Tissue Plasminogen Activator Promotes Postischemic Neutrophil Recruitment via Its Proteolytic and Nonproteolytic Properties

Bernd Uhl, Gabriele Zuchtriegel, Daniel Puhr-Westerheide, Marc Praetner, Markus Rehberg, Matthias Fabritius, Maximilian Hessenauer, Martin Holzer, Andrej Khandoga, Robert Fürst, Stefan Zahler, Fritz Krombach, Christoph A. Reichel

Objective—Neutrophil infiltration of the postischemic tissue considerably contributes to organ dysfunction on ischemia/reperfusion injury. Beyond its established role in fibrinolysis, tissue-type plasminogen activator (tPA) has recently been implicated in nonfibrinolytic processes. The role of this serine protease in the recruitment process of neutrophils remains largely obscure.

Approach and Results—Using in vivo microscopy on the postischemic cremaster muscle, neutrophil recruitment and microvascular leakage, but not fibrinogen deposition at the vessel wall, were significantly diminished in tPA−/− mice. Using cell transfer techniques, leukocyte and nonleukocyte tPA were found to mediate ischemia/reperfusion-elicted neutrophil responses. Intrascrotal but not intra-arterial application of recombinant tPA induced a dose-dependent increase in the recruitment of neutrophils, which was significantly higher compared with stimulation with a tPA mutant lacking catalytic activity. Whereas tPA-dependent transmigration of neutrophils was selectively reduced on the inhibition of plasmin or gelatinases, neutrophil intravascular adherence was significantly diminished on the blockade of mast cell activation or lipid mediator synthesis. Moreover, stimulation with tPA caused a significant elevation in the leakage of fluorescein isothiocyanate dextran to the perivascular tissue, which was completely abolished on neutrophil depletion. In vitro, tPA-elicited macromolecular leakage of endothelial cell layers was abrogated on the inhibition of its proteolytic activity.

Conclusions—Endogenously released tPA promotes neutrophil transmigration to reperfused tissue via proteolytic activation of plasmin and gelatinases. As a consequence, tPA on transmigrating neutrophils disrupts endothelial junctions allowing circulating tPA to extravasate to the perivascular tissue, which, in turn, amplifies neutrophil recruitment through the activation of mast cells and release of lipid mediators. (Arterioscler Thromb Vasc Biol. 2014;34:1495-1504.)

Key Words: fibrinolysis ■ inflammation ■ leukocytes ■ microvascular permeability ■ neutrophils ■ reperfusion injury ■ tissue plasminogen activator

Ischemia/reperfusion (I/R) considerably contributes to morbidity and mortality in a variety of pathologies, including myocardial infarction, hemorrhagic shock, and stroke.1 The recruitment of leukocytes to the postischemic tissue represents a key event in the pathogenesis of I/R injury.2,3 Infiltrating leukocytes release proinflammatory cytokines, reactive oxygen species, and proteases, thereby amplifying the ischemic tissue injury.1 Concomitantly, however, leukocytes also promote tissue regeneration and healing by releasing anti-inflammatory factors as well as by phagocytosing apoptotic and necrotic cells,7 illustrating the fundamental role of leukocyte recruitment in the postischemic inflammatory response.

Fibrinolysis is an elementary biological process, which enables the maintenance of tissue perfusion by preventing clot formation in blood vessels.8–10 Plasmin is the principal effector protease in the fibrinolytic system,9,10 which is activated by proteolytic processing of its zymogen plasminogen primarily through tissue-type plasminogen activator (tPA)11 and, to a much lesser degree, through urokinase-type plasminogen activator.8,12 tPA is a serine protease, which is constantly released from microvascular endothelial cells into the bloodstream as a single-chain molecule. Binding to fibrin substantially enhances the capacity of tPA to activate plasminogen, enabling localized fibrinolysis at the site of thrombus formation.11 Because

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Correspondence to Christoph Reichel, Department of Otorhinolaryngology, Head and Neck Surgery, Ludwig-Maximilians-Universität München, Marchioninistr 15, D-81366 Munich, Germany. E-mail christoph.reichel@med.uni-muenchen.de

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of these functional properties, recombinant tPA and biochemically modified variants of this protein (eg, alteplase, reteplase, or tenecteplase) are clinically used for the dissolution of fibrin clots in thromboembolic events.13

In addition to their prominent role in the fibrinolytic system, plasmin and its activators have been implicated in different (patho)physiological processes such as cell adhesion and migration.5,6,10 In the context of I/R, the single components of the fibrinolytic system are increasingly recognized as individual and autonomous mediators: Plasmin has been demonstrated to contribute to leukocyte infiltration of reperfused tissue largely via its proteolytic properties,14 whereas the serine protease urokinase-type plasminogen activator has been shown to regulate postischemic leukocyte responses independently of its proteolytic properties via receptor-mediated processes.15 Recently, it has been reported that also tPA is critically involved in the pathogenesis of I/R injury.16–19 The underlying mechanisms remained largely unclear.

Here, we demonstrate that endogenously released leukocyte and nonleukocyte tPA promote the recruitment of neutrophils to reperfused tissue via both its proteolytic and nonproteolytic properties, but without effects on fibrinogen deposition at the postischemic vessel wall: In the initial reperfusion phase, tPA is thought to selectively mediate transmigration of neutrophils through proteolytic activation of plasminogen and, in turn, of gelatinases. As a consequence, tPA on transmigrating neutrophils enhances microvasculare permeability, allowing circulating tPA to extravasate to the perivascular tissue. Extravasated tPA, in turn, amplifies postischemic neutrophil recruitment through the activation of perivascular mast cells and release of lipid mediators. Hence, we provide novel insights into the nonfibrinolytic properties of tPA uncovering a crosstalk between the fibrinolytic system and the inflammatory response, which specifically regulates distinct steps of the recruitment process of neutrophils.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Role of tPA in Postischemic Leukocyte Recruitment

In a first set of experiments, the role of tPA for the single steps in the recruitment process of leukocytes was characterized in the postischemic mouse cremaster muscle by using in vivo transillumination microscopy (Figure 1A). Surgical preparation of the cremaster muscle induced leukocyte rolling in postcapillary venules. At baseline conditions before induction of ischemia as well as after 60 and 120 minutes of reperfusion, no significant differences were observed in the numbers of rolling leukocytes among experimental groups (data not shown).

At baseline conditions, the number of leukocytes attached to the inner vessel wall of postcapillary venules was low and did not differ among experimental groups (Figure 1B). In contrast, after induction of reperfusion, there was a significant elevation in the numbers of firmly adherent leukocytes as compared with sham-operated animals. This elevation was significantly diminished in tPA-deficient mice after 120 minutes, but not after 60 minutes of reperfusion.

Before ischemia, only few transmigrated leukocytes were found within the perivenular tissue (Figure 1C). In contrast, the number of transmigrated leukocytes significantly increased after the onset of reperfusion as compared with sham-operated mice. This increase was significantly attenuated in tPA-deficient mice after 60 and 120 minutes of reperfusion.

Role of Leukocyte Versus Nonleukocyte tPA in Postischemic Leukocyte Recruitment

In additional experiments, the expression of tPA in the cremaster muscle of wild-type (WT) mice was analyzed. Immunostaining and confocal microscopy of cremasteric tissue whole mounts revealed that tPA is primarily localized on PECAM-1+ Gr-1− endothelial cells of postischemic microvessels (Figure 1D). In addition, a strong expression of tPA was found on the surface of Gr-1+ neutrophils/monocytes. As negative controls, the expression of tPA was not detected in tPA−/− mice as well as in WT mice receiving a control antibody instead of the anti-tPA antibody (Figure IV in the online-only Data Supplement).

Using flow cytometry, the expression of tPA was confirmed in isolated murine endothelial cells and neutrophils (Figure 1E).

To evaluate the relative contribution of leukocyte versus nonleukocyte tPA for postischemic leukocyte responses, cell transfer techniques were used (Figure 1F and 1G). In postcapillary venules of the cremaster muscle, the numbers of adherent and transmigrated WT donor cells were significantly reduced in tPA-deficient recipient mice as compared with WT mice receiving cells from WT donors after 60 and 120 minutes of reperfusion. Similarly, the number of adherent cells of tPA-deficient donors was significantly diminished in WT recipients after 60 and 120 minutes of reperfusion, whereas the number of transmigrated cells of tPA-deficient donors was significantly diminished in WT recipients only after 120 minutes of reperfusion.

To measure neutrophil activation, surface expression of CD62L/L-selectin and of the integrin CD11b/Mac-1 was determined on isolated bone marrow neutrophils before delivery into recipient mice by flow cytometry. As expected, surface expression of CD62L/L-selectin on unstimulated neutrophils isolated from the bone marrow of WT mice was high, and surface expression of CD11b/Mac-1 was low. On stimulation with the potent neutrophil-activating mediator formylmethionyl-leucyl-phenylalanine (1, 10, or 100 ng), however, there was a significant and dose-dependent increase in the surface expression of CD11b/Mac-1 as well as in the surface shedding of CD62L/L-selectin (Figure II in the online-only
These data indicate that neutrophils, which were isolated from the bone marrow, rest in a low activation state before delivery into recipient mice.

**Role of tPA in Postischemic Microvascular Fibrinogen Deposition**

Because tPA is the principal activating protease in plasmin-mediated fibrinolysis,11,20 the role of fibrin(ogen) and its degradation products for postischemic leukocyte responses was determined. Functional antibody blockade of fibrin(ogen) and its degradation products significantly diminished I/R-elicited intravascular firm adherence (B) and transmigration (C) were quantified in cremasteric postcapillary venules of sham-operated WT mice and of WT or tPA-deficient mice undergoing I/R. Representative confocal microscopy images of tPA expression (red) in neutrophils/monocytes (Gr-1; green) and in a postcapillary venule (platelet endothelial cell adhesion molecule 1 [PECAM-1]; yellow) of the cremaster muscle (scale bar: 10 μm; D). Representative flow cytometry plots for the expression of tPA (E) on isolated murine endothelial cells (EC) and neutrophils (N). Adherence (F) and transmigration (G) of calcein AM–labeled bone marrow leukocytes were quantified in the postischemic cremaster muscle using in vivo fluorescence microscopy. HPF indicates high-power field. Panels show results for WT mice receiving leukocytes from WT or tPA-deficient donors as well as for tPA-deficient mice receiving leukocytes from WT donors (mean±SEM for n=4–6 per group; #P<0.05 vs sham; *P<0.05 vs WT/WT→WT).

Data Supplement). Noteworthy, it cannot be excluded that the fluorescence signal at the vessel wall might be in part caused by flowing Alexa488-labeled fibrinogen and that this background signal might prevent discernment of differences in adherent fibrin(ogen) between WT and tPA-deficient mice.

**Effect of Recombinant Murine tPA and tPA Mutants on Leukocyte Recruitment**

To directly investigate the effect of tPA (Figure 2G) on the single steps of the leukocyte extravasation cascade, recombinant murine tPA was applied either into the systemic circulation or into the extravascular compartment. Intra-arterial application of tPA did not significantly alter the number of rolling (Figure 2A), firmly adherent (Figure 2C), or transmigrated leukocytes (Figure 2E). In contrast, intrascrotal stimulation with tPA caused a dose-dependent increase in the numbers of firmly adherent and transmigrated leukocytes as compared with PBS-treated control animals,
whereas leukocyte rolling was not significantly changed. Because the highest dose of tPA applied (1.0 μg) induced robust leukocyte responses, this dose was used in additional experiments.

In this context, the effect of different tPA mutants on the single steps of the leukocyte extravasation cascade was analyzed. As demonstrated above, intrascrotal stimulation
with native tPA induced a significant elevation in intravascular adherence (Figure 2D) and transmigration of leukocytes (Figure 2F), but did not alter leukocyte rolling (Figure 2B). This elevation was significantly lower in animals receiving MTPA-S481A (nonenzymatic murine tPA) or MTPA-ALANC (nonenzymatic and noncleavable murine tPA), but remained at a similar level on stimulation with MTPA-NC (noncleavable murine tPA).

Figure 3. Role of plasmin and gelatinases in tissue-type plasminogen activator (tPA)-elicited leukocyte responses. Leukocyte rolling, firm adherence, and transmigration were quantified in postcapillary venules of the mouse cremaster muscle after intrascrotal stimulation with recombinant murine tPA by using in vivo transillumination microscopy. Panels show results for PBS-treated control mice as well as for wild-type mice treated with ε-aminocaproic acid (EACA), tranexamic acid (TXA; A–C), matrix metalloproteinase (MMP) 2/9 inhibitor III (D–F), or vehicle undergoing intrascrotal stimulation with tPA or plasmin (mean±SEM for n=4 per group; #P<0.05 vs control; *P<0.05 vs vehicle).

Figure 4. Effect of tissue-type plasminogen activator (tPA) on the activation of mast cells and RNA synthesis of lipid mediator–generating enzymes. As a measure of mast cell activation, the number of cremasteric ruthenium red–positive mast cells was determined (A; arrows; scale bar, 50 μm). Results for sham-operated wild-type (WT) mice as well as for WT and tPA−/− mice undergoing ischemia/reperfusion (I/R; B). Results for WT mice undergoing intrascrotal stimulation with recombinant murine tPA, different tPA mutants, or compound 48/80 (C). Results for PBS-treated WT mice and for WT mice receiving cromolyn or drug vehicle undergoing intrascrotal stimulation with tPA (D). Tissue RNA expression of 5-lipoxygenase (5-LO) and lyso-platelet-activating factor acetyltransferase (LCAT) was determined in the cremaster muscle on I/R (E) or intrascrotal stimulation with tPA (F) using reverse transcription–polymerase chain reaction (mean±SEM for n=4 per group; #P<0.05 vs control/sham; *P<0.05 vs WT).
Role of Plasmin and Gelatinases in tPA-Elicited Leukocyte Recruitment

In additional experiments, we sought to analyze the mechanisms underlying tPA-dependent leukocyte recruitment in more detail. tPA is known to promote plasmin-dependent activation of gelatinases (matrix metalloproteinase [MMP]-2 and MMP-9). The application of ε-aminocaproic acid or tranexamic acid (lysine analogs that inhibit plasmin activity) or of MMP-2/MMP-9 inhibitor III (which inhibits the activity of gelatinases) selectively reduced tPA-elicited transmigration of leukocytes without effects on leukocyte intravascular rolling or adherence (Figure 3A–3C). As demonstrated before, intrascrotal stimulation with plasmin induced a significant elevation in the numbers of intravascularly adherent and transmigrated leukocytes, but did not alter the number of rolling leukocytes (Figure 3D–3F). On inhibition of gelatinases, the transmigration of leukocytes was selectively diminished.

In separate experiments, MTPA-S481A–elicited intravascular firm adherence, but not rolling and transmigration of neutrophils, was found to be significantly reduced on the application of tranexamic acid or ε-aminocaproic acid (which inhibits the binding of the kringle domain of tPA to the cells; Figure III in the online-only Data Supplement).

Role of Mast Cells, Protein Synthesis, and Lipid Mediator Generation in tPA-Elicited Leukocyte Recruitment

Previous in vitro studies identified mast cells as target cells of tPA. As a measure of activated mast cells, the number of ruthenium red–positive cells was determined in cremasteric tissue whole mounts (Figure 4A). On I/R, there was a significant elevation in the numbers of ruthenium red–positive cells as compared with sham-operated control animals, which was significantly reduced in tPA−/− animals (Figure 4B). Moreover, intrascrotal stimulation with native tPA or with tPA mutants lacking the catalytic domain or being resistant to cleavage by plasmin caused a marked and comparable increase in the number of ruthenium red–positive cells (Figure 4C). The application of cromolyn, an inhibitor of mast cell degranulation, completely abrogated the tPA-elicited increase in the numbers of ruthenium red–positive cells (Figure 4D). Using reverse transcription polymerase chain reaction, we further detected a significant elevation in tissue RNA expression of lyso-platelet-activating factor (PAF) acetyltransferase (LCAT; enzyme facilitating PAF synthesis) and a significant decrease in RNA expression of 5-lipoxygenase (5-LO; enzyme facilitating leukotriene synthesis) on I/R. Whereas the postischemic increase of LCAT RNA was only slightly, but not significantly, lower in tPA-deficient animals than in WT animals, RNA levels of 5-LO were significantly higher in tPA-deficient mice than in WT mice returning to values of sham-operated animals (Figure 4E). In line with these results, intrascrotal stimulation with tPA induced only a slight increase in tissue RNA expression of LCAT and a significant decrease in the RNA expression of 5-LO (Figure 4F) as compared with control animals receiving an intrascrotal injection of the inert protein albumin.

In additional experiments, we sought to characterize the functional relevance of protein synthesis, mast cell degranulation, and lipid mediator generation for tPA-dependent leukocyte responses: The application of actinomycin D (which inhibits protein synthesis) completely abolished tPA-elicited leukocyte responses (Figure 5A–5C). Noteworthy, treatment

![Figure 5](https://atvb.ahajournals.org/)

Figure 5. Role of mast cells, protein synthesis, and lipid mediators for tissue-type plasminogen activator (tPA)–elicited leukocyte responses. Leukocyte rolling, firm adherence, and transmigration were quantified in postcapillary venules of the mouse cremaster muscle after intrascrotal stimulation with recombinant murine tPA by using in vivo transillumination microscopy. Panels show results for leukocyte rolling, adherence, and transmigration in PBS-treated wild-type (WT) mice as well as in WT mice treated with actinomycin D (A–C), cromolyn, BN 52021, MK-886, indomethacin (D–F), or drug vehicle undergoing intrascrotal stimulation with tPA (means±SEM for n=4 per group; #P<0.05 vs sham/control; *P<0.05 vs vehicle).
with MK-886 (which inhibits the enzyme activity of 5-LO) as well as blockade of the PAF receptor (with BN52021), but not inhibition of prostaglandin synthesis (with the cyclooxygenase inhibitor indomethacin), significantly attenuated tPA-elicited leukocyte recruitment (Figure 5D–5F). Moreover, cromolyn prevented tPA-dependent leukocyte adherence (Figure 5E) and transmigration (Figure 5F) without affecting the number of rolling leukocytes (Figure 5D).

**Systemic Leukocyte Counts and Microhemodynamic Parameters**

Inner vessel diameters, blood flow velocities, and shear rates of analyzed postcapillary venules as well as systemic leukocyte counts were determined to ensure intergroup comparability (Table I in the online-only Data Supplement). No significant differences were detected among all experimental groups.

**Phenotyping of Transmigrated Leukocytes**

Phenotyping of transmigrated leukocytes was performed by immunostaining of paraffin-embedded tissue sections of the cremaster muscle. In response to I/R or on stimulation with tPA, >80% of transmigrated CD45-positive leukocytes were Ly-6G–positive neutrophils. The remaining 10% to 20% were F4/80-positive monocytes/macrophages.

**Role of tPA in Postischemic Microvascular Leakage**

The breakdown of microvascular barrier function is another critical event in the pathogenesis of I/R injury. As a measure...
of microvascular permeability, the leakage of macromolecule fluorescein isothiocyanate dextran into the perivascular tissue was determined by using fluorescence in vivo microscopy on the mouse cremaster muscle (Figure 6A). On I/R (30/150 minutes), there was a significant elevation in the leakage of fluorescein isothiocyanate dextran as compared with sham-operated mice (Figure 6B). This elevation was significantly reduced in tPA-deficient mice. Furthermore, direct stimulation with tPA or different tPA mutants (Figure 6C) caused a dose-dependent increase in the leakage of fluorescein isothiocyanate dextran, which was almost completely abolished in animals depleted of neutrophils (Figure 6D).

To further characterize the potential direct effects of tPA on microvascular barrier dysfunction, macromolecular permeability of microvascular endothelial cell layers was analyzed in vitro. The exposure to tPA induced a concentration-dependent increase in the macromolecular permeability of microvascular endothelial cell layers (Figure 6E), which was completely abrogated on the inhibition of enzymatic activity (by the addition of tPA inhibitor plasminogen activator inhibitor type-1; Figure 6F), but not significantly altered on the blockade of tPA receptor low-density lipoprotein receptor–related protein (by the addition of receptor-associated protein).

Discussion

I/R injury is the most common cause for organ dysfunction and failure after myocardial infarction, hemorrhagic shock, and stroke.1 In addition to its fundamental role in the fibrinolytic system, tPA has recently been implicated in the pathogenesis of I/R injury.16–19 The underlying mechanisms remained largely unclear.

Leukocyte recruitment from the microvasculature to the site of inflammation is a key event in the inflammatory response on I/R.2–6 In a first set of experiments, we sought to evaluate the role of tPA in the leukocyte extravasation process. Using near-infrared transillumination in vivo microscopy on the mouse cremaster muscle, we found that neutrophil infiltration of postischemic tissue was significantly diminished in tPA-deficient animals. These results confirm recent observations documenting that tPA promotes neutrophil recruitment in I/R injury of kidney or lung.17,19 Moreover, our data revealed that tPA selectively mediates transendothelial migration of neutrophils in the initial reperfusion phase, whereas at later time points, tPA is already involved in leukocyte intravascular accumulation. These findings point to a highly coordinated involvement of tPA in the leukocyte extravasation process.

Endothelial cells constantly release tPA into the bloodstream, where it maintains the blood flow by preventing thrombus formation.11 Accordingly, tPA was primarily detected on the endothelium of cremasteric microvessels. Notably, high expression levels of tPA were also identified on the surface of neutrophils. Whether leukocyte or nonleukocyte tPA promotes the recruitment of neutrophils to the postischemic tissue remained unknown. To address this question, we performed cell transfer experiments. Our in vivo microscopy data indicate that leukocyte and nonleukocyte tPA equally contribute to I/R-elicited intravascular adherence and transmigration of neutrophils.

In postischemic tissues, endothelial cells acquire a procoagulant phenotype, which results in fibrinogen deposition on the microvascular endothelium.22,23 Following previous observations,24 we demonstrate that fibrinogen or its degradation products are involved in neutrophil recruitment to the reperfused tissue. Because tPA is the principal activator of plasmin-mediated fibrinolysis, we hypothesized that tPA might modulate postischemic leukocyte responses through the effects on microvascular fibrinogen deposition. Interestingly, however, fibrinogen accumulation in the cremasteric microvasculature was not significantly altered by tPA deficiency, strongly suggesting that tPA mediates I/R-elicited extravasation of neutrophils independently of its fibrinolytic properties.

To further elucidate the mechanisms underlying tPA-dependent neutrophil recruitment, we directly analyzed the effect of tPA on the single steps of the leukocyte extravasation cascade. The administration of recombinant murine tPA into the systemic circulation did not induce significant leukocyte responses. In this regard, physiological inhibitors of tPA, such as plasminogen activator inhibitor type-1, are thought to limit excessive tPA activity in the vascular compartment, thereby preventing hyperfibrinolytic effects of this serine protease. In the postischemic inflammatory response, however, microvascular permeability immediately increases, enabling circulating tPA to extravasate to the perivascular tissue. Here, we demonstrate that extravascular administration of tPA causes a dose-dependent elevation in the numbers of intravascularly adherent and transmigrated neutrophils. Moreover, we found that this elevation was significantly higher as compared with stimulation with a tPA mutant lacking its catalytic domain. These data indicate that neutrophil responses elicited by tPA are mediated via both its proteolytic and nonproteolytic properties. Because urokinase-type plasminogen activator has previously been demonstrated to support extravasation of neutrophils largely through nonproteolytic, receptor-mediated mechanisms,19 our observations unravel the individual roles of the plasminogen activators urokinase-type plasminogen activator and tPA in the leukocyte recruitment process.

On binding to fibrin, single-chain tPA is converted by plasmin into a double-chain molecule, thereby enabling efficient fibrinolysis.15 In terms of leukocyte recruitment, we found that native tPA exhibits similar effects as compared with a tPA mutant resistant to cleavage by plasmin. Plasmin-dependent proteolytic processing of single-chain tPA is, therefore, thought to be dispensable for tPA-dependent neutrophil responses.

Previously, it has been reported that tPA itself is not able to attract neutrophils.17,19 Consequently, extravasated tPA might mediate neutrophil recruitment via indirect effects. Because of their close vicinity to microvessels and their ability to produce a variety of inflammatory factors (eg, PAF, leukotrienes, or prostaglandins),25,26 tissue mast cells might serve as target cells of extravasated tPA. In the present study, we found that postischemic mast cell activation was significantly reduced in tPA−/− animals. Moreover, we show that extravascular administration of tPA potently activates perivascular tissue mast cells, extending in vitro observations because tPA directly induced degranulation of cultured mast cells.21 In accordance with these results, we also demonstrate that the inhibition of...
mast cell activation almost completely abolished tPA-elicited neutrophil responses, indicating that extravasated tPA supports postischemic neutrophil recruitment indirectly via intermediate activation of perivascular tissue mast cells.

In addition to its proteolytic effects, tPA has recently been supposed to exhibit nonproteolytic properties.27,28 Here, we demonstrate that neutrophil recruitment elicited by the nonproteolytic properties of tPA is at least partially dependent on the binding of tPA to cells via its kringle domains. Furthermore, our data suggest that tPA promotes leukocyte recruitment in the postischemic inflammatory response through the generation of PAF and leukotrienes (eg, via activation of mast cells and recruitment of neutrophils, which constitutively express the lipid mediator—generating enzymes LCAT and 5-LO), but without inducing de novo RNA synthesis of LCAT and 5-LO. Hence, our observations indicate that extravasated tPA amplifies intravascular accumulation and transmigration of neutrophils to postischemic tissue via the activation of mast cells and release of lipid mediators.

Besides its effector function in the fibrinolytic system, plasmin is thought to activate MMPs (eg, gelatinases [MMP-2 and MMP-9]) by proteolytic processing.10 According to their ability to cleave junctional proteins (eg, occludin, claudin-5, or vascular endothelial-cadherin) and to degrade components of the perivenular basement (eg, collagen IV, laminin), gelatinases have been implicated in the transmigration of leukocytes.29,30 In our experiments, we found that tPA-dependent transmigration of neutrophils was significantly diminished on blockade of plasmin or gelatinases, whereas intravascular leukocyte accumulation remained unaltered. In addition, we show that plasmin-dependent neutrophil transmigration was selectively reduced on the blockade of gelatinases, collectively indicating that tPA mediates neutrophil transmigration through proteolytic activation of plasmin and, in turn, of gelatinases.

During their transmigration, neutrophils are thought to induce the opening of endothelial junctions31 as well to initiate remodeling processes within the perivenular basement membrane.14,21,30,32 Subsequently, microvascular permeability rapidly increases, ultimately leading to tissue edema, which represents another hallmark of I/R injury. With respect to our previous findings, tPA might, therefore, also be involved in the evolution of postischemic microvascular barrier dysfunction. We found that I/R-elicited microvascular leakage was significantly diminished in tPA−/− mice, supporting previous reports documenting that tPA promotes I/R-elicited lung edema.19 Moreover, we demonstrate that direct stimulation with tPA induced a significant elevation in microvascular permeability, which was nearly abolished in neutrophil-depleted animals. These data indicate that tPA-dependent microvascular leakage is mediated by neutrophils, extending previous observations because therapeutic application of recombinant tPA enhanced leakage of the blood–brain barrier in experimental stroke.23 Furthermore, we show that exposure to tPA induced a significant increase in the macromolecular permeability of microvascular endothelial cell layers in vitro, which was completely abrogated on the inhibition of protease activity, but remained unaffected on blockade of low-density lipoprotein receptor–related protein, which is an endothelially expressed receptor for tPA and other ligands. Consequently, tPA on extravasating neutrophils is thought to promote the breakdown of the microvascular barrier during I/R via its proteolytic properties and independently of low-density lipoprotein receptor–related protein. Interestingly, tPA-elicited leakage of the blood–brain barrier strictly required low-density lipoprotein receptor–related protein–dependent signaling,27,29,33,34 unveiling a divergent regulation of microvascular permeability in the central nervous system and in peripheral tissues.

In conclusion, our experimental data demonstrate that endogenously released leukocyte and nonleukocyte tPA coordinate to promote the extravasation of neutrophils to postischemic tissue via both its proteolytic and nonproteolytic properties, but without effects on microvascular fibrinogen deposition: In the initial reperfusion phase, tPA is thought to selectively mediate transmigration of neutrophils through proteolytic activation of plasminogen(ogen) and, in turn, of gelatinases. As a consequence of these events, tPA on transmigrating neutrophils enhances microvascular permeability, thereby enabling circulating tPA to extravasate to the perivascular tissue. Subsequently, extravasated tPA amplies postischemic neutrophil recruitment through the activation of perivascular mast cells and release of lipid mediators. Our findings provide novel insights into the nonfibrinolytic properties of tPA uncovering a crosstalk between the fibrinolytic system and the postischemic inflammatory response, which specifically regulates distinct steps in the recruitment process of neutrophils.

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Disclosures
None.

References


**Significance**

Neutrophil recruitment to the perivascular tissue considerably contributes to organ dysfunction and failure on ischemia/reperfusion injury in myocardial infarction, stroke, and transplantation. Beyond its established role in fibrinolysis, tissue-type plasminogen activator has recently been implicated in nonfibrinolytic processes. The role of this serine protease in the recruitment process of neutrophils remained largely obscure. Here, we demonstrate that endogenously released tissue-type plasminogen activator mediates transmigration of neutrophils to postischemic tissue via proteolytic activation of plasminogen and gelatinases, but independently of microvascular fibrinogen deposition. Subsequently, transmigrating neutrophils disrupt endothelial junctions, allowing tissue-type plasminogen activator to extravasate to the perivascular tissue, which, in turn, amplifies postischemic neutrophil responses through the activation of mast cells and release of lipid mediators. Hence, we provide novel insights into the nonfibrinolytic properties of tissue-type plasminogen activator uncovering a crosstalk between the fibrinolytic system and the inflammatory response, which specifically regulates distinct steps of the postischemic recruitment process of neutrophils.
Tissue Plasminogen Activator Promotes Postischemic Neutrophil Recruitment via Its Proteolytic and Nonproteolytic Properties
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Materials and Methods

Animals
Male C57BL/6J mice were purchased from Charles River (Sulzfeld, Germany). Male tPA−/− mice were generated as described \(^1\) and backcrossed for 10 generations to the C57BL/6J background. All experiments were performed with male mice at the age of 10 – 20 weeks. Animals were housed under conventional conditions with free access to food and water. All experiments were performed according to German legislation for the protection of animals.

M. cremaster assay
The surgical preparation of the cremaster muscle was performed as originally described by Baez with minor modifications \(^2, 3\). Mice were anesthetized using a ketamine/xylazine mixture (100 mg/kg ketamine and 10 mg/kg xylazine), administrated by intraperitoneal (i.p.) injection. The left femoral artery was cannulated in a retrograde manner for administration of microspheres and drugs (see below). The right cremaster muscle was exposed through a ventral incision of the scrotum. The muscle was opened ventrally in a relatively avascular zone, using careful electrocautery to stop any bleeding, and spread over the transparent pedestal of a custom-made microscopic stage. Epididymis and testicle were detached from the cremaster muscle and placed into the abdominal cavity. Throughout the procedure as well as after surgical preparation during in vivo microscopy, the muscle was superfused with warm-buffered saline.

In vivo microscopy
The setup for in vivo microscopy was centered around an Olympus BX 50 upright microscope (Olympus Microscopy, Hamburg, Germany), equipped for stroboscopic fluorescence epi-illumination microscopy. Light from a 75-W xenon source was narrowed to a near-monochromatic beam of a wavelength of 700 nm by a galvanometric scanner (Polychrome II, TILL Photonics, Graefelfing, Germany) and directed onto the specimen via a FITC filter cube equipped with dichroic and emission filters (DCLP 500, LP515, Olympus). Microscopy images were obtained with Olympus water immersion lenses [20x/numerical aperture (NA) 0.5 and 10x/NA 0.3] and recorded with an analog black-and-white charge-coupled device (CCD) video camera (Cohu 4920, Cohu, San Diego, CA, USA) and an analog video recorder (AG-7350-E, Panasonic, Tokyo, Japan). Oblique illumination was obtained by positioning a mirroring surface (reflector) directly below the specimen and tilting its angle relative to the horizontal plane. The reflector consisted of a round cover glass (thickness, 0.19–0.22 mm; diameter, 11.8 mm), which was coated with aluminum vapor (Freichel, Kaufbeuren, Germany) and brought into direct contact with the overlying specimen as described previously \(^3\). For measurement of centerline blood flow velocity, green fluorescent microspheres (2 µm diameter, Molecular Probes, Leiden, The Netherlands) were injected via the femoral artery catheter, and their passage through the vessels of interest was recorded using the FITC filter cube under appropriate stroboscopic illumination (exposure, 1 ms; cycle time, 10 ms; λ=488 nm), integrating video images for sufficient time (>80 ms) to allow for the recording of several images of the same bead on one frame. Beads that were flowing freely along the centerline of the vessels were used to determine blood flow velocity (see below).

Quantification of leukocyte kinetics and microhemodynamic parameters
For off-line analysis of parameters describing the sequential steps of leukocyte extravasation, we used the Cap-Image image analysis software (Dr. Zeintl, Heidelberg, Germany). Rolling leukocytes were defined as those moving slower than the associated blood flow and quantified as described previously \(^3\). Firmly adherent cells were determined as those resting in the associated blood flow for more than 30 s and related to the luminal surface per 100 µm vessel length. Transmigrated cells were counted in regions of interest (ROI), covering 75 µm on both sides of a vessel over 100 µm vessel length. By measuring the distance between several images of one fluorescent bead under stroboscopic illumination, centerline blood flow velocity was determined. From measured vessel diameters and centerline blood flow velocity, apparent wall shear rates was calculated, assuming a parabolic flow velocity profile over the vessel cross-section.
**Quantification of fluorescent leukocyte responses**

To investigate the contribution of leukocyte and non-leukocyte tPA to postischemic leukocyte responses, a cell-transfer technique was used as described previously. Briefly, bone marrow leukocytes were isolated from donor mice by flushing the femur and tibia bones with PBS. Cells were then sieved and counted, resuspended in PBS containing BSA (0.25%), and incubated with calcine-AM (10 µM final concentration at 37°C for 30 minutes). After 2 washes, the cells were injected intravenously into recipient mice via the right jugular vein (10⁷ cells/mouse) 120 min prior to the surgical preparation. Fluorescent cells were counted in 175 regions of interest (HPF) per animal this being equivalent to the total quantifiable area of an exteriorized cremaster muscle in the present studies. Results are shown as the number of adherent or transmigrated calcein-labeled cells/HPF.

**Inhibitors and antibodies**

An anti-Ly-6G mAb (clone 1A8; 100 µg i.p.; 24 h prior to induction of inflammation; BD Biosciences, San Jose, CA, USA) was used for the depletion of neutrophils. A blocking polyclonal goat antibody directed against mouse fibrin(ogen) and its degradation products (4 mg/kg; 5 min prior to induction of inflammation) was used for inhibition interaction with fibrin(ogen) and its degradation products. Actinomycin D (inhibitor of RNA transcription; 0.2 mg/kg i.s.; co-administered with inflammatory mediators; Sigma-Aldrich) was used to inhibit protein synthesis. BN 52021 (20 mg/kg i.a.; 5 min prior to induction of inflammation; Sigma-Aldrich) is a PAF receptor antagonist; Indomethacin (4 mg/kg i.a.; 5 min prior to induction of inflammation; Sigma-Aldrich) is a COX inhibitor. MK-886 (1 mg/kg i.a.; 5 min prior to induction of inflammation; Sigma-Aldrich) is an inhibitor of 5-LO. Sodium cromoglycate (cromolyn; 200 mg/kg i.a.; 5 min prior to induction of inflammation; Sigma-Aldrich) is an inhibitor of mast cell degranulation. Formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma Aldrich) is a neutrophil attractant. Receptor-associated protein (RAP; Molecular Innovations; Novi, MI) is a LRP antagonist. Tranexamic acid (TXA; 100 mg/kg i.a.; 5 min prior to induction of inflammation; Sigma Aldrich) and ε-aminocaproic acid (EACA; 100 mg/kg i.a.; 5 min prior to induction of inflammation; Sigma Aldrich) are plasmin inhibitors. MMP-2/9 inhibitor III (250 µg i.a.; 5 min prior to induction of inflammation; Calbiochem, Darmstadt, Germany) is a specific inhibitor of gelatinases (MMP-2 and 9). Recombinant murine tPA (0.01 µg, 0.1 µg, 1.0 µg in 0.2 mL PBS i.a. or i.s.; Molecular Innovations), MTPA-S481A (murine tPA, non-enzymatic; 1.0 µg in 0.2 mL PBS i.s.; Molecular Innovations), MTPA-NC (murine tPA, non-cleavable; 1.0 µg in 0.2 mL PBS i.s.; Molecular Innovations), MTPA-ALANC (murine tPA, non-enzymatic and non-cleavable in 0.2 mL PBS i.s.; Molecular Innovations), or plasmin (1.0 µg in 0.2 mL PBS i.s.; Molecular Innovations) were used to induce leukocyte recruitment to the cremaster muscle.

**Experimental groups**

Animals were assigned randomly to the following groups: Sham-operated wild-type (WT) mice, WT mice, and tPA- mice as well as WT mice receiving an antibody directed against fibrin(ogen) and its degradation products or control antibody undergoing I/R (30/120 min). In another set of experiments, control mice received PBS or different doses of recombinant murine tPA administrated either by intraarterial (i.a.; 0.1, 1.0, or 10.0 µg; diluted in 0.2 ml PBS supplemented with 0.01% BSA) or by intrascrotal (i.s.) injection (0.01, 0.1, 1.0 µg; diluted in 0.2 ml PBS supplemented with 0.01% BSA). Additional experiments were performed in mice receiving an i.s. injection of different tPA mutants (1.0 µg; diluted in 0.2 ml PBS supplemented with 0.01% BSA). In further experiments, TXA, EACA, MMP-2/9 inhibitor III, cromolyn, actinomycin D, indomethacin, BN 52021, MK-886, anti-Ly-6G mAb, or corresponding drug vehicle/isotype control antibody were applied prior to i.s. injection of murine tPA. In separate groups, MMP-2/9 inhibitor III was applied prior to i.s. injection of plasmin (1.0 µg; diluted in 0.2 ml PBS supplemented with 0.01% BSA), and TXA or EACA were administered prior to i.s. injection of MTPA-S481A.

**Experimental Protocols**

For the analysis of postischemic leukocyte responses, three postcapillary vessel segments in a central area of the spread out cremaster muscle were randomly chosen. After having obtained baseline recordings of leukocyte rolling, firm adhesion, and transmigration in all three vessel segments,
ischemia was induced by clamping all supplying vessels at the base of the cremaster muscle using a vascular clamp (Martin, Tuttingen, Germany). After 30 min of ischemia, the vascular clamp was removed and reperfusion was restored for 160 min. Measurements were repeated at 60 and 120 min after onset of reperfusion.

In further experiments, leukocyte recruitment to the cremaster muscle was induced by i.a./i.s. injection of varying concentrations of recombinant murine tPA, by different tPA mutants, or by plasmin (see above). After 240 min, 5 vessel segments were randomly chosen in a central area of the spread-out cremaster muscle. After having obtained recordings of migration parameters, blood flow velocity was determined. In selected experiments, FITC dextran was subsequently infused i.a. for the analysis of microvascular permeability (see below). After in vivo microscopy, tissue samples of the cremaster muscle were taken for immunohistochemistry. Blood samples were collected by cardiac puncture for the determination of systemic leukocyte counts using a Coulter AcT Counter (Coulter Corp, Miami, FL). Anesthetized animals were then euthanized by exsanguination.

**Microvascular permeability**

Analysis of microvascular permeability was performed as described previously. Briefly, the macromolecule FITC-dextran (5 mg in 0.1 ml saline, Mr 150,000, Sigma-Aldrich) was infused i.a. after determination of centerline blood flow velocity (see above). Five postcapillary vessel segments as well as the surrounding perivascular tissue were excited at 488 nm, and emission >515 nm was recorded by a CCD camera (Sensicam, PCO, Kelheim, Germany) 30 min after injection of FITC-dextran using an appropriate emission filter (LP 515). Mean gray values of fluorescence intensity were measured by digital image analysis (TILLvisION 4.0, TILL Photonics) in six randomly selected ROIs (50x50 µm²), localized 50 µm distant from the postcapillary venule under investigation. The average of mean gray values was calculated.

**Microvascular fibrinogen deposition**

In a separate set of experiments (n=4), deposition of fibrinogen on microvascular endothelial cells was determined by in vivo microscopy as demonstrated before with minor modifications. Briefly, Alexa 488-conjugated human fibrinogen (17 mg/kg; Molecular Probes, Eugene, OR) was administered i.a. 5 min after induction of ischemia. To assess the microvascular distribution of fibrinogen, three to five postcapillary venules were randomly analyzed in sham-operated WT mice as well as in WT and tPA-/- animals undergoing I/R. Deposition of Alexa 488-conjugated human fibrinogen was analyzed by measuring fluorescence intensity profiles using Image J software (NIH, Bethesda, USA).

**Immunohistochemistry**

To determine the phenotype of transmigrated leukocytes, immunostaining of paraffin embedded serial tissue sections of cremaster muscle was performed. Sections were incubated with primary rat anti-mouse anti-Ly-6G, anti-CD45 (BD Biosciences), or anti-F4/80 (Serotec, Oxford, UK) IgG antibodies. Then, the paraffin sections were stained with a commercially available immunohistochemistry kit (Super Sensitive Link-Label IHC detection system, BioGenex, San Ramon, CA, USA), obtaining an easily detectable reddish end product. Finally, the sections were counterstained with Mayer’s hemalaun. The number of extravascularly localized Ly-6G, CD45-, or F4/80-positive cells was quantified by light microscopy (magnification 400x) on six sections (10 observation fields per section) from six individual animals per experimental group in a blinded manner, respectively. The numbers of transmigrated Ly-6G-positive cells (neutrophils) and F4/80-positive cells (monocytes/macrophages) are expressed as the percentage of total CD45-positive leukocytes.

**Mast cell activation**

To determine mast cell activation in vivo, ruthenium red staining of the cremaster muscle was performed in selected experiments as described previously. Briefly, exteriorized cremaster muscles were superfused for 60 min with a 0.001% solution of ruthenium red (Sigma Aldrich). The number of ruthenium red-positive cells was quantified by light microscopy (objective magnification 10x) in cremaster muscle whole mounts (10 observation fields per whole mount) in a blinded manner. As a positive control for mast cell staining, exteriorized cremaster muscles of untreated mice were superfused for 30 min with the mast cell activator compound 48/80 (CMP 48/80; 1 µg ml⁻¹; Sigma Aldrich).
**Flow cytometry**

In separate experiments, bone marrow leukocytes were incubated with PBS or varying concentrations of fMLP (1 – 100 ng/ml). Expression of CD62L and CD11b on neutrophils was evaluated by flow cytometry (Gallios; Beckman Coulter). Neutrophils were identified by high expression of CD45, CD11b, and Gr-1 as well as by low expression of CD115.

**Confocal microscopy**

For the analysis of tPA expression, excised mouse cremaster muscles were fixed in 2 % paraformaldehyde. Tissues were then blocked and permeabilized in PBS, supplemented with 10 % goat serum (Sigma) and 0.5 % Triton X-100 (Sigma). After incubation at 4°C for 12 hours with antibodies directed against tPA (goat anti-mouse; Santa Cruz), Gr-1 (rat anti-mouse; Ebioscience), or PECAM-1 (rat anti-mouse; Santa Cruz), tissues were incubated for 3 hours at room temperature with an Alexa Fluor 488-linked rabbit anti-goat antibody and Alexa Fluor 555-linked goat-anti-rat antibody (Invitrogen). Immunostained tissues were mounted in PermaFluor (Beckman Coulter, Fullerton, CA) on glass slides. Confocal z-stacks typically covering 30 µm (z-spacing 0.5 µm) were acquired using a Leica SP5 confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) with an oil-immersion lens (Leica; 63x; NA 1.40).

**RT-PCR**

5-lipoxygenase (5-LO) and lyso-PAF-acetyltransferase (LPCAT) mRNA expression: Total RNA contents were extracted using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturers' instruction manual. Reverse transcription was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Hamburg, Germany). Real time PCR was conducted with the TaqMan® Universal Master Mix Kit in a 7300 Real-Time PCR System (all Applied Biosystems, Hamburg, Germany). Primers and probes were obtained from ABI (Foster City, California, USA) TaqMan Gene Expression Assay catalog (5-LO: Mm01182750_m1; LPCAT: Mm00557141_m1) and from Biomers (Ulm, Germany) GAPDH forward: 5′-tgc agt ggc aaa gtg gag at-3′. GAPDH reverse: 5′- tgc cgt gag tgg agg cat act-3′ (bp 1-1254; gene bank accession number NM_008084); GAPDH TaqMan probe: 5′- FAM –cca tca acg acc cct tca tgg acc tc- BHQ-3′. GAPDH was used as an internal housekeeping gene. Calculation of the mRNA content was performed by a mathematical model developed by Pfaffl and colleagues.

**In vitro permeability**

Human microvascular endothelial cells (CDC/EU.HMEC-1) were seeded on collagen G-coated Transwell® plate inserts (polyester membrane, pore size 0.4 µm; Corning, Amsterdam, The Netherlands) and cultured in endothelial cell growth medium (Promocell, Heidelberg, Germany) + 10% FCS (Promocell) until the cells were confluent. Cells were either left untreated (control) or treated with the indicated concentration of human tPA. In separate experiments, cells were pre-treated with RAP (100 ng/ml) for 15 min and then tPA (100 ng/ml) was applied or cell were treated with a combination of tPA and PAI-1 (100 ng/ml, each), which was pre-mixed and incubated for 5 min in a separate tube at room temperature. FITC-dextran (40 kDa, 1 mg/ml; Sigma-Aldrich) was added to the upper compartment. At t = 0, 5, 10, 15, 30, and 60 min, samples were taken from the lower compartment for fluorescence measurements (ex 485 nm; em 535 nm) using a fluorescence plate reader (SpectraFluor Plus, Tecan). Data at t = 30 min were used for statistical analysis. All experiments were performed in triplicates.

**Statistics**

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). After testing normal distribution of the data (using the Shapiro-Wilk test), the ANOVA on ranks test followed by the Student-Newman-Keuls test (>2 groups) or the Rank sum test (2 groups) were used for the estimation of stochastic probability in intergroup comparisons. Mean values and SEM are given. P values 0.05 were considered significant.
References


Supplemental Figure I. Role of tPA for postischemic microvascular fibrinogen deposition. Using in vivo transillumination microscopy, leukocyte rolling, firm adherence, and transmigration were quantified in postcapillary venules of the mouse cremaster muscle after I/R. Panels show results for sham-operated WT mice as well as for WT mice treated with a blocking antibody directed against fibrin(ogen) and its degradation products or control antibody undergoing I/R (A, B). Representative in vivo fluorescence microscopy image of Alexa488-conjugated fibrinogen deposition on endothelial cells of a postcapillary venule in the postischemic cremaster muscle. The corresponding fluorescence intensity profile (measured along the white line) is shown (C). Panels show results for the extension into the vessel lumen (D) as well as the fluorescence intensity (E) of deposited Alexa488-conjugated fibrinogen (mean±SEM for n=3-6 per group; *p<0.05 vs. WT/control antibody).
Supplemental Figure II. Surface expression of CD62L and CD11b on bone marrow neutrophils. As a measure of cell activation, surface expression levels of CD62L and CD11b were measured on neutrophils isolated from the bone marrow of WT mice by using flow cytometry (A, B). Panels show results for PBS-treated control neutrophils as well as for neutrophils undergoing stimulation with fMLP (1, 10, or 100 ng; mean±SEM for n=4; #p<0.05 vs. control).
Supplemental Figure III. Effect of TXA and EACA on leukocyte responses elicited by MTPA-S481A. Using in vivo transillumination microscopy, leukocyte rolling, firm adherence, and transmigration were quantified in postcapillary venules of the cremaster muscle of WT mice receiving the lysine analogues TXA, EACA, or vehicle undergoing intrascrotal stimulation with the non-proteolytic tPA mutant MTPA-S481A (A, B, C; mean±SEM for n=5 per group; *p<0.05 vs. vehicle).
Supplemental Figure IV. Specificity of immunostaining for tPA. Representative confocal microscopy images in low magnification (scale bar: 100 µm) of immunostaining for PECAM-1 (endothelial cells and leukocytes, green) and tPA (red) in the cremaster muscle of WT mice, tPA-deficient mice, or WT mice receiving a control antibody instead of the anti-tPA antibody.
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<td>0.8 ± 0.1</td>
<td>1253.9 ± 94.2</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>MTPA-S481A</td>
<td>EACA</td>
<td>25.3 ± 0.3</td>
<td>0.8 ± 0.1</td>
<td>1280.5 ± 76.8</td>
<td>2.2 ± 0.3</td>
</tr>
</tbody>
</table>
Supplemental Table I. Microhemodynamic parameters and systemic leukocyte counts. Systemic leukocyte counts as well as microhemodynamic parameters, including inner vessel diameter, blood flow velocity, and wall shear rate were obtained as detailed in Material and Methods (mean±SEM for n=3-6 per group).