Mac-1 (CD11b/CD18) Links Inflammation and Thrombosis After Glomerular Injury

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Background—Inflammation and thrombosis coexist in several disorders. Although it is recognized that leukocytes may induce a procoagulant state at sites of inflammation, the critical molecular determinants of this process remain largely unknown.

Methods and Results—To examine mechanisms of inflammation-induced thrombosis, we developed a murine model of thrombotic glomerulonephritis (TGN), a known cause of acute renal failure in patients. This model, induced by lipopolysaccharide and antibody to the glomerular basement membrane, led to rapid glomerular neutrophil recruitment, thrombotic glomerular lesions with endothelial cell injury, and renal dysfunction. In mice immunodepleted of neutrophils or lacking the leukocyte-specific integrin Mac-1, neutrophil recruitment, endothelial injury, glomerular thrombosis, and acute renal failure were markedly attenuated despite the robust generation of renal cytokines. Neutrophil elastase is a likely effector of Mac-1 because its activity was reduced in Mac-1–deficient mice and the phenotype in mice deficient in Mac-1 or neutrophil elastase was similar. Platelets accumulated in glomerular capillaries within 4 hours of TGN before evidence of thrombosis. Platelet immunodepletion before TGN markedly exacerbated hematuria (hemorrhage), inflammation, and injury, whereas thrombocytopenic Mac-1–deficient mice remained resistant to disease, indicating that initial glomerular platelet deposition protects the vessel wall from neutrophil-mediated sequelae. The subsequent thrombosis relied on the interaction of Mac-1 on recruited neutrophils with glycoprotein Ibα on platelets as antibody-mediated disruption of this interaction attenuated TGN without affecting renal neutrophil accumulation.

Conclusions—These observations establish Mac-1 on neutrophils as a critical molecular link between inflammation and thrombosis and suggest it as an attractive target for antithrombotic therapy. (Circulation. 2009;120:1255-1265.)

Key Words: cell adhesion molecules ■ inflammation ■ kidney ■ leukocytes ■ thrombosis

Coagulation and inflammation are closely related entities in many diseases. There is abundant evidence that these 2 processes intersect at multiple points, which raises the possibility that antinflammatory therapeutics may be used to manage thrombotic disorders. This requires a better understanding of the molecular players that link leukocyte activation to the coagulation cascade. Glomerular thrombus formation is often found in severe human glomerulonephritides and is a leading cause of acute renal failure. Thrombotic microangiopathy (TMA), which describes a particular histopathological lesion as opposed to a single clinical pathological entity, occurs in various clinical settings including hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura, transplant rejection, systemic lupus erythematosus, and glomerulonephritis exacerbated by infection. TMA is characterized by endothelial cell swelling and detachment mainly in arterioles and capillaries, inflammatory cell infiltration, and intraluminal platelet thrombosis leading to organ damage. Functional manifestations of TMA are clinically classified as HUS/thrombotic thrombocytopenic purpura. Microvascular endothelial cell injury is still considered the most likely inciting factor in TMA. This may be triggered by bacterial-derived endotoxins/toxins, viruses (HIV), immune complexes, and drugs such as chemotherapeutic agents that
likely activate neutrophils. However, the pathogenic role of neutrophils and molecular mechanisms underlying their potential contribution to the observed procoagulant state in TMA and other thrombotic disorders remains largely unknown.

**Clinical Perspective on p 1265**

In the present study, we developed a mouse model of thrombotic glomerulonephritis (TGN) induced by the sequential injections of anti-glomerular basement membrane (GBM) antibody and lipopolysaccharide that was characterized by renal endothelial cell damage, occlusive microvascular thrombosis, renal failure, and hematuria. These parameters are prominent in patients with HUS/thrombotic thrombocytopenic purpura. The use of genetically modified mice, cell immunodepletion approaches, and functional blocking antibodies in this model allowed us to examine the molecular basis of inflammation-induced thrombosis in vivo and explore the pathogenesis of this clinically important disease. We demonstrate that neutrophils play a primary role in disease pathogenesis. We provide evidence that Mac-1, a member of the leukocyte-specific CD18 integrin family, promotes neutrophil recruitment, endothelial injury, glomerular thrombosis, and acute renal failure by regulating the release and/or activity of the serine proteinase neutrophil elastase (NE). We show that platelets initially preserve the integrity of the glomerular microvasculature after Mac-1-mediated neutrophil recruitment and injury, whereas subsequent neutrophil engagement of platelet glycoprotein (GP)Ibα via Mac-1 potently induces thrombosis.

**Methods**

**Mice**

Gene-deleted mice backcrossed to C57Bl/6 are denoted as B6 with an F number designating the number of generations the animals were backcrossed to C57Bl/6. Mac-1−/− (Mac-1+/−) mice are B6F1 and were bred and maintained in the Viral Antigen Free facility at the Harvard Medical School. NE-deficient mice (B6F10) were bred in the Viral Antigen Free facility at Harvard School of Public Health animal housing facility and maintained in the Longwood Medical Research Center animal housing facility at Harvard Medical School, NE-deficient mice (B6F10) were bred in the Viral Antigen Free facility at Harvard School of Public Health animal housing facility and maintained in the Longwood Medical Research Center facility, Age-matched wild-type (WT) C57Bl/6 mice (Jackson Laboratory, Bar Harbor, Me) were used for all of the aforementioned C57Bl/6 mice gene−/−. Experimental procedures were approved by the Animal Care and Use Committee of Harvard Medical School, Boston, Mass.

**Generation of TGN**

Rabbit antibody against mice GBM (anti-GBM serum) was prepared by immunizing rabbits with mouse GBM (Covance Research Products Inc). Control rabbit serum was produced from Sigma, St Louis, Mo. Anti-GBM serum was incubated at 36°C for 30 minutes to inactivate complement and was filter sterilized. Male C57Bl/6 mice were injected twice via tail vein with 300 µL anti-GBM serum or normal rabbit serum at 1-hour intervals. Fifty micrograms of lipopolysaccharide from phenolic extracts of Salmonella typhimurium/H9262 were injected into mice by CO2 inhalation, and both kidneys were harvested for histological analysis at the indicated times after lipopolysaccharide injection. All experimental procedures were performed in 6- to 8-week-old male mice because animals >10 weeks of age were more resistant to disease induction.

**Immunodepletion of Neutrophils and Platelets and Anti-M2 Antibody Treatment**

For immunodepletion of neutrophils, a rat anti-mouse monoclonal antibody against the neutrophil maturation antigen Gr-1 (BD Biosciences/PharMingen) was used. WT mice were injected intraperitoneally twice with 100 µg of anti-Gr-1 antibody in 200 µL PBS at 24 hours before and after disease induction. A rat IgG2b (R&D Systems, Minneapolis, Minn) was used as an isotype control. The platelet-depleting anti-GPIbα antibody and isotype rat IgG control were purchased from Empret (Wurzburg, Germany). WT or Mac-1 knockout mice were injected intravenously with 40 µg anti-GPIbα or rat IgG in PBS 12 hours before disease induction. WT mice were injected intravenously with 100 µg of affinity-purified, peptide-specific polyclonal antibody (termed anti-M2) to the Mac-1 binding site for GPIbα or nonimmune rabbit IgG antibody 1 hour before the first injection of anti-GBM.

**Peripheral Blood Cell Count and Differentials**

Blood collected in EDTA was used in MBC biochemistry (Tokyo, Japan) to generate a complete blood count with cellular differential.

**Functional Assessment of Renal Injury**

Serum values for creatinine, blood urea nitrogen (BUN), and lactate dehydrogenase (LDH) were obtained with a Hitachi 7700 Automatic Analyzer at MBC (Tokyo, Japan). Hematuria grading was conducted by dipstick analysis with the use of Uro-paper (Eiken Chemical Co, Tokyo, Japan).

**Histological and Immunohistochemical Analysis of Renal Tissue**

Coronal sections (4 µm) of paraffin-embedded kidneys were stained with periodic acid–Schiff (PAS) or Masson trichrome reagent for analysis of glomerular and interstitial injury. Prevalence of glomerular capillary thrombosis (%) equaled total number of glomeruli with thrombus in at least 1 glomerular capillary per total number of glomeruli. Fibrin deposition was detected by phosphotungstic acid–hematoxylin (PTAH) staining of paraffin renal sections.

**Chloroacetate Esterase Reaction for Neutrophil Enumeration**

Paraffin sections from kidneys were deparaffinized and incubated in freshly prepared chloroacetate solution containing 0.0125% Naphthol AS-D (Sigma) and 0.0625% Fast Blue BB salt (Sigma) in phosphate buffer (pH 7.3) for 1.5 hours in the dark. The number of esterase-positive neutrophils in at least 50 glomeruli per section was calculated and reported as the total number of neutrophils per glomerulus cross section.

**Multiple Cytokine and Chemokine Analysis**

Homogenates from mice kidney at indicated times were subjected to the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, Calif) to measure the concentration of 32 cytokines: granulocyte colony-stimulating factor, granulocyte colony-stimulating factor, interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IL-18, interferon-γ, tumor necrosis factor-α, monocyte chemotactic protein-1, RANTES (regulated upon activation, normal T cell...
expressed and secreted), macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, eotaxin, keratinocyte-derived chemokine, fibroblast growth factor basic, leukemia inhibitory factor, macrophage colony-stimulating factor, monokine induced by interferon-gamma, macrophage inflammatory protein-2, platelet-derived growth factor-BB, and vascular endothelial growth factor.

**Analysis of Renal E-Selectin**
E-selectin levels in kidney homogenates were measured by an E-selectin assay kit (R&D Systems). Protein concentrations were determined by the Lowry assay with the Bio-Rad DC protein assay dye reagent.

**Measurement of Granulocyte Elastase Digests of Plasma Fibrirogen and Fibrin**
To obtain plasma, blood sampled from the retroorbital plexus was collected into plastic tubes containing sodium citrate and centrifuged. Fibrin degradation products specifically degraded by granulocyte-elastase (e-XDP) were measured by the E-XDP Test (Mitsubishi Kagaku Iatron Co, Tokyo, Japan), which consists of Tris-saline buffer (R1) and latex reagent (R2) containing IF-123–coated latex beads. IF-123 specifically recognizes e-XDP.13 The latex agglutination assay was performed with the use of an automatic analyzer LPIA-NV7 (Mitsubishi Kagaku Iatron Co). The test sample in Tris-saline buffer was mixed in a reaction cuvette at 37°C. Latex reagent was added to this mixture, and the absorbance at 800 nm was measured every 15 seconds for 7 minutes. The V value (latex agglutination velocity) was defined as Δabsorbance at 800 nm/min. The rate of agglutination was expressed in terms of ΔA800/min as a function of the concentration of e-XDP.

**Statistical Analysis**
Results are expressed as mean±SEM for data resulting from in vivo analyses of mice. In all cases, an unpaired t test was used to compare 2 groups, *P<0.05 was considered statistically significant. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Development of a Murine Model of TGN**
TGN was induced by the injection of lipopolysaccharide and anti-GBM serum (nephrotoxic serum), which reproducibly produced severe glomerular thrombosis within 72 hours (Figure 1A). Glomerular neutrophil accumulation was prominent and occurred as early as 4 hours after disease induction, whereas inflammatory cell infiltration into the tubulointerstitial area and fibrosis were minimal (data not shown). Glomerular crescent formation and sclerosis were absent, although tubular damage was observed, as characterized by tubular dilation and casts. This was associated with rapidly progressive renal failure, as evidenced by a significant elevation of serum creatinine and BUN (Figure 1B). Replacement of nephrotoxic serum with normal rabbit serum failed to induce TGN, suggesting that a combination of anti-GBM serum and lipopolysaccharide is required for disease induction (Figure 1A and 1B). The observed histopathological changes and renal dysfunction were also dependent on lipopolysaccharide, as anti-GBM antibody alone resulted only in acute neutrophil accumulation and mild proteinuria14 (data not shown). Thus, lipopolysaccharide and in situ glomerular antibody deposits together trigger TGN.

To examine the contribution of neutrophils to TGN, neutrophils were immunodepleted with anti-Gr-1 monoclonal antibody 24 hours before disease induction (13.3±6.0 versus 548.8±196.4 neutrophils per microliter for Gr-1 monoclonal antibody [n=4] and control [n=4] groups, respectively, 24 hours after treatment). Neutrophil-depleted mice exhibited a marked reduction in glomerular thrombosis that correlated with significantly reduced indices of renal failure. These data suggest a primary role for neutrophils in the initiation of TGN (Figure 1C and 1D).

**Mac-1 Is Essential for the Development of TGN**
Mac-1−/− and WT cohorts were subjected to TGN. Mac-1−/− mice had minimal glomerular thrombosis and significantly reduced fibrin deposition (Figure 2A). The integrity of the glomerular microvasculature, a primary site of injury in microangiopathic disorders in humans, was evaluated by examining CD34, a marker of capillary endothelial cells that is transcriptionally downregulated in inflammatory settings.15,16 Immunohistochemically, TGN led to a significant reduction of CD34 in the glomerular capillaries of WT mice, which is the primary site of injury in this model, whereas its expression in the interstitium was similar to that in untreated mice. In contrast, CD34 remained intact in Mac-1−/− mice subjected to TGN (Figure 2B). In addition, the cytokine-inducible, endothelial-specific adhesion molecule E-selectin was elevated in the renal tissue of WT mice, indicating endothelial activation, whereas this was much less pronounced in Mac-1−/− animals (Figure 2C). Together these data indicate a requirement for Mac-1 in endothelial activation and damage after TGN. Indices of renal dysfunction were significantly less pronounced in Mac-1−/− versus WT animals. Hematuria, a marker of hemorrhage, was milder in Mac-1−/− mice compared with WT counterparts (Figure 2D), and Mac-1−/− mice were resistant to TGN-induced renal failure, as evidenced by a significant attenuation in the elevation of serum creatinine, BUN, and LDH (Figure 2E). The protection from TGN in Mac-1−/− mice was associated with a marked reduction in glomerular neutrophil accumulation at both 4 and 24 hours after disease induction (Figure 2F). To assess whether changes in renal cytokine levels contribute to the observed protection in Mac-1−/− mice, multiplex cytokine analysis of renal tissue 4 hours after induction of TGN was conducted. Among 32 cytokines/chemokines measured, 16 were induced, and these were comparable in WT and Mac-1−/− mice (Table 1).

Hematologic abnormalities associated with TGN were less severe in Mac-1−/− mice. Mac-1 deficiency led to a partial attenuation of the observed thrombocytopenia in WT mice that likely reflects less platelet consumption. Mild anemia was present in both WT and Mac-1−/− mice, suggesting that it is not a consequence or predictor of glomerular thrombosis. Circulating white blood cell counts decreased within hours after induction of TGN in both WT and Mac-1−/− mice, but the recovery at day 4 was greater in Mac-1−/− mice than in WT counterparts, possibly as a compensation for reduced efficiency of renal neutrophil accumulation (Figure 1 in the online-only Data Supplement).

Together, the data indicate that Mac-1−/− mice exhibit reduced neutrophil accumulation and reduced susceptibility to TMA-associated endothelial injury, glomerular pathology,
thrombosis, renal failure, and thrombocytopenia despite an abundance of renal cytokines and chemokines.

A Role for NE in Renal Damage
Mac-1 engagement can lead to NE release,17,18 suggesting it as a possible effector of Mac-1. Mice deficient in NE subjected to TGN exhibited a reduction in disease indices compared with WT cohorts. Glomerular neutrophil recruitment was reduced in NE-deficient mice, which phenocopies the Mac-1−/− mice (Table 2). To determine whether NE was downstream of Mac-1, we compared NE activity in plasma of WT and Mac-1−/− mice by quantifying levels of NE-digested fibrinogen degradation products (e-XDP).13 A significant reduction in NE-derived fibrinogen products was observed in plasma samples of Mac-1−/− compared with WT mice at day 1 after disease induction (Table 3).

Platelet Immunodepletion Accelerates TGN in WT Mice, But Thrombocytopenic Mac-1−/− Mice Remain Resistant to Disease
It is widely recognized that platelets play important roles in the pathogenesis of thrombotic diseases, but there is also convincing evidence that inflammation is a potent trigger of hemorrhage in the absence of platelets.19 Here we evaluated accumulation of platelets and their role in TGN development. Platelets deposited in glomerular capillaries within 4 hours of TGN induction (Figure 3A), and this was dependent on Mac-1 (intensity of GPIb staining, WT/control: 0.047 ± 0.01; WT/TGN: 0.557 ± 0.032 [median, 0.576]; Mac-1−/−/TGN: 0.313 ± 0.073 [median, 0.303]; P < 0.038 for WT/TGN versus Mac-1−/−/TGN). Next, platelets were immunodepleted with anti-GBM antibody 12 hours before disease induction. This regimen maintained low circulating platelet...
counts up to 24 hours after TGN induction (0 hour, <2.0% of control; 24 hours, <5.0% of control), as described previously. TGN induction in thrombocytopenic animals accelerated renal injury, resulting in lethality in a proportion of WT animals within 72 hours. Thus, mice were evaluated 48 hours after disease induction. A significant increase in histological parameters of glomerular injury (Figure 3B) and glomerular endothelial damage (as assessed by CD34 staining) (Figure A) was observed in Mac-1−/− mice subjected to TGN compared with WT mice. Hematuria (D) and serum creatinine (sCr), BUN, and LDH levels (E) were significantly reduced in Mac-1−/− mice subjected to TGN compared with WT. F, At indicated times after induction of TGN, kidneys were harvested, and the number of polymorphonuclear neutrophils (PMNs) per glomerular cross section was determined. A representative kidney section from WT and Mac-1−/− mice 4 hours after induction of nephritis is shown. Neutrophils (stained blue, arrow) were recruited only into the glomerulus with no observable interstitial infiltration. Data represent mean±SEM. *P<0.05 compared with WT subjected to TGN; #P<0.05 compared with untreated WT mice. Bar=30 μm.
was observed in thrombocytopenic animals. An increase in hemorrhage (hematuria), a rapid elevation of BUN, serum creatinine, and LDH, and increased glomerular polymorphonuclear neutrophil accumulation were also observed (Figure 3D through 3F). Platelet immunodepletion 24 hours after induction of TGN did not exacerbate disease and indeed led to a trend of reduced indices of renal failure (Figure II in the online-only Data Supplement). The accumulation of platelets within hours of TGN induction, before any evidence of thrombosis, coupled with our data that elimination of platelets before TGN increases glomerular injury, suggests that early platelet deposition is cytoprotective.

Others have provided compelling evidence that inflammation induces hemorrhage in the background of thrombocytopenia, but the contribution of inflammatory cells to this process has not been evaluated directly. Thus, we examined the role of Mac-1 in thrombocytopenia-induced exacerbation of TGN. GPIbα antibody treatment before TGN immunodepleted platelets in both WT and Mac-1−/− mice (platelet counts: WT, <2%; Mac-1−/−, <3% of control 12 hours after anti-GPIbα injection). Notably, platelet immunodepleted Mac-1−/− animals remained completely resistant to TGN-induced renal failure (Figure 4A). This indicates that Mac-1–mediated vessel injury is counteracted by platelets.

Mac-1 Interaction With Platelet GPIbα Promotes Thrombosis

Mac-1 binds GPIbα on platelets. This interaction is required for neutrophil adhesion and transmigration at sites of endothelial denudation and platelet deposition after wire-induced vascular injury. Here, an antibody targeting the GPIbα binding site on CD11b (termed anti-M2) was injected in WT mice to assess the contribution of Mac-1/GPIbα interaction to the development of TGN. Anti-M2 treatment had no effect on glomerular neutrophil accumulation (Figure 4B). Despite this, a significant reduction in thrombosis (Figure 4B) and indices of renal failure (Figure 4C) was observed in anti-M2 versus IgG isotype control-treated WT animals. Because anti-M2 did not affect neutrophil recruitment, the data suggest a direct role for Mac-1 interaction with GPIbα on platelets in the initiation of glomerular thrombosis.

Discussion

The major finding of our work is that Mac-1 represents a critical molecular link between inflammation and thrombosis in a model of TGN that recapitulates features of the human disease. Our studies introduce the concept that Mac-1–mediated neutrophil recruitment and neutrophil-platelet interactions are key steps in inflammation-induced thrombosis leading to organ damage in TGN. We demonstrate that Mac-1 supports neutrophil recruitment likely through regulation of

Figure 2. (Continued).
NE release and/or activity and stimulates the accumulation of platelets that initially function to preserve vessel integrity in the context of neutrophil-mediated vascular damage. However, subsequent interaction of neutrophil Mac-1 with GpIbα on deposited platelets promotes thrombosis, a major aspect of renal injury in this model (Figure 5).

We developed a model of TMA in mice that has functional outcomes and laboratory features that resembled HUS in humans so that the molecular cross talk between inflammation and thrombosis, 2 prominent features of HUS, and the pathogenesis of this disease can be explored. Many aspects of HUS were recapitulated in our model, such as endothelial injury, glomerular thrombosis, thrombocytopenia, neutrophilia, renal failure, and LDH elevation. However, anemia, a major characteristic of HUS, was not significant in this mouse model, as was also the case in published rat models.21,22 This may be because the glomerular intravascular hemolysis is not severe enough to manifest as systemic anemia because glomerular thrombosis is localized and less extensive in the rodent models compared with the human disease. Glomerular endothelial cell damage is a predominant feature of TMA in humans. CD34 served as a sensitive readout of glomerular thrombosis in TGN correlated with an increase in renal levels of E-selectin, a selective marker of endothelial activation. Anti-GBM antibody or lipopolysaccharide alone was not sufficient

| Table 2. NE-Deficient Mice Are Protected From TGN |

<table>
<thead>
<tr>
<th>PAH</th>
<th>WT</th>
<th>NE−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematuria grade</td>
<td>2.66±0.14</td>
<td>1.43±0.30*</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.30±0.05</td>
<td>0.17±0.02*</td>
</tr>
<tr>
<td>BUN, mg/dL</td>
<td>153.70±28.70</td>
<td>78.90±9.80*</td>
</tr>
<tr>
<td>LDHx10³, IU/L</td>
<td>2.00±0.26</td>
<td>1.11±0.12*</td>
</tr>
<tr>
<td>PMNs/glomerulus cross-section</td>
<td>5.74±1.04</td>
<td>0.91±0.15*</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. TGN was induced in NE-deficient mice. Quantification of glomerular PAS, hematuria, functional markers (BUN, serum creatinine, LDH), and glomerular polymorphonuclear neutrophil (PMN) accumulation in WT and NE−/− mice are given. All analyses were done at 96 hours after TGN except glomerular neutrophil recruitment, which was evaluated at 4 hours after TGN induction.

*P<0.05 compared with WT.

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Table 1. Intrarenal Cytokine Profile in WT and Mac-1−/−-Deficient Mice at 4 Hours After Induction of TGN

<table>
<thead>
<tr>
<th></th>
<th>WT Control</th>
<th>WT TGN</th>
<th>Mac-1−/− Control</th>
<th>Mac-1−/− TGN</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>22.2±6.0</td>
<td>47.3±4.7*</td>
<td>17.4±1.6</td>
<td>48.0±2.6†</td>
</tr>
<tr>
<td>IL-1β</td>
<td>151.4±15.8</td>
<td>208.5±13.9*</td>
<td>102.8±9.7</td>
<td>229.1±13.1†</td>
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<tr>
<td>IL-6</td>
<td>22.6±4.7</td>
<td>181.2±46.9*</td>
<td>15.6±1.2</td>
<td>253.7±35.2†</td>
</tr>
<tr>
<td>IL-12 (p40)</td>
<td>7.3±1.0</td>
<td>124.9±26.8*</td>
<td>6.1±0.8</td>
<td>95.3±15.3†</td>
</tr>
<tr>
<td>G-CSF</td>
<td>1.1±0.3</td>
<td>310.1±43.8*</td>
<td>0.6±0.1</td>
<td>369.1±58.4†</td>
</tr>
<tr>
<td>KC</td>
<td>4.0±1.1</td>
<td>1282.1±293.5*</td>
<td>3.1±0.2</td>
<td>1682.4±491.2†</td>
</tr>
<tr>
<td>RANTES</td>
<td>64.6±10.9</td>
<td>550.9±85.7*</td>
<td>52.5±4.9</td>
<td>546.5±26.5†</td>
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<tr>
<td>MCP-1</td>
<td>42.0±6.7</td>
<td>411.7±85.7*</td>
<td>35.1±2.9</td>
<td>488.5±120.9†</td>
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<tr>
<td>MIP-1β</td>
<td>26.3±5.1</td>
<td>56.4±5.4*</td>
<td>18.2±2.3</td>
<td>56.2±3.2†</td>
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<td>IL-15</td>
<td>181.2±13.8</td>
<td>340.4±53.8*</td>
<td>88.9±25.0</td>
<td>290.5±51.1†</td>
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<tr>
<td>FGF basic</td>
<td>326.2±43.9</td>
<td>2329.5±794.5*</td>
<td>730.1±288.8</td>
<td>2131.5±458.4†</td>
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<tr>
<td>LIF</td>
<td>7.6±0.8</td>
<td>58.5±7.2*</td>
<td>3.85±0.58</td>
<td>64.9±10.0†</td>
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<tr>
<td>M-CSF</td>
<td>19.7±1.3</td>
<td>88.1±6.5*</td>
<td>20.8±0.3</td>
<td>95.2±12.6†</td>
</tr>
<tr>
<td>MIG</td>
<td>2628.4±209.6</td>
<td>63 889.8±13 826.4*</td>
<td>2777.3±901.6</td>
<td>62 931.0±4478.1†</td>
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<tr>
<td>MIP-2</td>
<td>6.4±0.6</td>
<td>265.1±41.1*</td>
<td>4.2±0.7</td>
<td>342.7±77.3†</td>
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<td>PDGF-BB</td>
<td>1300.8±149.9</td>
<td>7742.9±1491.0*</td>
<td>3808.4±728.0</td>
<td>8805.2±1370.8†</td>
</tr>
</tbody>
</table>

Data are mean ± SEM; n=4 per group. G-CSF indicates granulocyte colony-stimulating factor; RANTES, regulated upon activation, normal T cell expressed and secreted; MCP-1, monocyte chemotactic protein-1; MIP-1β, macrophage inflammatory protein-1β; FGF, fibroblast growth factor; LIF, leukemia inhibitory factor; M-CSF, macrophage colony-stimulating factor; MIP-2, macrophage inflammatory protein-2; and PDGF, platelet-derived growth factor. TGN was induced in WT or Mac-1−/− mice, and kidneys were harvested 4 hours later. Cytokine concentrations in pg/mg of total protein in kidney homogenates were measured by the Bio-Plex System.

*P<0.05 compared with WT.
†P<0.05 compared with Mac-1−/− control.
to reduce glomerular endothelial CD34, indicating a positive correlation of this marker and TMA-related pathology. Our model relied on immune complexes formed in situ. Another murine model recently developed with immune complexes to a planted antigen also resulted in renal endothelial injury and TMA, suggesting a close link between immune complexes and TMA-related endothelial damage.23

Neutrophils were a prerequisite for development of thrombosis in TGN, and Mac-1 specifically promoted TGN. Mac-1−/− mice subjected to TGN retained CD34 and had reduced renal E-selectin, which indicates a primary role for Mac-1 on neutrophils in endothelial activation and injury in this model. A deficiency in Mac-1 or NE resulted in a significant attenuation of neutrophil influx. Elastase activity was decreased in plasma of Mac-1−/− mice, leading us to propose that Mac-1 engagement results in the local release of NE,17,18 which then stimulates the secretion of endothelial chemottractants24 or the generation of chemotactant cleavage products25 that promote neutrophil recruitment. Previous studies suggest that the role of Mac-1 in neutrophil accumulation is context dependent. Mac-1 is not required for neutrophil recruitment in immune complex–induced nephrotoxic nephritis, the reverse arthus reaction (J. Hirahaski, MD, PhD, and T.N. Mayadas, PhD, unpublished data, 2008), or complement-induced vasculitis.17 It is required for sustaining adhesion in a heterologous model of anti-GBM–induced nephritis that is independent of NE14 and has a codominant role with its sister integrin lymphocyte function-associated antigen-1 in neutrophil accumulation in some other models.26

Platelets accumulated in glomerular capillary within hours of induction of TGN, and this was Mac-1 dependent. It is possible that reactive oxygen species generated by recruited neutrophils trigger the release of von Willebrand factor from endothelial cells, which leads to platelet adherence.27 Platelet immunodepletion before TGN increased hematuria and renal injury, suggesting that platelets are cytoprotective and prevent excessive inflammation. This is in contrast to reports in the field,28,29 but in all of these cases anticoagulant therapies were initiated after disease was induced. Consistent with this, platelet immunodepletion after TGN induction also reduced disease indices. What is the cytoprotective mechanism of platelets? Platelets are well established to preserve vessel integrity. A supportive role for platelets is shown after organ perfusion30,31 and in sprouting vessels in tumors.32

Figure 3. Immunodepletion of platelets accelerates TGN. A, TGN (lipopolysaccharide [LPS] + anti-GBM serum [αGBM]) was induced in WT mice, and renal tissue was harvested 4 hours later from these mice and untreated WT mice (normal) to immunohistochemically assess platelet accumulation with the use of antibody that recognizes the αIIb subunit of αIIb/3α on platelets. B to F, WT mice were intravenously injected with anti-GPIbα (αGPIbα) (40 μg per mouse) or control IgG (Con) 12 hours before TGN induction. Glomerular PAS and fibrin deposition (PTAH staining) were significantly increased (B), and glomerular CD34 expression was reduced (C) in platelet-depleted mice in comparison with controls. The hematuria grade (D) was greater, as were biochemical markers for renal function (BUN, serum creatinine [sCr]) and general organ damage (LDH) (E). The number of polymorphonuclear neutrophils (PMNs) per glomerulus cross-section (F) was also increased in platelet-depleted mice. Data are mean±SEM. *P<0.05 compared with control IgG group. Bar=30 μm.
Clinically, patients with a drop in platelet counts, as in idiopathic thrombocytopenic purpura, develop spontaneous bleeding, whereas others with equally low platelet counts do not, suggesting that additional factors dictate the propensity for bleeding. One explanation is that thrombocytopenia increases susceptibility to inflammation-induced hemorrhage. A recent study showed that although thrombocytopenia alone did not lead to hemorrhage, acute inflammation induced in the skin, brain, or lung of thrombocytopenic animals resulted in massive bleeding at the inflammatory site that was independent of platelet adhesion receptors required for platelet plug formation. Our studies indicate a potent function for Mac-1–initiated inflammation in inducing hemorrhage in the context of thrombocytopenia, and thus identify an important point of intersection between inflammation and coagulation.

Platelet-derived factors may change the quality of the vessel wall, thus making it less vulnerable to inflammation-mediated damage, and/or platelet-neutrophil interactions may promote the transcellular biosynthesis of lipoxins, which are potent stop signals for polymorphonuclear neutrophil trafficking. This is a fruitful area for future investigation.

Thrombosis was consistently reduced in Mac-1−/− mice after induction of TGN. Mac-1 on recruited neutrophils could promote thrombosis through several pathways by virtue of its capacity to bind numerous ligands. It binds factor X, which can be activated by neutrophil serine proteinases and thus provides an alternative mechanism for thrombin generation. It also binds platelet counterreceptors GP1b and junctional adhesion molecule-3 directly and indirectly interacts with fibrinogen bound to αIIbβ3. Our data indicate that Mac-1 promotes thrombosis by mediating neutrophil engagement of platelet GP1b. Blocking the GP1b binding site on Mac-1 with an anti-M2 antibody has been shown previously to limit vascular injury by inhibiting neutrophil recruitment to platelets deposited in the injured vessel wall. Here, blockade of Mac-1/GP1bα interaction inhibited events downstream of neutrophil recruitment because this parameter was unaffected in mice treated with anti-M2. To our knowledge, this is the first in vivo evidence that Mac-1 interaction with platelets directly promotes thrombosis. Mac-1 binding to GP1bα on platelets may provide a physical proximity that permits transcellular metabolic cooperation and the efficient delivery of proteinases such as elastase, which activate platelets through limited proteolytic cleavage of αIIbβ3. In support of this, the interaction of active Mac-1 on neutrophil micro-
inflammatory cytokines in renal tissue that are known to cause activation of T and B cells, macrophages, endothelial cells, complement, and the coagulation cascade. This triggers neutrophil recruitment through Mac-1, which further promotes endothelial cell activation. Engagement of Mac-1 results in NE release, which enhances neutrophil influx. Recruited neutrophils promote platelet accumulation. Platelets initially protect the vessel wall from neutrophil-mediated sequelae. However, subsequent interaction of Mac-1 on recruited neutrophils with GPIbα (Gp1b) on platelets triggers thrombosis, which is responsible for vessel occlusion and organ damage.

In conclusion, we demonstrate that Mac-1 on neutrophils represents a critical molecular link between inflammation and coagulation leading to organ damage. The protective effect of Mac-1 deficiency was apparent despite the strong induction of a number of proinflammatory cytokines in renal tissue that are known to cause activation of T and B cells, macrophages, endothelial cells, complement, and the coagulation cascade. A likely effector of Mac-1–mediated neutrophil recruitment is the serine protease NE that was required for neutrophil recruitment and ensuing vessel damage and whose activity was significantly diminished in the absence of Mac-1. Inflammation-induced hemorrhage in the context of thrombocytopenia was Mac-1 dependent, suggesting that platelets support vessel wall integrity in the context of Mac-1–mediated injury. On the other hand, the interaction of Mac-1 on recruited neutrophils with GPIbα on platelets is a major pathway for the subsequent development of thrombosis. These data suggest the possibility of targeting Mac-1 as a novel therapeutic modality to preserve vascular integrity and attenuate thrombosis in thrombotic disorders such as TGN.

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Disclosures

None.

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Complete blood count and differential. Blood samples taken from wild-type (closed circles) and Mac-1^-/- (open circles) mice at the indicated times after induction of TGN were analyzed for white blood cell (WBC), RBC (Hb), neutrophil (PMN) and platelet counts.
### Platelet immunodepletion after TGN induction did not increase disease indices.

WT mice were intravenously injected with anti-GPlβa (40 μg/mice) or control IgG (Con IgG) 24 hrs after TGN induction. Biochemical markers for renal function (BUN, sCr) and general organ damage (LDH) were comparable between the two groups. Data are the mean ± SD.
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