Interleukin-6–Signal Transducer and Activator of Transcription-3 Signaling Mediates Aortic Dissections Induced by Angiotensin II via the T-Helper Lymphocyte 17–Interleukin 17 Axis in C57BL/6 Mice

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Objective—Dysregulated angiotensin II (Ang II) signaling induces local vascular interleukin-6 (IL-6) secretion, producing leukocyte infiltration and life-threatening aortic dissections. Precise mechanisms by which IL-6 signaling induces leukocyte recruitment remain unknown. T-helper 17 lymphocytes (Th17) have been implicated in vascular pathology, but their role in the development of aortic dissections is poorly understood. Here, we tested the relationship of IL-6–signal transducer and activator of transcription-3 signaling with Th17-induced inflammation in the formation of Ang II–induced dissections in C57BL/6 mice.

Approach and Results—Ang II infusion induced aortic dissections and CD4+ interleukin 17A (IL-17A)–expressing Th17 cell accumulation in C57BL/6 mice. A blunted local Th17 activation, macrophage recruitment, and reduced incidence of aortic dissections were seen in IL-6−/− mice. To determine the pathological roles of Th17 lymphocytes, we treated Ang II–infused mice with IL-17A–neutralizing antibody or infused Ang II in genetically deficient IL-17A mice and found decreased aortic chemokine monocytic chemotactic protein-1 production and macrophage recruitment, leading to a reduction in aortic dissections. This effect was independent of blood pressure in IL-17A–neutralizing antibody experiment. Application of a cell-permeable signal transducer and activator of transcription-3 inhibitor to downregulate the IL-6 pathway decreased aortic dilation and Th17 cell recruitment. We also observed increased aortic Th17 infiltration and IL-17 mRNA expression in patients with thoracic aortic dissections. Finally, we found that Ang II–mediated aortic dissections occurred independent of blood pressure changes.

Conclusions—Our results indicate that the IL-6–signal transducer and activator of transcription-3 signaling pathway converges on Th17 recruitment and IL-17A signaling upstream of macrophage recruitment, mediating aortic dissections. (Arterioscler Thromb Vasc Biol. 2013;33:1612-1621.)

Key Words: angiotensin II ■ aortic dissection, familial ■ inflammation ■ interleukin-6 ■ T-lymphocytes, helper ■ vascular inflammation

Angiotensin II (Ang II) is the major effector peptide of the renin–angiotensin system, and its signaling via the type 1 Ang II receptor induces vascular contractility, hypertrophy, and extracellular remodeling.1 More recently, Ang II has been shown to induce inflammation, a process mediated by monocyte/macrophage cell recruitment into the adventitial and medial layers of large arteries. Importantly, both human and experimental animal studies have suggested a role for Ang II in the development of aortic dissections.2

Vascular inflammation is a stereotypic process producing recruitment of activated leukocytes, monocytes/macrophages, and lymphocytes into all layers of the vascular wall.3,4 Circulating leukocytes are recruited from the circulation into the vessel wall either through the intimal (inside-out) or adventitial (outside-in) surfaces, through a coordinated process of demargination, tissue infiltration, and local cellular activation.5,6 Of these, monocytes/macrophages mediate the final pathological consequences of vascular inflammation. Ang II–stimulated monocytes are major generators of reactive oxygen species stress, producers of matrix metalloproteases, and secretors of additional cytokines in the vessel wall.7-9 These effects result in extracellular matrix degradation, enhanced reactivity to inflammatory agents, endothelial dysfunction, and vascular dissection.10 The mechanisms that...
Ang II–induced cytokines play in this process of local vascular inflammation are not well understood.

Interleukin-6 (IL-6) is the most highly upregulated cytokine in Ang II–stimulated vessels yet identified, and it has been identified as an independent biomarker of vascular atherosclerotic risk and aneurysmal rupture. IL-6 is a member of a superfamily of cardioactive cytokines, whose members include cardioprotein, interleukin-11 and -12, and granulocyte-colony stimulating factor that bind to unique \( \alpha \)-receptors and whose actions are mediated through a common glycoprotein 130 signal transducer converging on the signal transducer and activator of transcription (STAT)-3. Although our findings suggest that IL-6 is necessary for macrophage activation in the early stages of vascular inflammation leading to aortic dissection, IL-6 lacks chemotactic activity, and therefore, its effects on monocyte recruitment have not been fully explained.

Earlier studies have linked vascular effects of Ang II as mediated by lymphocyte populations. Not only are \( T \) and B lymphocytes found in Ang II–induced vascular diseases, but the effect of Ang II on hypertension, vasomotor dysfunction, oxidative stress, arteriolar thrombosis, and atherosclerosis is prevented with total T-lymphocyte deficiency. These studies suggest that IL-6 is necessary for macrophage activation in these processes, thus preventing the incidence of vascular inflammation and associated outcomes.

We have previously reported that chronic subcutaneous injection of Ang II (2500 ng/kg per minute) induced aortic dissections (defined as intramural hematoma in the suprarenal aorta) in 35% to 50% of aged mice. In this study, aortic hematomas/dissections were demonstrated with aortic ultrasonography and tissue histochemistry. In wild-type (WT) mice in the C57BL/6 background, histochemical analysis of cross sections in the suprarenal abdominal aorta consistently showed adventitial thickening and blood-filled false lumens located in the tunica adventitia in the Ang II–treated mice (Figure I in the online only Data Supplement). Approximately 40% of Ang II–infused mice developed areas of focal hemorrhages, visualized as false lumens indicating aortic dissection. All mice that developed dissections maintained an aortic size 50% greater than control aortas.

We reproduced our earlier studies of Ang II infusions conducted in IL-6−/− mice in the C57BL/6 background, where we observed a significantly reduced early incidence of aortic dissections after 7 days (31% in WT, \( n=16 \), versus 0% in IL-6−/−, \( n=12 \), respectively; 7 days; \( P<0.05 \)). This reduction in the incidence of dissections was not accounted for by changes in the systolic blood pressure of IL-6−/− mice. Here, Ang II induced a pressor effect of 30 mm Hg after 7 days in WT mice (from mean 102±3 mm Hg to a mean of 132±6 mm Hg; \( n=10 \); \( P=0.002 \); Figure II in the online only Data Supplement), which was not statistically different from the pressor response in IL-6−/− mice of the same background (mean 103±4 mm Hg to 119±7 mm Hg; \( n=10 \); \( P=0.048 \)).

Earlier studies have shown that Ang II induces Th17 recruitment in hyperlipidemic vascular tissues, a cell type mediating hypertension, endothelial dysfunction, and atherosclerosis in the apolipoprotein E–deficient background. To establish whether Ang II induces Th17 cell recruitment into the aortic wall in normolipidemic mice, we measured the abundance of IL-17A mRNA in the aorta and found that it was increased 4-fold relative to sham-infused mice (Figure 1A; \( P<0.01 \)). IL-17A–positive immunostaining was found in both the medial and adventitial layers (Figure 1A; \( P<0.01 \)).

To confirm that Ang II induced the aortic accumulation of the Th17 cell population, we conducted flow cytometric analysis of the dissociated aortae staining for both CD4 and IL-17A. Ang II induced a significant 5-fold recruitment of the CD4+IL-17A+ Th17 cell population (Figure 1B; \( P<0.01 \)). The retinoic acid receptor–related orphan receptor (ROR)\( \gamma \)-T directs Th17 cell differentiation. To further confirm recruitment of Th17 cells, we quantified CD4+ROR\( \gamma \)-T+ cells by flow cytometry. A similar 5-fold increase in ROR\( \gamma \)-T+ cells (Figure 1C; \( P<0.05 \)) was also observed in response to Ang II infusion.

We next tested whether local macrophage and T-lymphocyte recruitment were affected in the IL-6−/− background. Flow cytometric staining of aortic CD11b+ macrophages indicated that Ang II–induced macrophage recruitment was abolished in IL-6−/− mice (Figure 2A). We also tested whether IL-6 deficiency affected expression of IL-17A. A 5-fold increase in aortic wall of IL-17A mRNA was produced by Ang II in the IL-6−/− phenotype, whereas IL-17A transcript level was decreased in the IL-6−/− background (Figure 2B; 5-fold versus 2-fold, Ang II–treated WT versus Ang II–treated IL-6−/−; \( P<0.01 \)). We also observed by flow cytometry that aortic CD4+ IL-17A+ cells were decreased with IL-6 deficiency (Figure 2C; 7.3% versus 5.8%, Ang II–treated WT versus IL-6−/−). These data indicate
that IL-6 deficiency reduces Ang II–induced aortic IL-17A expression and accumulation of Th17 lymphocytes.

IL-17A Neutralization Reduced Aortic Inflammation and Dissections Induced by Ang II

Because IL-6 plays a pivotal role in Th17 differentiation, we hypothesized that the reduced inflammatory phenotype observed in Ang II–treated IL-6−/− mice was attributable, at least in part, to decreased Th17 activation. To test the pathogenic role of IL-17A, we infused Ang II in mice treated with an IL-17A–neutralizing antibody (NAb) or an isotype control antibody. First, we confirmed by ELISA that the IL-17A NAb reduced IL-17A secretion from aortic explants in tissue culture (Figure 3A).

We found that a 2-week IL-17A NAb treatment significantly reduced Ang II–induced aortic dissections (Figure 3B; 33% in Ang II plus isotype control antibody, n=12, versus 0% in Ang II plus IL-17A NAb, n=13; P<0.01). Also, IL-17A neutralization reduced aortic dilation (Figure 3B; P<0.05 at day 12) and reduced aortic adventitial thickening. IL-17A NAb also abolished Ang II–induced aortic Th17 recruitment (Figure 3C; 12% in isotype control antibody treatment versus 4% with IL-17A NAb treatment; P<0.05). Neutralization of IL-17A also reduced the aortic macrophage population (Figure 3D and 3E), indicating that IL-17A plays a role upstream of macrophage recruitment in Ang II–induced inflammation.

IL-17A has been implicated in the Ang II–induced pressor response because IL-17A deficiency blunts the increase in blood pressure from Ang II infusion. To determine whether IL17A neutralization produced a similar confounding pressor effect, we measured systolic blood pressures. We observed that at both baseline and after Ang II infusion, pressor effects were indistinguishable in untreated WT mice versus the IL17A NAb–treated mice. The mean systolic blood pressures of untreated WT mice changed from 96±4 mm Hg to 121±4 mm Hg after Ang II treatment, whereas the mean systolic blood pressure of the IL17A NAb–treated mice changed from 106±2 mm Hg to 135±8 mm Hg (Figure 3F). The pairwise differences in baseline and Ang II–induced blood pressures were not significant by antibody treatment. These data indicate that the IL17 NAb reduction in aortic dissections was independent of the pressor response.

IL-17A Deficiency Blunted Inflammatory Responses and Aortic Dissections

We next used IL-17A–deficient mice to test the role of Th17 lymphocytes in Ang II–induced vascular inflammation and aortic dissection. We found that, compared with WT mice, age-matched IL-17A−/− mice had a significantly lower incidence of Ang II–induced early (7 days) and late (14 days) aortic dissections (Figure 4A; 41% and 50% in C57BL/6 versus 0%
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and 8% in IL-17A−/−mice, respectively; n=12; *P<0.05). In addition, IL-17A−/− mice developed aortic dissections later (12 days), suggesting that IL-17A plays an early pathogenic role in the formation of aortic dissection and dilation (Figure 4A; diameters of suprarenal aorta in C57BL/6 and IL-17A−/− were 1.3 versus 1.0 mm at 6 days; *P<0.05). We confirmed the absence of Th17 cells in IL-17A−/− mice (Figure 4B; 12% in C57BL/6 versus 2% in IL-17A−/−), suggesting that abnormal Th17 homing in these mice may account for protection against aortic aneurysms.

We further examined cytokine secretion from aortic tissue in response to Ang II treatment. Multiplex cytokine/chemokine measurements in aortic explant tissue culture media demonstrated that Ang II enhanced expression of monocytic chemotactic protein-1, which was decreased in the IL-17A−/− background (Figure 4C). In addition, aortic macrophage recruitment in response to Ang II was decreased in IL-17A−/− mice (Figure 4D; 21% in WT versus 7% in IL-17A−/−). To determine the pressor response of the IL-17A−/− mice, systolic blood pressure was monitored. Here, we observed that IL-17A−/− mice had reduced resting blood pressure compared with WT controls (92±2 versus 101±2 mm Hg; P=0.015) and demonstrated a weak pressor response after Ang II infusion (104±5 versus 120±4 mm Hg; P=ns); the mean systolic pressure of the Ang II–infused IL-17A−/− mice was lower than the mean pressure of Ang II–infused WT controls (P=0.04; Figure 4E).

IL-6–STAT3 Signaling Mediated Ang II–Induced Th17 Lymphocyte Formation

IL-6 signaling via the glycoprotein 130 transducer activates intracellular signaling mediated by the STAT3 or nuclear factor-IL6 pathway.16 Our previous work has shown that STAT3 is activated in aortic monocytes in a manner that is absolutely dependent on IL-6.27 To test the role of IL-6–STAT3 signaling, we synthesized a peptide derivative of the STAT3 second helix, a domain that binds specifically with STAT3 but not with STAT1,31 fused to penetratin,32 that we and others have shown is a potent cell-permeant inhibitor of STAT3 action (Figure IV in the online-only Data Supplement).31,33 We found that the subcutaneous infusion of penetratin-STAT3 inhibitory peptide significantly reduced Ang II–induced aortic suppressor of cytokine signaling-3 mRNA expression (Figure 5A) as well as the suprarenal aortic dilation in WT mice (1.38±0.2 mm in Ang II–treated versus 1.19±0.05 mm in Ang II+penetratin-STAT3 inhibitory peptide–treated mice; *P<0.05; Figure 5B). The effect of penetratin-STAT3 inhibitory peptide on the formation of Th17 lymphocytes was measured in splenic lymphocytes. Here, we observed that Ang II induced a dramatic formation of Th17 cells, where 22% of the splenic lymphocytes were CD4+IL17+Th17 lymphocytes, and this number was significantly reduced to 13% in the presence of the penetratin-STAT3 inhibitory peptide (P<0.05; Figure 5C). Together, these data indicate that STAT3 is a critical intracellular signal for Ang II–induced Th17 formation.

Figure 2. Interleukin-6 (IL-6) deficiency reduced angiotensin II (Ang II)–induced macrophage and T-helper (Th) 17 recruitment. Age-matched wild-type (WT) and IL-6−/− mice were treated with Ang II or saline (sham) for 14 days. A, Flow cytometric analysis of CD11b-positive macrophages was performed using disassociated aortic cells, and the number of CD11b-positive cells was measured. Black curve, sham-treated WT; blue curve, Ang II–treated WT; red curve, sham-treated IL-6−/−; green curve, Ang II–treated IL-6−/− (n=4 in each group). B, Interleukin-17A (IL-17A) expression was analyzed using quantitative reverse transcription polymerase chain reaction. White bar, sham-treated WT; black bar, Ang II–treated WT; cross bars, sham-treated IL-6−/−; gray bar, Ang II–treated IL-6−/− (n=3–5 in each group; *P<0.05 and **P<0.01). C, Flow cytometric analysis of CD4-positive and IL-17A–positive cells was performed, and number of double-positive cells was measured. Representative panels corresponding to each group are shown (n=6). IL-6−/− showed abated Th17 recruitment to the aorta.

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Th17 Lymphocyte Recruitment in Patients With Thoracic Aortic Aneurysms

Previous work has shown that macrophages and T lymphocytes are present in human aortic aneurysms. To determine whether aortic Th17 recruitment is increased in humans with thoracic aortic aneurysm and dissection, we quantified IL-17A expression using immunohistochemistry in thoracic aortic samples from patients with transforming growth factor (TGF)-β receptor mutation (TGFBR2 R460C). We observed IL-17A immunostaining predominantly at the media–adventitia border (Figure 6A–6F). Rarely, IL-17A immunostaining was observed in the medial or intimal layers. Compared with controls, ascending aortic samples from patients with type A dissections caused by TGFBR2 mutation showed significant enhancement in IL-17A–expressing cell recruitment (4±2 cells/field versus 82±23 cells/field, control versus thoracic aortic aneurysm and dissection, respectively; *P<0.01; Figure 6G).

To confirm local accumulation of Th17 cells, total RNA was extracted from the same samples and subjected to quantitative reverse transcription polymerase chain reaction for human interleukin-17 mRNA. We observed a 2.8-fold increase in human interleukin-17 mRNA in thoracic aortic aneurysm and dissection samples relative to control (*P<0.05; Figure 6H).

These results extend the pathophysiological relevance of our observations that IL-17A–expressing Th17 cells are recruited into the aortic wall and mediate aortic dissections in a mouse model by suggesting that Th17 cells may also be important contributors to human aortic aneurysms and dissections.

Ang II–Induced Aortic Dissections Independent of its Vasopressor Effects

Other laboratories have demonstrated that the acceleration of atherosclerosis and abdominal aortic aneurysm in Ang II–infused mice is independent of Ang II–mediated increase in blood pressure. To extend these observations to Ang II–induced aortic dissections, we examined the effect of Ang II...
on dissections in mice made normotensive using the vasodilator hydralazine. WT mice were treated with hydralazine (online- only Data Supplement Materials) before and throughout infusion with Ang II. Under these conditions, the hydralazine-treated mice did not exhibit an Ang II–induced pressor response (mean of 101±3 mm Hg to a mean of 96±5 mm Hg; \( P \)=ns; Figure 7). However, 33% of the normotensive hydralazine-treated mice developed aortic dissections, which was similar to 45% incidence of dissection in mice that were made hypertensive with Ang II (\( P \)=ns; Figure 7B). These data indicate that Ang II–induced aortic dissections are independent of the systolic pressor response.

**Discussion**

Ang II is a potent inducer of vascular inflammation, IL-6 production, and monocyte recruitment and activation. In this study, we have found that IL-6 signaling converges on the recruitment of Th17 cells, a cell type necessary for the development of Ang II–induced aortic inflammation and dissections via its involvement in macrophage recruitment to the aortic wall. This work extends our previous study that identified IL-6–STAT3 signaling in monocyte activation to macrophages by providing a unifying mechanistic pathway where monocyte/macrophage recruitment into the aortic wall is coordinated by CD4+IL-17A+Th17 lymphocytes. To our knowledge, this is the first application of a genetic deletion of IL17A in normolipidemic C57BL/6 mice to study the role of IL17A in aortic dissection and dilation induced by Ang II.

Lacking direct chemotactic activity, the role of IL-6 in mediating inflammation has been elusive. Previously, we demonstrated that IL-6 signaling in Ang II–stimulated vascular disease was mediated by macrophage activation, a process involving phospho-Tyr STAT3 formation, loss of F4/80 cell surface staining, and induction of matrix-modifying matrix metalloproteases. Interpreted together, these data indicate that IL-6 is locally produced in sufficient concentrations to induce intracellular signaling, which directly leads to monocyte to macrophage differentiation. Our results here are surprising because they suggest that a second major target of aortic IL-6 secretion is the naive Th0 lymphocyte population, which is stimulated toward Th17 differentiation. Our findings that significant induction of Th17 lymphocyte in the spleen of Ang II–infused animals suggest that Ang II signaling significantly alters the lymphocyte
population systemically. We interpret our data to mean that IL-6 is a potent regulator of aortic IL-17A production: it mediates the differentiation of Th cells into IL-17–producing Th17 cells found in the aorta by stimulating STAT3 activity.

Our data suggest that IL-17A expression is largely dependent on IL-6 signaling. However, a small induction of IL17 remains in aortas of IL-6−/− mice (Figure 2B). Although many studies have shown that IL-6 is required for T-cell lineage development into Th17 cells, some innate immune cells are not dependent on IL-6 induction of RORγt and IL-17. For example, subsets of γδT cells and invariant natural killer T cells that do not undergo T-cell receptor selection constitutively express RORγT and can preferentially develop into IL-17–producing cells. Similarly, stimulation of γδT cells with IL-1β or interleukin-23 promotes IL-17 secretion and does not require IL-6.

Furthermore, analysis of human abdominal aortic aneurysmal tissue has indicated the presence of an oligoclonal population of γδT cells; these data suggest γδT cells are involved in aortic inflammation. One possible explanation of our findings is that Ang II infusion also stimulates innate γδT and invariant natural killer T cells to produce IL-17A even in the absence of IL-6. This IL-6–independent induction of IL-17A production would lead to monocyte chemotaxis and may account for the delayed inflammation and dissection observed in IL-6–deficient mice.

Several recent studies have used hyperlipidemic apolipoprotein E–deficient mice to study the effect of total T-lymphocyte or Th17 cell deficiency on aneurysm formation. Although these studies indicated that total T-cell or Th17 deficiency was not sufficient to attenuate Ang II–induced aneurysm formation in apolipoprotein E–deficient mice after 28 days of Ang II infusion, our results clearly suggest that defects in Th17 development protect against early development of aortic dissection and dilation by reducing vascular leukocyte infiltration and cytokine/chemokine expression. This setting, reduced leukocyte infiltration may result in fewer medial breaks, an initial precursor lesion that is followed by aortic dissection and later, aneurysm formation (Figure I in the online-only Data Supplement). Th17 cells have been implicated in the pathogenesis of autoimmune and inflammatory diseases and more recently in cardiovascular disease. Increased circulating Th17 cells and Th17 cell infiltration into the aorta are found in Ang II–induced hypertension, and IL-17A deficiency blunts these responses and prevents hypertension. Our data indicate that Th17 cells are enriched in the medial–adventitial border of suprarenal aortas (Figure 1A, bottom), indicating their accumulation at the principal site of dissection in this model. Others have shown that Th17 cells, as well as IL-17 expression in atherosclerosis, are increased, and blockade of IL-17A reduced aortic macrophage infiltration, cytokine secretion, and atherosclerotic plaque formation. Our data are consistent with these observations, where we observe reduced monocytic chemotactic protein-1 expression in IL-17A–deficient mice. Interestingly, other studies have shown that IL-6 expression is itself induced by IL-17A and reduced by blockade of IL-17A signaling, suggesting an auto-amplification loop where the proinflammatory effects of IL-6 are enhanced by activated Th17 cells. These studies highlight an important proinflammatory role for T cells, especially the Th17 subset, in vascular inflammation and dissection.
Our flow cytometry data suggest that CD4+ T cells are a primary source of IL-17A in aortic tissue, but there is a component of IL-17A that comes from CD4-negative cell types (Figure 1B). Although Th17 cells are classically the main producers of IL-17A,41 other cells, such as γδ T cells,42 lymphoid tissue inducer–like cells,43 and invariant natural killer T cells,44 have also been shown to produce IL-17A. It is possible that Ang II stimulates IL-17A production in these cell types, in addition to CD4+ cells. Further work will be required to determine cell-specific contribution to IL-17A production and formation of aortic dissection.

Monocyte/macrophage recruitment and differentiation play key pathogenic roles in Ang II–induced aortic aneurysms.45 Discussed above, IL-17A contributes to inflammatory processes by promoting monocyte chemotaxis, adhesion, and migration. Indeed, reduced monocyte recruitment is seen on neutralization of IL-17A (Figure 3E). It has been recently reported that IL-17 induces monocyte migration partially through monocytic chemotactic protein-1 induction, consistent with our results of reduced monocytic chemotactic protein-1 expression in IL-17A−/− mice (Figure 4).46,47 IL-17A treatment of aorta from atherosclerotic mice promoted aortic chemokine (C-X-C motif) ligand 1 expression and monocyte adhesion.24 Together, these results highlight an important role of Th17/IL-17A in the pathogenesis of aortic inflammation by promoting cytokine production and monocyte recruitment.

Besides vascular inflammation, hypertension is another well-established propathogenic factor for many cardiovascular diseases, including aortic dissections. Furthermore, we and others have previously shown that 2500 ng/kg per minute dose of Ang II administered subcutaneously by osmotic mini-pump results in hypertension.27 Many laboratories have also demonstrated that infusion of a lower dose of Ang II (1000 ng/kg per minute) than that used in our studies promotes abdominal aortic aneurysms, independent of hypertension in hypercholesterolemic mice.14,48 In this study, we examined the pressor effects of Ang II on IL-6 knockout mice. Interestingly, although Lee et al28 reported that IL-6 mediates the pressor effect of Ang II, we observed a pressor effect in IL-6−/−, which was not statistically different from that of WT mice (Figure II in the online-only Data Supplement). These differences may be a result of strain effects or differences in Ang II dosing. Nevertheless, the

![Figure 6. Interleukin-17A (IL-17A)–positive cell accumulation was observed in patients with thoracic aortic aneurysms and dissections (TAAD). In thoracic aortic samples from patients with transforming growth factor (TGF)−β receptor mutation (TGFβR2 R460C), IL-17A was detected by immunofluorescence microscopy. Positive staining is shown in green, and counterstaining with 4',6-diamidino-2-phenylindole (DAPI) is shown in blue. Representative images of aortic sections from control patients (A–C) and patients with TGFβR2 mutations (D–F) are shown. G, Quantification of IL-17A–positive cells in human aortic samples. IL-17A–positive cells per visual field were counted under a microscope at ×200 magnification. White bar, control patients; black bar, patients with TGFβR2 mutations and type A dissection (n=3 in each group; **P<0.01). H, Quantitative reverse transcription polymerase chain reaction analysis of human interleukin (hIL)-17 mRNA normalized to GAPDH. Fold change of hIL-17 mRNA in TAAD patients relative to control patients is presented.]

![Figure 7. Angiotensin II (Ang II) induction of dissections is independent of systolic blood pressure (BP). Wild-type (WT) mice were infused with Ang II for 7 days in the absence or presence of hydralazine. A, Baseline and post–Ang II infusion systolic BPs were measured using the tail-cuff method. Black bars, Ang II–treated WT; gray bars, Ang II– and hydralazine-treated WT (n= 9 to 11 mice per group; **P<0.01). B, The percent of dissections in each group was determined at the end of the study. ns indicates no significance.]

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finding that Ang II induced similar pressor responses suggests that the permissive role of IL-6 in aortic dissections is largely independent of a blunted pressor effect.

Our study expands our understanding of the inflammatory process in aortic dissections by identifying Th17 cells as a central coordinator of aortic inflammation. Because IL-17A acts on vascular tissue and induces the production of proinflammatory cytokines and chemokines\(^{23,24,46}\) and reactive oxygen species,\(^{25}\) we hypothesized that IL-17A–expressing Th17 cells may mediate recruitment of leukocytes to sites of inflammation in the aorta. Vascular cells, including endothelial cells,\(^{49}\) smooth muscle cells,\(^{25}\) and monocytes,\(^{22}\) express IL-17RA, the major component of the receptor complex for IL-17A and IL-17F.\(^{50}\) Recent in vitro and in vivo studies suggest an important role of IL-17A in mediating monocyte chemotaxis in different systems.\(^{22,47}\) Patients with rheumatoid arthritis who were treated with IL-17A antibody showed inhibited monocyte chemotaxis.\(^{46}\) LDL receptor deficient (Ldlr\(^{-/-}\)) mice with IL-17R signaling disruption in bone marrow–derived cells resulted in reduced IL-6 production and attenuated atherosclerosis.\(^{25}\) Apolipoprotein E-deficient mice with IL-17RA deficiency demonstrated decreased production of proinflammatory cytokines/chemokine and reduced recruitment of macrophages, T cells, and neutrophils.\(^{22}\) Consistent with these findings, our results indicate that abnormalities in Th17 activation caused by IL-17A deficiency or IL-6 deficiency lead to a reduction in macrophage recruitment and cytokine/chemokine expression in the wall of the aorta.

Previous work has shown that IL-17A–deficient mice have a blunted long-term pressor response to Ang II infusion.\(^{20}\) In our studies, we surprisingly found IL17 NAb did not affect resting or pressor responses to Ang II; however, the IL-17A\(^{-/-}\) mice had a reduced resting systolic blood pressure and a blunted Ang II pressor response. These divergent observations on the pressor responses may suggest that there may be developmental role of IL17A on vascular tone in IL17\(^{-/-}\) mice that is not apparent with short-term IL17 neutralization. Furthermore, the divergent observations on the pressor responses between IL17A\(^{-/-}\) and IL-6\(^{-/-}\) background may suggest that there may be compensation for IL-6 deficiency via other IL-6 superfamily of cytokines (interleukin-11, leukemia inhibitory factor, oncostatin-M, cardiotropin, and others) that sustain IL-17 cells and the pressor response.

The TGFBR2 R460C mutation is an activating receptor mutation, producing both tonic TGFβ pathway stimulation\(^{51}\) and lymphocytic inflammation.\(^{3}\) This tonic TGFβ signaling is shared by the well-established Marfan FBN1 mutation, a mutation that releases latent TGFβ from the extracellular matrix. Currently, there is evidence that TGFβ and Ang II cross talk plays an important role in vascular pathology. For example, the pathology induced by TGFβ signaling in Marfan disease is significantly attenuated by Ang II antagonism.\(^{23}\) We, therefore, suspect that pathology in patients with TGFBR2 R460C mutation may be, in part, the consequence of Ang II–IL6 signaling. Our findings for Th17 cell accumulation in these patients suggest that investigation of the Ang II–IL6–Th17 pathway may be further warranted.

In summary, our data suggest that the Th17–IL-17 axis, which is regulated by IL-6–STAT3 signaling, is an important effector arm of Ang II–mediated vascular inflammation. It functions upstream of monocyte/macrophage activation and is independent of Ang II pressor response. Finally, the data indicate that the Th17–IL-17 pathway has direct correlates in human aortic aneurysms and dissections.

**Acknowledgments**

We acknowledge Dr. Heidi Spratt for professional statistical analysis of data, the University of Texas Medical Branch (UTMB) Histopathology and Flow Cytometry Core Facility at UTMB, Dr. Stefan Serabyn for synthesizing angiotensin II and penetratin-STAT3 inhibitory peptide, and Dr. Ken Fujise for providing us access to the CODA blood pressure apparatus.

**Sources of Funding**

This work was supported by the National Institutes of Health (P50 HL083794 to A.R. Brasier and D.M. Milewicz, HL70925 and UL1TR000071 to A.R. Brasier, DK079053 to R.G. Tilton) and the Ted Nash Long Life Foundation to A.R. Brasier.

**Disclosures**

None.

**References**

Angiotensin II is a potent activator of inflammatory signaling in vascular tissue, leading to aortic dissections. Previous work has shown that interleukin-6 is an important effector arm for angiotensin II–induced monocyte/macrophage recruitment and vascular disease. We show here that interleukin-6 acts as a key signaling pathway, inducing the formation and recruitment of T-helper (Th) 17 using mice genetically deficient in interleukin-17 and administration of neutralizing antibody to interleukin-17. Furthermore, induction of the Th17 axis requires the signal transducer and activator of transcription-3 because administration of a cell-permeant inhibitor of signal transducer and activator of transcription-3 blocks Th17 formation and aortic dissections. Correlations of Th17 activity are identified in human ascending aortic dissections. These data suggest that one of the actions of interleukin-6 signaling is to modulate adaptive immunity through specific Th subsets. These findings have broad implications for the diagnosis and treatment of aortic dissections.
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*Arterioscler Thromb Vasc Biol.* 2013;33:1612-1621; originally published online May 16, 2013; doi: 10.1161/ATVBAHA.112.301049

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplementary Figure I. Aortic pathology of Ang II infusion in C57BL/6 mice. Ang II-treated mice developed features of aortic remodeling as early as day 3 and continued over the 14-day infusion. As previously demonstrated, moderate early (2-7 days) aortic dilatation of up to 30% increase in aortic diameter was observed in all mice. Effects of dilation in ~65% of the Ang II-treated mice (as shown in (A) returned to normal level at later stage of infusion (8-14 days). Around 35% of the Ang II-infused mice developed areas of focal hemorrhages, indicating aortic dissection (as shown in B). Aortic cross sections in the suprarenal abdominal region clearly showed Ang II–induced adventitial thickening in all animals (A and B) and blood-filled false lumen formation located in the tunica adventitia in 35% of the Ang II-treated mice (B). Scale bar: 50 µM. (C) Rarely, aortic dissection occurred in the thoracic aorta and extended to the abdominal aorta. Cross-section of the dissected aorta showed medial breaks (black arrows), adventitial remodeling (blue arrows) and presence of a hematoma (asterisk). Larger images were captured at 200x and smaller images were captured at 400x.
Supplementary Figure II. Effect of IL-6 deficiency on Ang II induced hypertension. Systolic blood pressure was measured via a non-invasive tail-cuff method in WT and IL-6-deficient mice at baseline and 7 day of Ang II infusion as indicated. n=10 mice per group. *, p<0.05; ns, no significance.
Supplementary Figure III. Dose response curve of IL-6 stimulated STAT3 transcription in presence of penetratin-STAT3 inhibitor peptide (PSTAT3ip). Transiently transfected HepG2 cells were stimulated with IL-6 (8 ng/ml) or left untreated for 24 h with indicated doses of PSTAT3ip before simultaneous harvest and reporter gene assay. The reporter gene was the multimerized human acute phase response element driving luciferase [(hAPRE1)$_5$-LUC]. Data are presented as normalized luciferase units.
**Animal care and use:** All animal experiments were approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee. Mice were housed in the UTMB Animal Resource Center in accordance with the NIH Guidelines for the Care and Use of Animals in Research. Male C57BL/6J wild-type (WT) and IL-6/- mice (obtained as breeding pairs and bred in-house; C57BL/6J background) were purchased from Jackson Laboratory (Bar Harbor, Me., USA); IL-17/- mice were generated as described in Nakae et al.¹ and back-crossed into the C57BL/6J background. They were generously provided by David G Harrison, M.D. from Emory University. Mice were maintained on a 12-hour light/dark cycle and were allowed food and water ad libitum. In all experiments, age-matched mice (4 to 10 month-old) were used unless indicated otherwise. For Ang II infusion, anesthetized mice were implanted with subcutaneous Alzet osmotic minipumps (Alzet Duract Corp.) delivering either saline (sham) or Ang II (synthesized by the University of Texas Medical Branch Peptide Synthesis Core) at 2,500 ng/kg/min for 10 or 14 days. Ang II-infused mice were treated with 100 µg/mouse i.p. anti-IL-17 (MAB421; R&D Systems) or IgG2A isotype control antibody (MAB006; R&D Systems) at days 0, 4, 8, 12 after starting Ang II infusion.²³

**Ultrasonography:** During Ang II infusion, animals were imaged every 3-5 days with noninvasive sonographic techniques. Serial ultrasound imaging provided visualization of the progression of vessel dilatation and presence of intramural hematomas. Mice were sedated with 1 % inhaled isoflurane (Baxter Healthcare Corporation, Deerfield, IL, USA) delivered via nose cone and were positioned supine and imaged using a Vevo 770 ultrasound system for small animals (Visualsonics). Abdominal aortas were imaged by two masked investigators in the both transverse and longitudinal axis view with 704 and 707B transducers. Measurements were obtained in triplicate at the level of suprarenal aorta.

**Histological Analysis:** Ketamine-anesthetized mice were perfused with PBS via the left ventricle to remove blood from tissue. The entire aorta was excised and placed in sterile PBS. Peri-adventitial fat was removed under scope magnification. For histological staining, aortas were fixed in 4 % formalin, dehydrated, and then embedded in paraffin. Aortic tissue was cut at 6 µm serial sections and stained with Movat staining kit (Market Lab Inc) or elastin staining kit (Sigma-Aldrich) according to manufacturers' protocols. Presence of aortic dissection featured by intramural hematoma was identified under dissecting microscope and was recorded. Aortic cell numbers were measured on at least 5 random visual fields on sections from 3 different animals under microscope by two masked investigators.

**Aortic explant and cytokine analysis:** Briefly, following euthanasia and after vascular perfusion with PBS buffer, the entire aorta was dissected and immediately placed in 0.5 ml DMEM medium (Cellgro) containing 1× ITS (Sigma-Aldrich) and 0.1 % BSA (Sigma-Aldrich) and incubated in a tissue culture hood at 37°C for 4 hrs. Culture medium was frozen at -80°C until assayed for IL-17A and MCP-1 using a multiplex ELISA kit (Lincoplex/Millipore mouse or human adipocyte/cytokine panel) according to the manufacturers' instructions. Cytokine concentrations were determined relative to recombinant standards.

**Aortic digestion and flow cytometry:** Aortas were dissected, cleaned, minced and placed in 1 ml digestion solution containing 0.6 units/ml Liberase Blendzyme 3 (Roche) and 50 µg/ml porcine pancreatic elastase (Sigma-Aldrich) in a base solution of DMEM. Aortic tissue was digested at 37°C with agitation for 1-2 h. After digestion, cells were filtered using a 70 µM cell strainer (BD Biosciences) and washed in FACS buffer (0.5 % BSA and 0.02 % NaN3 in DMEM) at 300 g for 5 min. When necessary, red blood cells were removed using RBC lysis solution (Qiagen). For IL-17A staining, cells were digested and incubated in the presence of PMA (50 ng/mL), ionomycin (1 µmol/L) and brefeldin A (5 µg/mL) for total of 4 hours. For cell membrane antigen staining, murine Fc receptors were blocked using antibodies against mouse CD16/32.

1. Nakae et al.
2. et al.
3. Nakae et al.
antigens (eBioscience) for 10 min on ice. Cells were then incubated with fluorochrome-conjugated primary antibodies anti-CD11b (M1/70; eBiosciences) and anti-CD4 (GK1.5; eBiosciences) for 30-45 min on ice in the dark. Corresponding isotype control antibodies were added at the same concentrations as the antibodies of interest. Cells were then fixed and permeabilized in fixation/permeabilization solution (eBiosciences) for 30 min at 4°C. After washing, cells were stained with fluorochrome-conjugated anti-IL-17A (eBio17B7; eBiosciences) and anti-RORγT (AFKJ5.9; eBiosciences) for 30 min on ice. After incubation, samples were washed 3 times in FACS buffer, centrifuged at 500 g for 5 min, fixed in 0.5 % PFA, and analyzed by FACSCanto (BD Biosciences). Debris and dead cells, defined by forward scatter, were excluded from analysis. Data were analyzed with FlowJo software.

**IHC and immunofluorescence:** Formalin-fixed, paraffin-embedded sections from suprarenal aortas were rehydrated using serial concentrations of ethanol. When necessary, antigen retrieval was performed with antigen unmasking solution (Vector Laboratories) according to manufacturer’s instructions. Sections were blocked using 0.1 % Triton-X, 5 % normal serum of the species producing the secondary antibodies for 1 hr at room temperature. Incubations with primary antibodies were performed at the following concentrations overnight at 4°C: 1:200 rat anti-macrophage (MOMA-2; Abcam), and 1:100 rabbit anti-IL-17A (E-19; Santa Cruz). After washing, secondary antibodies were added at a dilution of 1:500 for 1 hr at room temperature. For immunofluorescence, secondary antibodies were highly cross-absorbed Alexa Fluor 488-conjugated secondary Ab (Invitrogen), and slides were counterstained with DAPI (Vector Laboratories). For IHC, biotinylated goat anti-rat or anti-rabbit antibodies were used as secondary antibodies, followed by staining with ABC kits per manufacturer’s instruction (Vectastain; Vector Laboratories). DAB (Vector Laboratories) was used as substrate and slides were then counterstained with Hematoxylin. For human aortic tissue samples, discarded aortic tissue and de-identified clinical data from patients with MFS and TGFBRII mutations were obtained with informed consent under a protocol approved by the University of Texas Health Science Center Institutional Review Board. PFA-fixed, paraffin-embedded sections were taken from the ascending aorta above the sinuses of Valsalva. Sections were stained using a 1:100 dilution of goat anti-IL17A (E-19; Santa Cruz). Secondary antibody-only controls were used to determine staining specificity.

**Quantitative real-time PCR (Q-RT-PCR):** Aortas were freshly isolated and frozen at -80°C for later use. Individual frozen aortas were pulverized in liquid nitrogen inside plastic pouches (4.5 mls thick; Kapak SealPAK), resuspended in TriReagent (Sigma-Aldrich), and further homogenized on ice in glass dounce homogenizers (Wheaton). RNA was extracted according to the manufacturer’s instructions of TriReagent. RNA then was quantified by Nanodrop (Thermo Scientific) and samples were included when the 260/280 nm ratio was >1.6. Five μg RNA was reverse transcribed using Superscript III (Invitrogen) according to provided directions. Real-time PCR reactions were performed in triplicate using 1 μl of resulting cDNA per 20 μl reaction volume containing iQ SYBR Green Supermix (Bio-Rad). The housekeeping gene GAPDH was used as an internal control. Primers were purchased from SABioscience for the following RNAs: mouse ccl2 (PPM03151F), mouse il17a (PPM03023A) and mouse SOCS3 (PPM05161B). PCR was performed on the CFX96 system (Bio-Rad) according to preset protocol. mRNA was analyzed by the ΔΔCt method.

**Systolic blood pressure measurement:** Systolic blood pressure (BP) was measured via a non-invasive tail-cuff method (Kent Scientific) one week prior to pump implantation (baseline) and for at least three consecutive days during angiotensin II infusion. To determine the role of blood pressure on development of aortic dissections, mice were started on hydralazine in drinking water at 3 days prior to Ang II pump implantation and maintained on hydralazine-water
during the 7 days of Ang II infusion. Since hydralazine is a very potent vasodilator and it suppressed the BP significantly, we varied the hydralazine dose between 20 mg/L and 10 mg/L to maintain the BP close to baseline. From day -3 to day 1, mice were kept on 10 mg/L hydralazine; from day 1 to 4 on 20 mg/L hydralazine and from day 5 to 6 on 15 mg/L hydralazine. BP was measured on each day except day 5. Measurements are reported as the mean ± SEM.

**Human Sample Collection:** The Institutional Review Board at the University of Texas Health Science Center at Houston approved this study. Informed consent was obtained from patients with TGFBR2 mutation. Control aortic tissues were obtained from the International Institute for the Advancement of Medicine from individuals who died of non-vascular disease and age- and gender-matched to the patients as closely as possible.

**Data analysis:** Data are reported as mean ± SEM. Differences between 2 groups were analyzed by Student’s t test (2-tail, assuming unequal variances). One-way ANOVA was performed when comparing multiple groups followed by Tukey’s post-hoc test to determine significance. BP measurements were analyzed by repeated measures ANOVA and pair-wise comparison was performed with Tukey’s. Fisher’s exact test was performed on the data for aortic dissections to determine significance at different time points because of small group size. In all cases, p < 0.05 was considered significant.

**References**


