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A critical role for both CD40 and VLA5 in angiotensin II–mediated thrombosis and inflammation

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ABSTRACT: Angiotensin II (Ang-II)–induced hypertension is associated with accelerated thrombus formation in arterioles and leukocyte recruitment in venules. The mechanisms that underlie the prothrombotic and proinflammatory responses to chronic Ang-II administration remain poorly understood. We evaluated the role of CD40/CD40 ligand (CD40L) signaling in Ang-II–mediated microvascular responses and assessed whether and how soluble CD40L (sCD40L) contributes to this response. Intravital video microscopy was performed to analyze leukocyte recruitment and dihydrorhodamine-123 oxidation in postcapillary venules. Thrombus formation in cremaster muscle arterioles was induced by using the light/dye endothelial cell injury model. Wild-type (WT), $CD40^{-/-}$, and $CD40L^{-/-}$ mice received Ang-II for 14 d *via* osmotic minipumps. Some mice were treated with either recombinant sCD40L or the VLA5 (very late antigen 5; $\alpha5\beta1$) antagonist, ATN-161. Our results demonstrate that $CD40^{-/-}$, $CD40L^{-/-}$, and WT mice that were treated with ATN-161 were protected against the thrombotic and inflammatory effects of Ang-II infusion. Infusion of sCD40L into $CD40^{-/-}$ or $CD40L^{-/-}$ mice restored the prothrombotic effect of Ang-II infusion. Mice that were treated with ATN-161 and infused with sCD40L were protected against accelerated thrombosis. Collectively, these novel findings suggest that the mechanisms that underlie Ang-II–dependent thrombotic and inflammatory responses link to the signaling of CD40L *via* both CD40 and VLA5.—Senchenkova, E. Y., Russell, J., Vital, S. A., Yildirim, A., Orr, A. W., Granger, D. N., Gavins, F. N. E. A critical role for both CD40 and VLA5 in angiotensin II–mediated thrombosis and inflammation. FASEB J. 32, 3448–3456 (2018). www.fasebj.org

KEY WORDS: sCD40L thrombosis · leukocytes · platelets · endothelium · hypertension

Hypertension is a common clinical condition that is associated with high morbidity and mortality (1). Angiotensin II (Ang-II) is known to play a major role in different forms of hypertension. It has been implicated as a key inducer of the proinflammatory and prothrombotic phenotype assumed by the vasculature (2, 3). A role for Ang-II is evidenced in large arteries as an Ang-II–dependent accumulation of inflammatory cells (*e.g.*, leukocytes and platelets), increased production of reactive oxygen species (ROS), impaired vasomotor function, and a predisposition to thrombus development (2–5). Ang-II–dependent changes also occur in the microvasculature, where chronic infusion of Ang-II results in elevated oxidative stress; increased adhesive interactions between leukocytes, platelets and endothelial cells; and accelerated thrombus development (6–9). Additional support for Ang-II as a mediator of inflammation and thrombosis in the vasculature is provided by several studies that have demonstrated the ability of Ang-II receptor blockers to blunt leukocyte adhesion and thrombosis in different Ang-II–dependent models of human disease, including hypertension (6–9) and hypercholesterolemia (10). Moreover, there is clinical evidence that the treatment of patients with hypertension with Ang-II receptor blockers or angiotensin-converting enzyme inhibitors is associated with a lower incidence of thrombotic events (3, 4, 11).

Whereas mechanisms that underlie the Ang-II–dependent inflammatory and thrombotic responses in the vasculature remain poorly understood, we have previously found that Ang-II, but not elevated blood pressure, per se, mediates enhanced thrombosis within the cremasteric microvasculature that occurs in arterioles, but

ABBREVIATIONS: Ang-II, angiotensin II; CD40L, CD40 ligand; DHR, dihydrorhodamine-123; GPIIb/IIIa, glycoprotein IIb/IIIa; Mac-1, macrophage 1 antigen; ROS, reactive oxygen species; sCD40L, soluble CD40L; VLA5, very late antigen 5; WT, wild type

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not in venules (6–9). In addition, Ang-II activates a variety of cell populations, including endothelial cells, leukocytes, and platelets, thereby contributing to both inflammation and coagulation/thrombosis (7). Endothelial injury and circulating cell activation leads to the production of prothrombotic and proinflammatory mediators (2, 4, 6–9). In this regard, increased oxidative stress and enhanced homotypic—platelet–platelet—and heterotypic—leukocyte–endothelial cell and platelet–leukocyte—cell adhesion/aggregation are important consequences of Ang-II–mediated cell activation (5, 7). These activated cells also release cytokines and soluble forms of adhesion mediators that can amplify and perpetuate the proinflammatory and prothrombotic phenotype assumed by the vasculature in response to Ang-II (2).

CD40 ligand (CD40L; also known as CD154 or gp39)—released by activated platelets and lymphocytes—has been implicated as a mediator of chronic Ang-II–induced inflammatory changes and altered blood coagulation profile—for example, reduced bleeding time, enhanced tissue factor–induced thrombin generation by platelets—in the mouse aorta *in vitro* (12, 13). These observations are consistent with clinical reports that have described elevated levels of soluble CD40L (sCD40L) in the plasma of patients with hypertension (14) and in animal studies that have demonstrated a role for CD40/CD40L, including sCD40L, signaling in thrombus development (15, 16). Both cell-associated and circulating sCD40L also induce a proinflammatory and prothrombotic phenotype in response to other risk factors, such as hypercholesterolemia (17).

Whereas platelets are a major source of sCD40L (12, 13), they also represent a major target for cell-associated sCD40L (18). In response to CD40L stimulation, platelets exhibit signs of activation (*e.g.*, increased surface expression of P-selectin expression and α-granule release) and are more sensitive to agonist (*e.g.*, ADP, thrombin, or collagen)-induced aggregation (18). Other cells, including endothelial cells and leukocytes, are also activated by CD40L. The activation response in different cell types results from the engagement of CD40L with its primary receptor, CD40, or with other receptors, including the fibrinogen/vitronectin receptor, VLA5 [very late antigen 5; α-5 β-1 (α5β1)], the platelet integrin receptor, glycoprotein IIb/IIIa (GPIIb/IIIa; αIIbβ3), TNF-5 (TNF receptor 5), and the integrin receptor, macrophage 1 antigen (Mac-1; CD11b/CD18, $\alpha_M\beta_2$) (12, 19).

We hypothesized that the Ang-II–induced hypertension associated with accelerated thrombotic responses and inflammatory cell recruitment within the microcirculation is linked to CD40L-mediated effects; therefore, in the present study, we investigated *in vivo*—using a combination of pharmacologic and genetic approaches—the potential contribution of CD40L to Ang-II–induced thrombosis and inflammation in an *in vivo* model of thrombus formation and leukocyte recruitment. We demonstrated that Ang-II elicits a proinflammatory and prothrombotic phenotype in the cremasteric microvasculature *via* a mechanism that involves signaling between sCD40L and both CD40 and VLA5. Together, these results suggest a key role for Ang-II in the acceleration of prothrombotic/proinflammatory responses within the microvasculature and potential therapeutic modulation of key participants CD40- and VLA5-dependent signaling to Ang-II–associated prothrombotic and proinflammatory responses.

MATERIALS AND METHODS

Mice

Male wild-type (WT; C57Bl6), CD40 knockout ($CD40^{-/-}$; B6.129P2-*Tnfrsf5tm1Kik*/J), and CD40L knockout ($CD40L^{-/-}$; B6.129S2-*Cd40tm1Imx*/J) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA), as previously described (15). Mice had access to a standard chow pellet diet and tap water *ad libitum* and were maintained on a 12-h light/dark cycle during which room temperature was maintained at 21–23°C. All mouse experiments were conducted in accordance with experimental procedures approved by the Louisiana State University Health Science Center Institutional Animal Care and Use Committee, and were in compliance with the guidelines of the American Physiologic Society.

Drug treatments

In some experimental groups, mice were treated either VLA5 antagonist (ATN161; gift of Dr. Andrew Mazar, Northwestern University, Evanston, IL, USA), an inhibitor of integrin α5β1, administered in 5-mg/kg, intraperitoneal injections 5 times over 14 d, or sCD40L (PeproTech, Rocky Hill, NJ, USA; 10 ng/mouse) by intravenous injection 30 min before data collection or 0.5 ng/min for 7 d using osmotic Alzet Mini-Pump (0.5 μl/h, Model 1007D; Alzet, Cupertino, CA, USA).

Osmotic pump implantation

Ang-II (1 μg/kg/min)–loaded micro-osmotic pumps (Model 1002) were implanted up to 14 d s.c. (intrascapular region) under isofluorane anesthesia by using sterile procedures as previously described (6–9, 20). Systolic blood pressure values were obtained by using a computerized tail cuff system (SC-1000; Hatteras Instruments, Cary, NC, USA) before Ang-II infusion and 14 d later (7). All mice that underwent implantation of the Ang-II–loaded pump exhibited elevated blood pressure (Supplemental Table 2). Some experimental WT mice were implanted with micro-osmotic pumps (Model 1007D; Alzet) that were loaded with murine recombinant sCD40L (0.5 ng/min) for 7 d.

Light/dye-induced thrombosis

Mice were anesthetized with sodium pentobarbital—5 mg/kg body weight *via* intraperitoneal injection—and the right jugular vein was cannulated for administration of FITC-dextran (5 mg/kg, i.v.; Sigma-Aldrich, St. Louis, MO, USA). Cremaster muscle was prepared for intravital microscopic observation, as previously described (6–9). Body temperature was maintained at 35–37 °C. After a 20-min stabilization period, FITC-dextran was infused and allowed to circulate for 10 min. In selected arterioles (30–40 μm diameter), thrombus formation was elicited by continuous epi-illumination from a 175-W xenon light source (Lambda LS; Sutter Instrument, Novato, CA, USA) and a fluorescein filter cube (HQ-FITC; Chroma, Rockingham, VT, USA). Thrombus formation was quantified by using onset time (time of the onset of platelet deposition on the vessel wall) and cessation time (time required for complete blood flow cessation) (6).

Leukocyte adhesion in cremaster muscle venules

Mice were anesthetized with ketamine hydrochloride (150 mg/kg body weight, i.p.) and xylazine (7.5 mg/kg body weight, i.p.), and cremaster muscle was prepared for intravital microscopic observation, as previously described (7). Three venular segments—100 μm in length and 22–36 μm in diameter—were selected for evaluation. The number of firmly adherent—stationary for ≥30 s—leukocytes per venular segment were measured, averaged for each animal, and expressed as the number per square millimeter.

Dihydrorhodamine-123 oxidation

WT, WT Ang-II, $CD40^{-/-}$ Ang-II, and $CD40L^{-/-}$ Ang-II mice were anesthetized with ketamine hydrochloride (150 mg/kg body weight, i.p.) and xylazine (7.5 mg/kg body weight, i.p.), and cremaster muscle was prepared for intravital microscopic observation of ROS production by using the known technique of recording dihydrorhodamine-123 (DHR) oxidation and *in vivo* visualization, as previously described (7). In brief, 3 venular segments—100 μm in length and 22–36 μm in diameter—were selected for evaluation in each mouse. Recordings were taken before [background intensity (I_{Bgrd})] and after 15 min of superfusion with the oxidant-sensitive fluorochrome, dihydrorhodamine-123 (DHR; 1 mM in bicarbonate-buffered saline; Sigma-Aldrich), which was excited with a xenon light source. Tissue was washed with bicarbonate-buffered saline, and the fluorescent image of each section was recorded (I_{DHR}). Images were captured onto a computer, and an area 100-μm long and 7-μm wide along the vessel wall was analyzed in each vascular segment by using ImageJ 1.62 software (National Institutes of Health, Bethesda, MD, USA). The ratio of I_{DHR}:I_{Bgrd} was calculated for each vascular segment, and the average ratio for each animal was determined (7).

ELISA

A commercially available sCD40L ELISA (Abcam, Cambridge, MA, USA) was used to detect CD40L levels in the serum of WT mice, and were implanted with the Ang-II–loaded pumps per the manufacturer's instructions (Alzet).

Statistical analyses

Data within groups were compared by using 1-way ANOVA with a Newman-Keuls *post hoc* correction for multiple comparisons. Analysis was performed by using Prism 6 software (GraphPad Software, La Jolla, CA, USA). Data are shown as means ± SEM. Values of $P < 0.05$ were considered statistically significant.

RESULTS

$CD40^{-/-}$ and $CD40L^{-/-}$ mice exhibit protection against both Ang-II–induced arterial thrombosis and inflammatory cell recruitment within cremaster muscle microvessels

Platelets and T cells express CD40L and its soluble mediator, sCD40L, both of which are known to be prothrombotic markers (6, 17, 18). We assessed the role of CD40L and its receptor, CD40, in arteriolar thrombosis (venule thrombus formation was not associated with Ang-II–induced hypertension) (6). We have previously shown that both $CD40^{-/-}$ and $CD40L^{-/-}$ mice exhibited prolonged thrombus cessation time in cremaster muscle arterioles (15). Thus, $CD40^{-/-}$ and $CD40L^{-/-}$ mice were used to assess the contribution of each component of the CD40/CD40L dyad, as previously described (14, 15). **Figure 1*A*, *B*** shows that hypertensive $CD40^{-/-}$ and $CD40L^{-/-}$ mice both exhibited a more dramatic restoration in onset and cessation times *vs.* their WT counterparts [80% ($CD40^{-/-}$ Ang-II) and 79% ($CD40L^{-/-}$ Ang-II) increases compared with WT Ang-II]. These protective effects were also observed in leukocyte adherence and DHR oxidation. As shown in Fig. 1*C*, the number of adherent leukocytes was significantly reduced in $CD40^{-/-}$ and $CD40L^{-/-}$ mice (WT control: 161.3 ± 50.7; WT Ang-II: 813.4 ± 18.3; $CD40^{-/-}$ Ang-II: 268.2 ± 52.7; and $CD40L^{-/-}$ Ang-II: 408.1 ± 47.0 leukocytes/mm^2), which correlated with a reduction in DHR oxidation (WT control: 1.6 ± 0.7; WT Ang-II: 31.1 ± 7.8; $CD40^{-/-}$ Ang-II: 2.8 ± 1.8; and $CD40L^{-/-}$ Ang-II: 8.2 ± 1.5; Fig. 1*D*).

Elevated levels of sCD40L in WT mice recapitulate enhanced microvascular thrombus formation similar to that observed in Ang-II–induced hypertension

The soluble form of CD40 (CD40L) promotes platelet–platelet and leukocyte–platelet endothelial cell interactions (17, 18). Furthermore, sCD40L, which is shed from the surface of activated platelets, has been previously reported to promote inflammation and thrombosis/coagulation *in vitro* and *in vivo* (14, 15). As such, we investigated the role of sCD40L in a prothrombotic/proinflammatory phenotype associated with Ang-II–induced hypertension. **Figure 2*A*** demonstrates that WT mice that were chronically infused with Ang-II exhibited elevated serum levels of sCD40L compared with their WT counterparts (0.45 ± 0.078 ng/ml *vs.* 0.07 ± 0.021 ng/ml, respectively). To confirm that sCD40L levels were within the pathophysiologic range, we administered sCD40L. Figure 2*A* indicates that sCD40L levels had no significant difference compared with levels of CD40L measured in WT mice that were chronically infused with Ang-II (0.53 ± 0.09 ng/ml *vs.* 0.45 ± 0.078 ng/ml, respectively), which suggests that sCD40L levels were within the pathophysiologic range. In addition, results in Fig. 2*B* demonstrate that the chronic infusion of sCD40L in WT mice elicited a prothrombotic response that was similar to that observed in WT mice that were subjected to chronic Ang-II infusion, with both responses being significantly quicker than that of WT control. These results, combined with data that show that Ang-II induces elevated sCD40L levels, suggest a link between Ang-II–accelerated thrombosis and CD40L–CD40 dyad.

Infusion of recombinant sCD40L abolishes the protective effect of CD40 and CD40L deficiency against Ang-II–enhanced thrombus formation

We next evaluated whether sCD40L may account for the roles of CD40/CD40L in Ang-II–dependent thrombosis

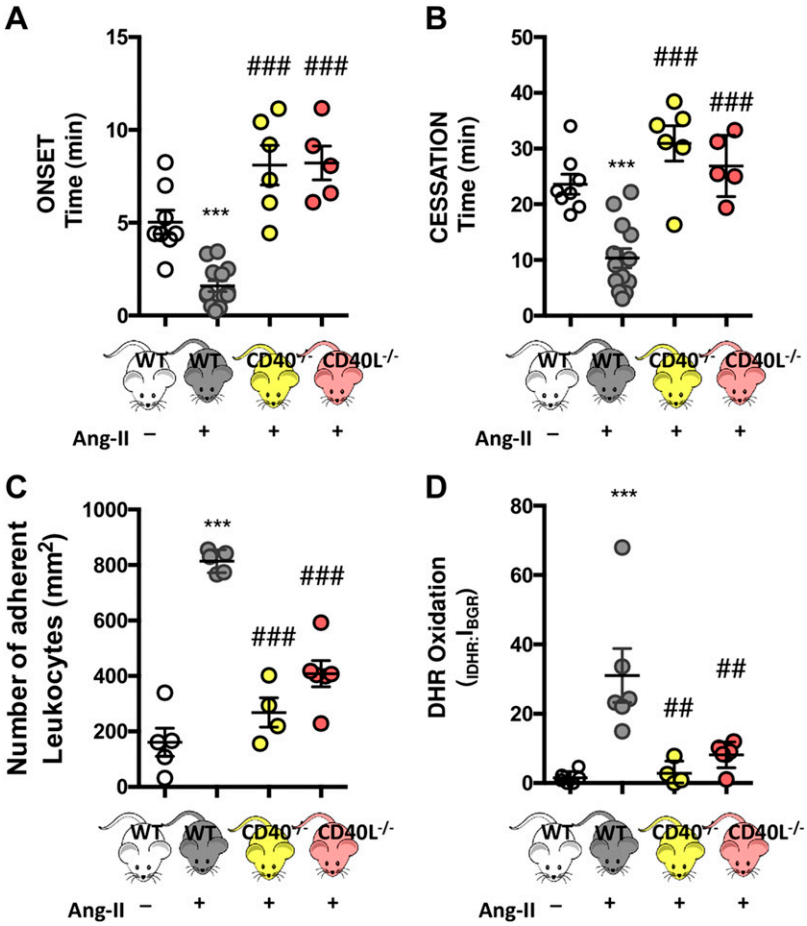

Figure 1. Effects of CD40 and CD40L deficiency on thrombus formation, leukocyte recruitment, and oxidant production in cremaster muscle. WT, $CD40^{-/-}$, or $CD40L^{-/-}$ mice were implanted with Ang-II (1 μg/kg/min)– or control-loaded micro-osmotic pumps for up to 14 d before undergoing: *1*) light/dye-induced thrombosis model coupled with intravital fluorescence microscopy, *2*) intravital fluorescence microscopy, or *3*) DHR oxidation coupled with intravital fluorescence microscopy. *A*, *B*) Time of onset (initial platelet deposition; *A*) and time of blood flow cessation (occlusion; *B*) during the light/dye-induced thrombosis model. *C*, *D*) Intravital microscopy was performed and shows the number of adherent (stationary for ≥30 s) leukocytes (*C*) and DHR oxidation (quantified as ratio of I_{DHR}:I_{Bgrd}; *D*). Data are presented as means ± SEM of 4–13 mice per group. $^{***}P < 0.001$ *vs.* WT saline, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* WT Ang-II.

by injecting sCD40L into $CD40^{-/-}$ and CD40L mice with chronically elevated levels of Ang-II. Infusion of sCD40L into $CD40^{-/-}$ or $CD40L^{-/-}$ mice that were implanted with the Ang-II–loaded pump elicited a prothrombotic response that was similar to that observed during chronic Ang-II infusion (**Fig. 3**). In addition, acute administration of sCD40L abolished the protective effects against Ang-II–induced thrombosis that were detected in both $CD40^{-/-}$ and $CD40L^{-/-}$ mice (Fig. 1). These results suggest that CD40L (sCD40L) may engage with a different receptor than CD40 to mediate the prothrombotic response.

VLA5 antagonism attenuates thrombosis in chronic infused Ang-II–treated mice

CD40L is able to bind to different receptors, including CD40, GPIIb/IIIa, Mac-1, and VLA5 (11, 18); however, GPIIb/IIIa is not expressed on leukocytes (21), and Mac-1 does not affect thrombus formation in mice, (22) which suggests that neither receptor likely mediates the pleiotropic effects of sCD40L. Thus, in our final series of experiments, we focused on VLA5 to address whether VLA5, which is expressed on leukocytes, platelets, and endothelial cells, plays a role in mediating responses that are evoked by CD40L in Ang-II–induced thrombosis. **Figure 4*A*** demonstrates that the VLA5 antagonist, ATN-161, was highly effective in preventing accelerated platelet deposition (onset of the thrombosis) that was elicited by chronic Ang-II infusion. In addition, antagonism of VLA5 was also highly protective against blood flow cessation in cremaster arterioles (Fig. 4*B*).

VLA5 antagonist, ATN-161, protects against enhanced, exacerbated leukocyte adhesion and oxidant production associated with Ang-II–induced hypertension

Having demonstrated the protective effects of VLA5 antagonism on thrombosis and knowing the crosstalk between arteriolar thrombosis and inflammation, we next focused on whether VLA5 also plays a role in mediating inflammation that is associated with Ang-II infusion. Figure 4*C* demonstrates that VLA5 prevented increased adherence of leukocytes and oxidant production (Fig. 4*D*) in postcapillary venules elicited by Ang-II infusion. VLA5 antagonism did not protect mice against Ang-II–induced elevation of systolic blood pressure (Supplemental Table 2).

Antagonism of VLA5 in WT mice prevents sCD40L-induced accelerated thrombus formation

In the final part of the study, we wanted to address the question of whether sCD40L mediates thrombus formation *in vivo via* VLA5. As such, we used a VLA5 antagonist, ATN-161, and infused mice with sCD40L to accelerate

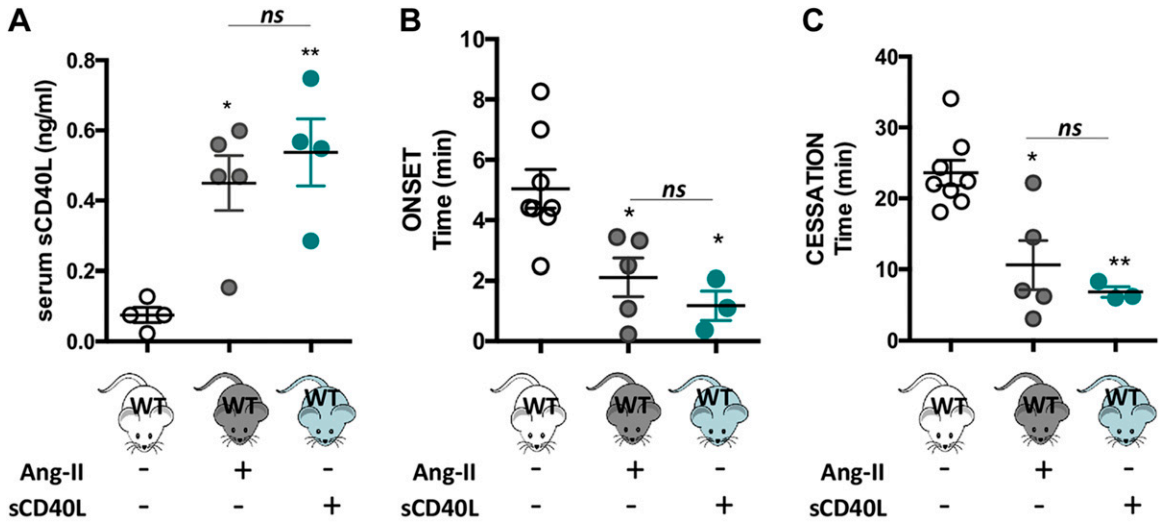

Figure 2. Serum levels of sCD40L and effect of chronic sCD40L administration on thrombosis in Ang-II–induced hypertension in WT mice. WT mice were implanted with Ang-II (1 μg/kg/min)– or control-loaded micro-osmotic pumps for up to 14 d. *A*) Serum levels of sCD40L were measured in WT mice, WT mice that were implanted with Ang-II–loaded pumps, or WT mice that were treated with sCD40L [5 μg for 7 d infused (0.5 ng/min) *via* micro-osmotic pumps]. For thrombosis experiments, mice were treated with recombinant murine sCD40L [5 μg for 7 d infused (0.5 ng/min) *via* micro-osmotic pumps], and light/dye-induced thrombosis model coupled with intravital fluorescence microscopy was performed. *B*, *C*) Time of onset (initial platelet deposition; *B*) and time of complete blood flow cessation (occlusion; *C*) was quantified. Data are presented as means ± SEM of 3–8 mice per group; ns, no significant difference between groups. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* WT saline.

thrombus formation in arterioles. **Figure 5** shows that blocking VLA5 with the specific VLA5 antagonist, ATN-161, completely prevented sCD40L-mediated thrombosis (onset and cessation time) in WT mice.

DISCUSSION

There are several novel, key findings from this study: *1*) Ang-II elicits a proinflammatory and prothrombotic phenotype in the cremasteric microvasculature that involves CD40/CD40L; *2*) *in vivo*, the proinflammatory and prothrombotic phenotype associated with Ang-II chronic infusion is attenuated with VLA5 antagonism; and *3*) Ang-II–dependent prothrombotic and proinflammatory responses are linked to the signaling of CD40L *via* both CD40 and VLA5.

Elevated blood pressure—a known risk factor for cardiovascular diseases—is associated with an increased risk of thrombosis that involves an interaction between the renin-angiotensin system and hemostasis. As such, patients with hypertension are at risk for stroke and myocardial infarction, with thromboembolic events manifested not only in large arteries and veins, but also in the microvasculature (4, 6–9, 23). Platelets play a major role in these events, and platelet activation/reactivity, coagulation, fibrinolysis, and leukocyte and vessel wall activation are

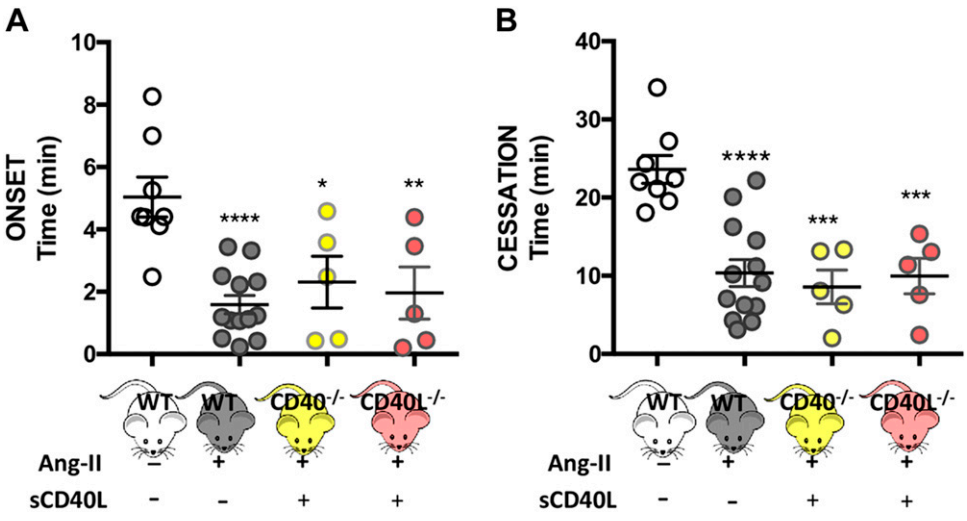

Figure 3. Effects of sCD40L on light/dye-induced thrombus formation in arterioles of WT, $CD40^{-/-}$, and $CD40L^{-/-}$ mice that were implanted with Ang-II–loaded pump. WT, $CD40^{-/-}$, or $CD40L^{-/-}$ mice were implanted with Ang-II (1 μg/kg/min)– or control-loaded micro-osmotic pumps for up to 14 d. Mice were treated with sCD40L (10 ng/mouse, i.v., 30 min before data collection), and the light/dye-induced thrombosis model coupled with intravital fluorescence microscopy was performed. *A*, *B*) Time of onset (initial platelet deposition; *A*) and time of complete blood flow cessation (occlusion; *B*) was quantified. Data are presented as means ± SEM of 5–13 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* WT saline, $^{****}P < 0.0001$.

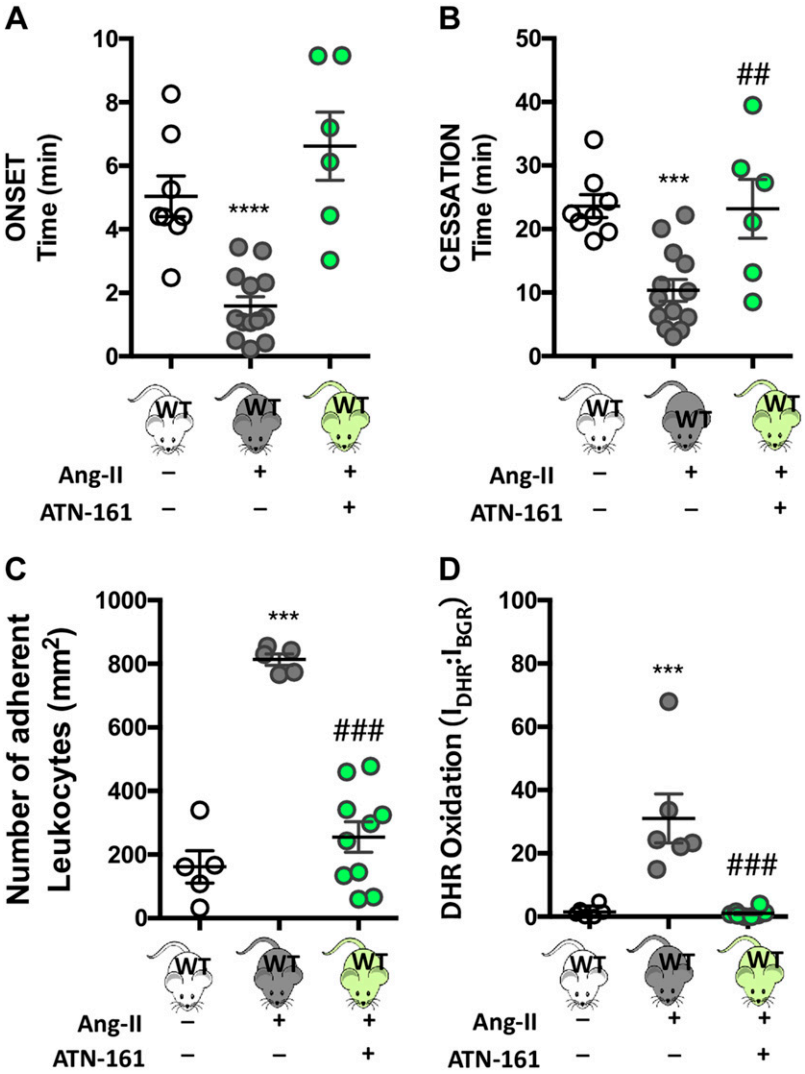

Figure 4. Effect of pharmacologic blocking of VLA5 with ATN-161 in the cremaster microvasculature of WT mice. WT mice were implanted with Ang-II (1 μg/kg/min)– or control-loaded micro-osmotic pumps for up to 14 d. Mice were treated with VLA5 antagonist (ATN161; 5 mg/kg, i.p., 5 times over 14 d) before undergoing: *1*) light/dye-induced thrombosis model coupled with intravital fluorescence microscopy, *2*) intravital fluorescence microscopy, or *3*) DHR oxidation coupled with intravital fluorescence microscopy. *A*, *B*) Time of onset (initial platelet deposition; *A*) and time of complete blood flow cessation (occlusion; *B*) during the light/dye-induced thrombosis model. *C*, *D*) Intravital microscopy was performed and shows the number of adherent (stationary for ≥30 s) leukocytes (*C*) and DHR oxidation (quantified as ratio of I_{DHR}:I_{Bgrd}; *D*). Data are presented as means ± SEM of 5–13 mice per group. $^{***}P < 0.001$, $P < 0.0001$ *vs.* WT saline; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* WT Ang-II, $^{****}P < 0.0001$.

commonly associated with hypertension (8, 24, 25), although the mechanisms that underlie these hypertension-associated complications remain poorly understood. We have previously shown in our laboratory that Ang-II–induced thrombosis in arterioles (not venules) is associated with a role for $CD4^+$ T cells, even though venules exhibited increased leukocyte adhesion (7, 9). Both platelets and T cells are sources of sCD40L (16), and, as such, in this study we wanted to determine whether sCD40L played a possible functional role in

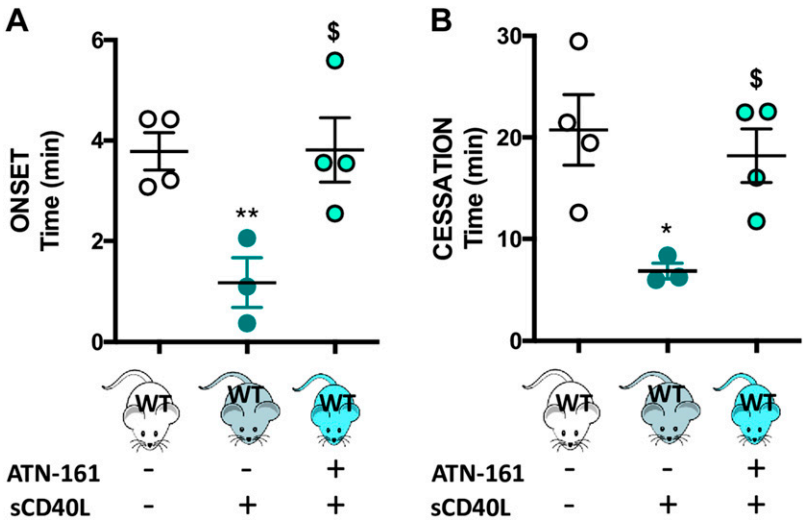

Figure 5. Effects of VLA5 antagonism and sCD40L administration on light/dye thrombus formation in arterioles of WT mice. WT mice were treated with VLA5 antagonist (ATN161; 5 mg/kg, i.p., 5 times over 14 d) and sCD40L (10 ng/mouse, i.v., 30 min before data collection). The light/dye-induced thrombosis model was performed. Time of onset (initial platelet deposition; *A*) and time of complete blood flow cessation (occlusion; *B*) were quantified. Data are presented as means ± SEM of 3–4 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* WT, $^{\$\$}P < 0.01$ *vs.* WT sCD40L.

Ang-II–induced inflammation and thrombosis *in vivo*, in particular, focusing on arteriolar thrombosis and venular leukocyte recruitment.

Platelets express substantial levels of CD40 and CD40L, and, upon activation, CD40L can be cleaved from the membrane by extracellular proteases, yielding a functional, extracellular peptide usually indicated as sCD40L (11, 15, 24). In addition, elevated plasma levels of CD40L have been reported in patients with cardiovascular risk factors, such as hypercholesterolemia, hypertension, and diabetes (25–29), and has been proposed as a biomarker for patients with acute coronary syndromes with increased risk of recurrent myocardial infarction and death (28, 29). Collectively, with *in vitro* evidence that underlies Ang-II prothrombotic actions (29, 25), we proposed that Ang-II may contribute to the activation of CD40/CD40L signaling that accompanies these conditions in *in vivo* thrombus formation within the microvasculature. It has been proposed that CD40/CD40L signaling may play a role in the induction of the prothrombotic/proinflammatory phenotype associated with Ang-II–dependent hypertension. Here, we determined whether and how the CD40/CD40L dyad contributes to thrombus development in the microvasculature of mice with Ang-II–dependent hypertension.

Ang-II has previously been demonstrated to promote leukocyte and platelet recruitment, enhance ROS production in postcapillary venules (7, 20), and accelerate light/dye-induced thrombus formation in arterioles (6–9, 11). The findings of the present study indicate that all of these microvascular responses to chronic Ang-II administration are largely prevented in mice that are genetically deficient in either CD40 or CD40L, which suggests that CD40/CD40L signaling is critical for the induction of both the proinflammatory and prothrombotic phenotype by Ang-II.

A role for CD40/CD40L signaling in inflammatory cell recruitment and thrombus formation has been demonstrated by using $CD40^{-/-}$ and/or $CD40L^{-/-}$ mice in a variety of other animal models of human diseases, including hypercholesterolemia (17), sepsis (15), and inflammatory bowel disease (15, 30). Moreover, aortic endothelial cells derived from $CD40L^{-/-}$ mice that were chronically exposed to Ang-II have been shown to exhibit blunted increases in the expression of $p47^{phox}$ (NADPH oxidase), P-selectin, and VCAM-1; reduced production of superoxide; and a decrease in T-lymphocyte contribution (7, 9, 16, 31, 32, 33). It has also been shown that $CD40L^{-/-}$ mice exhibited less tissue factor–triggered thrombin generation, an attenuated platelet reactivity, and a longer tail bleed time compared with WT controls (16). Our results, coupled with findings from previous reports, indicate that CD40/CD40L signaling contributes to Ang-II–mediated responses in both large vessels (aorta) and microvessels, and that the previously described protection against Ang-II–mediated changes in hemostatic and platelet function by CD40L deficiency is accompanied by, and presumably results in, the inhibition of Ang-II–dependent thrombosis and inflammatory cell recruitment *in vivo*.

Clinical reports describe blunted increases in plasma CD40L in patients who were treated with the Ang-II receptor antagonist, losartan (26, 27), and experimental models have revealed that Ang-II elicits an increased expression of CD40 and CD40L in different cell populations (25, 31). Furthermore, CD40L deficiency protects against endothelial dysfunction, platelet hyper-reactivity, and hypercoagulability that accompanies chronic Ang-II infusion (31). As such, we investigated these findings further to address whether sCD40L could contribute to the Ang-II–accelerated microvascular thrombosis response. This study provides 2 lines of evidence that support this possibility. First, we noted that the chronic infusion of sCD40L in WT mice recapitulated the enhanced light/dye-induced thrombus formation in arterioles that was similar to that observed in mice that were exposed to chronic Ang-II infusion. Second, we observed that the protection against Ang-II–induced thrombosis that was observed in $CD40L^{-/-}$ mice was reversed by acute administration of sCD40L. These observations are consistent with reports that describe a role for both sCD40L and membrane-bound CD40L in thrombus formation and stabilization (16). Our findings are also in agreement with previous reports that have shown a reversal of the protection against thrombus formation in $CD40L^{-/-}$ mice after the administration of exogenous sCD40L (14). The prothrombotic effect of sCD40L may reflect the known ability of this molecule to activate both endothelial cells and platelets (30) and to synergize with thrombin (31), actions which seem to result from the engagement of sCD40L with CD40.

An interesting outcome in the present study was that the administration of sCD40L was equally as effective in reversing the protection against Ang-II–induced thrombosis afforded by a deficiency in either CD40L or CD40. The effect of sCD40L administration in $CD40^{-/-}$ mice suggests that the prothrombotic action of sCD40L involves its interaction with a receptor other than CD40. Whereas many of the inflammatory and thrombotic actions of CD40L have been attributed to its direct interaction with CD40, there is evidence to document the existence of signaling mechanisms that involve the engagement of CD40L with other receptors, such as GPIIb/IIIa ($\alpha IIb\beta 3$) on platelets (16), Mac-1 (CD11b/CD18) on leukocytes (32), and receptor VLA5 ($\alpha v\beta 1$) on endothelial cells (33). Of interest, although VLA5 acts as a receptor for both fibronectin and sCD40L, they cannot bind to VLA5 simultaneously because of the fact that VLA5 is not constitutively active and, therefore, has to undergo conformational change for it to become an active integrin (34).

By using the VLA5 antagonist, ATN161, we provide evidence in this study that suggests a major role for VLA5 in mediating the prothrombotic effects of chronic Ang-II administration. Of equal interest is our observation that VLA5 blockade also largely prevents the effects of the recruitment of adherent leukocytes and increased ROS production elicited in postcapillary venules by Ang-II. Because we have previously reported that ROS production, mediated *via* NADPH oxidase, is required for the prothrombotic and proinflammatory effects of Ang-II (5–9, 11), it is possible that the involvement of both CD40 and VLA5 in the Ang-II–dependent thrombosis response may be explained by a shared ability to promote ROS production and inflammation in the microvasculature.

Support for the involvement of both receptors—CD40 and VLA5—in the observed microvascular responses to Ang-II is also provided by the observation that CD40L can bind the 2 receptors concomitantly and activate a shared signaling pathway (33). To investigate this further and assess the role of VLA5 in sCD40L-mediated thrombosis, we treated Ang-II–infused mice with the VLA5 antagonist and sCD40L. We found that sCD40L was not effective in inducing the prothrombotic phenotype in arterioles with the VLA5 antagonist, which suggests a role for VLA5 in mediating sCD40L prothrombotic effect in microcirculation. Whereas WT mice express both CD40 and VLA5 receptors, $CD40^{-/-}$ mice express only VLA5 receptors, which are available at elevated CD40L levels in serum. Our data suggest that Ang-II activates its receptors (AT1 and AT2) on blood cells and endothelial cells and mediates sCD40L elevation, which interacts with CD40 and VLA5. The molecular mechanisms that underpin Ang-II–mediated prothrombotic/proinflammatory responses are linked to sCD40L and, specifically, to VLA5.

Binding of sCD40L to the inactive form of VLA5 is independent of its binding to αIIbβ3 and its classic CD40 receptors. This CD40–VLA5 activated complex induces downstream signaling events, such as MAPK, p38, and ERK1/2, and has also been shown to synergize in the release of inflammatory mediators, such as matrix metalloproteinase-2 and -9, which suggests a crosstalk between these CD40L and VLA5 receptors (35). More recently, it has also been shown that the CD40L–VLA5 interaction promotes the survival of malignant T cells—as demonstrated by the activation of key survival signaling pathways, such as MAPKs (p38 and ERK1/2) and PI3K—and resistance to Fas-mediated apoptosis, an important hallmark of T-cell malignancies (36).

In summary, our results demonstrate that chronic Ang-II infusion leads to aberrant inflammatory and thrombotic responses in the microcirculation. Our findings shed light on the CD40L–CD40 dyad, implicating a major role for CD40 and sCD40L in the development of a prothrombotic and proinflammatory phenotype during chronic Ang-II infusion. Furthermore, this study demonstrates that the CD40L-dependent responses to Ang-II involve the interaction of CD40L with both CD40 and VLA5. Here, we found, for the first time to our knowledge, that VLA5 contributes to the thromboinflammatory phenotype assumed by the microvasculature during Ang-II–induced hypertension. Together, these novel data regarding the contribution of VLA5 to Ang-II–mediated thrombosis and inflammatory cell recruitment provide a potential therapeutic target strategy for the treatment of patients with hypertension. FJ

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AUTHOR CONTRIBUTIONS

E. Y. Senchenkova, J. Russell, S. A. Vital, and A. Yildirim collected experimental data; E. Y. Senchenkova, J. Russell, S. A. Vital, A. Yildirim, D. N. Granger, and F. N. E. Gavins analyzed the data; E. Y. Senchenkova, A. W. Orr, D. N. Granger, and F. N. E. Gavins wrote the paper; and D. N. Granger and F. N. E. Gavins supervised the research.

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