experiments with murine cells, rat anti-mouse CD11b, CD41, CD42b and CD162 and rat IgG_1 control were from BD Pharmingen. Rat anti-mouse CD18 was from Immunotech. Hamster anti-mouse CD29 and hamster IgG control were from eBioscience (Frankfurt, Germany).

For flow chamber experiments, blocking mouse anti-human EMMPRIN/CD147 mAb (clone UM8D6) was from Ancell (Bayport, MN, USA). The anti-GPVI mAb 5C4 was generated as previously described [25].

For mouse *in vivo* experiments, blocking anti-GPVI mAb (clone 1F5-1-11) was generated as previously described [26]. Rat isotype-control IgG (clone 14B7) served as a control. The mAb against GPIbα (p0p/B) was prepared as a Fab fragment as previously described [27,28]. Non-immune rat IgG Fab served as a control.

Cells

Washed human platelets were isolated from whole blood as previously described, and adjusted to a final concentration of 2×10^8 mL^{-1} in Hepes Tyrode buffer [29]. Human monocytes were isolated as previously described [30]. Briefly, mononuclear cells were isolated by centrifugation of citrate–phosphate–dextrose–adenine-anticoagulated blood on a Ficoll gradient (20 min, $800 \times g$, room temperature). Mononuclear cells were cultured on plastic dishes (0.5×10^6 cells mL^{-1}) in very-low-endotoxin RPMI-1640 (Biochrom, Berlin, Germany) supplemented with 10% low-toxic fetal calf serum (Clonetics, Solingen, Germany). After 24 h, non-adherent cells were removed by gentle washing. The remaining cells (85–90% CD14-positive monocytes) were resuspended in EDTA (0.05% phosphate buffered saline [PBS]) and washed in PBS. ANA-1 is a murine monocyte cell line that is derived from C57BL/6 mice [31] ANA-1 cells were purchased from the European Collection of Cell Cultures (Porton Down, UK) and maintained in suspension culture.

Gene silencing of EMMPRIN

Small interfering RNA (siRNA)-mediated gene silencing of EMMPRIN/basigin was performed as previously described [20]. The siRNA sequence for human EMMPRIN was GAC GGC CAT GCT GGT CTG CAA (dXdY-overhang), and the siRNA sequence for the mouse homologous protein basigin was provided by Qiagen (Hilden, Germany). Non-silencing control siRNA (Qiagen) served as a negative control.

Intravital microscopy

ANA-1 cells were transfected with EMMPRIN-silencing siRNA or non-silencing control siRNA. Cells were labeled with 5-carboxyfluorescein-diacetate succinimidyl ester (DCF) and adjusted to 5×10^6 cells in 300 μL of PBS [25]. Mice were anesthetized by isoflurane inhalation combined with intraperitoneal injection of midazolam, fentanyl, and medetomidin. Polyethylene catheters were introduced into the left jugular vein of recipient mice, and 5×10^6 fluorescently tagged ANA-1 cells were introduced intravenously. For arterial injury, the common carotid artery was dissected free and ligated vigorously near the carotid bifurcation for 5 min as previously described [25]. Interaction of ANA-1 cells with the vascular wall of carotid arteries was visualized in situ by intravital videofluorescence microscopy before and after vascular injury. We used an Olympus BX51WI-microscope (× 20 water immersion objective, XLUMPlanFI 20 ×/0.95W; Olympus, Hamburg, Germany) together with an MT20 illumination system and CELL^R image acquisition software (Olympus). Adherent cells were indicated as number of adherent ANA-1 cells per unit surface area (mm^2).

To assess platelet thrombus formation at the site of injury, we performed another set of experiments. Platelets were labeled with DCF, and 1.5×10^5 platelets in a volume of 250 μL were injected intravenously into the jugular vein. Immediately thereafter, carotid injury was induced by ligature as described above. Platelet thrombus formation was imaged by intravital microscopy with the Olympus BX51WI microscope. Platelet thrombus area at the site of injury was quantified for 30 min in 5-min time intervals, and calculated as thrombus area (in μm^2) per mm^2 vessel surface area. All images were evaluated with a computer-assisted image analysis program (CAPIMAGE 7.4; Ingenieurbuero Dr. Zeintl, Heidelberg, Germany).

Flow cytometry

In vivo *formation of PMAs* DCF-tagged ANA-1 cells (5×10^6) were injected intravenously into anesthetized mice. After 10 min, heparinized whole blood was collected by cardiac puncture of the left ventricle. Whole blood was stained with a Cy5-labeled anti-GPIIb (CD41) mAb (MWReg 30; BD Pharmingen). PMA formation was determined by flow cytometric analysis (FACS Calibur; Becton Dickinson, Heidelberg, Germany) of platelet (CD41)-positive cells within the ANA-1 population, the latter being defined by DCF positivity. To analyze the role of GPIb, we pretreated C57BL6/J mice with 4 mg kg^{-1} body weight anti-GPIb p0p/B Fab fragments or control IgG Fab [32]. To investigate the role of GPVI, we applied 8 mg kg^{-1} body weight function-blocking anti-GPVI mAb (clone IF5-1-11) or a non-GPVI-binding control IgG (clone 14B7) intraperitoneally 120 min before ANA-1 cell injection [27]. In experiments with *Itga2b*-deficient mice, we identified platelets by their surface expression of GPIbα (CD42b). Mouse platelet counts were acquired with a Sysmex cell counter (Sysmex, Hamburg, Germany).

Validation of blood sampling technique To exclude the possibility that blood sampling by ventricular puncture artificially induced platelet activation, we compared the technique of cardiac puncture eith blood sampling from the facial vein [33]. This assay is described in detail in Data S1. As cardiac puncture allowed us to collect larger amounts of the circulating blood from the mice, we used this technique to achieve a higher number of cells (counts) for flow cytometric analysis.

Determination of monocyte surface markers following siRNA treament Expression of surface markers was determined on ANA-1 cells and human monocytes that were treated with EMMPRIN-silencing siRNA or non-silencing control siRNA (Fig. S2). We applied mAbs directed against either EMMPRIN, CD11b, CD18, CD29, or CD162, and flow cytometric analysis was performed.

Flow chamber

Monocyte recruitment to adherent platelets under flow Experiments were basically performed as previously described [34]. In brief, glass coverslips were coated with collagen (type 1, rat tail) and placed into a flow chamber (FCS2; Biotechs, Butler, PA, USA). The flow chamber was mounted on an inverted fluorescence microscope (Zeiss Axiovert, Jena, Germany). Isolated washed human platelets were perfused for 10 min at 1000 s^{-1}, which is equivalent to the shear forces found in medium-sized arteries under physiologic conditions, to achieve high surface coverage of firmly adherent platelets [32,34]. Thereafter, platelets were superfused for another 10 min with isolated human monocytes that were either left untreated (medium) or treated with: (i) EMMPRIN-silencing siRNA; (ii) non-silencing control siRNA; (iii) mouse anti-human EMMPRIN/CD147 mAb; or (iv) control IgG_1. For monocyte superfusion, we applied either arterial (1000 s^{-1}) or venous (200 s^{-1}) wall shear rates [35,36].

In another set of experiments, isolated washed human platelets were perfused for 10 min at 1000 s^{-1} to achieve high platelet surface coverage. Surface-adherent platelets were then incubated with: (i) rat anti-human blocking GPVI mAb (10 μg mL^{-1}); or (ii) control IgG. Thereafter, platelets were superfused for another 10 min at 1000 s^{-1} or 200 s^{-1} with human monocytes that were treated with EMMPRIN-silencing siRNA or control siRNA. Transient interaction (rolling)

and firm adhesion of monocytes on the platelet surface were studied in real time. The number of firmly adherent monocytes was assessed by counting the cells that did not move or detach from the surface within 20 s. All images were videotaped and evaluated by blinded staff. In addition, we performed a control experiment investigating the activation of adherent platelets in the presence of GPVI mAb (Fig. S6). This assay is described in detail in Data S1.

PMA formation under flow Formation of PMAs under arterial flow conditions in vitro was assessed in a closed flow chamber system [37]. Chamber slides were precoated for 30 min with 1% bovine serum albumin to block firm adhesion. Human monocytes and platelets were isolated and washed, and added to Tyrodes solution. Before experiments, monocytes were preincubated with human EMMPRIN/CD147 mAb (clone UM8D6) or control IgG_1 for 30 min. Thereafter, platelet and monocyte suspensions were mixed, resulting in a final concentration of 1×10^8 platelets mL^{-1} and 1×10^5 monocytes mL^{-1}. Cell suspensions (preincubated with either IgG_1 or anti-EMMPRIN mAb) were perfused for 30 min at 1000 s^{-1}. In another experiment, platelets were preactivated with ADP (5 μM) for 5 min. Cell suspensions were perfused in parallel, with a duplicate setup and multichannel flow chamber slides (μ-slides; Ibidi, Martinsried, Germany), thus excluding time-dependent effects on platelets and monocytes. In addition, we applied an air-driven continuous flow pump system (Ibidi) to minimize mechanical stress on suspended cells. After perfusion, cell suspensions were analyzed by flow cytometry with CD14/CD41 antibodies to assess PMA formation, as described above.

Statistics

Results were reported as mean ± standard deviation, and were analyzed by Student's *t*-test or one-way ANOVA with a Tukey post hoc test where appropriate. The number of individual experiments is indicated by *n*. $P < 0.05$ was considered to be significant.

Results

EMMPRIN mediates firm adhesion of monocytes to platelets under arterial and venous flow conditions

We applied a flow chamber assay to directly analyze the interactions of platelets and monocytes under flow conditions. First, we allowed human platelets to adhere to collagen, resulting in high surface coverage of firmly adherent platelets [38]. Subsequent perfusion of isolated human monocytes over immobilized platelets resulted in robust monocyte adhesion (Fig. 1A). Blocking of EMMPRIN substantially inhibited firm adhesion of monocytes to platelets as compared with pretreatment with isotype-matched control antibody (Fig. 1B,D). Interestingly, a significant effect of EMMPRIN inhibition was observed under both venous (200 s^{-1}) and arterial (1000 s^{-1}) flow conditions (Fig. 1B,D). To confirm the results obtained in the presence of a blocking antibody, EMMPRIN surface expression on monocytes was next inhibited by pretreatment with EMMPRIN-specific siRNA (Fig. S1). Control siRNA treatment served as a negative control. Importantly, gene silencing of EMMPRIN also significantly attenuated monocyte adhesion to immobilized platelets at wall shear rates of 200 s^{-1} and 1000 s^{-1} (Fig. 1A,B,D). siRNA treatment showed no effect on surface expression of PSGL-1, lymphocyte function-associated antigen-1, β_1-integrin, and β_2-integrin (Fig. S2).

Interestingly, neither pretreatment of monocytes with activity-blocking antibody nor EMMPRIN silencing showed a significant effect on monocyte rolling when they were perfused over immobilized platelets under arterial shear conditions (Fig. 1C). Under venous flow conditions, we found a minor effect of EMMPRIN antibody blockade on monocyte rolling. However, this effect was not seen when EMMPRIN was silenced by siRNA pretreament (Fig. 1E).

EMMPRIN fosters monocyte recruitment to the vascular wall in vivo

The above results prompted us to study the effect of EMMPRIN on platelet-mediated monocyte recruitment to the vascular wall *in vivo*, using intravital epifluorescence microscopy. For this, surface expression of EMMPRIN was inhibited by specific siRNA pretreatment in ANA-1 cells. Firm adhesion to the vascular wall was quantified before and after ligature-induced injury of the mouse carotid artery. Ligature injury induced platelet thrombus formation at the site of the vascular lesion within minutes (Fig. S3). This represents an established trigger for subsequent recruitment of circulating leukocytes [39]. In wild-type mice, inhibition of EMMPRIN surface expression substantially reduced firm adhesion to the injured vascular wall as compared with control siRNA-treated monocytes (Fig. 2A,B). To further investigate the role of platelets in this context, we utilized mice deficient in the transcription factor NF-E2. NF-$E2^{-/-}$ mice exhibit defective platelet release from megakaryocytes, which results in the absence of platelets from circulating blood (Fig. S4) [23,24]. Notably, in NF-$E2^{-/-}$ mice, not only the number of recruited monocytes but also the effect of EMMPRIN was strongly reduced (Fig. 2C). These findings reinforce the importance of platelets for monocyte recruitment to the vascular wall after arterial injury. Furthermore, here we have identified EMMPRIN as a novel player in this process that augments monocyte recruitment *in vivo*. However, the effect of EMMPRIN in supporting monocyte adhesion seems to mostly depend on the presence of platelets.

It is of note that recruitment of ANA-1 cells to the vascular wall is a very rare event in the absence of vessel injury, e.g. 5–10 min before induction of the carotid ligature (Fig. 2B). However, we cannot exclude the possibility that a minor population of ANA-1 cells is recruited via EMMPRIN, independently of GPVI or platelets (Fig. 2A).

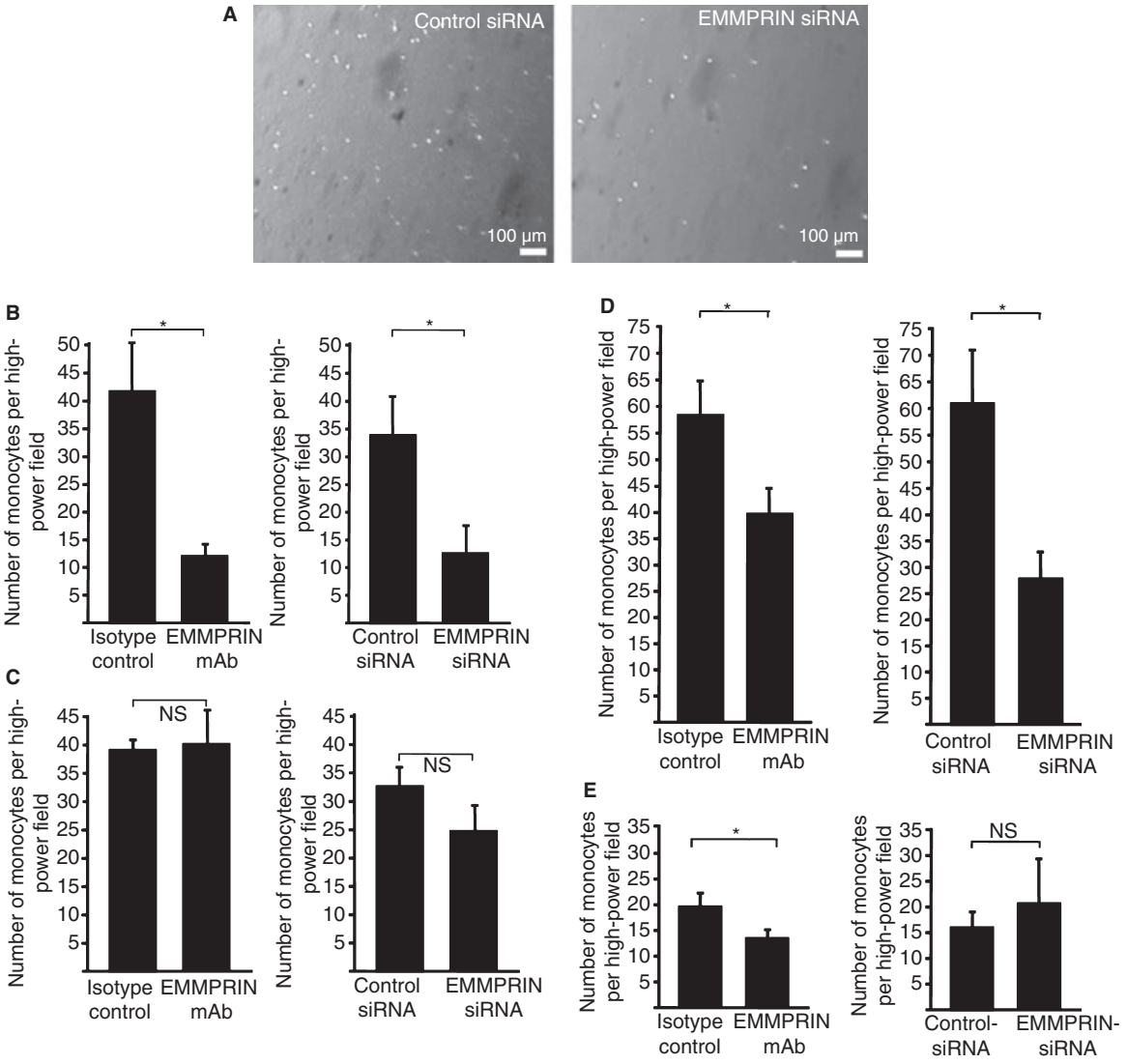

Fig. 1. Extracellular matrix metalloproteinase inducer (EMMPRIN) induces firm monocyte adhesion under arterial and venous shear conditions in vitro. Isolated human monocytes were perfused over collagen-immobilized platelets for 10 min at a shear rate of 1000 s^{-1} (A–C) or 200 s^{-1} (D, E). Before perfusion experiments, monocytes were treated with either: (i) EMMPRIN-silencing small interfering RNA (siRNA); (ii) non-silencing control siRNA; (iii) mouse anti-human EMMPRIN/CD147 mAb (UM8D6); or (iv) control IgG_1. (A) Representative micrographs of adherent monocytes pretreated with EMMPRIN-silencing siRNA or non-silencing control siRNA, and perfused at 1000 s^{-1}. Quantitative results are shown as number of firmly adherent monocytes (B, D) or number of monocytes interacting transiently with platelets (C, E) per high-power field. Mean ± standard deviation; $n = 5–7$; $^*P < 0.05$ as compared with control, NS, not statistically significant.

EMMPRIN mediates the formation of PMAs in vitro and in vivo

Platelet–monocyte interaction and aggregate formation contributes to arterial thrombosis and exacerbates atherosclerotic lesion formation [5,40]. Therefore, we next investigated the role of EMMPRIN in platelet–monocyte interactions *in vivo*. We intravenously infused EMMPRIN siRNA-pretreated or control siRNA-pretreated monocytic cells into wild-type mice, and PMAs were quantified by flow cytometry. Importantly, inhibition of EMMPRIN surface expression in monocytes significantly reduced the number of platelet–ANA-1 aggregates (Fig. 3A,B). To ensure that EMMPRIN-dependent aggregate formation also takes place in the absence of other whole blood constituents (e.g. fibrinogen), we investigated platelet–monocyte interactions under flow conditions in vitro. We applied a recently established closed flow chamber model that allows the recirculation of cells under conditions of unidirectional arterial flow and minimizes cell activation [37]. Here, we coperfused isolated human platelets and human monocytes at 1000 s^{-1}, and quantified PMA formation by flow cytometry. Importantly, blockade of EMMPRIN reduced PMA formation in the Tyrodes-buffered medium (Fig. 3C). This indicates that an EMMPRIN-dependent mechanism directly modulates the interaction of platelets and monocytes, independently of bridging molecules (e.g. plasma fibrinogen).

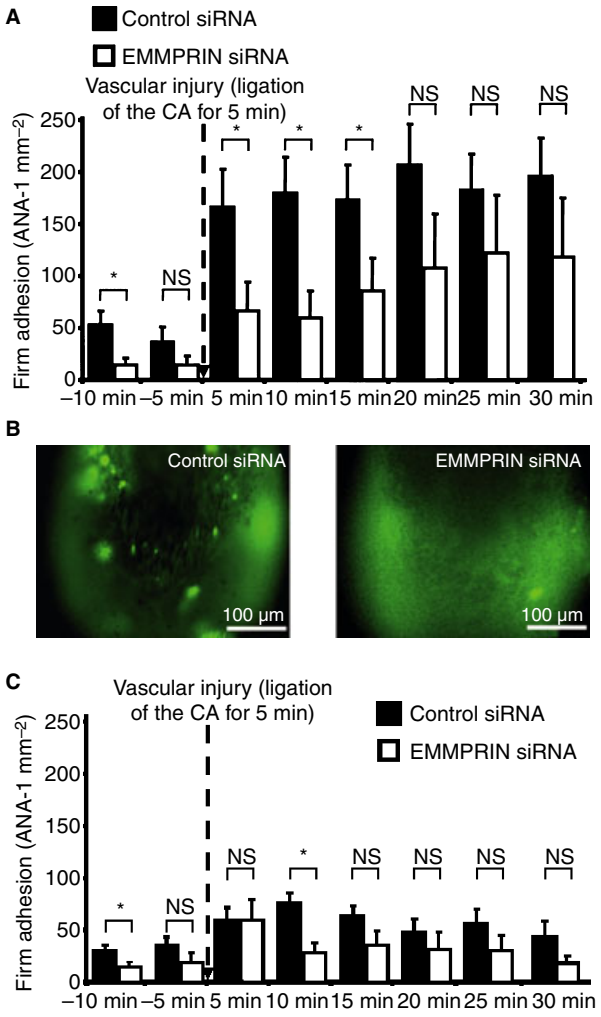

Fig. 2. Extracellular matrix metalloproteinase inducer (EMMPRIN) supports monocyte recruitment to the vascular wall *in vivo*. Adhesion of murine ANA-1 cells was visualized in the carotid artery (CA) by intravital video fluorescence microscopy before and after vascular injury. Before injection, ANA-1 cells were transfected with EMMPRIN-silencing small interfering RNA (siRNA) or non-silencing control siRNA. Cells were labeled with 5-carboxyfluorescein diacetate succinimidyl ester and injected intravenously into wild-type C57/B6 mice (A, B) and nuclear factor-erythroid 2-deficient mice (C), which lack circulating platelets. Microscopic images obtained 10 min after injury are presented in (B). The number of adherent monocytes was quantified at consecutive time points, and is given per mm^2 of vascular surface. Mean ± standard deviation; $n = 8$; $^*P < 0.05$ for EMMPRIN siRNA vs. control siRNA; NS, not statistically significant.

In the same model, preactivation of platelets, which increased EMMPRIN surface expression [21], not only enhanced aggregation of isolated human platelets and monocytes, but also showed a stronger inhibitory effect of EMMPRIN blockade on PMA formation (Fig. 3D).

EMMPRIN mediates platelet–monocyte interactions independently of integrins and P-selectin

It is well known that monocytes interact with platelets via PSGL-1/P-selectin signaling and also by binding of MAC-1 ($\alpha_M\beta_2$) to GPIbα or GPIIbIIIa [40]. Thus, we next investigated the significance of EMMPRIN *in vivo* under functional blockade of GPIbα, or in the genetic absence of P-selectin ($CD62P^{-/-}$) or the platelet surface receptor GPIIb (*Itga2b*$^{-/-}$). As the integrin subunit α_{IIb} solely dimerizes with β_3-integrin, the *Itga2b*$^{-/-}$ mouse model reveals a specific deficiency in the GPIIb–IIIa receptor, which is exclusively expressed on platelets and megakaryocytes. We intravenously infused EMMPRIN siRNA-pretreated or control siRNA-pretreated ANA-1 cells either into (i) wild-type mice, (ii) $CD62P^{-/-}$ mice, (iii) *Itga2b*$^{-/-}$ mice or (iv) wild-type mice pretreated with either GPIbα-function blocking antibody or control Fab antibody. Importantly, the effect of EMMPRIN was persistent in all groups, and silencing of EMMPRIN reduced PMA formation by between approximately 40% and 70% (Fig. 4). It is of note that blood collection from the cardiac ventricle did not artificially induce platelet activation or platelet–ANA-1 aggregate formation (Fig. S5). Furthermore, treatment with anti-GPIbα Fab antibody did not alter platelet numbers, as described previously [41]. Thus, EMMPRIN-mediated interaction of monocytes and platelets is not dependent on P-selectin, GPIIb integrin, or GPIbα.

EMMPRIN is a transmembranous member of the immunoglobulin superfamily, and EMMPRIN on one cell is known to bind to EMMPRIN on adjacent cells in a transcellular homophilic process. We earlier identified EMMPRIN on platelets, and found that its surface expression is increased upon platelet activation [21]. This was recently confirmed by others in a prospective clinical study linking platelet activation and EMMPRIN surface expression in patients with coronary artery disease [42]. Furthermore, cells transfected with EMMPRIN tether to EMMPRIN-coated matrices under flow, indicating that EMMPRIN might also serve as an adhesion receptor and mediate homophilic cell interactions [43]. Therefore, we addressed the question of whether EMMPRIN expressed by activated platelets might serve as an adhesion receptor for flowing monocytes. To investigate this, we applied the above-described (Fig. 1) flow chamber assay to analyze the interactions of platelets and monocytes in vitro. Again, we allowed platelets to adhere to collagen, which has previously been shown to induce EMMPRIN expression on platelets [21]. Collagen-adherent human platelets were incubated with either a function-blocking EMMPRIN antibody or control IgG_1, and superfused with human monocytes pretreated with EMMPRIN siRNA or control. Blockade of platelet EMMPRIN did not reduce binding of monocytes to platelets at 1000 s^{-1} (Fig. 5A). However, when venous shear rates of 200 s^{-1} were applied, we now observed a significant effect on firm monocyte adhesion by blockade of EMMPRIN. Importantly, monocyte recruitment to platelets could be inhibited either by blockade of EMMPRIN on collagen-adherent platelets or by pretreatment of monocytes with EMMPRIN-silencing siRNA prior to perfusion (Fig. 5B). Thus, our findings indicate direct binding of monocyte EMMPRIN to EMMPRIN on the platelet surface. However, such homophilic platelet–monocyte interactions seemed to be relevant only at lower shear rates.

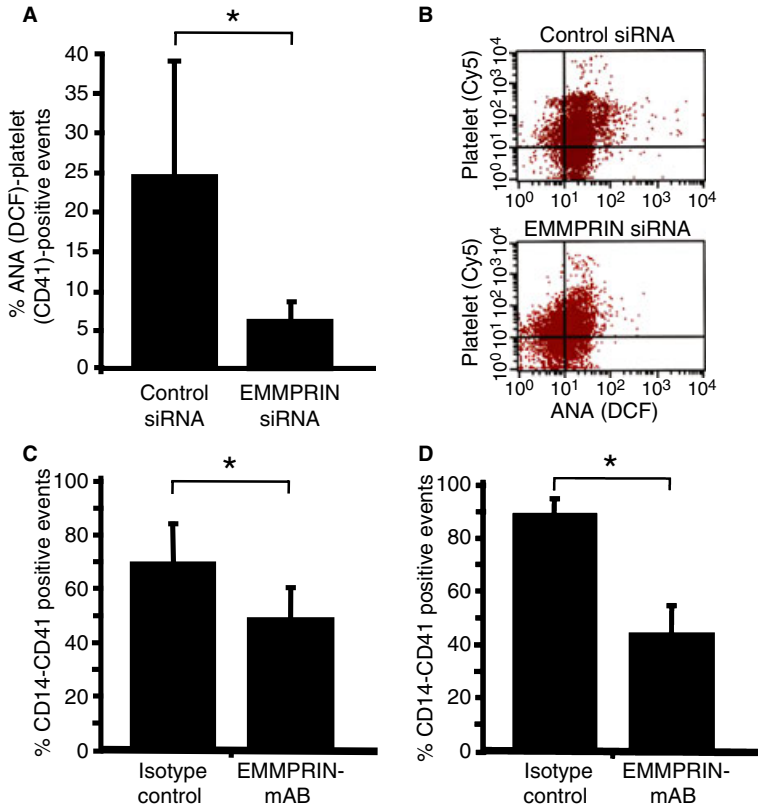

Fig. 3. Extracellular matrix metalloproteinase inducer (EMMPRIN) mediates formation of platelet–monocyte aggregates. (A) ANA-1 cells tagged with the fluorescent dye 5-carboxyfluorescein-diacetate succinimidyl ester (DCF) were injected intravenously into wild-type mice, and blood collection was performed after 10 min. Platelet–monocyte aggregate formation was determined by flow cytometric analysis of platelet (Cy5)-positive cells within the ANA-1 population, the latter being defined by DCF positivity. Mean ± standard deviation (SD); $n = 8$; $^*P < 0.05$ as compared with control small interfering RNA (siRNA). (B) Dot blot representation of flow cytometry data. Six thousand events are displayed. (C, D) Aggregate formation of isolated platelets and monocytes under arterial flow conditions (shear rate: 1000 s^{-1}) was assessed in a closed flow chamber system to study circulating cells. Monocytes were pretreated with blocking EMMPRIN mAb or control IgG_1. Platelets were either unstimulated (C) or preactivated with ADP (D). Platelet–monocyte aggregate formation was determined by flow cytometric analysis of platelet (CD41)-positive cells within the monocyte population, the latter being defined by CD14 positivity. Mean ± SD; $n = 6$; $^*P < 0.05$ as compared with isotype control.

Platelet GPVI mediates binding of monocyte EMMPRIN in vitro and in vivo

Only recently has GPVI been identified as a putative receptor for EMMPRIN [43]. In detail, it has been shown that GPVI-transfected Chinese hamster ovary cells tether to an EMMPRIN-coated surface under flow [43]. Here, we investigated the role of GPVI in platelet–monocyte interactions and PMA formation. Therefore, we used the above-described flow chamber model, applying arterial and venous shear rates. Instead of EMMPRIN mAb, we now used an anti-GPVI mAb to block GPVI on surface-adherent platelets before subsequent perfusion of isolated human monocytes. Importantly, functional blockade of GPVI on adherent platelets reduced firm monocyte adhesion to platelets at high shear rates (Fig. 6A). Furthermore, under arterial flow conditions, blockade of platelet GPVI inhibited the effect of EMMPRIN silencing on monocyte adhesion to platelets (Fig. 6A). These findings support the notion that monocytes interact via EMMPRIN with platelet GPVI under arterial shear conditions. In contrast, blockade of platelet GPVI did not significantly reduce monocyte adhesion to platelets when venous shear conditions were applied, and also the effect of EMMPRIN siRNA was not altered (Fig. 6B). Thus, in this model, platelet GPVI seemed to be dispensable at lower shear rates. It is of note that incubation of surface-adherent platelets with anti-GPVI mAb did not alter platelet activation and EMMPRIN surface expression as compared with the IgG control (Fig. S6A,B).

We next investigated the role of GPVI in EMMPRIN-dependent PMA formation *in vivo*. We intravenously infused EMMPRIN siRNA-pretreated or control siRNA-pretreated ANA-1 cells into wild-type mice pretreated with a GPVI function-blocking antibody. Interestingly, the effect of EMMPRIN was virtually abrogated following application of the GPVI mAb (Fig. 6C). Thus, in contrast to other adhesion molecules, e.g. GPIIb integrin or GPIbα, GPVI plays a critical role in mediating EMMPRIN-dependent platelet–monocyte interactions *in vivo*. It is of note that antibody treatment had no effect on platelet numbers (Fig. S7).

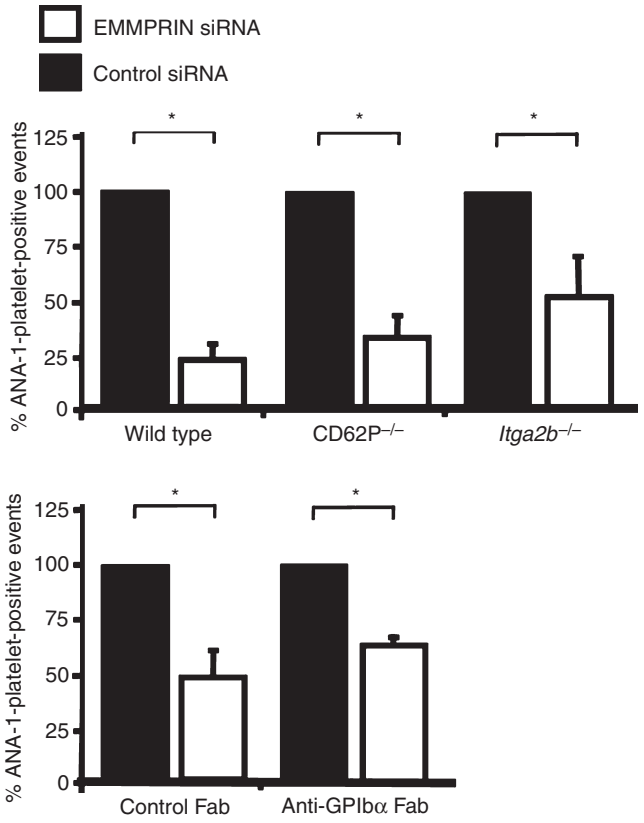

Fig. 4. Extracellular matrix metalloproteinase inducer (EMMPRIN) mediates platelet monocyte interactions in the absence of P-selectin, glycoprotein (GP)IIb integrin, and GPIbα. *In vivo* platelet–ANA-1 aggregate formation was assessed in mice. EMMPRIN small interfering RNA (siRNA)-pretreated or control siRNA-pretreated ANA-1 cells were intravenously infused into either wild-type mice, CD62P$^{-/-}$ mice, GPIIb integrin-deficient mice (*Itga2b*$^{-/-}$), or wild-type mice pretreated with a GPIbα function-blocking Fab antibody or control Fab. Platelet–monocyte aggregate formation was determined after 10 min by flow cytometric analysis of platelet-positive cells within the ANA-1 population, the latter being defined by 5-carboxyfluorescein-diacetate succinimidyl ester positivity. Platelets were detected by surface expression of CD41, or in *Itga2b*$^{-/-}$ mice by expression of GPIbα (CD42b). Mean ± standard deviation; $n = 4$–8; $^*P < 0.05$ as compared with control siRNA.

Discussion

Platelet–monocyte interactions are found in various inflammatory conditions and in acute ischemic events. Although some ligand–receptor pairs that modulate PMA formation have been identified, additional molecules may be involved in this process [44]. Here, we identified a novel role of EMMPRIN in the interaction of platelets and monocytes in circulating blood and following vascular injury. In detail, we show: (i) that EMMPRIN mediates firm monocyte adhesion to surface-adherent platelets in vitro at arterial and venous shear rates; (ii) that EMMPRIN augments platelet-mediated monocyte adhesion to the vascular wall *in vivo*; (iii) that EMMPRIN fosters the formation of platelet–ANA-1 complexes within the circulation *in vivo*; and (iv) that platelet GPVI represents a critical counter-receptor that mediates platelet adhesion to monocyte EMMPRIN both in vitro and *in vivo*.

Recruitment of monocytes to the vascular wall and their subsequent proteolysis-mediated migration into the developing plaque is crucial at all stages of atherogenesis, from the fatty streak to plaque rupture, with its clinical sequelae of myocardial infarction and stroke. During atherosclerotic lesion development, platelet recruitment is an early finding and precedes leukocyte adhesion [3]. Once platelets become adherent, they support monocyte recruitment either directly by providing a sticky surface, or indirectly by triggering an inflammatory response of the endothelium. Adherent platelets exhibit increased surface expression of P-selectin, which serves as a ligand for PSGL-1 on monocytes. Besides PSGL-1–CD62P interaction, firm adherence of monocytes to platelets has up to now mainly been attributed to interaction of monocytic MAC-1 ($\alpha_M\beta_2$) with platelet GPIbα, and to fibrinogen-bridged binding of MAC-1 to platelet GPIIb/IIIa [45–50]. Kuijper *et al.* [51] reported that blocking of P-selectin or its monocyte ligand PSGL-1 on platelets inhibited about half of the firm adhesion of monocytes to surface-bound platelets under arterial flow conditions. These data suggest that additional adhesion receptors could be involved in firm monocyte adhesion to surface-bound platelets [44].

Very recent in vitro data have indicated that EMMPRIN may act as an adhesion molecule [43]. We show here that both human monocytes and murine ANA-1 cells utilize EMMPRIN for interaction with platelets under flow conditions in vitro and in the mouse circulation *in vivo*. Thereby, EMMPRIN not only mediates firm adhesion to surface-adherent platelets, but also induces the formation of circulating PMA. Circulating platelet–leukocyte aggregates play a critical role in inflammatory conditions [4], and have been found in autoimmune diseases [52], acute lung injury [53], and coronary artery disease [54]. Although interactions with different leukocyte populations, e.g. neutrophils, have been described, circulating activated platelets preferentially bind monocytes over other subsets [55]. Interestingly, the half-life of detectable circulating PMAs is much longer than the time required for very rapid loss of P-selectin from the platelet surface [56,57]. This is another observation supporting the notion that, in addition to P-selectin, other adhesion molecules are involved in platelet–monocyte interactions. In the presence of functional EMMPRIN (ANA-1 cells treated with control siRNA), we observed numerous ANA-1–platelet aggregates *in vivo*, even in the absence of P-selectin (CD62P$^{-/-}$). However, when EMMPRIN-silenced ANA-1 cells were infused into CD62P$^{-/-}$ mice, we observed a strong reduction in the number of circulating aggregates. Likewise, the effect of EMMPRIN persisted under functional blockade of GPIbα with Fab fragments, as well as in mice in which GPIIb integrin was genetically ablated. Thus, monocyte EMMPRIN triggers interactions with platelets by a mechanism that is not dependent on the common adhesion molecules (P-selectin, GPIbα, or GPIIbIIIa).

Together with previous data from our group [21] and others [42] showing that platelets expressed EMMPRIN by an activation-dependent mechanism, we also investigated EMMPRIN–EMMPRIN-mediated monocyte adhesion to platelets.

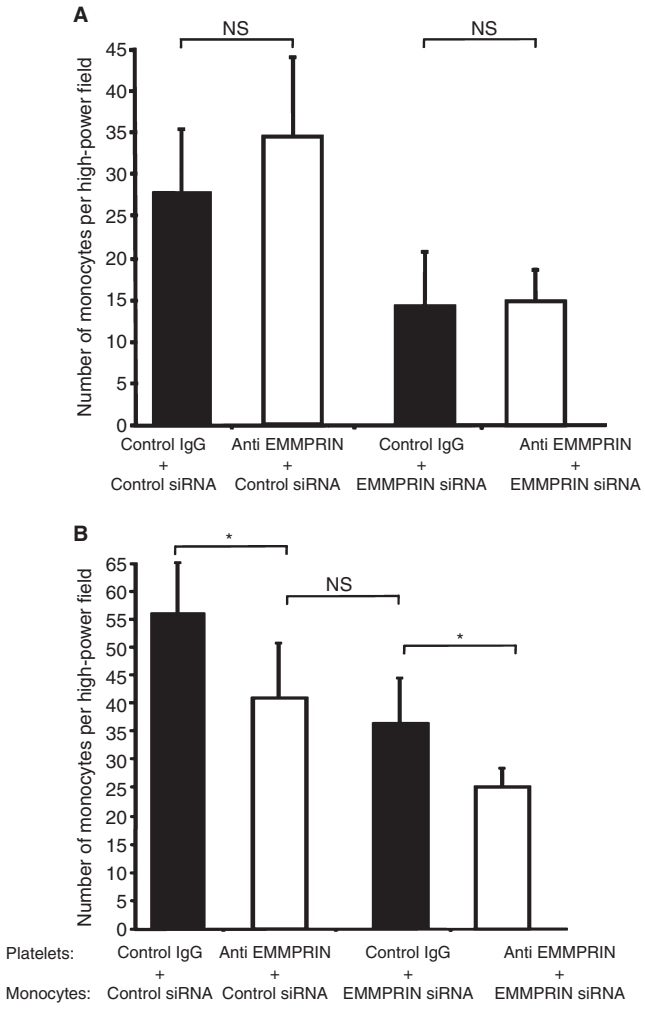

Fig. 5. Homophilic interactions of monocyte and platelet extracellular matrix metalloproteinase inducer (EMMPRIN) mediate firm monocyte adhesion at venous shear rates. In a flow chamber model, isolated platelets were perfused over a collagen matrix, and surface-adherent platelets were then incubated with either blocking EMMPRIN mAb or control IgG_1. Platelets were then superfused for 10 min at (A) 1000 s^{-1} or (B) 200 s^{-1} with human monocytes that were treated with EMMPRIN-silencing small interfering RNA (siRNA) or control siRNA. The number of firmly adherent monocytes was quantified. Inhibition of EMMPRIN on either monocytes or surface-adherent platelets reduced monocyte adhesion at 200 s^{-1}. Mean ± standard deviation; $n = 5–7$; $^*P < 0.05$; NS, not statistically significant.

At shear rates of 200 s^{-1}, adhesion of human monocytes, which were superfused over surface-adherent activated platelets, was decreased by function blocking of EMMPRIN on the platelet surface. Thus, homophilic interactions of EMMPRIN expressed on both platelets and monocytes contributed to monocyte adhesion under venous flow conditions. Interestingly, we observed the strongest reduction of monocyte adhesion in the presence of both functional blockade of platelet EMMPRIN and silencing of monocyte EMMPRIN. One reason for this finding could be that siRNA treatment did not silence monocyte EMMPRIN entirely, or vice versa, EMMPRIN blockade on adherent platelets was not complete following antibody incubation.

Interestingly, we found that platelet GPVI is critical for EMMPRIN-mediated platelet–ANA-1 aggregate formation *in vivo*. Blockade of GPVI virtually abrogated the effect of EMMPRIN silencing in mice. However, when we perfused isolated monocytes over isolated platelets in vitro, inhibition of platelet GPVI reduced monocyte adhesion under arterial flow conditions but seemed to be dispensable at lower shear rates. It is therefore tempting to speculate, at least for perfusion of isolated cells in vitro, that a homophilic interaction between platelet and monocyte EMMPRIN reduced the importance of platelet GPVI under conditions of low shear stress.

The platelet collagen receptor GPVI plays a crucial role in platelet activation, adhesion, and aggregation [58]. It triggers platelet thrombus formation at the site of vascular injury [27], and inhibition of GPVI reduces atheroprogression *in vivo* [38]. Interestingly, in patients with coronary artery disease and acute cardiac ischemia, both EMMPRIN and GPVI are upregulated. We recently found increased expression of EMMPRIN on circulating monocytes in patients with myocardial infarction [20]. Likewise, GPVI is upregulated on the surface of circulating activated platelets in acute coronary syndrome [59]. On the basis of these findings it is tempting to speculate that, in addition to well-established adhesion receptor pairs, EMMPRIN–GPVI-mediated binding may have increased relevance under inflammatory and acute ischemic conditions by supporting the interaction of platelets and monocytes.

Surface-adherent and activated platelets mediate rapid monocyte recruitment following vascular injury, whereas the extracellular matrix itself has only a low capacity to mediate monocyte arrest under arterial shear conditions [51]. We show here that, *in vivo*, EMMPRIN supports the adhesion of monocytes to the site of endothelial rupture. Thus, in the presence of functional EMMPRIN, significantly more ANA-1 cells were recruited. We also showed that platelet thrombus formation at the site of vessel injury occurred rapidly following ligature induction. Importantly, in NF-E2-deficient mice, which lack circulating platelets, recruitment of monocytes was strongly reduced in both EMMPRIN siRNA-treated monocytes and control cells. Thus, EMMPRIN-dependent recruitment of monocytes to the vascular wall *in vivo* is controlled by platelets. However, the effect of EMMPRIN appeared to diminish over time, and was no longer significant 20 min after ligature application. It is well known that platelets accumulate at sites of vascular injury, where they become activated and aggregate. In addition, we have previously shown that tissue factor-dependent luminal fibrin formation strongly increases 20–30 min after injury [60]. Thus, it is possible that, at that time, the effect of EMMPRIN in our experimental setup may have been overcome by coagulation factors exposed intraluminally and also by additional ligand–receptor interactions (e.g. PSGL-1 interaction with P-selectin expressed on activated platelets), all of which contribute to local monocyte recruitment. Furthermore, recent evidence suggests that surface levels of platelet GPVI, like those of GPIbα, are controlled by distinct mechanisms,

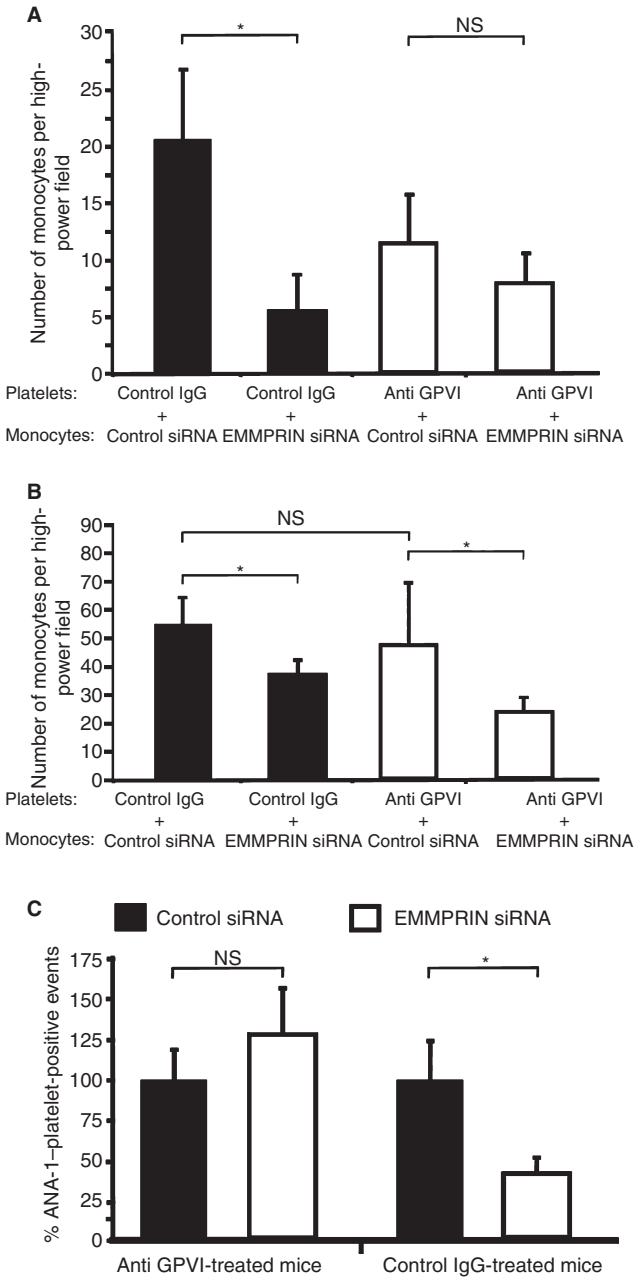

Fig. 6. Role of glycoprotein (GP)VI in extracellular matrix metalloproteinase inducer (EMMPRIN)-mediated platelet–monocyte interactions. In a flow chamber model, isolated platelets were perfused over a collagen matrix, and surface-adherent platelets were then incubated with either blocking GPVI mAb or control IgG. Platelets were then superfused for 10 min at (A) 1000 s^{-1} or (B) 200 s^{-1} with human monocytes that were treated with EMMPRIN-silencing small interfering RNA (siRNA) or control siRNA. The number of firmly adherent monocytes was quantified. Blockade of the GPVI receptor on adherent platelets reduced monocyte adhesion at 200 s^{-1}. Mean ± standard deviation (SD); $n = 8$; $^*P < 0.05$. (C) *In vivo* platelet–monocyte aggregate formation was assessed in mice pretreated with a GPVI function-blocking antibody or control IgG. 5-Carboxyfluorescein-diacetate succinimidyl ester (DCF)-tagged ANA-1 cells were transfected with EMMPRIN-silencing siRNA or non-silencing control siRNA, and injected intravenously into wild-type mice. Platelet–monocyte aggregate formation was determined after 10 min by flow cytometric analysis of platelet (CD41)-positive cells within the ANA-1 population, the latter being defined by DCF positivity. Mean ± SD; $n = 4$–5; NS, not statistically significant.

such as shedding by metalloproteinases [61] or by as yet unknown platelet-expressed proteases [62]. Thus, downregulation or redistribution of GPVI on the platelet surface might also account for this observation. Finally, there was a trend for reduced firm adhesion of EMMPRIN-silenced monocytes in NF-E2$^{-/-}$ mice postinjury. Thus, it might be possible that a minor population of ANA-1 cells is recruited via EMMPRIN, independently of GPVI or platelets.

In conclusion, we identified a novel role of monocyte EMMPRIN in firm adhesion to platelets, PMA formation and monocyte recruitment following vascular injury. Our present data link EMMPRIN more closely to inflammatory diseases such as atherosclerosis. We recently determined that proatherogenic stimuli upregulate the expression of EMMPRIN on monocytes [18,19]. Interestingly, EMMPRIN is also expressed in human atheroma, and plays a role in atherogenic cell differentiation and remodeling of the extracellular matrix [63]. Thus, EMMPRIN may play roles in both the differentiation of monocytes and in destabilization of atheromatous plaque. Therefore, EMMPRIN might be a new pharmacologic target for – besides reducing protease activation – inhibiting platelet–monocyte interactions and monocyte recruitment to the vascular wall in inflammatory conditions such as atherosclerosis.

Addendum

C. Schulz: designed and performed research, analyzed data, and wrote the article; M.-L. von Brühl: performed research, analyzed data, and wrote the article; V. Barocke: performed research and analyzed data; P. Cullen: analyzed data and wrote the article; K. Mayer: performed research; R. Okrojek: performed research; A. Steinhart: performed research; Z. Ahmad: performed research; E. Kremmer: contributed vital analytic tools; B. Nieswandt: contributed vital analytic tools; J. Frampton: contributed vital analytic tools; S. Massberg: designed research and wrote the article; R. Schmidt: designed research, analyzed data, and wrote the article. All authors read and approved the final manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Gene silencing of EMMPRIN.
Figure S2. Flow cytometric analysis of ANA-1 cells and human monocytes.
Figure S3. *In vivo* platelet thrombus formation in response to ligature injury.
Figure S4. Platelet counts in wild-type and NF-E2$^{-/-}$ mice.
Figure S5. Validation of the blood withdrawal technique.
Figure S6. Activation of adherent platelets in the presence of GPVI mAb and control IgG.
Figure S7. Platelet counts in GPVI mAb-treated mice.
Data S1. Methods.

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