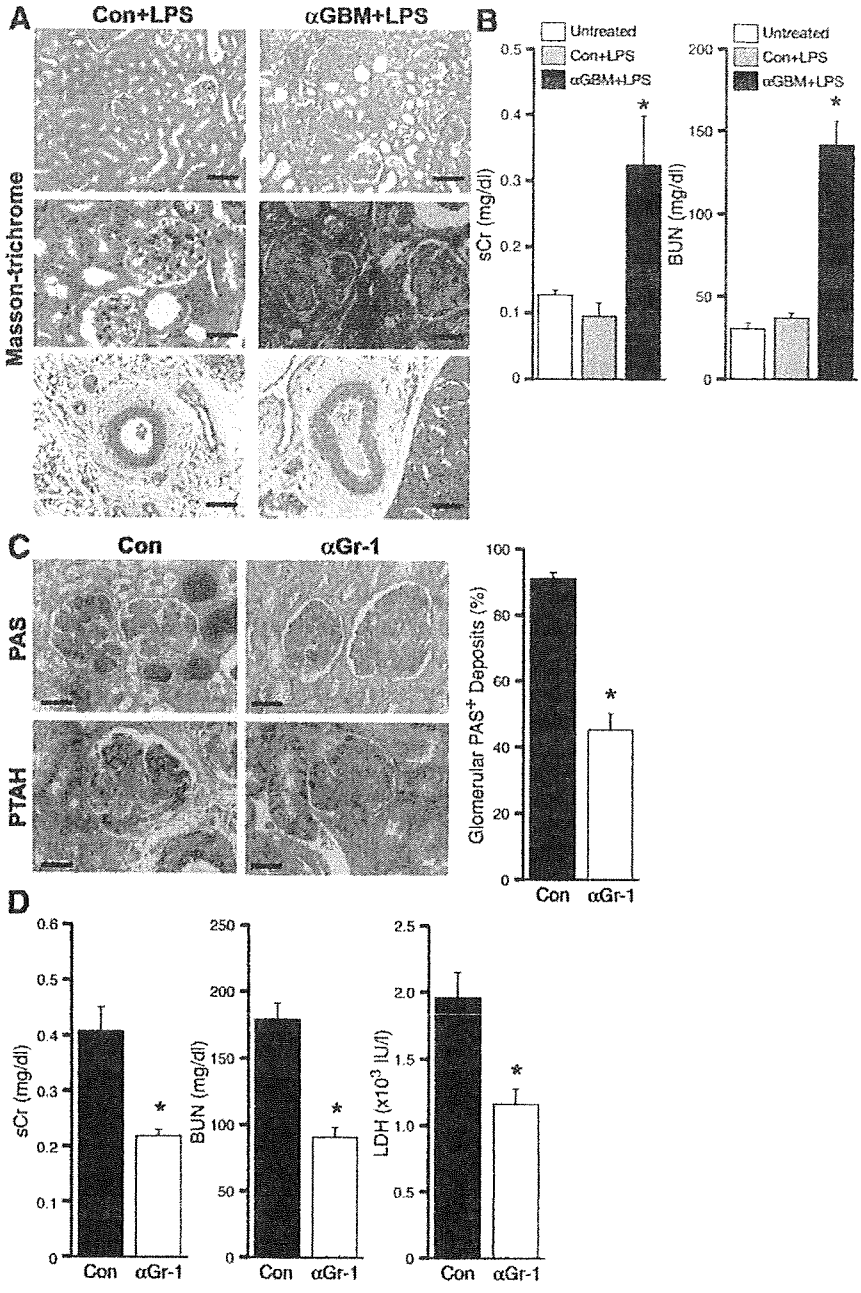

Figure 1. Development of a murine TGN model. A, Kidneys were harvested from mice injected with control (Con) rabbit serum with lipopolysaccharide (LPS) or 2 intravenous injections of anti-GBM serum (αGBM) at 1 hour intervals followed by lipopolysaccharide. Representative Masson trichrome–stained sections of kidneys harvested 96 hours later of TGN are shown. Only mice given anti-GBM serum antibody plus lipopolysaccharide exhibited massive glomerular thrombosis, tubular dilatation and casts (top and middle panels), and plugging of large vessels (bottom panel). Bar=100 μm (top, bottom), 30 μm (middle). B, Serum creatinine (sCr) and BUN levels were evaluated in WT mice before (untreated) and 96 hours after injection with control or anti-GBM serum plus lipopolysaccharide. *$P<0.05$ compared with control. C, Mice treated with control IgG (control) or neutrophil immunodepleting anti-Gr-1 (αGr-1) were subjected to TGN. Shown are representative PAS- and PTAH-stained sections that demonstrate a partial reduction in glomerular thrombus formation (PAS) and fibrin deposition (PTAH) in anti-Gr-1–treated mice. Scoring of PAS-positive glomeruli is presented. Bar=30 μm. D, Neutrophil immunodepletion partially attenuated serum creatinine and BUN, biochemical markers for renal function, and LDH, a general marker for organ damage. Data represent mean±SEM. *$P<0.05$ compared with control.

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–Deficient 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 GPIbα 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-GP1bα antibody 12 hours before disease induction. This regimen maintained low circulating platelet

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 antiinflammatory 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,[1,2] 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.[3] Microvascular endothelial cell injury is still considered the most likely inciting factor in TMA.[4] This may be triggered by bacterial-derived endotoxins/toxins, viruses (HIV), immune complexes, and drugs such as chemotherapeutic agents that

Received March 21, 2008; accepted July 14, 2009.

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Guest Editor for this article was Douglas L. Mann, MD.

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.109.873695/DC1.

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Circulation **is available at http://circ.ahajournals.org** **DOI: 10.1161/CIRCULATIONAHA.109.873695**

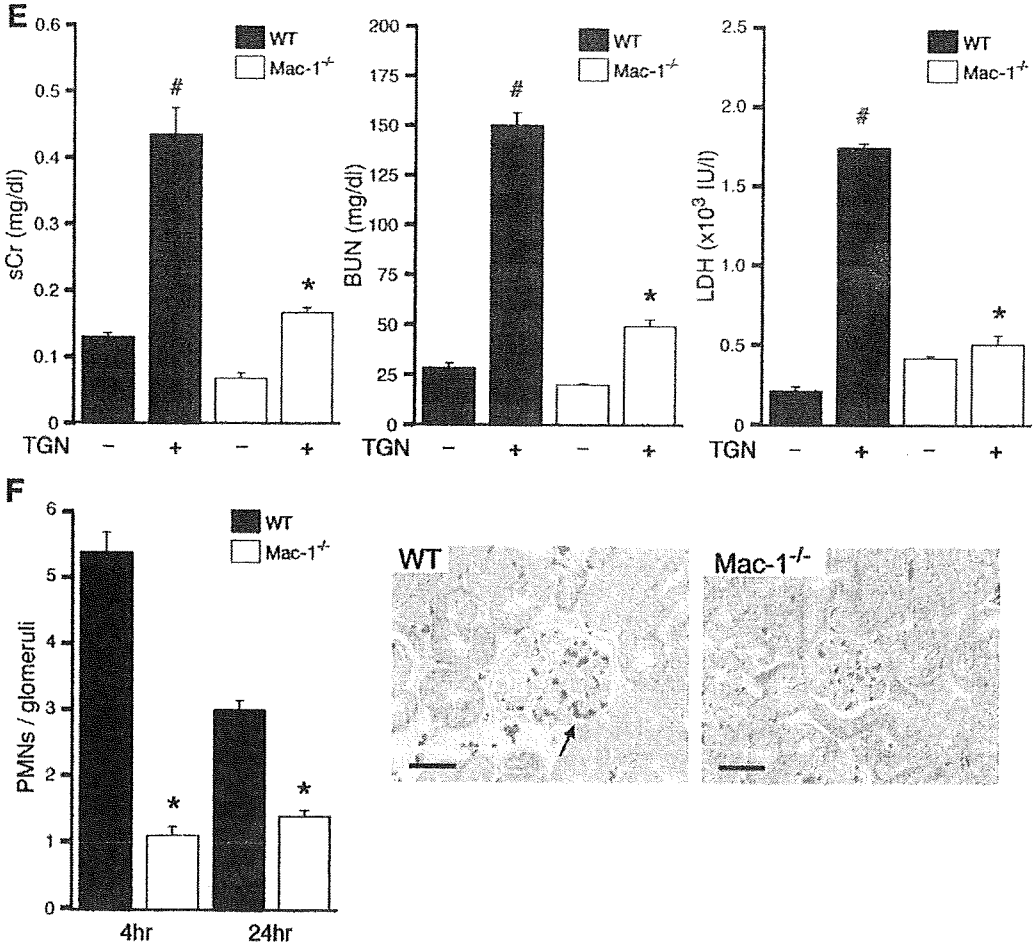

Figure 2. (Continued).

3C) 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,[19] 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. GP1bα 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.[11] Here, an antibody targeting the GPIbα binding site on CD11b (termed anti-M2)[11] 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

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CLINICAL PERSPECTIVE

Under inflammatory conditions such as infection, autoimmune diseases, and atherosclerosis, thrombosis is an important clinical manifestation that can lead to fatal organ damage. Thrombotic microangiopathy occurs in various clinical settings including hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, transplant rejection, systemic lupus erythematosus, and glomerulonephritis exacerbated by infection. In this article, we report that Mac-1, a leukocyte-specific CD18 integrin, links inflammation and thrombosis in thrombotic microangiopathy. Mac-1 is responsible for vascular endothelial damage and links neutrophil-platelet interaction through glycoprotein Ibα on platelets. Blockade of Mac-1/glycoprotein Ibα interaction prevented inflammation-induced glomerular thrombosis and preserved renal function. In conclusion, we propose that Mac-1 is a novel therapeutic target that may prevent thrombosis in inflammatory diseases and thus preserve vascular integrity.

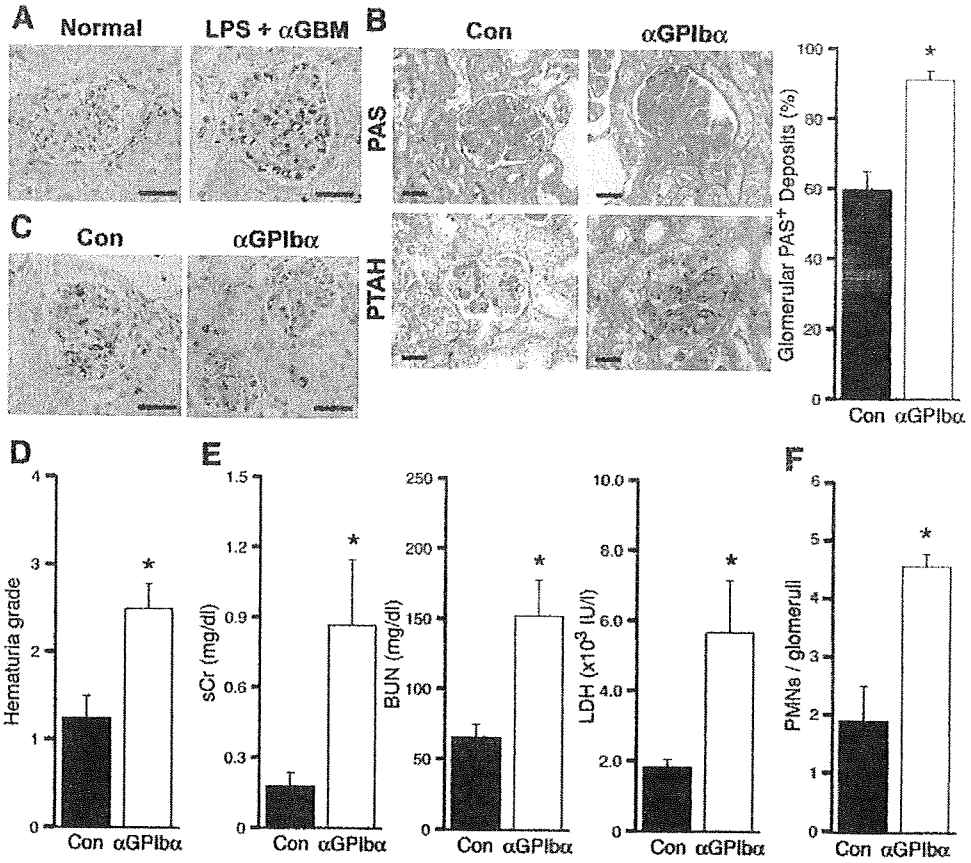

Figure 3. Immunodepletion of platelets accelerates TGN. A, TGN (lipopolysaccharide [LPS]+anti-GBM serum [αGBM]) was induced in WT mice, and renal tissue was harvested 4 hours later from these mice and untreated WT mice (normal) to immunohistochemically assess platelet accumulation with the use of antibody that recognizes the αIIb subunit of αIIbβ3 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.

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 chemoattractants[24] or the generation of chemoattractant cleavage products[25] 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 NE[14] 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 perfusion[30,31] and in sprouting vessels in tumors.[32]

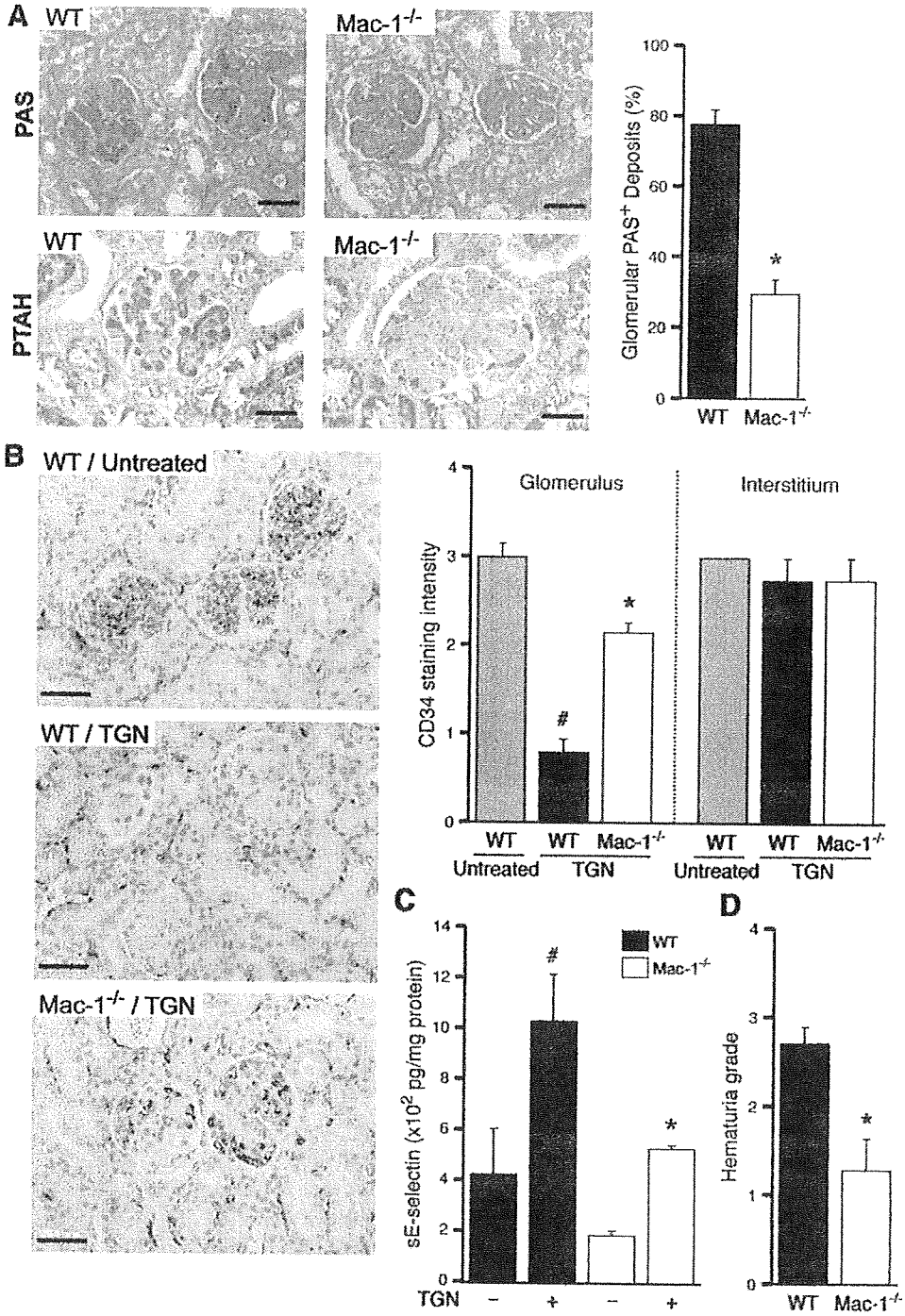

Figure 2. Mac-1–deficient mice are protected from developing TGN. TGN was induced in WT and Mac-1$^{-/-}$ mice and analyzed 96 hours after induction of disease. A, Analysis of PAS- and PTAH-stained kidney sections and scoring of PAS+ deposits revealed a significant reduction in severity of TGN in Mac-1$^{-/-}$ mice compared with WT. Bar=30 μm. B, Immunohistochemical analysis of CD34 on renal tissue of WT untreated mice and WT and Mac-1$^{-/-}$ mice subjected to TGN. A reduction in glomerular CD34, but not interstitial CD34, was observed in WT/TGN mice compared with untreated WT, whereas Mac-1$^{-/-}$/TGN mice retained glomerular CD34. Bar=50 μm. C, E-selectin in renal tissue of indicated animals was measured and showed a decrease in Mac-1$^{-/-}$ mice compared with WT mice subjected to TGN. 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.

counts up to 24 hours after TGN induction (0 hour, <2.0% of control; 24 hours, <5.0% of control), as described previously.[20] 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

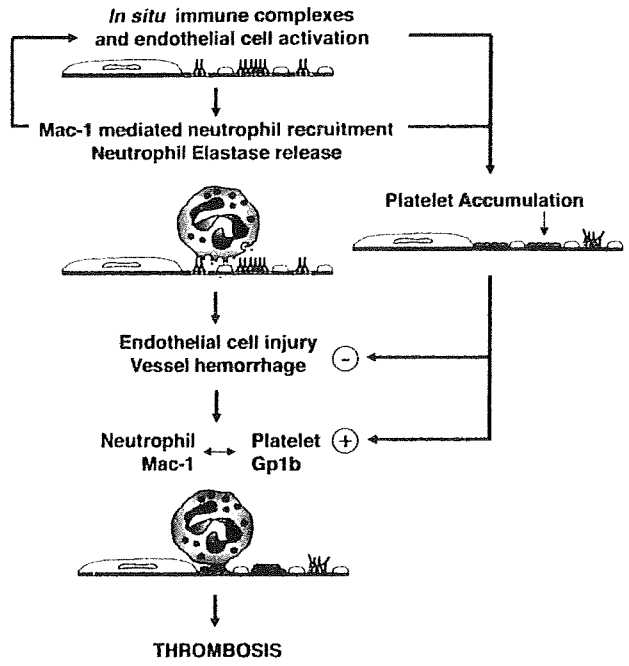

Figure 5. Model of Mac-1–mediated inflammation induced thrombosis in TGN. Anti-GBM antibody in combination with lipopolysaccharide results in in situ immune complex formation and endothelial cell activation. This triggers neutrophil recruitment through Mac-1, which further promotes endothelial cell activation. 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.

particles with GPIbα was recently shown to promote platelet activation in vitro.[40]

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 proteinase 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 GP1bα 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.

Acknowledgments

We are grateful to Bio-Rad for support with the Bio-Plex protein array system.

Sources of Funding

This work was supported by the Ministry of Education, Culture, Sports, Science, and Technology, Japan (17209034 [Dr Fujita], 17790547 [Dr Hirahashi]), the Uehara Memorial Foundation (Japan) (Dr Hirahashi), Arthritis Foundation (Dr Tsuboi), a grant from the Ministry of Health, Labour, and Welfare in Japan (Dr Suzuki), and National Institutes of Health grants RO1 AR050800, HL065095, and DK077111(Dr Mayadas).

Disclosures

None.

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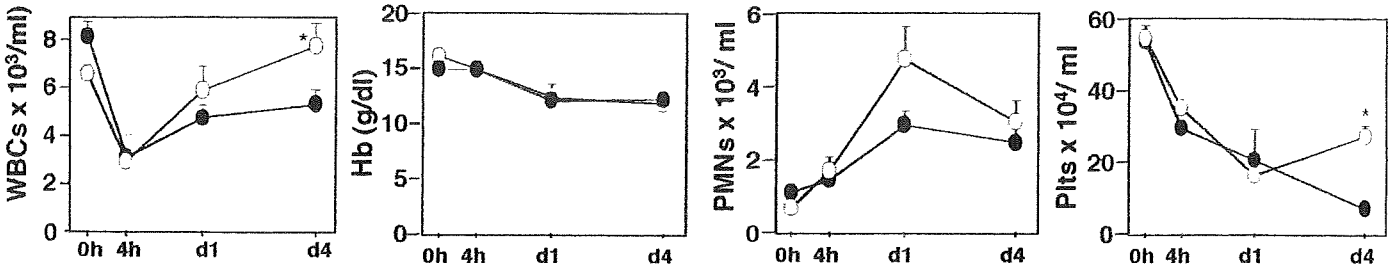
Figure S2

	Con IgG (n=4)	αGPIbα (n=4)	P-value
BUN (mg/dl)	105.7 ± 1.1	86.8 ± 8.4	0.067
sCr (mg/dl)	0.28 ± 0.01	0.24 ± 0.03	0.320
LDH x 10^3 (IU/l)	1.85 ± 0.18	1.79 ± 0.12	0.765

Platelet immunodepletion after TGN induction did not increase disease indices. WT mice were intravenously injected with anti-GPIbα (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

Figure S1



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.

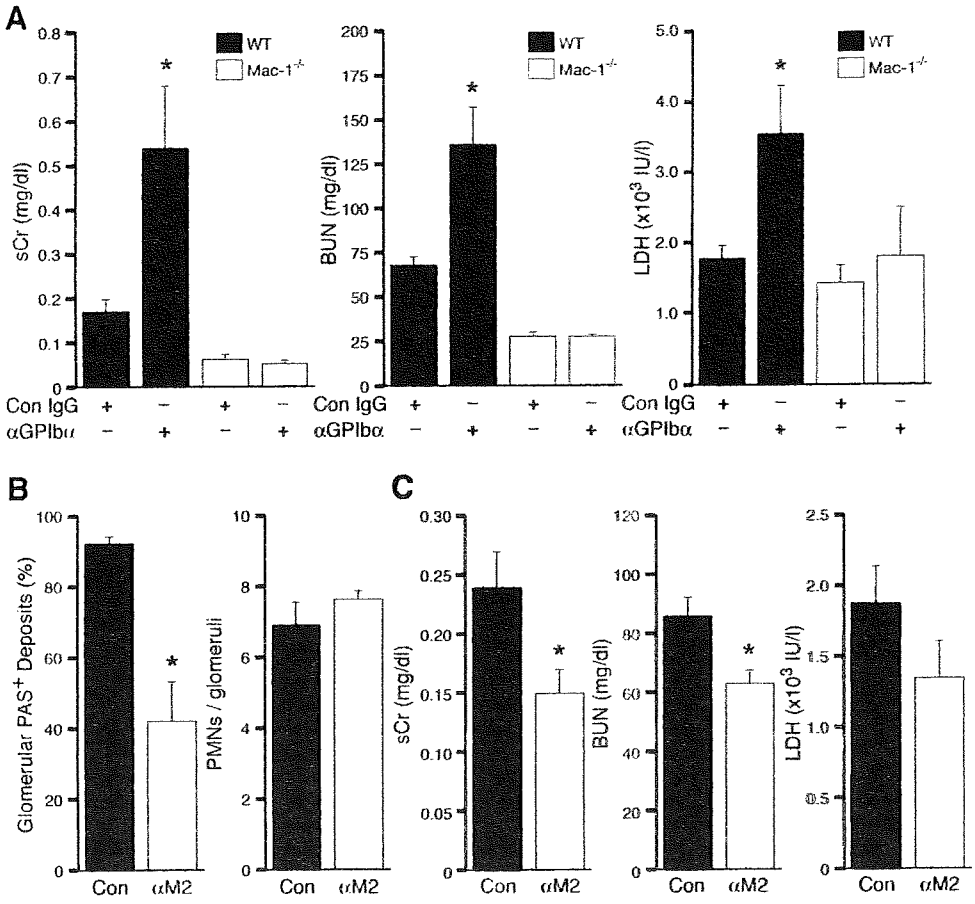


Figure 4. Thrombocytopenic Mac-1–deficient mice remain resistant to TGN. Mac-1 interaction with platelet GPIbα promotes thrombosis. A, TGN was induced in WT or Mac-1$^{-/-}$ mice treated with IgG control (Con) or anti-GPIbα (αGPIbα) to immunodeplete platelets. Serum creatinine (sCr), BUN, and LDH were significantly elevated only in thrombocytopenic WT mice after induction of TGN. Thrombocytopenic Mac-1$^{-/-}$ mice exhibited no increase in indices of renal failure after induction of TGN. B and C, TGN was induced in WT mice treated with a polyclonal anti-M2 (αM2) or rabbit IgG control. B, PAS deposition (left panel) and neutrophil (PMNs) accumulation (right panel) were evaluated in renal tissue. A significant reduction in PAS deposition but no decrease in glomerular neutrophil accumulation was observed in anti-M2 versus IgG control animals. C, Indices of renal failure including serum creatinine, BUN, and LDH were reduced in anti-M2–treated animals compared with control. Data represent mean±SEM. *$P<0.05$ compared with WT.

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.[33] One explanation is that thrombocytopenia increases susceptibility to inflammation-induced hemorrhage. A recent study showed that although thrombocytopenia alone did not lead to hemorrhage,[34] 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.[19] 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,[35] and/or platelet-neutrophil interactions may promote the transcellular biosynthesis of lipoxins, which are potent stop signals for polymorphonuclear neutrophil trafficking.[36] 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 provide an alternative mechanism for thrombin generation.[37] It also binds platelet counterreceptors GP1bα and junctional adhesion molecule-3 directly[37,38] and indirectly interacts with fibrinogen bound to αIIbβ3.[39] Our data indicate that Mac-1 promotes thrombosis by mediating neutrophil engagement of platelet GPIbα. Blocking the GPIbα binding site on Mac-1 with anti-M2 antibody has been shown previously to limit vascular injury by inhibiting neutrophil recruitment to platelets deposited in the injured vessel wall.[11] Here, blockade of Mac-1/GPIbα 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 GPIbα 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.[27] In support of this, the interaction of active Mac-1 on neutrophil micro-