Role of T Lymphocytes in Angiotensin II–Mediated Microvascular Thrombosis

Elena Y. Senchenkova, Janice Russell, Elvira Kurmaeva, Dmitry Ostanin, D. Neil Granger

Abstract—Clinical trials and animal studies have revealed a role for the renin-angiotensin system in the enhanced thrombus development that is associated with hypertension. Because T lymphocytes have been implicated in the vascular dysfunction and blood pressure elevation associated with increased angiotensin II (Ang II) levels, we evaluated the role of the adaptive immune system in mediating the enhanced thrombosis during Ang II–induced hypertension. Light/dye-induced thrombosis was induced in cremaster arterioles of wild-type, immunodeficient Rag-1−/−, CD8−/−, or CD4−/− lymphocyte-deficient and NADPH oxidase (gp91phox)−deficient mice implanted with an Ang II–loaded pump for 2 weeks. Chronic Ang II infusion enhanced arteriolar thrombosis in wild-type mice but not in Rag-1−/−, CD4−/− T-cell–deficient, or gp91phox−/− mice. CD8+ T-cell−/− mice exhibited partial protection. Adoptive transfer of T cells derived from wild-type or gp91phox−/− mice into Rag-1−/− restored the prothrombotic phenotype induced by Ang II. T lymphocytes (CD4+ and, to a lesser extent, CD8+) play a major role in mediating the accelerated microvascular thrombosis associated with Ang II–induced hypertension. NADPH oxidase–derived reactive oxygen species, produced by cells other than T lymphocytes, also appear critical for the Ang II–enhanced, T cell–dependent thrombosis response. (Hypertension. 2011;58:959-965.) • Online Data Supplement

Key Words: thrombosis ■ angiotensin II ■ T lymphocytes ■ NADPH oxidase

T he risk factors for cardiovascular disease (CVD), including hypertension, have been shown to produce structural and functional alterations in large and microscopic blood vessels that ultimately lead to end-organ damage.1-3 Although a variety of mechanisms have been proposed to explain the vascular dysfunction induced by CVD risk factors, recent attention has focused on the pro-oxidative, proinflammatory, and prothrombogenic phenotype that is assumed by the vasculature in the presence of ≥1 risk factor. Characteristic features of the altered vascular phenotype induced by CVD risk factors include an accumulation of leukocytes and platelets on the vessel wall (with subsequent transendothelial migration of leukocytes), increased production of reactive oxygen species by vascular endothelium and circulating blood cells, impaired vasomotor and endothelial barrier functions, and enhanced thrombus formation.1 Because the aforementioned responses are also elicited in different acute and chronic inflammatory diseases, it has been suggested that CVD risk factors exert their deleterious effects on the vasculature through activation of the innate and/or adaptive immune systems, which have also been linked to oxidative stress and hypercoagulation/thrombosis.1-4

Angiotensin II (Ang II) has been implicated as a potential initiator of the inflammatory phenotype and vascular dysfunction that are associated with different CVD risk factors.1-4-6 Animal studies have revealed that acute or chronic administration of Ang II induces reactive oxygen species production, impairs vasomotor function,5,7,8 promotes the adhesion of leukocytes and platelets to endothelial cells lining the microvasculature,9 and enhances thrombus formation.10,11 The oxidative stress elicited by Ang II has been attributed to activation of NAD(P)H oxidase, which is expressed in a variety of cells, either circulating in blood or composed of the vessel wall.8,12 A role for Ang II in mediating the vasomotor dysfunction and increased incidence of thrombotic events in human hypertension is supported by clinical studies showing a reversal of these responses in patients treated with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers.13 Recent animal studies have revealed that the phenotypic changes in the vasculature caused by Ang II are linked to activation of the adaptive immune system.5,14 The elevated blood pressure, vasomotor dysfunction, and oxidative stress elicited by Ang II in wild-type (WT) mice are not observed in T-lymphocyte–deficient Rag-1−/− mice, whereas adoptive transfer of WT T cells into Rag-1−/− restores the Ang II phenotype. The T-cell–dependent vascular alterations induced by Ang II also appear to be linked to NADPH oxidase activity in T cells inasmuch as adoptive transfer of T cells from p47phox−/− (a protein subunit of NADPH oxidase)–deficient mice into Rag-1−/− only partially restores the Ang II phenotype.5,14
Clinical and experimental evidence implicates Ang II in the genesis of thrombosis in both large and microscopic blood vessels. Ang II has been shown to activate the coagulation pathway, inhibit fibrinolysis, promote platelet aggregation, and enhance the rate of thrombus development in the microvasculature. Although different cell (platelets and endothelial cells) and receptor populations (eg, Ang II type 2 and 4 receptors) have been implicated in Ang II–enhanced thrombosis, it remains unclear whether the adaptive immune system also contributes to this response. In light of growing evidence that links adaptive immunity to hemostasis and the known association between immune cells and thrombosis, it remains unclear whether the adaptive immune system also contributes to this response. Hence, the overall objective of this study was to test the hypothesis that T-lymphocyte–associated NADPH oxidase contributes to the accelerated microvascular thrombosis associated with Ang II–induced hypertension. Our findings implicate both activated effector/memory CD4+ T lymphocytes (CD44high/CD62Llow) and NADPH oxidase (in cells other than T cells) in the accelerated microvascular thrombosis associated with chronically elevated Ang II levels.

Materials and Methods

Animals
C57BL/6 (WT; n = 47), CD8+−/− (B6.129S2-Cd8atm1Mak/J; n = 6), CD4+−/− (B6.129S2-Cd4tm1Mak/J; n = 6), gp91phox−/− (B6.129S6-Cybbtm1Din/J; n = 15), and Rag-1−/− (B6.129S7-Rag1tm1Mom/J; n = 29), interferon (IFN)-γ−/− (B6.129S7-Ifngtm1Tr/J; n = 5), and tumor necrosis factor (TNF)-α−/− (B6.129-Tnfrsf1atm1Med/J; n = 5) mice were obtained from Jackson Laboratories (Bar Harbor, ME) at age 6 to 8 weeks. All of the animal experiments were performed according to the criteria outlined by the National Institutes of Health and were approved by the Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee.

Ang II Infusion
Saline or Ang II (1 μg/kg per minute)–loaded micro-osmotic pumps (ALZET, Cupertino, CA, model 1002) were implanted ≤14 days subcutaneously (intracerebral region) under isoflurane anesthesia using sterile procedures, as described previously. Systolic blood pressure (SBP) values were obtained using a computerized tail-cuff method.

Deoxycorticosterone Acetate Salt–Induced Hypertension
To determine whether enhanced microvascular thrombosis is also evident in a low renin model of hypertension, the deoxycorticosterone acetate (DOCA) salt model was used in WT mice (n = 16). The mice, anesthetized with ketamine (150.0 mg/kg, IP) and xylazine (7.5 mg/kg, IP), underwent a uninephrectomy and SC implantation of a 50-mg (21-day release) DOCA pellet (Innovative Research of America, Sarasota, FL) in the midscapular region. After surgery, the mice received the analgesic carprofen (5 mg/kg, SC) and were then placed on 1.0% saline/0.2% KCl drinking solution for 3 weeks. Sham controls were produced by removing a kidney but without implantation of a DOCA pellet. The sham group was placed on regular drinking water plus 0.2% potassium chloride. SBP was monitored using the tail-cuff method.

T-Cell Transfer
T lymphocytes were isolated from the spleen of WT (C57BL/6) or gp91phox−/− donor mice using a Dynal Mouse T Cell Negative Isolation kit (Invitrogen Dynal AS, Oslo, Norway) and injected (~107 cells in 0.2 mL of PBS, IV) into Rag-1−/− recipients before Ang II pump implantation.

Cremaster Muscle Procedure
On day 15 after Ang II– or saline-loaded pump implantation or day 21 after uninephrectomy (both sham and DOCA salt), the mice were anesthetized with pentobarbital (50 mg/kg, IP). The right carotid artery was cannulated for measurement of arterial blood pressure, and the right jugular vein was cannulated for drug administration. The cremaster muscle was prepared for intravital microscopic observation as described previously, and arterioles with diameters between 30 to 36 μm and a wall shear rate >500 seconds−1 were selected for study.

Light/Dye-Induced Thrombosis
Thrombus formation was evaluated in cremaster arterioles as described previously. After light/dye injury (induced by activation of intravascular fluorescein isothiocyanate–dextran), the arteriole under study was continuously epi-illuminated and thrombus formation was quantified by determining the time of onset of platelet deposition/aggregation within the microvessels (onset time) and the time required for complete flow cessation for ≥30 seconds (cessation time). Epi-illumination was discontinued once blood flow ceased in the vessel under study. Typically, 1 to 3 thrombi were induced in each mouse, and the results of each vessel were averaged.

Flow cytometric analysis of activated T-cell phenotype in peripheral blood and spleen was performed as described previously. For a more detailed method, see the online Data Supplement (available at http://hyper.ahajournals.org).

Experimental Groups
Light/dye-induced thrombus development was evaluated in arterioles of each of the following experimental groups: (1) WT mice + saline pump (n = 7); (2) WT mice + Ang II pump (n = 9); (3) WT mice + uninephrectomy (sham controls; n = 5); (4) WT+uninephrectomy + DOCA salt (n = 6); (5) lymphocyte-deficient Rag-1−/− mice + saline (n = 7) or Ang II pump (n = 6); (6) Rag-1−/− mice + Ang II pump reconstituted with T cells from WT donors (n = 6); (7) Rag-1−/− mice + Ang II pump reconstituted with T cells from gp91phox−/− donors (n = 4); (8) gp91phox−/− mice + saline (n = 8) or Ang II pump (n = 6); (9) CD8+ T-cell−/− mice + Ang II pump (n = 6); (10) CD4+ T-cell−/− mice + Ang II pump (n = 6); (11) IFN-γ−/− + Ang II pump (n = 5); and (12) TNF-α−/− + Ang II pump (n = 5).

Data Analysis
All of the data are presented as mean ± SEM. Group comparisons were made using a 1-way ANOVA followed by the Newman-Keuls post hoc test. Statistical significance was set at P < 0.05.

Results

Ang II Enhances Light/Dye-Induced Thrombosis in Cremaster Muscle Arterioles
Figure 1 summarizes the thrombosis responses to light/dye injury in 2 distinct murine models of hypertension, that is, Ang II- and DOCA salt–induced hypertension. After 2 weeks of Ang II infusion, SBP was increased to 153.8 ± 8.6 mm Hg in WT mice compared with 107 ± 2.2 mm Hg in WT mice with a saline pump. Enhanced thrombus development during chronic Ang II infusion is evidenced by the reductions of the.
onset time (thrombus initiation) and time for flow cessation (propagation/stabilization). However, mice with DOCA salt-induced hypertension (SBP: 142.7±7.3 mm Hg) did not exhibit an altered thrombosis response to light/dye injury when compared with sham (uninephrectomy alone) controls.

**Lymphocyte Deficiency Protects Against Ang II–Enhanced Microvascular Thrombosis**

Figure 2 compares the effects of chronic Ang II infusion on light/dye-induced thrombus development in WT and lymphocyte-deficient Rag-1−/− (SBP: 158.7±8.6 mm Hg) mice. Unlike in WT mice, Ang II did not accelerate the rate of thrombus formation in Rag-1−/− mice. However, after adoptive transfer of WT T lymphocytes for a period of 2 weeks in Rag-1−/− (SBP: 173±4.8 mm Hg) mice, the prothrombotic phenotype induced by Ang II infusion was fully restored, which suggests that T cells play a major role in Ang II–mediated thrombosis.

**CD4+ T Cells, and to a Lesser Extent CD8+ T Cells, Mediate the T-Lymphocyte–Dependent Enhancement of Thrombosis Associated With Ang II–Induced Hypertension**

To determine which T-lymphocyte subpopulation accounts for the T-cell–dependent thrombotic response to Ang II, we compared thrombus development in mice that are genetically deficient in either CD4+ or CD8+ T cells with their WT counterparts (Figure 3). The CD4+ T-cell–deficient mice (SBP: 170.7±4.8 mm Hg) responded to chronic Ang II infusion in a manner similar to that noted in Rag-1−/− mice, that is, the acceleration of time of onset and time to flow cessation induced by Ang II was completely prevented. Although CD8+ T-cell–deficient mice (SBP: 161.4±11.3 mm Hg) were also protected against Ang II thrombosis, this was only reflected in the restored time of onset, with no improvement in the time to flow cessation, suggesting that CD8+ T-cell−/− mice assume a prothrombogenic phenotype during chronic Ang II infusion.

Phenotypic analysis of T cells in peripheral blood and spleen revealed an ≈15% reduction (42.00±2.00% versus 49.35±0.85%) in CD4+ T cells within T-cell receptor alpha, beta subset in spleens of Ang II–treated mice compared with controls (data not shown). A 35.5% increase (17.2±0.9% versus 12.7±1.2%) in the percentage of activated/effector memory CD4+ lymphocytes was detected in the spleen, whereas 68% (10.4±1.6% versus 6.2±0.5%) more activated/effector/memory CD4+ cells (CD62LnegCD44high) were measured in blood of Ang II–treated mice compared with the WT control group (Figure S1, available in the online Data Supplement). No significant changes were noted in the activation state of CD8+ T cells in both spleen and blood between control and Ang II–treated mice (Figure S2).

Intracellular production of IFN-γ, interleukin (IL) 4, and IL-17 cytokines by splenic CD4+ T cells isolated from control and Ang II–treated mice was not different (Figure S3). This is consistent with the observation that Ang II–enhanced, light/dye-induced thrombosis did not differ between IFN-γ−deficient and WT mice (Figure S4). However, partial protection against Ang II–enhanced thrombosis (as reflected by an improved time of onset of thrombosis) was detected in mice deficient in TNFα receptors (Figure S4).

**NADPH Oxidase, Associated With Cells Other Than T Lymphocytes, Contributes to Ang II–Accelerated Thrombus Development**

The role of NADPH in Ang II–enhanced microvascular thrombosis was evaluated using mice deficient in the critical NADPH oxidase subunit, gp91phox−/− (SBP: 141.4±6.6 mm Hg; Figure 4A) These mutant mice exhibited complete protection against the prothrombotic effects of Ang II infusion, as evidenced by the

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**Figure 1.** Light/dye-induced thrombus formation in cremaster muscle arteries of wild-type (WT) mice with either (A) angiotensin II (Ang II)–or (B) deoxycorticosterone acetate (DOCA) salt–induced hypertension. Control groups for the Ang II model include WT mice and WT mice implanted with a saline-loaded pump (WT-saline). The DOCA salt model included a control group exposed to uninephrectomy alone (WT-sham). *P<0.01 vs the WT-saline group.

**Figure 2.** Role of T lymphocytes in angiotensin II (Ang II)–enhanced, light/dye-induced thrombus formation. Wild-type (WT)-saline indicates WT mice implanted with saline-loaded pumps; WT-Ang II, WT mice implanted with Ang II–loaded pumps; Rag-1−/−-saline, immunodeficient Rag-1−/− mice implanted with saline-loaded pump; Rag-1−/−-Ang II−/− mice reconstituted with T cells obtained from WT donor mice. *P<0.01 vs WT-saline; &P<0.01 vs Rag-1−/−-Ang II.
normalized values for time of onset and time to flow cessation. To determine whether NADPH oxidase in T lymphocytes accounts for the protection observed in gp91phox−/− mice, adoptive transfer experiments were performed wherein T cells derived from either WT or gp91phox−/− mice were administered to Rag-1−/− mice with Ang II pumps (SBP: 163.3±2.4 mm Hg; Figure 4B). These experiments revealed that Rag-1−/− mice reconstituted with T cells derived from either WT or gp91phox−/− mice exhibited a fully restored thrombosis response to Ang II, suggesting that NADPH oxidase in T cells is not critical for Ang II–induced thrombosis.

**Discussion**

The renin-angiotensin system has been implicated in different characteristic responses of the vasculature to chronic arterial hypertension, including vasomotor dysfunction, inflammation, oxidative stress, and thrombogenesis. Although there is a large and growing body of evidence that suggests a causal link between the elevated blood pressure and vasomotor dysfunction induced by Ang II with inflammation and oxidative stress, the contributions of the latter responses to the enhanced thrombus formation associated with Ang II–dependent hypertension remains unclear. In the present study, we provide evidence for the involvement of the adaptive immune system, as well as NADPH oxidase–dependent oxidative stress, in the accelerated thrombus development that occurs in response to chronically elevated Ang II levels.

We have reported previously that chronic Ang II infusion in mice results in accelerated thrombus formation in the microvasculature after light/dye injury. The Ang II–mediated thrombogenic response, which is more evident in arterioles than venules, involves the activation of several receptor populations, including angiotensin (type 2 and 4), endothelin 1 (ET-1A), and bradykinin (BK-1) receptors. The elevated blood pressure that accompanies Ang II infusion was considered an unlikely cause of the accelerated thrombosis, because Ang II type 1 receptor blockade (or genetic deficiency) ablates the Ang II–induced hypertension without altering thrombus development. The present study provides additional evidence that hypertension, per se, is not a major determinant of the accelerated thrombosis observed during chronic Ang II infusion. A comparison of the light/dye-induced thrombosis responses between mice with DOCA salt hypertension (a low renin model) and Ang II–induced hypertension demonstrated that the accelerated thrombus response was unique to the Ang II model, despite comparable elevations in blood pressure in the 2 models of hypertension. The results of a previous study in a rat model of hypertension tend to support our findings. Capers et al noted that Ang II infusion–induced hypertension was associated with a 10-fold increase in aortic

**Figure 3.** Role of CD4+ and CD8+ T lymphocytes in angiotensin II (Ang II)–enhanced, light/dye-induced thrombus formation. Wild-type (WT)-saline indicates WT mice implanted with saline-loaded pumps; WT-Ang II, WT mice implanted with Ang II-loaded pumps; CD8+ T-cell−/−-Ang II, CD8+ T-cell–deficient mice implanted with Ang II pumps; CD4+ T-cell−/−-Ang II, CD4+ lymphocyte–deficient mice implanted with Ang II–loaded pumps. *P<0.01 vs WT-saline; &P<0.01 vs WT-Ang II.

**Figure 4.** Contribution of NADPH oxidase to angiotensin II (Ang II)–enhanced, light/dye-induced thrombus formation. A, Wild-type (WT)-saline indicates WT mice implanted with saline-loaded pumps; WT-Ang II, WT mice implanted with Ang II–loaded pumps; gp91phox−/−-saline, gp91phox−/−-deficient mice implanted with saline pump; gp91phox−/−-Ang II, gp91phox−/− mice implanted with Ang II pump (B); Rag-1−/−-Ang II, Rag-1−/− mice implanted with Ang II–loaded pumps; Rag-1−/−-WT T cells, Rag-1−/− mice with WT T cells obtained from WT donor mice; Rag-1−/−-gp91phox−/− T cells, Rag-1−/− mice with WT T cells obtained from gp91phox−/− mice. A, *P<0.01 vs WT-saline; &P<0.05 vs WT-Ang II; B, **P<0.01 vs WT-Ang II; &P<0.01 vs WT-Ang II.
expression of thrombin receptor mRNA, whereas no changes in mRNA levels were detected in Dahl salt-sensitive (low renin) hypertensive rats. Furthermore, they demonstrated that treatment of the Ang II–infused rats with superoxide dismutase prevented the upregulation of thrombin receptor mRNA, implicating oxidative stress in this response. The latter observation is interesting in view of documented evidence that both the Ang II and low renin models of hypertension are associated with oxidative stress.5,14

Recent reports have attributed a major role to T lymphocytes in mediating the elevated blood pressure and impaired endothelium-dependent vasodilation that are associated with chronic Ang II infusion.5,22,23 These reports indicate that, unlike their WT counterparts, immunodeficient Rag-1−/− mice do not exhibit hypertension and vasomotor dysfunction in response to elevated Ang II levels, suggesting an immunologic basis for the altered vascular tone and reactivity induced by chronic Ang II exposure. The results of the present study suggest that the adaptive immune system also contributes to the enhanced thrombogenesis induced by Ang II, with CD4+ T cells and, to a lesser extent, CD8+ T cells mediating this response. Our conclusions regarding the involvement of T lymphocytes in Ang II–enhanced thrombogenesis is based on 4 lines of evidence: (1) absence of an accelerated thrombosis response to Ang II in immunodeficient Rag-1−/− mice; (2) restoration of the Ang II–mediated thrombosis in Rag-1−/− mice after adoptive transfer of T cells derived from WT mice; (3) absence of Ang II–enhanced thrombosis in mice that are genetically deficient in CD4+ T cells, with a partial reduction noted in CD8+ T-cell–deficient mice; and (4) the activation status of CD4+ T cells in Ang II–treated mice. The larger number of activated CD4+ T cells in Ang II–treated mice is consistent with previous reports.5 The accumulation of activated/effector/memory CD4+ T cells in the spleen is consistent with CD4+ T-cell recruitment in peripheral organs5,24–26 of Ang II–treated mice compared with the WT controls. The interesting dual contribution of CD4+ and CD8+ T lymphocytes to the initiation phase (onset) of thrombus development is consistent with cross-talk between the 2 T-cell populations.27,28

The mechanism(s) that underlie the ability of the T cell to accelerate microvascular thrombosis in the presence of Ang II remain unclear. T cells, nevertheless, are known to directly interact and communicate with platelets,29–31 release cytokines that can activate coagulation cascade (eg, induce tissue factor), and promote thrombus formation.29,32,33 Chronic Ang II infusion is associated with increased plasma levels of IL-634,35 and an increased production of TNF-α by T lymphocytes.5 Furthermore, T-cell–derived cytokines, such as TNF-α and IL-1β, have been shown to accelerate light/dye-induced thrombus formation in arterioles.36,37 Our negative findings in Ang II–treated IFN-γ–deficient mice suggest that this cytokine is an unlikely mediator of this T-cell–dependent thrombosis response. However, we did detect partial protection against Ang II–induced thrombosis in TNF-α receptor–deficient mice, which implicates at least a small role for this cytokine. IL-6, which has been implicated in the pathogenesis of sepsis, also appears to be a potent stimulant of thrombo-

Oxidative stress and the enhanced production of superoxide, which accompanies chronic Ang II administration, are another potent procoagulant/prothrombotic stimulant.8,14 Reactive oxygen species, including superoxide and secondarily derived oxidants, are known to augment platelet aggregation responses, activate the coagulation cascade, inhibit anticoagulant mechanisms and fibrinolysis, and promote thrombus formation.28,30 The principal molecular target of Ang II that explains its pro-oxidative effect is NAD(P)H oxidase.8,12 Chronic Ang II administration results in an increased expression of Nox1, gp91phox, p47phox, and p22phox subunits of NADPH oxidase and leads to increased enzyme activity.5,40 The Ang II–mediated enzyme activation is evident in endothelial cells, vascular smooth muscle, and different blood cell populations, including T lymphocytes,12,41,42 Although vessel wall-associated NADPH oxidase has frequently been linked to the vasomotor and inflammatory effects of Ang II, recent evidence supports a role for T-lymphocyte–associated NAD(P)H oxidase in mediating these responses to Ang II.5

A novel finding of the present study is that NADPH oxidase also contributes to the prothrombotic actions of Ang II, as evidenced by our observation that gp91phox−/− mice do not exhibit the accelerated thrombosis response to Ang II that is observed in WT mice. We also assessed the possibility that NADPH oxidase associated with T cells is responsible for the Ang II–mediated thrombosis. Our finding that Rag-1−/− mice reconstituted with T cells derived from either WT or gp91phox−/− mice exhibited a fully restored thrombosis response to Ang II suggests that NADPH oxidase in T cells is not critical for Ang II–induced thrombosis. Although the cellular source of NADPH oxidase that mediates Ang II–enhanced thrombosis response remains unclear, vascular endothelium and a blood cell population other than T cells (eg, monocytes, platelets) are likely candidates. Irrespective of the cellular source of NADPH oxidase that contributes to Ang II–enhanced thrombogenesis, our observation that T-lymphocyte deficiency (Rag-1−/− and CD4+ T-cell−/− mice) and NADPH oxidase deficiency (gp91phox−/− mice) are equally effective in preventing the Ang II–mediated acceleration of thrombosis suggests that T cells and NADPH oxidase are series coupled effectors of the thrombogenic response. One possibility is that Ang II activates vascular wall NADPH oxidase, which, in turn, leads to activation of T cells, perhaps through the generation of neoantigens secondary to oxidative protein modification.43,44 Alternatively, Ang II may activate T cells, which, in turn, release mediators, such as cytokines, that increase the expression and activity of NADPH oxidase in endothelium or other blood cell populations. The latter possibility appears more likely because it has been reported that Rag-1−/− mice do not exhibit the increased vascular superoxide production observed in WT mice during chronic Ang II infusion.5 Further support is provided by reports describing the ability of T-cell–derived cytokines, such as IL-6 and TNF-α, to increase the expression of NADPH oxidase in vascular endothelium.45,46
Perspectives

Ang II exerts a prothrombogenic effect in both large arteries and arterioles. Immune cell activation and oxidative stress have been implicated in the altered vascular reactivity and accelerated atherothrombosis that accompanies Ang II–induced hypertension. The findings of this study are consistent with a role for activation of the adaptive immune system in the accelerated microvascular thrombosis that results from chronically elevated levels of Ang II. CD4+ T lymphocytes appear to make a major contribution in this regard. Reactive oxygen species generated from NADPH oxidase is also linked to the prothrombogenic action of Ang II in arterioles. These observations offer novel therapeutic targets for the prevention of thrombosis in patients with hypertension and/or other risk factors for CVD.

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Disclosures

None.

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On-line supplement

Materials and Method.

Flow Cytometry.

For flow cytometric studies, T-cells were isolated from spleen of control (n=5), AngII-treated (n=5) or Rag-1-/- mice treated with AngII and reconstituted with 10⁷ wild type T lymphocytes (one WT donor to one Rag-1-/- recipient mouse; n=5). Spleens harvested from donor mice were placed into FACS buffer (1X PBS supplemented with 4% fetal bovine serum) on ice. A single sell suspension was obtained by grinding spleen between the frosted sides of two frosted-microscope slides (Erie Scientific Company, Portsmouth, N.H. 03801). The cell suspension was filtered through a 70 µm cell strainer (BD Biosciences, CA), spun for 10 min @ 400g at 4-8 °C, and counted. T cells were isolated using a Dynal mouse T cell negative isolation kit (Invitrogen, Oslo, Norway). For lymphocyte phenotyping, approximately 1x10⁶ splenocytes were plated into a 96-well round bottom plate, with staining and flow cytometric analysis as previously described. For intracellular staining, T cells (5x10⁵ cells/well in 24-well plate in 1 ml complete RPMI-10 medium) were activated in vitro with plate-bound anti-CD3 and soluble anti-CD28 (1ug/ml) (BD Biosciences) antibodies for 2 days (42 hours). On the morning of the 2nd day, cells were harvested, washed and re-stimulated in uncoated 24-well plates in the presence of 40ng/mL phorbol 12-myristate 13-acetate (PMA), 2uM ionomycin and GolgiStop (BD Biosciences, San Jose, CA) for an additional 5-6 hrs. Cells were harvested, washed in PBS, and processed for surface staining, followed by intracellular staining using antibodies and reagents from eBioscience according to the manufacturer’s instructions (eBioscience, San Diego, CA). Samples were collected on a BD LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (ver. 7.2.5 for PC, Tree Star, Ashland, OR). Cells from whole blood were prepared according to the following method. Approximately 0.9 ml of blood was drawn from the carotid artery into a syringe with 0.1 ml of ammonium chloride/trizma base buffer (ACT buffer). After lysis of red blood cells (RBCs) in hypotonic buffer, cells were washed twice, resuspended, and plated into 96-well round bottom plates for staining and analysis using flow cytometry in a BD LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (ver. 7.2.5 for PC, Tree Star, Ashland, OR).

For lymphocyte phenotyping and activation analysis, the following antibodies were used: Anti-Mouse gamma delta TCR PE-Cy5 (Clone eBioGL3; isotype Armenian Hamster IgG; eBioscience), PE-Cy™7 Hamster Anti-Mouse TCR β chain (Clone H57-597, Isotype Armenian Hamster IgG2, λ1; BD Pharmingen™), FITC Rat anti-Mouse CD25 (Clone 3C7, Isotype Rat
(LEW) IgG2b, κ; BD Pharmingen™, Anti-Mouse CD8a Biotin (Clone 53-6.7, Isotype Rat IgG2a, κ; eBioscience), PE-Texas Red Streptavidin (BD Pharmingen), Pacific Blue™ Rat Anti-Mouse CD4 (Clone RM4-5, Isotype Rat IgG2a, κ; BD Pharmingen), Phosphoerythrin (PE) anti-Human/Mouse CD44 (Clone IM7, Isotype Rat IgG2b, κ; eBioscience), Allophycocyanin (APC) anti-mouse CD62L (Clone MEL-14, Isotype Rat IgG2a, κ; BD Pharmingen)

For intracellular staining the following antibodies from eBioscience were used: Flourescein isothiocyanate (FITC) anti-mouse interferon-gamma (IFN-γ) (Clone XMG1.2, Isotype Rat IgG1, κ; eBioscience), allophycocyanin (APC) anti-mouse interleukin-4 (IL-4) (Clone 11B11, Isotype Rat IgG1, κ; eBioscience), phycocerythrin (PE) anti-mouse interleukin-17A (IL-17A) (Clone eBio17B7, Isotype Rat IgG2a, κ; eBioscience), TCR PE-Cy5 (Clone eBioGL3; isotype Armenian Hamster IgG; eBioscience), PE-Cy™7 Hamster Anti-Mouse TCR β chain (Clone H57-597, Isotype Armenian Hamster IgG2, λ1; BD Pharmingen™), Anti-Mouse CD8a Biotin (Clone 53-6.7, Isotype Rat IgG2a, κ; eBioscience), PE-Texas Red Streptavidin (BD Pharmingen), Pacific Blue™ Rat Anti-Mouse CD4 (Clone RM4-5, Isotype Rat IgG2a, κ; BD Pharmingen), and LIVE/DEAD Fixable Blue (Invitrogen).

Results

**AngII induces activation of peripheral CD4+ lymphocytes and CD4+ cells in spleen (CD44^highCD62L^neg).** Figures S1 shows the phenotype of activated CD4^+ lymphocytes in WT mice following AngII infusion. These changes were not detected with CD8^+ lymphocytes (Figure S2). In Rag-1^-/- mice reconstituted with WT T cells, AngII-infusion resulted in higher levels of activated T cells for both CD4^+ and CD8^+ lymphocytes in spleen (12.7 ±1.2 vs 54.5 ±1.5 per cent for CD4^+ cells & 3.4±0.2 vs 34.0±1.4% for CD8^+ cells) and peripheral blood (6.2±0.5 vs 44.3±2.0 per cent for CD4^+ cells & 1.9±0.2 vs 33.5 ± 2.2% for CD8^+ cells).

Figure S1. Flow cytometric analysis of CD4+ lymphocytes in spleen and whole blood of control and AngII-treated mice. Representative graphs were initially gated on viable TCRαβ+CD4+ cells. Bars indicate percentages of CD4+ cells in spleen and whole blood with an activated/effector/memory phenotype (CD44^{high}CD62L^{neg}). * indicates p<0.05 in control vs. AngII-infused mice.
Figure S2. Flow cytometric analysis of CD8⁺ lymphocytes in spleen and whole blood of control and AngII-treated mice. Representative graphs were initially gated on viable TCRαβ⁺CD8α⁺ cells. Bars indicate percentages of CD8⁺ cells in spleen and whole blood with an activated/effector/memory phenotype (CD44⁺CD62L⁻) in control and AngII- infused mice.
Figure S3. Quantitative detection of intracellular IFNγ-positive CD4\(^+\) and CD8\(^+\) T-cells in control and AngII-infused mice after 2 weeks of AngII infusion. Intracellular staining was performed on T lymphocyte isolated from spleen following their activation in vitro with plate-bound antibodies to CD3 and soluble CD28 as described in Materials and Methods. No differences between the groups were detected. Levels of IL-17- and IL-4-positive T-cells (within both CD4\(^+\) and CD8\(^+\) subsets) were below 1.0% (not shown).

Figure S4. Role of IFNγ and TNFαr in angiotensin II-enhanced, light/dye-induced thrombus formation. A. Control– wild type mice implanted with saline-loaded pumps; WT-AngII – WT mice implanted with AngII-loaded pumps; IFNγ\(^{-/-}\)-AngII – IFNγ-deficient mice implanted with AngII pumps; B. TNFαr\(^{-/-}\)-AngII - TNFαr deficient mice implanted with AngII-loaded pumps. * indicates p<0.05 and **indicates p<0.01 vs WT-AngII.