Novel Methods for Assessment of Platelet and Leukocyte Function Under Flow – Application of Epifluorescence and Two-Photon Microscopy in a Small Volume Flow Chamber Model

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Abstract: Various models exist for the study of platelet and leukocyte function under flow conditions. Flow chambers offer the unique possibility to analyze cell-cell and cell-surface interactions at a great variety of conditions. However, working with small animals (i.e. mice) strongly limits the amount of isolated cells available for perfusion. Here, we present a flow chamber technique based on a small volume multichannel perfusion chamber. First, we studied the interaction of isolated murine platelets with diverse matrix proteins under flow in parallel perfusion experiments using epifluorescence microscopy. In addition, we evaluated real-time processes of platelet-leukocyte interaction and thrombus formation on an inflamed endothelial surface using two-photon microscopy (2PM). We show for the first time that high-speed 2PM allows the visualization of cell-surface-interactions at shear conditions typically found in precapillary vasculature. In summary, the flow chamber model introduced here represents a promising tool for the characterization of cell interactions in vascular research, especially when only small amounts of blood cells are available.

Keywords: Platelet adhesion, leukocyte adhesion, flow chamber, mouse models.

BACKGROUND

Interactions of platelets, leukocytes and the vascular wall play a pivotal role in multiple diseases such as atherosclerosis, thrombosis, arteriogenesis and also sepsis. Imaging of the interplay of circulating blood cells with endothelium or subendothelial matrices is, therefore, critical for understanding the pathophysiology and successful treatment of various diseases. Different imaging techniques exist to address adhesion, migration and cellular interactions of platelets and leukocytes. While *in vivo* microscopy clearly represents the essential method to study physiological processes and mechanisms of disease [1], significant additional information can be gained from *ex vivo* flow studies [2]. Thus, by applying flow chamber models the impact of various parameters can be analyzed in detail, for example: 1) shear rates, 2) rheological factors (hematocrit, buffer composition, temperature, etc.), and 3) vessel structure (simulation of bifurcations, branching). Most importantly, specific ligand-receptor interactions underlying the process of platelet / leukocyte rolling and firm adhesion can easily be clarified by *in vitro* flow models. Therefore, matrix proteins (collagen, fibrinogen, etc.) or living cells (endothelial monolayer, transfected cells) are coated onto coverslips. However, some disease models require utilization of genetically modified animals (i.e. transgenic mice). Here, application of flow chamber models was so far strongly limited by the availability of blood cells. Mice blood volume ranges between 85 and 95 ml/kg body weight (bw) with a plasma volume between 56 to 66 ml/kg bw [3]. Thus, terminal blood collection from mice will yield only 0.8-1.0 ml of whole blood [4]. We here report on a model that allows the utilization of small amounts of blood cells (i.e. from mice) for subsequent cell analysis under flow conditions.

Epifluorescence microscopy is well established to address platelet adhesion and thrombus formation at arterial shear conditions *in vivo* [5, 6]. Recently, two-photon microscopy (2PM) was introduced as a powerful tool for deep tissue *in vivo* imaging. This technique is established to assess slow *in vivo* processes that develop within minutes or hours such as leukocyte migration in the lymph node [7] and megakaryocytopoiesis in bone marrow [8]. As compared to confocal microscopy, 2PM provides the same 'optical-sectioning' capability, but has the advantages of greater imaging depth and minimal photobleaching and toxicity [9]. Only recently, high speed 2PM setups, in which the laser beam is split into multiple foci, have become available. We here analyzed the capability of 2PM for imaging of platelet-leukocyte-endothelium interactions under flow conditions in real-time.

METHODS

Animals

C57BL/6J mice were purchased from Charles River Laboratories. GPIIb (α_{IIb} integrin)-deficient mice were

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generated as described previously [10]. 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.

Blood Preparation

Murine platelets were isolated from citrate-anticoagulated whole blood as described [6]. Platelets were labelled with the fluorescent dye 5-carboxyfluorescein diacetate succinimidyl ester (DCF; Invitrogen). Thereafter, platelets were washed and resuspended in Tyrodes-HEPES buffer [11] obtaining a platelet count of 1×10^7 cells/ml. To provide a near-physiological distribution of platelets in the perfused fluid isolated erythrocytes (final hematocrit 35%) were added.

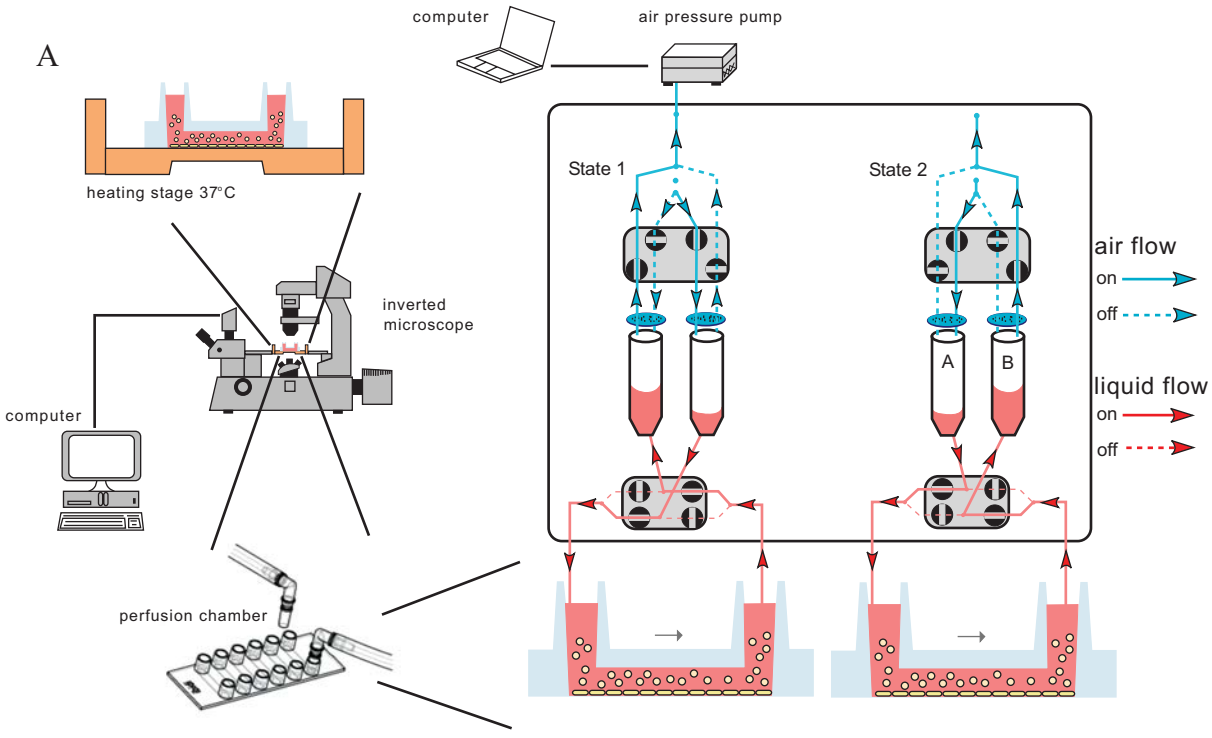
In experiments addressing both platelet and leukocyte function under flow, white blood cells were isolated from murine spleen [12] or whole blood (by Ficoll gradient centrifugation) as described previously [13]. For two-photon microscopy leukocytes (CMTMR 20µM, orange-red; Invitrogen) and platelets (DCF 20µM, green; Invitrogen) were differentially tagged with fluorescent dyes. Thereafter, cells were washed and leukocytes were added to the platelet suspension (prepared as described above) to obtain a leukocyte count of 1×10^5 cells/ml. The endothelial monolayer was stained directly with rhodamine-b [red; Sigma].

Flow Chamber Setup

Flow chamber slides (µ-slides, Ibidi - Integrated BioDiagnostics, Martinsried, Germany) consisted of 6 parallel channels with a volume of 30µl each. Channels were coated with different matrix proteins: murine fibrinogen (10 µg/mL, Invitrogen), human von Willebrand Factor (vWF) (40 µg/mL, Calbiochem), collagen type I from rat tail (40 µg/mL, BD Biosciences), or murine laminin (40 µg/mL, Sigma-Aldrich). Where indicated, collagen-coated channels were pre-incubated with soluble GPVI-Fc (800 µg/mL), or an equimolar amount of control Fc lacking the external domain of GPVI, 15 minutes before experiments [14]. Isolated platelets were perfused at 1000/s which represents arterial shear rates [15]. Platelet surface coverage was evaluated using a computer-assisted analysis program (Cap Image 7.4, Zeintl, Heidelberg, Germany).

In experiments addressing platelet-leukocyte-endothelium interactions, bEnd.3 murine endothelial cells (A.T.C.C. / Promochem) [16] were cultured directly in chamber slides and grown until confluence. Cells were either left in their resting state or treated with medium that contained 50 ng/mL tumor necrosis factor (TNF)-α and 20 ng/mL interferon (IFN)-α for 20 hours prior to the experiments [11]. Platelets and leukocytes were perfused together over bEnd.3 cells at an arterial shear rate of 500/s [15].

The flow chamber was placed into a heating stage to continuously maintain a temperature of 37°C, and mounted on an inverted fluorescence microscope (Axiovert, Zeiss, Germany). We used an air-driven continuous flow pump system (Ibidi) that featured computer-assisted direct control of the applied shear rates (Fig. **1**). Due to the fact that the flow is generated *via* air pressure, the mechanical stress on the suspended cells is minimized. The pump connects the multichannel slides in both directions via short tubes with reservoirs that contain the cell suspensions (Fig. **1**). By alternately switching the air-valves in defined time intervals



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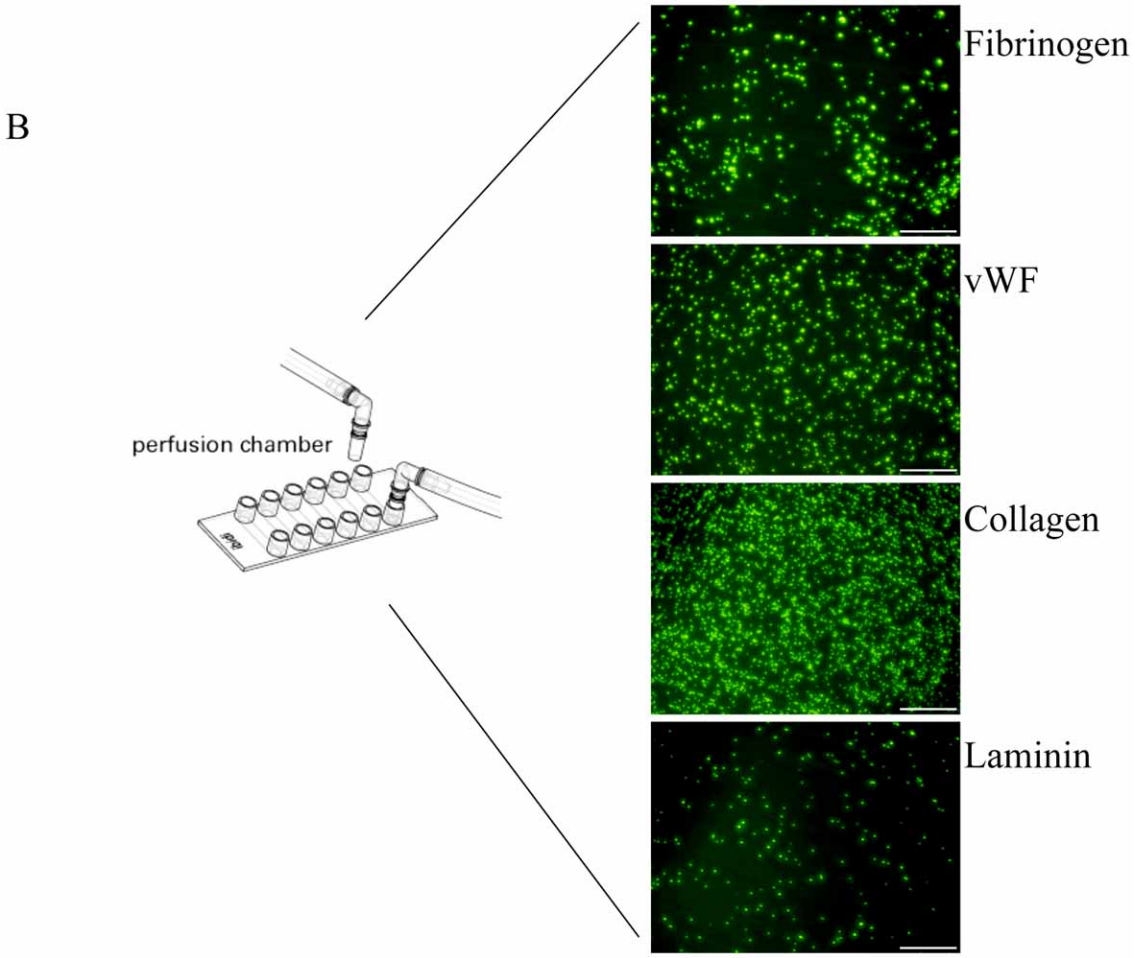

Fig. (1). Flow chamber model

(**A**) Schematic of the flow chamber setup. The flow chamber is placed in a heating stage and mounted on an inverted fluorescence microscope. The chamber slides each consist of 6 channels that are connected *via* tubes to reservoirs and the air-driven pump system. The flow pump is linked to a computer that controls alternate switching of the valves maintaining an unidirectional flow. Schematic is reprinted by approval of Ibidi (Martinsried, Germany). (**B**) Platelet adhesion was assessed by perfusion of murine platelets over different matrix proteins for 10 minutes at a shear rate of 1000/s. Adhesion to laminin, which indicates non-specific binding, was only minimal. Bars, 100 μm.

and applying negative pressure to the reservoirs the pump systems achieves a continuous unidirectional flow.

Fluorescent-tagged cells were visualized under flow using either a Zeiss Axiotech microscope (20x water immersion objective, W 20x/0.5, Zeiss) with a 100W HBO mercury lamp for epi- illumination as reported previously [5, 11]. For two-photon microscopy we used a high-speed 2-photon microscope (TriM Scope, LaVision BioTec, Bielefeld, Germany). The TriM Scope was combined with an upright Olympus microscope and 2 photo multiplier tubes (Hamamatsu Photonics, Herrsching am Ammersee, Germany) for detection. The frame rate was 800Hz @ 1024x1024 pix.

Statistics

Data are presented as mean ± SEM and analyzed using Student's t-test. A $P < 0.05$ was considered statistically significant.

RESULTS

In the here presented flow chamber setup (Fig. **1A**) several variables of the perfusion system where specifically optimized resulting in minimal volumes of circulating fluids: small chamber volume (30μl each), recirculation of the perfused cell suspension, and by maintaining only minimal fluid volume in the connecting tubes and reservoirs. In addition, the usage of multichannel chamber slides enabled us to directly compare the influence of different coatings on platelet function in parallel, thus excluding time-dependent effects on the cell preparation. Perfusion of isolated murine platelets at arterial shear rates over coated extracellular matrix proteins led to strong platelet adhesion. Platelet adhesion and aggregate formation on collagen, fibrinogen and von Willebrand factor (vWF) exhibited surface-specific adhesion profiles (Fig. **1B**). Importantly, adhesion to laminin, which indicates non-specific binding, was only minimal. Next we addressed the specificity of platelet adhesion to coated surfaces in our flow chamber model. In

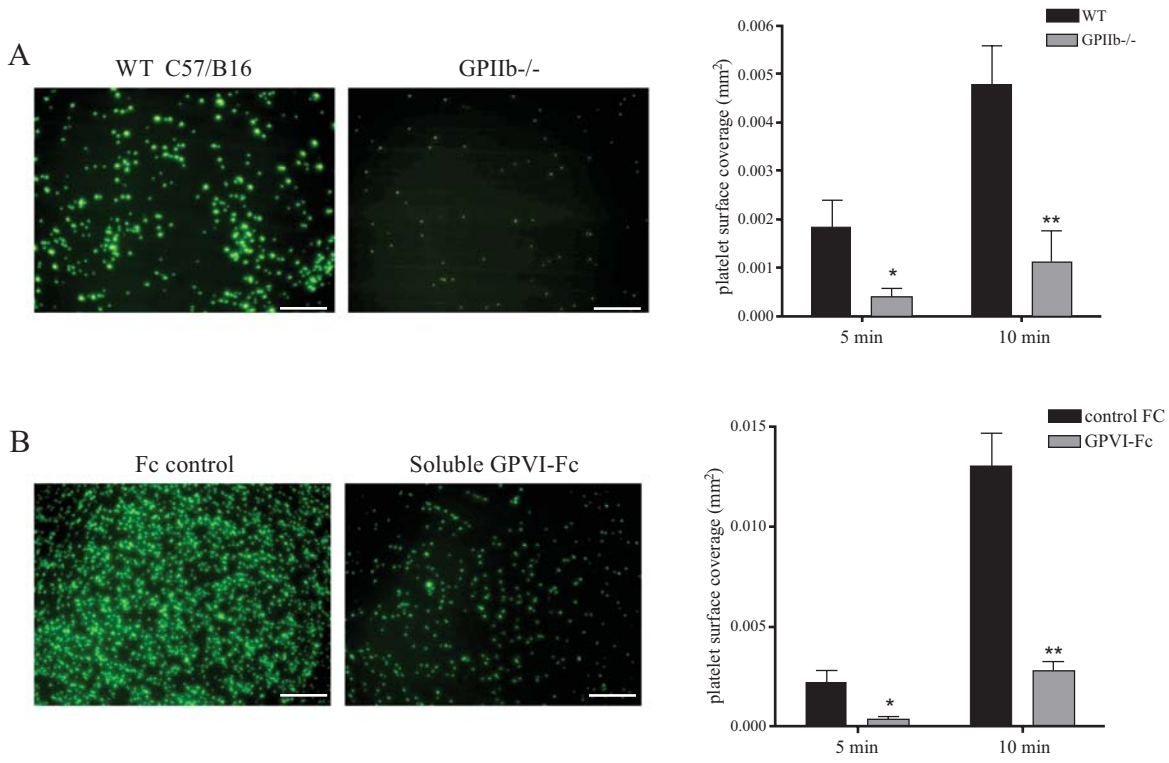

Fig. (2). Specificity of cell-surface interaction

Platelet adhesion was assessed by perfusion of murine platelets over fibrinogen or collagen for 10 minutes at a shear rate of 1000/s. Bars, 100 μm. (**A**) The fibrinogen coated surface was perfused with platelets from wildtype or GPIIb-deficient mice. Adhesion of platelets isolated from GPIIb$^{-/-}$ mice is strongly reduced (n=6, mean ± SEM, *P < 0.05 for WT versus GPIIb$^{-/-}$ at 5 min, **P < 0.01 at 10 min). (**B**) The collagen surface was pretreated with soluble GPVI-Fc (800 μg/mL) or an equimolar amount of control Fc and then perfused with wildtype platelets. Platelet adhesion is strongly reduced in the presence of GPVI-Fc but not control Fc (n=6, mean ± SEM, *P < 0.05 for control Fc versus GPVI-Fc at 5 min, **P < 0.001 at 10 min).

contrast to wildtype animals platelet adhesion to fibrinogen from glycoprotein (GP)IIb-deficient mice, that lack a functional fibrinogen receptor [10], was virtually abrogated. In addition, blocking binding receptors for GPVI, the major collagen receptor, by usage of soluble GPVI-Fc [14] strongly reduced platelet adhesion to the collagen matrix (Fig. **2**). Thus, in the here presented flow chamber model adhesion of platelets to extracellular matrix proteins is highly surface-specific.

We have previously shown that flow chamber models can be utilized to mimic inflammation of the vascular endothelium and to dissect the ligand-receptor interactions involved in leukocyte adhesion [8]. Two-photon microscopy is a novel method to study migration and homing of leukocytes or hematopoietic stem cells [7]. Only recently, the 2PM technique has been introduced to study cell behaviour under flow [17]. Thus we tested here whether this method is also feasible to study interaction of blood cells and endothelium in a flow chamber model. Importantly, behaviour under flow of murine platelets and leukocytes could be monitored in real-time (Fig. **3**). Further, platelet firm adhesion and thrombus formation, but also accumulation of leukocytes adjacent to adherent plateletes were observed on activated endothelium (Fig. **3**). Thus, the here presented flow chamber model offers the possibility to study function of blood cells in detail, and to assess their interaction under pathophysiological conditions such as inflammation.

DISCUSSION

Flow chambers are the most widely used devices for studying cell behaviour under flow. However, high amounts of cell material may be necessary in common models, especially when arterial shear rates are applied [18]. In addition, preparation of cells *in vitro* strongly limits the time span in which reproducible results are obtained [19]. Thus, earlier flow chambers allowed only very limited numbers of experiments from a single preparation of cells [19]. We here introduce a novel flow chamber model that utilizes multi-channel chamber slides to compare directly the interactions of live blood cells with diverse matrices including mono-layers of endothelial cells. All chambers were perfused in parallel, thus time-dependent effects on cells and matrices were excluded. In addition, the presented flow model depends on only small amounts of isolated blood cells, and therefore is adequate for small animal models (i.e. transgenic

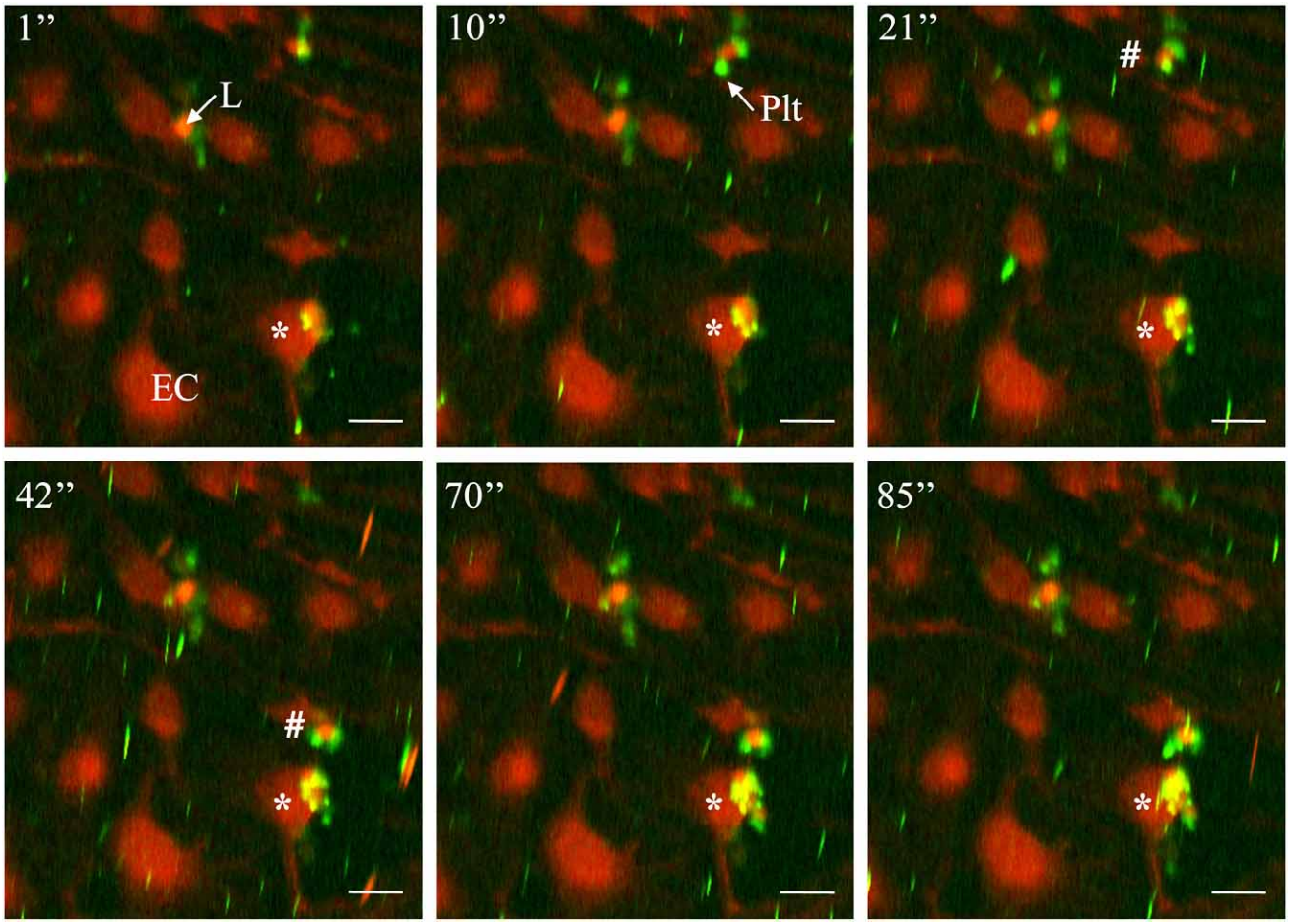

Fig. (3). Analysis of murine platelet-leukocyte interactions under flow

Differentially tagged platelets (DCF, green), leukocytes (CMTMR, orange-red) and endothelial cells (rhodamine-b, red) were perfused at 500/s and visualized by two-photon microscopy. Depicted are images from a representative flow chamber perfusion experiment, which are presented as a time course (1 to 85 seconds; also see “supplemental video”). Bars, 20 μm. Platelets accumulate on the activated endothelial surface and, thereby, mediate leukocyte adhesion. Platelets-leukocyte aggregates may also interact transiently (#) with endothelium. (*) indicates platelet thrombus formation.

mice). In detail, all 6 chambers within the multichannel slide were perfused with blood cells that were isolated from only a single mouse (i.e. GPIIb$^{-/-}$). We show that perfusion of isolated platelets at arterial shear rates leads to surface-specific platelet adhesion. Interferences with GPIIb integrin or GPVI leads to strong reduction of platelet thrombus formation. These effects are well comparable to previous publications that assessed thrombus formation in vascular injury models *in vivo* [10, 14].

Leukocyte adhesion on vascular endothelium represents a critical step during atherosclerotic lesion formation [20]. While the molecular determinants of leukocyte accumulation on inflamed tissues via postcapillary venules have been well characterized [21-23], the mechanisms that underlie leukocyte recruitment to atherosclerotic endothelium within inflamed arteries under high shear rates are poorly understood. Still, establishing assays that mimic pathophysiological conditions (i.e. inflammation) *in vivo* are a difficult task [24, 25]. As a consequence, previous flow models were often unrealistic and not suitable to study cell behaviour [24]. Models that have been described to more closely resemble *in vivo* conditions are mouse-driven *ex vivo* flow chamber models. Here, the murine heart continuously drives the blood flow through a chamber that becomes an external vessel connecting the arterial and venous systems of a live mouse [26, 27]. However, the method lacks the possibility to control different parameters such as shear rates or hemodynamic conditions as discussed above.

We [11] and others [25] have shown previously, that endothelial cells can be cultured on chamber slides, activated by cytokine-stimulation to obtain an inflammatory phenotype and then perfused with isolated human leukocytes. Due to the small amount of volume necessary for cell perfusion, we were able to establish a murine vascular inflammation model. Here, leukocytes and platelets were isolated from a single mouse and perfused over a monolayer of inflamed murine endothelial-like cells. As described previously for human cells, we observed substantial platelet and leukocyte accumulation on inflamed endothelium. Notably, we successfully applied two-photon microscopy for imaging of platelet-leukocyte-endothelium interactions under flow. Here, a strong advantage of 2PM is the simultaneous visua-

lization of fluorescence-tagged cells in a multicolour mode. As shown in the supplemental movie, platelets adhere rapidly on activated endothelial cells and form thrombi. Further, leukocytes interact intensively with platelets under flow, which mediate local adhesion of leukocytes at arterial shear rates. In line with these findings, we have previously shown that inhibition of the platelet adhesion receptors GPIIb or GPIbalpha strongly reduced platelet adhesion to inflammatory human endothelial cells (HUVEC) but also resulted in a significant reduction of leukocyte rolling and adhesion [11]. Vice versa, very few leukocytes were recruited to areas devoid of adherent platelets (Fig. **3**, suppl. movie). Thus, platelets adherent to inflammatory endothelial cells provide a "sticky" surface for subsequent leukocyte adhesion. At high shear conditions, platelet adhesion to EC is a prerequisite for leukocyte adhesion, and inhibition of platelets adhesion with specific antibodies is accompanied by a significant reduction in the number of adherent leukocytes. These findings are well comparable to observations of leukocyte-endothelium-interactions in mice *in vivo* [28, 29].

CONCLUSIONS

In summary, the here presented method can be applied to study physiological and pathophysiological processes mimicking the situation *in vivo*, and thus, may represent a promising tool for the characterization of cell interactions in vascular research.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

AUTHORS' CONTRIBUTIONS

C.S. designed the research, performed research, analyzed data and wrote the paper. E.H. performed research and analyzed data. F.G. performed research and analyzed data. M.O. performed research and analyzed data. M.v.B. contributed vital reagents and performed research. P.S. performed research and analyzed data. S.M. designed the research, analyzed data and wrote the paper.

ACKNOWLEDGEMENTS

We acknowledge the excellent technical assistance of Sandra Kerstan.

The study was supported by grants of the German Foundation of Heart Research and the Dr. Helmut Legerlotz-Stiftung (to C.S.), the Olympus Europa-Stiftung and grants of the Deutsche Forschungsgemeinschaft (MA 2186/3-1).

DECLARATION OF INTEREST

The authors report no financial conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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Received: March 16, 2009 Revised: June 09, 2009 Accepted: June 12, 2009