Novel Role of T Cells and IL-6 (Interleukin-6) in Angiotensin II–Induced Microvascular Dysfunction

Elena Y. Senchenkova, Janice Russell, Alper Yildirim, D. Neil Granger, Felicity N.E. Gavins

Abstract—Hypertension is an established risk factor for subsequent cardiovascular diseases, with Ang II (angiotensin II) playing a major role in mediating thrombotic and inflammatory abnormalities. Although T cells and IL-6 (interleukin-6) play an important role in adaptive immune responses, little is known about their role(s) in the thromboinflammatory responses associated with Ang II. Here we show using intravital microscopy coupled with the light/dye injury model that Rag-1 deficient (Rag-1$^{-/-}$) and IL-6 deficient (IL-6$^{-/-}$) mice are afforded protection against Ang II–induced thrombosis. Blocking IL-6 receptors (using CD126 and gp130 antibodies) significantly diminished Ang II–mediated thrombosis and inflammatory cell recruitment in mice. Furthermore, the adoptive transfer of IL-6$^{-/-}$-derived T cells into Rag-1$^{-/-}$ mice failed to accelerate Ang II–induced thrombosis compared with Rag-1$^{-/-}$ mice reconstituted with wild-type–derived T cells, suggesting T cell IL-6 mediates the thrombotic abnormalities associated Ang II hypertension. Interestingly, adoptive transfer of WT T cells into Rag-1$^{-/-}$/Ang II mice resulted in increased numbers of immature platelets, which constitutes a more active platelet population, that is, prothrombotic and proinflammatory. To translate our in vivo findings, we used clinical samples to demonstrate that IL-6 also predisposes platelets to an interaction with collagen receptors, thereby increasing the propensity for platelets to aggregate and cause thrombosis. In summary, we provide compelling evidence for the involvement of IL-6, IL-6R, and T-cell–dependent IL-6 signaling in Ang II–induced thromboinflammation, which may provide new therapeutic possibilities for drug discovery programs for the management of hypertension. **(*Hypertension*. 2019;73:829-838. DOI: 10.1161/HYPERTENSIONAHA.118.12286.)** • **Online Data Supplement**

Key Words: angiotensin II ■ hypertension ■ interleukin-6 ■ inflammation ■ thrombosis

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Hypertension is both a cardiovascular and cerebrovascular risk factor, predisposing hypertensive patients to both proinflammatory and prothrombotic vascular dysfunction.[1–3] Furthermore, it is known that the pleiotropic molecule Ang II (angiotensin II), the main effector of the renin-angiotensin system, plays a role in mediating elevated blood pressure and promoting vascular and endothelial cell dysfunction, hypertrophy, and oxidative stress.[1,4–10] Abnormalities in the functions of circulating immune cells which accompany the chronically elevated blood pressure associated with Ang II[5,7–9] and other models of hypertension (eg, high-salt diet model[9,10] and the spontaneous hypertension model[11,12]) has been well-documented. However, it is not the elevated blood pressure per se that is the driving factor for thrombosis[9,13] but rather Ang II itself.

T cells play an important role in adaptive immune responses, and we and others have shown a major role for these cells in Ang II–induced hypertension.[5,9,14,15] Additionally, immunodeficient mice lacking both T- and B-lymphocytes (Rag-1 knockout [Rag-1$^{-/-}$]) exhibit improvement in vasomotor dysfunction and leukocyte accumulation,[9,15–17] and adoptive transfer of T cells, but not B cells, into Rag-1$^{-/-}$ mice restored the prothrombotic phenotype induced by Ang II. These data confirm the major role that T cells play in mediating Ang II–induced accelerated microvascular thrombosis,[9] however, the mechanism by which T cells induces this prothrombotic environment remains unknown.

Both Ang II–induced hypertension and T cells have a strong relationship with the cytokine IL-6, which exerts its biological activities through 2 molecules: IL-6R (also known as IL-6Rα, gp80, or CD126) and gp130 (also referred to as IL-6Rβ or CD130).[18–21] In the context of Ang II hypertension, reports have shown IL-6 to contribute to increased blood pressure, inflammatory cell recruitment, endothelial dysfunction,[18,22–25] with a deficiency in either IL-6 or T cells providing protection.[26,27] However, not only is the role that IL-6 plays in Ang II–mediated thrombosis unknown, the actual role that T-cell–derived IL-6 signaling plays in the thromboinflammatory responses associated with Ang II remains poorly defined. Thus, we herein tested the hypothesis that the chronic prothrombotic phenotype associated with Ang II–induced hypertension is mediated by a mechanism that is dependent on both T cells and IL-6.

Our novel data reveal that IL-6 plays a major role in the T-cell–dependent thromboinflammatory responses elicited by chronic Ang II administration, with contributions by both

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From the Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center-Shreveport, Shreveport (E.Y.S., J.R., A.Y., D.N.G., F.N.E.G.); and Department of Physiology, Marmara University School of Medicine, Istanbul, Turkey (A.Y.).

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Correspondence to Felicity N.E. Gavins, Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center-Shreveport, 1501 Kings Hwy, Shreveport, LA 71130. Email fgavin@lsuhsc.edu

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IL-6Rα and gp130 receptors. We also found that IL-6 is able to potentiate platelets, predisposing them to stimulation/activation of the collagen receptor GPVI and contributing to platelet aggregation (by increasing GPIIbIIIa expression). In summary, T-cell–dependent IL-6 signaling mediates microvascular thrombotic and inflammatory responses associated with chronically elevated levels of Ang II. Our compelling data suggest that new therapeutic strategies for drug discovery programs based on T-cell–dependent IL-6 signaling pathways may provide a previously unknown therapeutic strategy for the management of the thromboinflammatory complications that accompany hypertension.

Materials and Methods

All data supporting the findings of this study are available within the article and its in the online-only Data Supplement or are available from the corresponding author on reasonable request. All studies were done blinded and performed male wild-type (WT, C57BL/6), IL-6 deficient (IL-6$^{-/-}$ B6.129S6-IL6$^{tml\ Kopf}$), and Rag-1 deficient (Rag-1$^{-/-}$; B6.129S7-Rag1$^{tmMom/J}$) mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 6 to 8 weeks of age. Housing and all of the animal experiments were performed in accordance with experimental procedures approved by the Louisiana State University Health Science Center Institutional Animal Care and Use Committee and in compliance with the guidelines of the American Physiological Society. (Table S1 in the online-only Data Supplement for groups.)

Human Samples

The study was approved by the institutional review board of the LSUHSC-S (STUDY00000261) and conducted in accordance with the Declaration of Helsinki. The consent form was discussed and after permission, blood was obtained from control volunteers (Materials in the online-only Data Supplement for details.

Drug Treatments

See Materials in the online-only Data Supplement for details.

Osmotic Pump Implantation

Ang II (1 µg/kg per minute) loaded micro-osmotic pumps (Alzet, Cupertino, CA, model 1002) were implanted for 14 days subcutaneously (intrascapular region) under isofluorane anesthesia.[7–9,17] See Materials in the online-only Data Supplement for more details.

Blood Pressure Measurement

Systemic arterial blood pressure was measured before and during the experiment as previously described.[28] See Materials in the online-only Data Supplement for more details.

T-Cell Isolation and Reconstitution

T-cell isolation and adoptive transfer were performed as described previously.[9,16,17] See Materials in the online-only Data Supplement for more details.

Intravital Microscopy

Light/Dye-Induced Thrombosis

Surgical procedures and light/dye-endothelial cell injury model were performed accordingly as described previously.[8,9] See Materials in the online-only Data Supplement for more details.

Intravital Microscopy

Cremaster Muscle

Separate groups of mice were subjected to intravital microscopy to evaluate leukocyte adhesion and emigration and platelet adhesion in cremaster muscle.[7,17] See Materials in the online-only Data Supplement for more details.

Immunoblockade of IL-6 Receptors

Rat anti-mouse IL-6Ra (CD126)-blocking antibody and mouse gp130 were injected immunoprecipitation in 100 µL saline at doses of 100 µg and 20 µg/mouse 24 hours before photo-illumination of the cremaster muscle microvessels.[24] Light/dye-induced thrombosis and leukocyte recruitment were recorded in separate groups of experimental mice.

Blood Cell Counts

Platelet and leukocyte counts were performed manually using hemocytometer (Reichert Hemacytometer, New York Microscope Company, NY). See Materials in the online-only Data Supplement for more details.

In Vivo Biotinylation Method for Platelet Lifespan Measurement

The in vivo biotinylation method[29,30] as used to establish a platelet lifespan and identify/quantify newly released platelets (platelet production) in WT-Saline and WT mice chronically infused with Ang II. Biotin and TO (thiazole orange) administration and labeling procedures were performed as previously described.[31,32] Newly released platelets were distinguished from other platelets based on SA (streptavidin conjugated with phycoerythrin (SA-PE; eBiosciences, San Diego, CA) binding to biotin. Newly released platelets were biotin negative by definition, representing a population CD41^{+}SA^{-}. The percentage of biotinylated platelets for every mouse during 5 days was determined by flow cytometry and lifespan converted into hours, as described previously.[29,31,33] See Materials in the online-only Data Supplement for more details.

Assessment of Activated Platelets by Flow Cytometry

Human venous blood was collected from healthy volunteers for platelet isolation. 1×10^{6} platelets were treated with 20 ng of IL-6 for 15 minutes labeled with P-selectin (CD62P) and CD41/CD61 (PAC-1 clone. Activated αIIbβ3) to assess platelet activation. Platelets were stimulated with either 0.01 U of thrombin or 1 ng of convulxin. See Materials in the online-only Data Supplement for more details.

Enzyme-Linked Immunosorbent Assay

A commercially available cytometric bead array kit was used to measure the concentration of IL-6 in serum as per the manufacturer's instructions. See Materials in the online-only Data Supplement for more details.

Statistical Analyses

All values are reported as mean±SEM. Data within groups were compared using a Student *t* test (2 groups) or an ANOVA (1-way ANOVA) with a Newman-Keuls post hoc correction for multiple comparisons. Analysis was performed using Graph Pad Prism5 software (San Diego). Data are shown as mean values±SEM. Differences were considered statistically significant at a value of $P<0.05$.

Results

Ang II Infusion Is Associated With Elevated Platelet Counts, Increased Levels of Immature Platelets and a Shortened Platelet Lifespan

Although it is known that immature platelets cause accelerated thrombosis and heightened inflammation, however, this paradigm is undetermined in the context of Ang II. We first sought to examine platelet counts, blood pressure, and thrombus formation following infusion with varying doses

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(0–1000 µg/kg per minute) of Ang II. We found that the 1000 ng/kg per minute and 1 µg/kg per minute dose were associated with increased circulating platelet counts and elevated blood pressure, which were coupled with heightened thrombosis 2 weeks after pump implantation (Figures S1A and S1B; Tables S2 and S3). These results suggested a potential role for platelets in the accelerated thrombosis associated with Ang II (Figures S1C and S1D). It is important to note that our findings here are not related to elevated blood pressure per se but rather Ang II–mediated effects on thrombus formation. Previously, we published data in an alternative model of hypertension (deoxycorticosterone acetate salt-induced hypertension), which had no effect on thrombus formation and CD40$^{-/-}$ mice implanted with Ang II–loaded pumps exhibit protection against Ang II–mediated thrombosis but remain hypertensive,[34] further supporting our findings here that elevated blood pressure per se is mediating the effects.

Next, we evaluated the presence of reticulated (immature) platelets (which constitutes a more active platelet population, that is, prothrombotic and proinflammatory) as a marker of accelerated thrombus formation. WT mice were infused with 1 µg/kg per minute for 14 days and platelet counts recorded concomitantly with immature platelet levels recorded after 1 week of either saline or Ang II infusion (Figure 1A and 1B). Ang II infusion resulted in increased total platelet counts and elevated levels of immature platelets coupled with increased platelet production (Figure 1C). Additionally, we found for the first time that Ang II chronic infusion caused a decrease in platelet lifespan (Figure 1D). Collectively, these results demonstrated that chronic Ang II infusion is associated with increased platelet numbers, elevated immature platelet counts (thrombocytosis), enhanced platelet production, and shorter platelet lifespan. These effects both reflect increased elimination of platelets from the circulation as a result of increased prothrombotic activity (immature platelets represent hyperactive platelet population and are indicators of accelerated prothrombotic responses).

Immunodeficiency and Immunoblocking of IL-6 Receptors Mitigate Ang II–Enhanced Thrombosis

Previously, we have demonstrated that IL-6 plays a dominant role in dextran sodium sulfate-induced colonic thromboinflammation.[31] As such, we wanted to assess whether IL-6 had a similar impact on thrombus formation in mice lacking IL-6 and chronically infused with Ang II. In our experiments, compared with WT/Ang II mice, IL-6$^{-/-}$ mice implanted with Ang II–loaded pumps demonstrated a more dramatic restoration in both onset (2× higher than WT mice) and cessation (2.89× higher than WT mice; Figure 2A and 2B), suggesting that IL-6 genetic deficiency results in an amelioration of Ang II–induced microvascular thrombosis in arterioles. These effects were also coupled with increased serum levels of IL-6 (4.0±1.5 versus 25.4±13.0 pg/mL WT and WT/Ang II, respectively; Figure S2).

Because Ang II–induced endothelial dysfunction has been linked to elevated IL-6 and having shown here that IL-6$^{-/-}$/Ang II mice afford protection against Ang II–induced thrombosis (Figure 2A and 2B), we next sought to determine the involvement of IL-6 receptors (IL-6Rα and gp130) in these interactions. Figure 2C and 2D shows the effect of exogenous administration of IL6Rα and gp130 mAbs, which block either the -α or -β subunit of IL-6 receptor. Interestingly, while

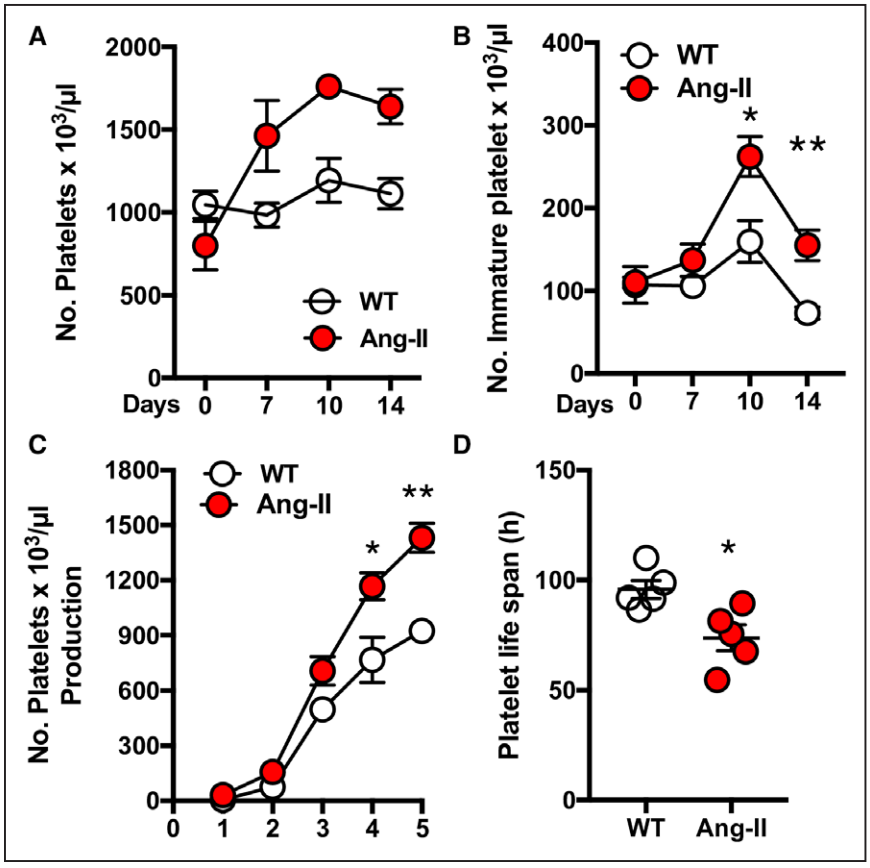

Figure 1. Ang II (angiotensin II) infusion is associated with elevated platelet counts, increased levels of immature platelet and a shortened platelet lifespan. Wild-type (WT) mice were implanted with either Ang II (1 µg/kg per minute) or control (saline) loaded micro-osmotic mini pumps for up to 14 d. Flow cytometry was performed to quantify platelet counts, immature platelet levels, newly released platelet levels, and platelet lifespan. **A**, Total platelet levels and (**B**) immature platelets levels after 1 wk of Ang II infusion (expressed as a platelet number per µL of whole blood on day 0, 7, 10, and 14). **C**, Platelets produced following Ang II infusion were monitored after day 7 and numbers of newly released platelets (biotin negative: CD41^{+}SA^{-}) were quantified daily. **D**, Platelet lifespan was determined in saline (WT) and Ang II–infused groups (expressed in hours). Data are mean±SEM of 3–6 mice per group. *P<0.05, **P<0.01 vs WT mice.

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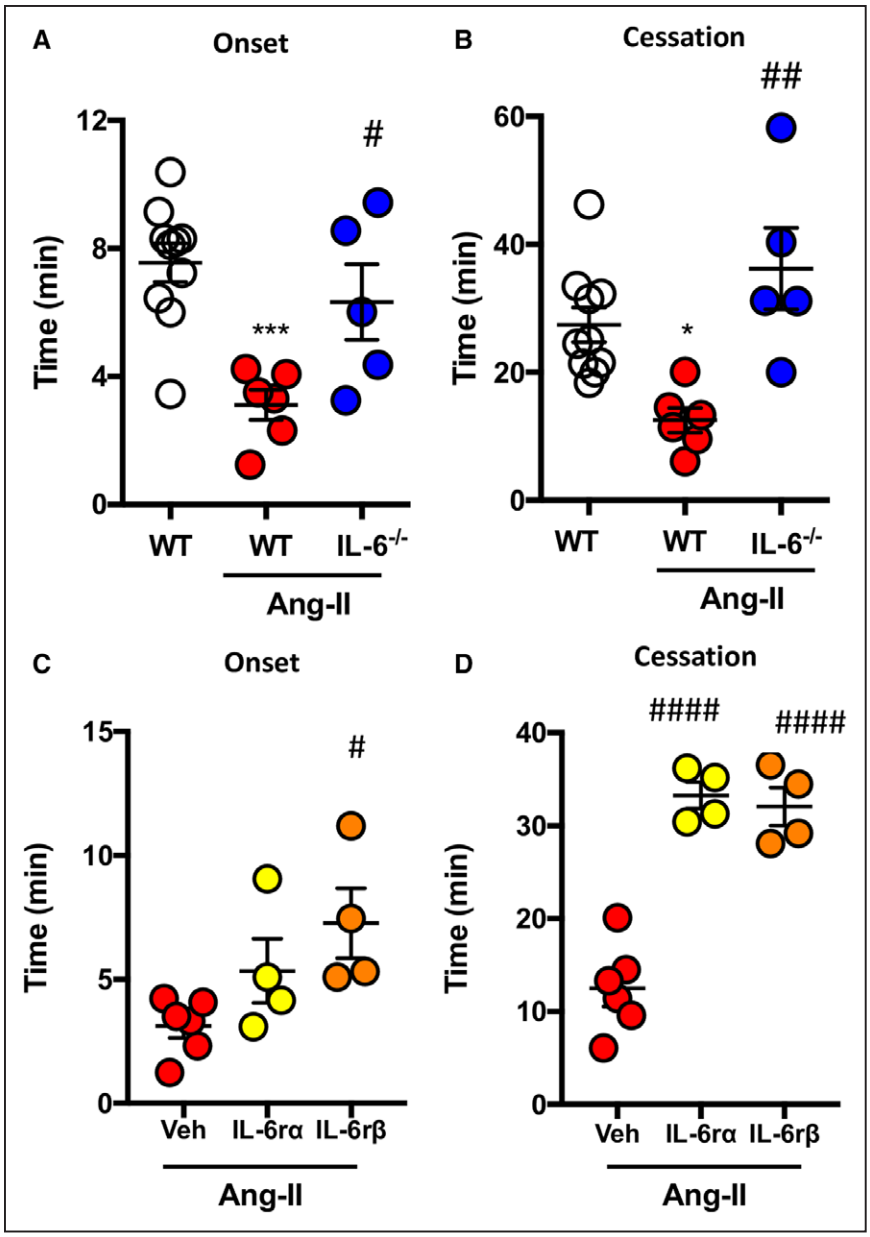

Figure 2. IL-6 (interleukin-6) deficiency protects against Ang II (angiotensin II)–induced microvascular thrombosis. Wild-type (WT) and IL-6 deficient (IL-6$^{-/-}$) mice were implanted with Ang II (1 μg/kg per minute) or control (saline) loaded micro-osmotic pumps for up to 14 d and light/dye-induced thrombosis model was performed. The following were quantified: (**A**) time in minutes (min) for onset (initial platelet deposition) and (**B**) cessation (occlusion) of blood flow. **C** and **D**, show the time for onset of a thrombus and cessation of blood in WT/Ang II and WT/Ang II groups treated 24 h before light/dye-induced thrombosis with IL-6Rα (20 μg/mouse) or IL-6Rβ (gp130; 100 μg/mouse). Data are mean±SEM of 5–10 mice per group. *P<0.05, ***P<0.001 vs WT mice. #P<0.05, ##P<0.01, ####P<0.0001 vs WT/Ang II mice (vehicle [Veh]).

blocking IL-6Rβ (gp130) prolonged both onset and cessation times in WT-Ang II mice (no affect in WT mice, data not shown), blocking of IL-6Rα affected onset only. These data suggest that IL-6Rα may play more of a role in thrombogenesis (ie, initial platelet recruitment to the vessel), whereas IL-6Rβ is involved in both thrombogenesis and thrombosis.

IL-6 Receptors Are Involved in Ang II–Enhanced Inflammatory Responses in Cremasteric Venules

Having shown the involvement of IL-6 receptors in thrombosis and knowing a crosstalk exists between thrombosis and inflammation, we next wanted to investigate whether blocking IL-6 receptors would reduce Ang II–induced inflammatory cell influx. Both IL-6Rα and gp130 immunoblocking blunted Ang II–enhanced platelet adhesion (assessed using intravital video microscopy), restoring levels back to levels seen in WT mice (Figure 3A). Figure 3B and 3C shows that WT-Ang II mice display heightened leukocyte adhesion and emigration when compared with controls, with the administration of IL-6Rα and gp130 mAbs significantly blunting enhanced leukocyte adhesion in cremaster muscle venules. In the case of emigrated leukocytes only gp130 mAb treatment, but not IL-6Rα mAb, was effective (Figure 3B and 3C). (No effects were observed in arterioles, data not shown.) These data suggest that immunoblockade of IL-6 receptors effectively protects against Ang II–induced platelet and immune cell accumulation within cremaster venules. Further supporting the concept that IL-6 is intimately involved in thromboinflammation associated with Ang II–induced hypertension.

T-Cell Adoptive Transfer Into Immunodeficient Rag-1/Ang II Mice Increases Immature Platelet Population

As we had provided clear evidence of the involvement of IL-6 in the thromboinflammatory state associated with Ang II and knowing that IL-6$^{-/-}$ mice have lower levels of lymphocytes,

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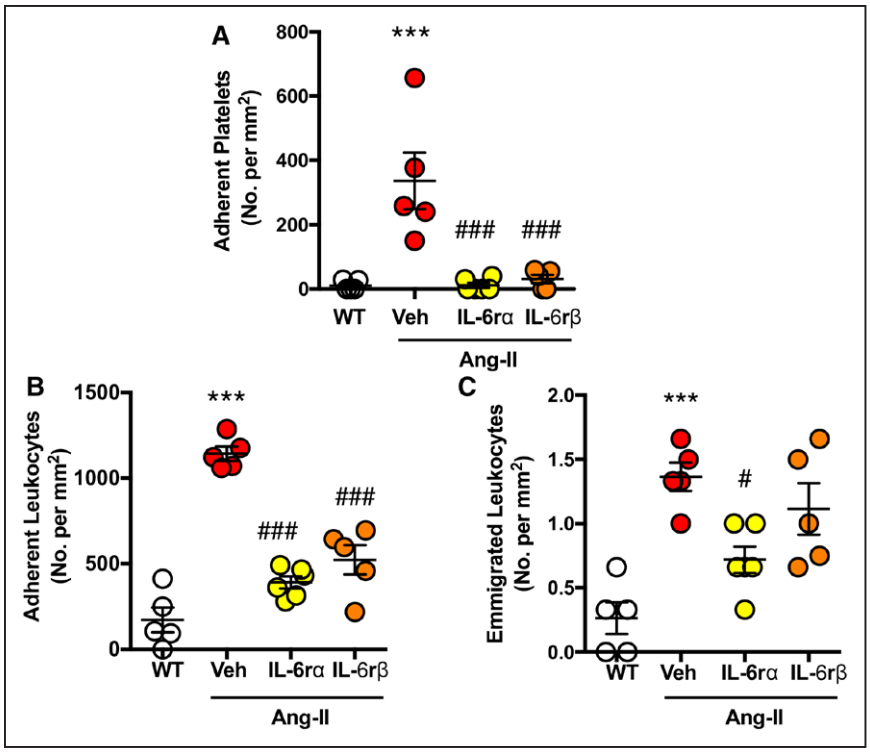

Figure 3. Blocking IL-6 (interleukin-6) receptors mitigates immune cell recruitment in Ang II (angiotensin II)–induced inflammation. Wild-type (WT) mice were implanted with Ang II (1 μg/kg per minute) or control (saline) loaded micro-osmotic pumps for up to 14 d before undergoing intravital microscopy (IVM) of the cremaster muscle microcirculation to quantify leukocyte and platelet interactions. WT/Ang II mice were treated 24 h before IVM with IL-6Rα (20 μg/mouse) or IL-6Rβ (gp130; 100 μg/mouse). The following parameters were quantified: (**A**) adherent platelets (≥2 s), (**B**) adherent leukocytes (stationary for ≥30 s), and (**C**) emigrated leukocytes. Data are mean±SEM of 5–6 mice per group. ***P<0.001 vs WT mice. #P<0.05, ###P<0.001 vs WT/Ang II mice (vehicle [Veh]).

which could in part explain their afforded protection (Figure 2), we next tested the role of T cells in our study (Figure 4A). Figure 4B shows for the first time that although Ang II–infused mice exhibit significantly elevated platelet numbers compared with WT mice, chronically elevated levels of Ang II failed to increase the number of circulating platelets in Rag-1$^{-/-}$/Ang II mice when compared with WT/Ang II mice, suggesting that lymphocyte deficiency is protective against Ang II–mediated elevation in platelet counts (WT: 1026.0±64.3; Ang II: 1746.0±101.9; Rag-1$^{-/-}$: 1261.0±139.4, and Rag-1$^{-/-}$/Ang II: 1137.0±111.1 platelets per 10^3/μL). These previously unknown findings about platelet counts concur with our earlier published findings that Rag-1$^{-/-}$/Ang II mice are protected from thrombosis.[9]

Because immunodeficient Rag-1$^{-/-}$/Ang II mice have lower circulating levels of platelets, we next sought to determine whether T cells were mediating the elevation in circulating platelet counts. We employed the use of adoptive transfer of WT T cells for a period of 2 weeks in Rag-1$^{-/-}$/Ang II mice. Figure 4B and 4C shows that the adoptive transfer of WT T cells into Rag-1$^{-/-}$/Ang II mice resulted in increased total platelet count and an increase in immature platelet count, representing a more active platelet population (ie, prothrombotic),[31,35–38] which may result in a majority of hyperactive platelets in the total platelet count compared with control. Additionally, we also confirmed that the Rag-1$^{-/-}$/Ang II mice reconstituted with WT-derived T cells, displayed elevated T-cell counts (Figure S3; 0.84±0.2% in Rag-1$^{-/-}$/Ang II versus 10.6±2.0 in Rag-1$^{-/-}$/Ang II reconstituted with WT-derived T cells). Thus, these results suggest that T cells contribute to the alteration in platelets responses.

Platelets are the primary participants of arterial thrombosis and increased immature platelets and accumulation within the vasculature leads to an increased risk of thrombotic events.

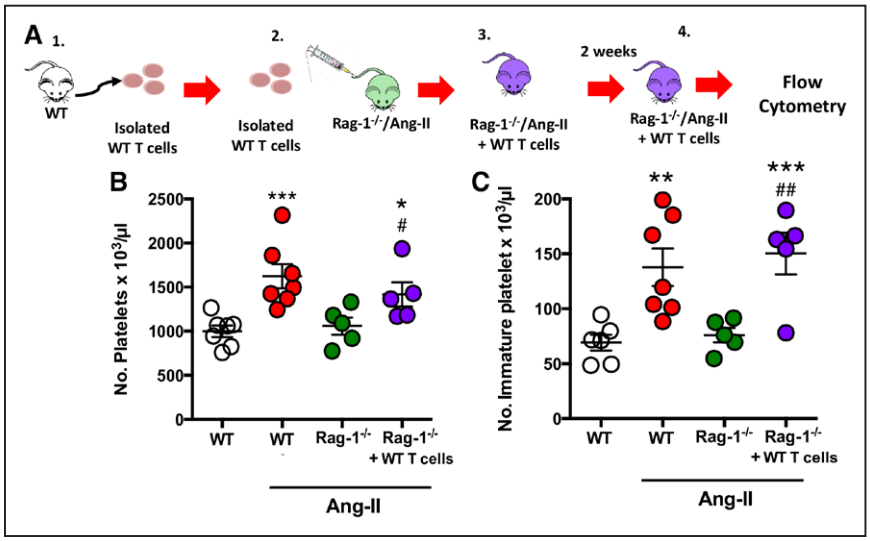

Figure 4. T-cell adoptive transfer into immunodeficient Rag-1$^{-/-}$/Ang II (angiotensin II) mice increases immature platelet population. Wild-type (WT) mice and Rag-1$^{-/-}$ mice were implanted with Ang II (1 μg/kg per minute) loaded micro-osmotic pumps for up to 14 d. A separate group of Rag-1$^{-/-}$/Ang II mice was also reconstituted with wild-type (WT)–derived T cells (Rag-1$^{-/-}$/Ang II+WT T cells). **A**, Schematic representation of (1) WT T cells being isolated; (2) Injected into Rag-1$^{-/-}$/Ang II mouse to make; (3) Rag-1$^{-/-}$/Ang II+WT T cell mouse; (4) After 2 weeks blood was collected from Rag-1$^{-/-}$/Ang II+WT T cell mouse and was subjected to platelet assessment by flow cytometry. **B**, Circulating platelet numbers and (**C**) the number of immature platelets was counted. Data are mean±SEM of 3–6 mice per group. *P<0.05, ***P<0.001 vs WT, and ##P<0.01 vs Rag-1$^{-/-}$/Ang II mice.

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To determine whether increased immature platelets and elevated T-cell count predisposes Rag-1$^{-/-}$/Ang II+WT T cells to Ang II–mediated thrombotic responses, we next used the light/dye-induced thrombosis model (Figure 5A). Figure 5B and 5C confirms our hypothesis, clearly demonstrating that these mice do indeed display a heightened thrombotic response (ie, shorter blood flow onset and cessation times) in cremaster arterioles (onset: Rag-1$^{-/-}$/Ang II 8.0±0.7 minutes versus Rag-1$^{-/-}$/Ang II-WT T cells 2.1±0.7 minutes and for cessation: Rag-1$^{-/-}$/Ang II 24.9±1.2 minutes versus Rag-1$^{-/-}$/Ang II-WT T cells 11.8±1.7 minutes). (No effect in venules. Data not shown.)

IL-6 Deficient T Cells Do Not Accelerate Thrombotic Responses Elicited by Ang II Infusion

Having assessed the novel antithromboinflammatory effects of IL-6 immunoblocking (as well as and IL-6 deficiency) and the role that T cells play in Ang II hypertension, we wanted to address whether T-cell–dependent IL-6 was actually driving the protection. Figure 5B and 5C shows that when recipient Rag-1$^{-/-}$/Ang II mice were reconstituted with T cells obtained from IL-6$^{-/-}$ donor mice they displayed protection against thrombosis (quantified as increased times of onset and cessation). These data suggest that T cell IL-6 mediates the thrombotic abnormalities associated Ang II hypertension.

IL-6 Primes Platelet Activation in Response to Collagen Receptor GPVI

Finally, to explore the translational relevance of the in vivo findings, we ascertained the direct effect of IL-6 on human isolated platelets following the GPVI collagen receptor agonist convulxin or the serine protease thrombin. Figure 6 shows that convulxin enhanced the surface levels of active $\alpha_{IIb}\beta_3$ (as assessed by PAC-1 binding), P-selectin (CD62P), and double positive active $\alpha_{IIb}\beta_3$-P-selectin (a population of platelets that are more likely to being involved in adhesion and aggregation) on platelets. Stimulation with IL-6 alone did not affect active $\alpha_{IIb}\beta_3$ or P-selectin but potentiated these markers of platelet activation when in the presence of convulxin. Interestingly, neither platelets incubated with IL-6 and stimulated with thrombin display any differences in percentage of $\alpha_{IIb}\beta_3$, P-selectin or versus thrombin stimulation alone (Figure S4). These data provide further evidence that not only is IL-6 able to enhance the prothrombotic ability of platelets in vivo, but it also predisposes platelets to an interaction with collagen receptors and in so doing, increases the propensity for platelets to aggregate and cause thrombosis.

Discussion

We present herein several novel key conceptual findings which we believe advances knowledge and understanding in the field of Ang II–mediated thromboinflammation. Specifically, we found that IL-6$^{-/-}$ mice afforded are protection against Ang II–induced thrombosis. Both IL-6Rα and IL-6Rβ are involved in Ang II–induced thrombosis, with IL-6 being able to potentiate platelets, predisposing the platelets to stimulation/activation of the collagen receptor GPVI, which is a primary receptor for adhesion (eg, increased P-selectin) and contributing to platelet aggregation increasing GPIIbIIIa expression). T cells obtained from IL-6$^{-/-}$ mice did not accelerate thrombosis, while WT-derived T cells induced thrombotic responses. Additionally, the adoptive transfer of WT T cells resulted in heightened platelet levels and increased numbers of immature platelets, which constitutes a more active platelet population and thereby predisposing a prothrombotic and proinflammatory environment elicited by Ang II infusion. Finally, chronic Ang II infusion also resulted in a shorter platelet lifespan.

Over the past decade, animal and clinical studies have revealed the involvement of both the innate and adaptive immune system in the development and pathophysiological consequences of cardiovascular disease and its risk factors such as hypertension.[5,14,15,36] It is well-documented that Ang II, the main effector hormone of renin-angiotensin system, induces hypertension and is a direct mediator of both thrombotic abnormalities and vascular inflammatory responses.

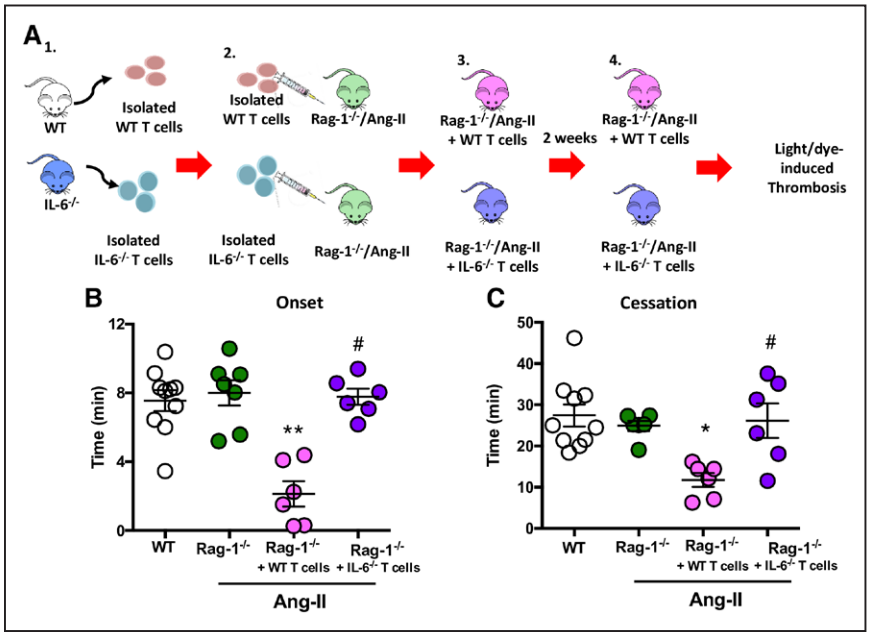

Figure 5. IL-6 (interleukin-6) deficient T cells adoptive transfer do not accelerate thrombotic responses elicited by Ang II (angiotensin II) infusion. Wild-type (WT) mice or immunodeficient Rag-1 (Rag-1$^{-/-}$) mice were implanted with Ang II (1 μg/kg per minute) loaded micro-osmotic pumps for up to 14 d. **A**, Schematic representation of (1) WT T or IL-6$^{-/-}$ T cells being isolated; (2) Injected into Rag-1$^{-/-}$/Ang II donor mouse to make; (3) Rag-1$^{-/-}$/Ang II+WT T cell mouse or Rag-1$^{-/-}$/Ang II+IL-6$^{-/-}$ T cell mouse; and (4) After 2 weeks (14 d) mice were subjected to the light/dye-induced thrombosis model. Time of (**B**) onset and (**C**) cessation was quantified in cremasteric arterioles. Data are mean±SEM of 6–10 mice per group. *P<0.05, **P<0.01 vs Rag-1$^{-/-}$/Ang II. #P<0.05 vs Rag-1$^{-/-}$/Ang II+WT T cells.

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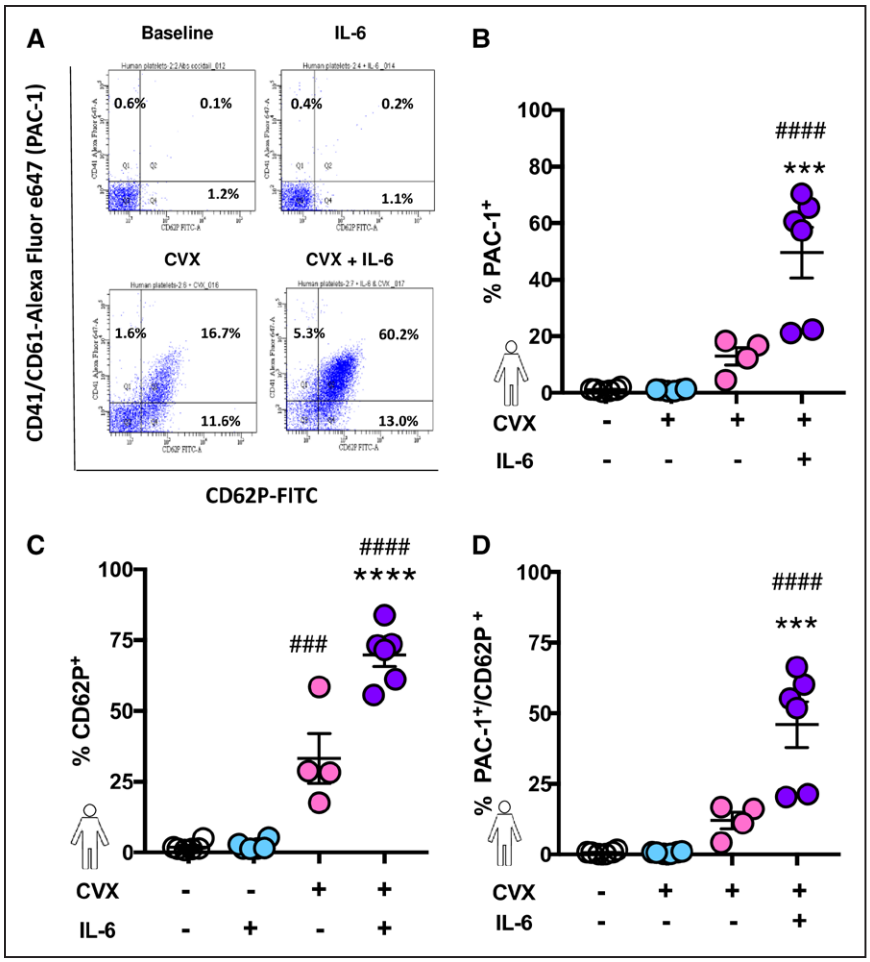

Figure 6. IL-6 (interleukin-6) increases platelet activation via GPVI pathway. Human platelets were isolated, washed and 1×10^6 preincubated with vehicle (saline) or IL-6 (20 ng) for 15 min at 37°C, and stimulated with and without convulxin (CVX; 1 ng per 1×10^6 platelets). **A**, Representative flow cytometric analysis for % expression of P-selectin (CD62P-FITC) and $\alpha_{IIb}\beta_3$ (CD41/CD61 Alexa eFluor 647) for baseline, IL-6 incubated platelets, CVX stimulated and IL-6 incubated and stimulated with CVX. The following were quantified: (**B**) % of activated integrin $\alpha_{IIb}\beta_3$ (PAC-1), (**C**) % P-selectin expression (CD62P), and (**D**) % of double positive platelet population for activated integrin $\alpha_{IIb}\beta_3$ (PAC-1) and P-selectin (CD62P) expression. Data are mean±SEM of 4–7 individual donors per group. ***P<0.001, ****P<0.0001 vs unstimulated platelets (baseline), ###P<0.001 and ####P<0.0001 vs CVX-stimulated platelets.

Several cytokines (eg, TNFα [tumor necrosis alpha], IFNγ [interferon gamma], IL-1β, and IL-6)[31,34,37] are known to contribute to the thromboinflammatory responses associated with acute and chronic inflammation, with IL-6 being considered a clinical biomarker of cardiovascular disease.[38,39] The normal range of IL-6 in plasma is about 5 to 7 pg/mL in human population[37] and its secretion is upregulated in response to Ang II, oxidative stress and vascular injury,[19,38–40] which concurs with increased IL-6 levels in these studies. However, the role that IL-6 plays in Ang II–mediated thrombosis, along with the role that T-cell–derived IL-6 signaling plays in thromboinflammatory responses associated with Ang II, is currently unkown. Here we questioned whether we could exploit these 2 immune players for drug discovery programs targeting both thrombotic and inflammatory responses in cardiovascular disease especially hypertension.

Cells which have IL-6R (CD126, gp80) are able to bind IL-6 directly and are able to shed their IL-6R after cleavage by protein kinase C, forming soluble IL-6R, which in turn may bind IL-6.[41,42] This complex (sIL-6R/IL-6) can cause homodimerization of gp130 and subsequent signal transduction in cells that lack the IL-6R.[41,43] Here we showed that that genetic IL-6 deletion affords protection against Ang II–mediated thrombus formation in cremaster arterioles. As IL-6 exerts its effects by interacting with its receptor complex composed of a specific α subunit, IL-6Rα (gp80, CD126, or IL-6R) and a signal transduction subunit gp130 (also referred to as IL-6Rβ or CD130),[18,21] we wanted to ascertain the contribution of IL-6Rα (classic cis signaling pathway) and gp130 from interacting with IL-6R/IL-6 (inhibiting trans-signaling pathway) to the Ang II thromboinflammatory responses. We found that acute immunoblocking of IL-6 receptors[21,44] in WT mice infused with Ang II elicited a protective effect, as demonstrated by decreased arterial thrombosis and reduced platelet recruitment and leukocyte adhesion in venules. The different responses observed in arterioles and venules may lie in both the composition of each vessel type, for example, shear rates, coupled with the differences associated with thrombotic versus inflammatory responses (eg, arterial thrombi are rich in aggregated platelets[45] and leukocyte and platelet accumulation typically occur in the venules, not arterioles). These data may suggest that either genetic deficiency of IL-6 or immunoblocking of IL6Rα and IL-6Rβ are protective against thromboinflammatory responses. Additionally, our findings concur with many other in vitro and in vivo models of inflammation showing IL-6 trans-signaling to regulates the expression of trafficking molecules that mediate leukocyte primary adhesion (E-selectin and VCAM-1 [vascular cell adhesion molecule-1]), chemokine activation (CCL2, CXCL10, CCL4, CCL5, CCL11, and CCL17), as well as secondary firm adhesion and trans-endothelial migration (ICAM-1 [intracellular adhesion molecule-1] and VCAM-1).[41]

From a clinical point of view several studies have already shown that blocking IL-6R using immunoblocking antibodies such as tocilizumab, siltuximab, sarilumab[46] prevents IL-6 from

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exerting its proinflammatory effects and as such have shown to be promising therapies for different pathological conditions such as rheumatoid arthritis and cancer, conditions which are accompanied by enhanced thrombotic complications, increased immature platelet population (ie, hyperactive platelets) and heighted inflammatory responses, for example, increased levels of IL-6.[46,47] Additionally, it has recently been published that blocking IL-6 signaling also improves pulmonary arterial hypertension, with IL-6 signaling considered as a new therapeutic target.[48,49] In the context of hypertension, several reports indicate that increased levels of IL-6 because of Ang II activate gp130-linked signaling and contribute to Ang II–induced hypertrophy.[19] Our study demonstrates that IL-6 levels are linked to increased immature platelet production and thrombosis and a reduction in levels of IL-6 results in a reduction of thrombotic complications. Future/ongoing studies are needed to fully address the role of IL-6 signaling in hypertension and the therapeutic potential of targeting the IL-6-STAT3 (IL-6–signal transducer and activator of transcription 3) axis for the management of thromboinflammatory diseases such as hypertension, beyond more traditional methods such as blood pressure and Ang II lowering.

Although we have previously demonstrated that Rag-1$^{-/-}$ mice which lack both T and B cells, exhibit a complete protection against Ang II accelerated thrombosis and that CD4^{+} T cells are involved in the thrombotic responses observed within the microvasculature,[9] the actual mechanism remained undefined. Interestingly, we found here that chronically elevated levels of Ang II resulted in not only increased platelet counts but resulted in heightened numbers of immature platelets (or reticulated platelets).[34,35] Rag-1$^{-/-}$ mice, however, were protected against this increase and adoptive transfer of T cells isolated from WT mice and transferred into Rag-1$^{-/-}$ recipients restored increased levels of immature platelets, suggesting that a reciprocal crosstalk exists between these 2 cell types: T cells and platelets.

Having shown previously that Ang II–induced microvascular thrombosis is mediated via T cells,[9] and having demonstrated here that a crosstalk exists between T cells and platelets in chronic Ang II infusion, we next sought to investigate the involvement of IL-6 in this immune cell relationship. It has been shown that IL-6 is required for the chemotactic activity and migration of T cells,[50,51] although its role in thrombosis was less well defined. Interestingly, we found for the first time that T cells isolated from IL-6$^{-/-}$ mice and injected into Rag-1$^{-/-}$ mice were protected against Ang II–induced thrombosis suggesting, that T cell IL-6 plays a key role in the thrombotic process.

Finally, having studied the effects of T cell IL-6 and the role they play in Ang II–induced thrombosis, we next turned our focus to the platelet to translate the findings to a clinical setting. As such, we assessed the effect of IL-6 on human platelet function (P-selectin and αIIbβ3 receptor expression) following stimulation with thrombin and the GPVI collagen receptor agonist convulxin. We found that IL-6 increased both P-selectin and αIIbβ3 receptor expression following convulxin treatment, but this effect was not produced with thrombin. It is well known that GPVI plays an important role in collagen-mediated platelet aggregation and adhesion especially in terms of conditions of arterial thrombosis where platelet activity induced by vascular inflammation is a known risk factor for arterial thrombosis.[52,53] Ang II causes heightened arterial thrombosis and platelet activation signaling plays a critical role in the function of platelets in hemostasis and thrombosis. Our novel findings showed that IL-6 is able to prime platelets predisposing the platelets to collagen receptor GPVI activation, which may lead to platelet adhesion and thrombotic responses via changes in P-selectin (critical for platelet activation and heterotypic and homotypic platelet interactions) and αIIbβ3 receptor (involved in aggregation and platelet plug stabilization) expression. These effects promote platelets to adhere and stabilize the platelet plug, thereby predisposing platelets to accumulate in arterioles. Indeed, IL-6 may be changing the intraplatelet GPVI receptor pools which are involved in platelet-collagen interactions.[54–56] GPVI is localized on the platelet surface plasma membrane and also on the membranes of the surfaces connected to the open canalicular system, an elaborate system of tunneling invaginations of the cell membrane unique to the platelet[57] and the α-granules in resting platelets. During platelet activation, the release of GPVI pools is redistributed (ie, ultrastructural changes occur which lead to an increase in GPVI on the activated platelet surface, accompanied by a decrease in interior expression),[56] a process in which IL-6 could be involved via its ability to initiate STAT3 dependent signaling which interacts with Syk and PLCγ2 (phospholipase Cγ2), thereby enhancing collagen-induced platelet activation and aggregation.[58,59] One could speculate that a critical implication of this molecular interaction could be to facilitate a crosstalk between collagen-induced and inflammatory cytokine-induced signal pathways on platelets predisposing them to adhesion and aggregation.

In summary, using genetic and pharmacological approaches, we have found that innate and adaptive immune responses to Ang II involve the interactions between platelets and T cells. We report a previously unknown effect of T-cell–dependent IL-6 to alter platelet and leukocyte adhesion and thrombus formation via interacting with its IL-6 receptors, demonstrating a key role of this cytokine as a mediator of Ang II–induced thromboinflammation. These novel and compelling data provide new therapeutic possibilities based on T-cell–dependent IL-6 signaling pathways for drug discovery programs for the management of hypertension.

Perspectives

The main effector of the renin-angiotensin system is Ang II, which is a critical determinant of the prothrombotic and proinflammatory environment associated with hypertension. The chronic prothrombotic phenotype associated with Ang II–induced hypertension is mediated by a mechanism that is dependent on both T cells and IL-6. Drug discovery programs based on T-cell–dependent IL-6 signaling pathways may provide a previously unknown therapeutic strategy for the management of the thromboinflammatory complications that accompany hypertension.

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Disclosures

None.

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Novelty and Significance

What Is New?

- The main effector of the renin-angiotensin system is Ang II (angiotensin II), which is a critical determinant of the prothrombotic and proinflammatory environment associated with hypertension. T cells and IL-6 (interleukin-6) play key roles in these processes driving forward the Ang II associated thromboinflammation.

What Is Relevant?

- The chronic prothrombotic phenotype associated with Ang II–induced hypertension is mediated by a mechanism that is dependent on both T cells and IL-6.

Summary

This study, using pharmacological and genetic approaches, coupled with murine and clinical samples is the first to show that T-cell–derived IL-6 signaling plays a key role in the thromboinflammatory responses associated with Ang II.

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