

Interferon- γ and the Interferon-Inducible Chemokine CXCL10 Protect Against Aneurysm Formation and Rupture

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Background—Vascular disease can manifest as stenotic plaques or ectatic aneurysms, although the mechanisms culminating in these divergent disease manifestations remain poorly understood. T-helper type 1 cytokines, including interferon- γ and CXCL10, have been strongly implicated in atherosclerotic plaque development.

Methods and Results—Here, we specifically examined their role in the formation of abdominal aortic aneurysms in the angiotensin II–induced murine model. Unexpectedly, we found increased suprarenal aortic diameters, abdominal aortic aneurysm incidence, and aneurysmal death in apolipoprotein E– and interferon- γ –deficient (*Apoe*^{-/-}/*Ifng*^{-/-}) mice compared with *Apoe*^{-/-} controls, although atherosclerotic luminal plaque formation was attenuated. The interferon- γ –inducible T-cell chemoattractant CXCL10 was highly induced by angiotensin II infusion in *Apoe*^{-/-} mice, but this induction was markedly attenuated in *Apoe*^{-/-}/*Ifng*^{-/-} mice. *Apoe*^{-/-}/*Cxcl10*^{-/-} mice had decreased luminal plaque but also increased aortic size, worse morphological grades of aneurysms, and a higher incidence of death due to aortic rupture than *Apoe*^{-/-} controls. Furthermore, abdominal aortic aneurysms in *Apoe*^{-/-}/*Cxcl10*^{-/-} mice were enriched for non-T-helper type 1–related signals, including transforming growth factor- β 1. Treatment of *Apoe*^{-/-}/*Cxcl10*^{-/-} mice with anti-transforming growth factor- β neutralizing antibody diminished angiotensin II–induced aortic dilation.

Conclusions—The present study defines a novel pathway in which interferon- γ and its effector, CXCL10, contribute to divergent pathways in abdominal aortic aneurysm versus plaque formation, inhibiting the former pathology but promoting the latter. Thus, efforts to develop antiinflammatory strategies for atherosclerosis must carefully consider potential effects on all manifestations of vascular disease. (*Circulation*. 2009;119:426-435.)

Key Words: aneurysm ■ atherosclerosis ■ immunology ■ inflammation ■ mice, knockout

The pathophysiological mechanisms that lead to stenotic plaques versus aneurysms, 2 distinct vascular lesions, remain poorly understood. Clinically, abdominal aortic aneurysms (AAAs) are more strongly correlated with a family history¹ and smoking² than are coronary stenoses. Diabetes mellitus, a strong risk factor for coronary plaques, actually protects against AAA formation in population-based studies.^{3,4}

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Chronic inflammation of the vascular wall is believed to contribute to both manifestations of arterial pathology.^{5,6} Atherosclerotic plaques are marked primarily by intimal infiltration of macrophages and T cells, at least at earlier disease stages. In contrast, aneurysmal segments are charac-

terized by macrophage, T-cell, and B-cell accumulation primarily in the media and adventitia at all stages of disease evolution.⁶ Human atherosclerotic stenoses specifically express mediators characteristic of a T-helper type 1 (Th1) immune response, including interferon (IFN)- γ , as well as the IFN- γ –inducible T-cell chemoattractant IP-10 (IFN- γ –inducible Protein of 10 kDa, now known as CXCL10).⁷ Characterization of mediators expressed by AAAs, however, has been inconsistent, which is potentially attributable to the different disease stages and anatomic areas studied. Tang et al⁸ found transmural accumulation of IFN- γ –producing T cells correlated with aortic dilation, although other groups have described a Th2-predominant immune response prevailing in human AAA.^{6,9}

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CD4⁺ T-cell deletion protects against AAA formation in a calcium chloride-induced AAA model in mice¹⁰; however, murine studies to date have not clarified whether adaptive cellular immunity of either the Th1 or Th2 system is detrimental or beneficial in aneurysmal disease. Although the Th1 cytokine IFN- γ contributes to atherosclerotic plaque formation,¹¹ there are conflicting reports on the role of IFN- γ and its receptor in the development of AAAs. IFN- γ deficiency resulted in a modest reduction of disease pathology in the calcium chloride-induced AAA model, whereas IFN- γ infusion restored the severity of the disease.¹⁰ In contrast, IFN- γ receptor deficiency augmented AAA formation in an aortic allograft model of AAA formation.¹² These seemingly contradictory studies may be less surprising in light of an emerging theme of proinflammatory and regulatory interplay of IFN- γ in inflammation and autoimmunity in other disease models, including arthritis¹³ and multiple sclerosis.^{14,15} IFN- γ appears to act as a master upstream regulator that modulates both proinflammatory and antiinflammatory processes depending on the disease stage and disease-specific cytokines.

Complete ablation of IFN- γ signaling, like global T-cell deletion, might thus disturb both the effector and regulatory arms of the immune system, potentially resulting in variable effects on vascular phenotypes. In contrast, a disruption that isolates specific downstream pathways might be particularly informative about the signals that contribute to AAAs. We therefore studied AAA formation both in IFN- γ -deficient mice and in mice deficient in the IFN- γ -inducible T-cell chemokine, CXCL10. These studies define a novel role for CXCL10 in AAA formation and more broadly suggest that cellular immunity may play different roles in 2 distinct manifestations of vascular disease, with important clinical implications.

Methods

Mice

Apolipoprotein E-deficient (*ApoE*^{-/-}) mice, interferon- γ -deficient (*Ifng*^{-/-}) mice (Jackson Laboratory, Bar Harbor, Me), and *Cxcl10*^{-/-} mice¹⁶ were backcrossed 10 times into a C57BL/6J background and interbred to generate the experimental genotypes, which were confirmed by polymerase chain reaction (PCR) genotyping. All mice received a standard laboratory diet (Harlan Teklad, Harlan Laboratories, Indianapolis, Ind). All animal procedures were approved by university animal care protocols at their respective institutions (University of Kentucky and Massachusetts General Hospital).

Infusion of Angiotensin II

Experimental mice were treated with angiotensin (Ang) II (500 or 1000 ng · kg⁻¹ · min⁻¹ as indicated; Sigma Chemical Co, St Louis, Mo) or normal saline via ALZET model 2004 osmotic pumps (DURECT Corp, Cupertino, Calif) that were implanted subcutaneously as described previously.¹⁷ After 4 weeks of infusion, mice were euthanized for blood collection and aorta harvest.

Lipid Analysis

A total of 0.5 to 1.0 mL of blood was aspirated from experimental mice by right ventricular puncture on euthanasia. Serum cholesterol concentrations were measured by enzymatic colorimetric assay (Wako Chemical Co, Richmond, Va). Lipoprotein cholesterol distribution was determined in individual serum samples (50 μ L) from mice after resolution on a Superose 6 column (GE Healthcare Biosciences, Piscataway, NJ).¹⁸

Blood Pressure Measurements

Systolic blood pressure was measured serially in conscious mice by use of a tail-cuff system (Visitech BP-2000, Visitech Systems Inc, Apex, NC, or Kent Scientific XBP1000, Torrington, Ct) during 3 training sessions at baseline and 4 weeks after placement of the Ang II pump.

Atherosclerotic Lesion Analysis

The size of atherosclerotic lesions was quantified with Image-Pro software (Media Cybernetics, Bethesda, Md).¹⁹ Discernable lesions on the luminal surface of the aorta were quantified from the aortic arch to the last intercostal artery branch in the thorax.

Quantification of Aneurysms and Morphometric Analysis

Aortic diameters and AAA incidence were determined as described previously.¹⁷ The maximum width of abdominal aortas was measured with computerized morphometry. Aneurysm incidence was quantified on the basis of a definition of an external suprarenal aorta width that was increased by 50% or more compared with saline-infused mice. In addition, we used a previously described classification system^{20,21} to categorize the morphological grade of the aneurysms: no aneurysm, type I (suprarenal dilation without thrombus), type II (remodeled suprarenal dilation with thrombus), type III (multiple aneurysms, including thoracic aneurysms and dissections), and death due to aneurysmal rupture. On necropsies of unexpected deaths, death due to rupture of an aneurysm was qualified by the presence of a retroperitoneal hematoma in addition to an AAA and/or the presence of a thoracic hematoma in addition to a thoracic aortic aneurysm or dissection. Measurements were conducted by 2 trained, independent observers blinded to genotype and treatment conditions.

Antibodies and Immunohistochemistry of Murine Lesions

For harvesting suprarenal aortas for immunohistochemistry, mice were perfused via left ventricular puncture with 4% paraformaldehyde under physiological pressure, and aortic segments were embedded in optimal cutting temperature compound (Tissue-Tek, Torrance, Calif). Serial 10- μ m sections were cut surrounding the cross section of widest diameter, and every fifth section was stained. Tissues were stained with hematoxylin and eosin (Fisher Scientific, Hampton, NH) for morphology. Immunohistochemistry was performed with antibodies to identify macrophages (Mac3, 1:20, BD Biosciences, San Jose, Calif), CD4⁺ T cells (CD4, 1:50, BD Pharmingen, San Jose, Calif), Thy-1.2⁺ T cells (CD90.2; BD Pharmingen), and vascular smooth muscle cells (α -actin, 1:100, Abcam, Cambridge, Mass), as well as the chemokine CXCL10 (1:100, R&D Systems, Minneapolis, Minn). Negative controls were prepared with substitution of the primary antibody with an isotype-matched control antibody. Appropriate biotinylated secondary antibodies were used, followed by detection with an ABC Development Kit (Vector Laboratories, Burlingame, Calif) and color development with DAB (Chemicon, Temecula, Calif) or AEC (Dako, Glostrup, Denmark). High-powered fields of stained sections were captured randomly with a SPOT digitizer (Diagnostic Instruments, Sterling Heights, Mich), and quantitative analysis was performed with IPLab software (BD Biosciences, Rockville, Md) by a single observer blinded to genotype and condition. Areas that were positively stained were divided by total lesion area to account for variability in lesion size.

To assess transforming growth factor (TGF)- β activity, we used a polyclonal antibody specific for the free and active form of TGF- β .^{22,23} To test the role of TGF- β in AAA formation in *ApoE*^{-/-} and *ApoE*^{-/-}/*Cxcl10*^{-/-} mice, mice were injected intraperitoneally 1 day before placement of the Ang II pump and 1 day after pump placement with a pan-specific neutralizing antibody against TGF- β 1, - β 2, and - β 3 (1 mg/kg; R&D Systems)²⁴ or isotype control.

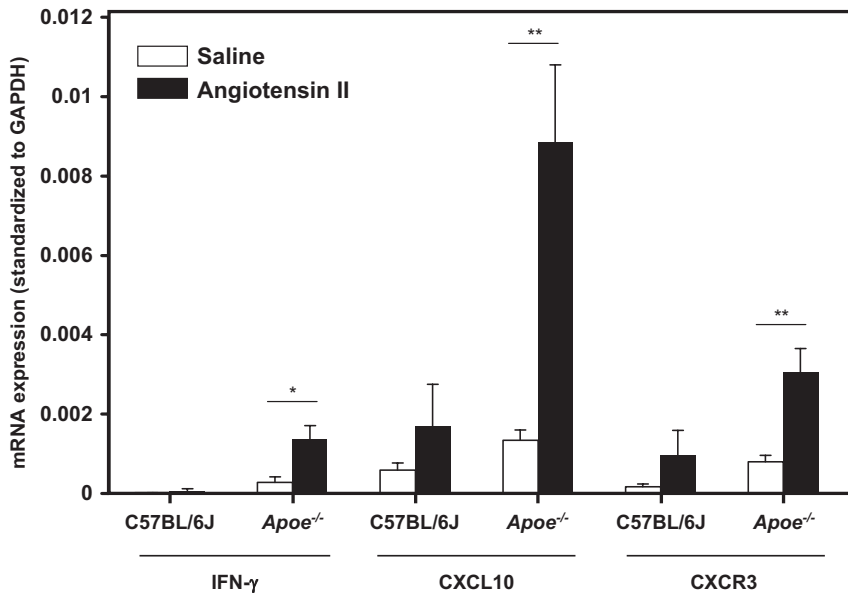


Figure 1. Ang II-treated *Apoe*^{-/-} mice have increased levels of Th1-associated cytokines. Quantitative real-time PCR of aortic segments from C57BL/6J and *Apoe*^{-/-} mice infused with Ang II (1000 ng · kg⁻¹ · min⁻¹; closed bars) vs saline (open bars). Data are standardized to GAPDH values. Th1-related cytokines: IFN- γ , CXCL10, and its receptor, CXCR3 (n \geq 6 per condition; **P*<0.05, ***P*<0.01).

RNA Isolation and Quantitative PCR

Total RNA was isolated from suprarenal aortas from mice perfused with buffer RLT (Applied Biosystems, Foster City, Calif) by mechanical homogenization with a roto-stator and RNeasy columns (Qiagen, Valencia, Calif).^{25,26} After DNaseI digestion, equivalent amounts of RNA from each sample were reverse transcribed with TaqMan reverse-transcription reagents, including oligo (dT)₁₅, random hexamers, and MultiScribe reverse transcriptase (Applied Biosystems). Quantitative reverse-transcription PCRs were conducted with the Multiplex qPCR system as described previously.^{25,26} Amplification plots were analyzed with MX4000 software, version 3.0. Gene expression was normalized to GAPDH or β -actin as an internal control.

Vascular Contractility Experiments

Abdominal aortas were removed and cleaned of adventitia while immersed in Krebs buffer. Measurement of force contraction was performed at 37°C as described previously.²⁷ Briefly, abdominal aortic segments (3 mm) were mounted by passing 2 tungsten wires through the arterial lumen and then were bathed in wells (Kent Scientific) filled with Krebs-Henseleit solution. Tension (1 g) was maintained continuously and recorded with a Tissue Force Analyzer 410 (Micro-Med Inc, Louisville, Ky). After 30 minutes of equilibration, abdominal aortic segments were immersed in potassium chloride (80 mmol/L) for 3 minutes. After washout, contractile activity was determined during incubation with phenylephrine (Sigma; 1 nmol/L to 1 mmol/L).

Statistical Analysis

Data were analyzed by 2-way ANOVA, Student *t* test, χ^2 , or Mann-Whitney rank sum with SigmaStat software (Aspire Software International, Ashburn, Va). Data were tested by use of parametric or nonparametric post hoc analysis, and multiple comparisons were performed with Tukey or Holm-Sidak tests, as appropriate. Percentage incidence of AAAs was analyzed by Fisher exact test. *P*<0.05 was considered statistically significant. All data are shown as mean \pm SEM.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Increased IFN- γ and CXCL10 mRNA Expression in Ang II-Infused *Apoe*^{-/-} Mice

Mice were placed on a normal diet for 20 weeks and then treated with Ang II (1000 ng · kg⁻¹ · min⁻¹) or vehicle control

via ALZET osmotic pumps for 4 weeks, which generates AAA in a reproducible manner and mimics many of the important features of the human disease.^{17,28} Ang II infusion did not generate any AAAs in control C57BL/6J mice (data not shown). Quantitative real-time PCR analysis of suprarenal aortic segments revealed significantly higher expression of the Th1-associated cytokines IFN- γ and CXCL10 (as well as its receptor, CXCR3) in the Ang II-exposed *Apoe*^{-/-} mice than in the saline-treated controls (Figure 1). C57BL/6J mice had similar trends for CXCL10 and CXCR3, but overall expression levels were markedly lower.

IFN- γ Deficiency Has Contrasting Effects on Ang II-Induced AAA and Atherosclerosis Formation

To address the role of IFN- γ in AAA formation, we infused 20-week-old *Apoe*^{-/-} mice that were either *Ifng*^{+/+} or *Ifng*^{-/-} with Ang II (1000 ng · kg⁻¹ · min⁻¹) or saline. Unexpectedly, 50% of the *Apoe*^{-/-}/*Ifng*^{-/-} mice died due to rupture of the abdominal aorta within 2 to 10 days of Ang II infusion (Figure 2; online-only Data Supplement Figure 1). In contrast, no deaths due to aneurysmal rupture occurred in the *Apoe*^{-/-}/*Ifng*^{+/+} group. Because of the high incidence of mortality in *Apoe*^{-/-}/*Ifng*^{-/-} mice, the infusion of Ang II was decreased to 500 ng · kg⁻¹ · min⁻¹ for subsequent studies of this genotype.

Total serum cholesterol concentrations were not altered by IFN- γ deficiency in either saline- or Ang II-infused mice (Table 1). IFN- γ deficiency did not alter systolic blood pressure before or during infusion of Ang II (data not shown). IFN- γ deficiency led to increased body weight in the *Apoe*^{-/-} background, although body weight was unaffected by Ang II infusion (Table 1). Adiponectin levels were not different between *Apoe*^{-/-}/*Ifng*^{-/-} mice and *Apoe*^{-/-} mice (n=9 of each; *P*=0.67), thus excluding one potentially confounding modifier of vascular pathology.²⁹

With the lower-dose infusion of Ang II (500 ng · kg⁻¹ · min⁻¹), we observed significantly increased suprarenal aortic diameters in *Apoe*^{-/-}/*Ifng*^{-/-} mice compared with *Apoe*^{-/-} controls (Figure 3A; *P*<0.05). IFN- γ -deficient mice also had

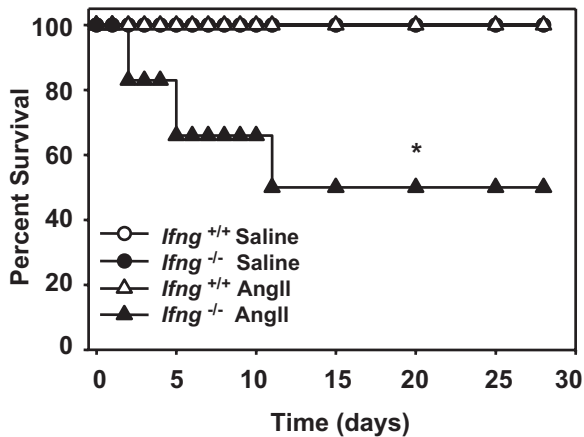


Figure 2. IFN- γ deficiency increased mortality due to aneurysmal rupture in Ang II-infused *Apoe*^{-/-} mice. Ang II (1000 ng · kg⁻¹ · min⁻¹) or saline was infused for 28 days via ALZET minipumps implanted subcutaneously into *Apoe*^{-/-}/*Ifng*^{+/+} or *Apoe*^{-/-}/*Ifng*^{-/-} mice. Data represent percentage survival in *Apoe*^{-/-}/*Ifng*^{+/+} (open symbols, n=11) or *Apoe*^{-/-}/*Ifng*^{-/-} (closed symbols, n=11) mice after infusion with saline (circles, n=5 mice/group) or Ang II (triangles, n=6 mice/group). **P*<0.05 for Ang II-infused *Apoe*^{-/-}/*Ifng*^{+/+} compared with *Apoe*^{-/-}/*Ifng*^{-/-}. No deaths occurred in either of the saline-treated groups or in the Ang II-infused *Ifng*^{+/+} mice, and thus, the symbols representing these groups are superimposed.

a concordant increase in the incidence of AAAs compared with *Ifng*^{+/+} controls (18 [62%] of 29 *Apoe*^{-/-}/*Ifng*^{-/-} mice versus 8 [32%] of 25 *Apoe*^{-/-} mice, *P*<0.05; Figure 3B). No AAAs were present in saline-infused control mice. Of note, IFN- γ deficiency did not produce any discernible differences in medial area or thickness of the suprarenal aorta (online-only Data Supplement Figures IIA and IIB), which rules out preexisting vascular differences between these genotypes that might predispose *Apoe*^{-/-}/*Ifng*^{-/-} mice to AAA. Furthermore, in functional assays, IFN- γ deficiency did not impart any significant differences in the ability of the abdominal aorta to respond to KCl or phenylephrine (online-only Data Supplement Table I). Thus, despite IFN- γ deficiency leading to dramatic differences in aneurysm formation in the suprarenal aorta, this was not associated with discernible structural or functional changes in the vessel wall.

Luminal atherosclerotic lesions were also quantified in mice infused with Ang II or saline. Minimal lesion development was noted in saline-infused mice fed a normal diet

Table 1. Analysis of Baseline Characteristics Between *Apoe*^{-/-} and *Apoe*^{-/-}/*Ifng*^{-/-} Mice

Infusion and IFN- γ Genotype	n	Cholesterol, mg/dL	Body Weight, g
Saline			
+/+	8	328±15	27.1±0.7
-/-	8	318±21	30.8±1.1*
Ang II 500 ng · kg ⁻¹ · min ⁻¹			
+/+	17	286±17	27.9±0.8
-/-	19	327±12	30.5±0.4*

Data represent mean±SEM.

**P*<0.001 for *Apoe*^{-/-}/*Ifng*^{+/+} compared with *Apoe*^{-/-}/*Ifng*^{-/-} mice within saline and Ang II-infused groups.

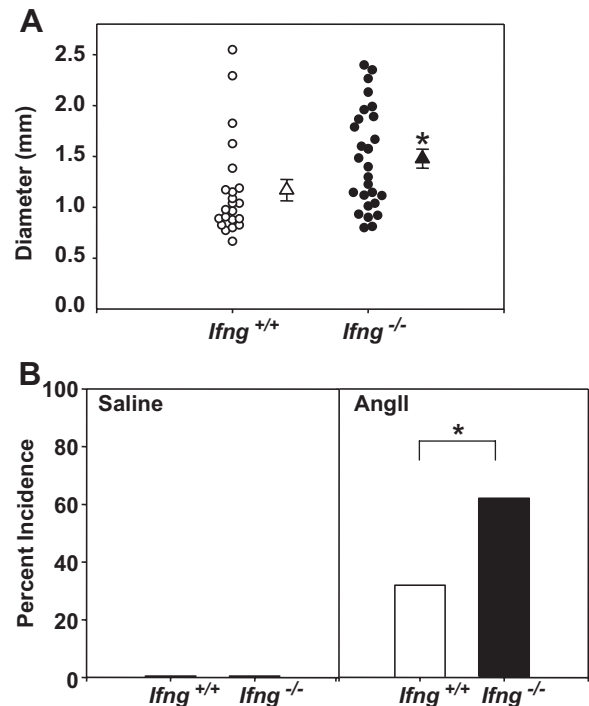


Figure 3. IFN- γ deficiency augmented Ang II-induced AAA formation. A, Circles represent suprarenal aortic diameter in individual mice, and triangles are mean±SEM of *Apoe*^{-/-}/*Ifng*^{+/+} (open symbols) and *Apoe*^{-/-}/*Ifng*^{-/-} (closed symbols) mice infused with Ang II (500 ng · kg⁻¹ · min⁻¹; *Apoe*^{-/-}/*Ifng*^{+/+} n=25; *Apoe*^{-/-}/*Ifng*^{-/-} n=29). Data were analyzed by Mann-Whitney rank sum test. **P*<0.05. B, Data represent percentage incidence of AAAs in *Apoe*^{-/-}/*Ifng*^{+/+} (open bars; saline n=8, Ang II n=25) and *Apoe*^{-/-}/*Ifng*^{-/-} (closed bars; saline n=8, Ang II n=29). Data were analyzed by Fisher exact test. **P*<0.05 for Ang II-infused *Apoe*^{-/-}/*Ifng*^{+/+} compared with *Apoe*^{-/-}/*Ifng*^{-/-} mice.

(Figure 4). Consistent with previous reports, Ang II infusion markedly enhanced atherosclerotic lesion size (Figure 4)^{17,30}; however, as in hyperlipidemia-induced atherosclerosis, Ang II-induced lesion formation was attenuated by IFN- γ deficiency (*P*<0.05). Thus, IFN- γ deficiency had a differential effect on atherosclerotic lesion formation as opposed to AAA formation.

CXCL10 Deficiency Has Contrasting Effects on Ang II-Induced AAA and Atherosclerosis Formation

CXCL10 is an IFN- γ -inducible effector T-cell chemokine that was highly upregulated by Ang II infusion in *Apoe*^{-/-} mice (Figure 1). CXCL10 expression appeared highest in the media and adventitia, which suggests that its role in the recruitment of T cells likely occurred from the adventitia and neovessels and not from the aortic lumen. Consistent with our prior studies,³¹ we observed downregulation of CXCL10 in spleens and vascular lesions of hyperlipidemic, IFN- γ -deficient mice (Figures 5A and 5B). Like *Apoe*^{-/-}/*Ifng*^{-/-} mice,³² CXCL10-deficient mice in the ApoE background on a high-fat diet were also recently found to have a >2-fold reduction in atherosclerotic plaques compared with controls.²⁶ We thus explored the role of CXCL10 in atherosclerotic plaque development and AAA formation in the Ang II model.

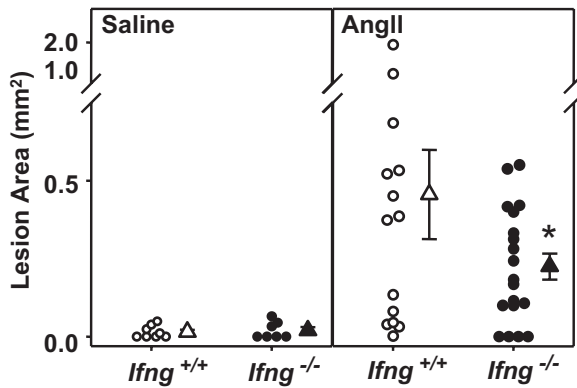


Figure 4. IFN- γ deficiency attenuated Ang II-induced atherosclerosis. Atherosclerotic lesion size was determined on the intimal surface of the aorta extending from the aortic arch to the last intercostal artery branch in the thorax in $Apoe^{-/-}/Ifng^{+/+}$ and $Apoe^{-/-}/Ifng^{-/-}$ mice infused with Ang II ($500 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) or saline for 28 days. Lesion size was determined irrespective of the presence or absence of AAA; only mice that died of AAA rupture were omitted from this analysis. Circles represent measurements from individual mice, and triangles are mean lesion area \pm SEM of $Apoe^{-/-}/Ifng^{+/+}$ (open symbols; saline $n=8$, Ang II $n=14$) and $Apoe^{-/-}/Ifng^{-/-}$ (closed symbols; saline $n=7$, Ang II $n=19$) mice. $*P<0.05$ for Ang II-infused $Apoe^{-/-}/Ifng^{+/+}$ compared with $Apoe^{-/-}/Ifng^{-/-}$ mice. Data were analyzed by 2-way ANOVA.

Twenty-week-old $Apoe^{-/-}$ and $Apoe^{-/-}/Cxcl10^{-/-}$ mice received Ang II ($1000 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) or saline for 28 days. After euthanasia, $Apoe^{-/-}$ and $Apoe^{-/-}/Cxcl10^{-/-}$ mice had similar lipid profiles (Table 2). The Ang II-infused $Apoe^{-/-}/Cxcl10^{-/-}$ mice weighed slightly more than their age-matched $Apoe^{-/-}$ controls (Table 2) but showed no differences in adiponectin levels ($n=7$ of each, $P=0.21$).

CXCL10 deletion protected against atherosclerotic luminal plaque formation in the Ang II model (Figure 5C), consistent with prior results with a high-fat diet.²⁶ Although luminal plaque formation was diminished, $Apoe^{-/-}/Cxcl10^{-/-}$ mice had a significantly higher death rate due to aortic rupture than the $Apoe^{-/-}$ controls in the Ang II-triggered AAA model (42% versus 11%, $P<0.01$; Figure 6A; representative necropsy sample in online-only Data Supplement Figure III). We were interested to find that the rate of rupture in $Apoe^{-/-}/Cxcl10^{-/-}$ mice was comparable to that observed in $Apoe^{-/-}/Ifng^{-/-}$ mice. Concordant with the increased mortality observed in $Apoe^{-/-}/Cxcl10^{-/-}$ double knockouts, we also documented more severe morphological changes throughout the aortas of these mice than in $Apoe^{-/-}$ controls (Figure 6B). Whereas $Apoe^{-/-}$ controls had localized suprarenal AAAs, the $Apoe^{-/-}/Cxcl10^{-/-}$ mice had thoracic aneurysms and hematomas with and without abdominal aneurysms, as well as large aneurysms with spiral dissections. Consistently, the infrarenal aorta had no aneurysmal pathology in either genotype (although occasionally, the aortic segment between the renal arteries was involved in conjunction with a suprarenal AAA), which is in agreement with previous reports.³⁴ We quantified these morphological differences using a previously reported classification grade that accounts for the complexity and multiplicity of the aneurysms.^{20,21} The distribution of grades was different between the 2 genotypes (Figure 6C; $P<0.005$), with the $Apoe^{-/-}/Cxcl10^{-/-}$ mice having signifi-

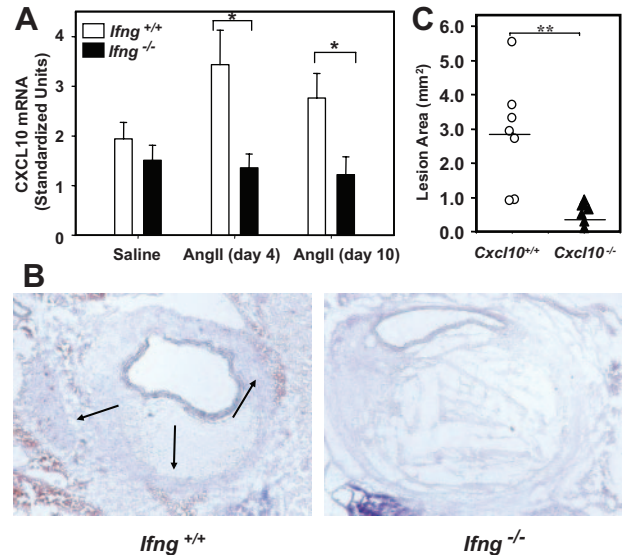


Figure 5. Decreased CXCL10 expression in IFN- γ -deficient mice; decreased atherosclerotic plaque in CXCL10-deficient mice. A, Quantitative real-time PCR of CXCL10 expression in the spleen from $Apoe^{-/-}/Ifng^{+/+}$ (open bars) and $Apoe^{-/-}/Ifng^{-/-}$ (solid bars) mice infused with Ang II ($500 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) for 4 or 10 days vs saline ($n=5$ mice/group, $*P<0.05$). B, Immunohistochemical analysis of CXCL10 in representative lesions from $Apoe^{-/-}/Ifng^{+/+}$ and $Apoe^{-/-}/Ifng^{-/-}$ mice. Arrows point to CXCL10 staining. C, Atherosclerotic lesion size was determined on the intimal surface of the aorta extending from the aortic arch to the last intercostal artery branch in the thorax in $Apoe^{-/-}/Cxcl10^{+/+}$ (circles, $n=7$) and $Apoe^{-/-}/Cxcl10^{-/-}$ (triangles, $n=7$) mice infused with Ang II ($1000 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) for 28 days. Lesion size was determined irrespective of the presence or absence of AAA; only mice that died of AAA rupture were omitted from this analysis. $**P<0.005$ for Ang II-infused $Apoe^{-/-}/Cxcl10^{+/+}$ compared with $Apoe^{-/-}/Cxcl10^{-/-}$ mice. Data were analyzed by 2-way ANOVA.

cantly more grade III aneurysms or death due to rupture of the aorta. Similar to the findings in IFN- γ -deficient mice, we also observed significantly increased suprarenal diameters and suprarenal/thoracic-to-infrarenal aortic area ratios in $Apoe^{-/-}/Cxcl10^{-/-}$ mice compared with $Apoe^{-/-}$ controls (Figure 6D and 6E). Of note, these analyses may underestimate differences between the 2 genotypes, because mortality was substantially increased in $Apoe^{-/-}/Cxcl10^{-/-}$ mice, and therefore, some aneurysms were not incorporated into the analyses represented by Figure 6D and 6E.

Taken together, the $Apoe^{-/-}/Cxcl10^{-/-}$ mice had qualitatively and quantitatively worse aneurysmal disease, with

Table 2. Analysis of Baseline Characteristics Between $Apoe^{-/-}$ and $Apoe^{-/-}/Cxcl10^{-/-}$ Mice

CXCL10 Genotype	n	Cholesterol, mg/dL	Body Weight, g
Saline			
+/+	5	437 \pm 48	24.3 \pm 0.4
-/-	4	510 \pm 63	25.2 \pm 2.5
Ang II 1000 ng \cdot kg ⁻¹ \cdot min ⁻¹			
+/+	30	535 \pm 33	26.6 \pm 0.8
-/-	23	455 \pm 30	29.2 \pm 0.9*

Data represent mean \pm SEM.

* $P<0.05$ for $Apoe^{-/-}/Cxcl10^{+/+}$ compared with $Apoe^{-/-}/Cxcl10^{-/-}$ mice within saline and Ang II-infused groups.

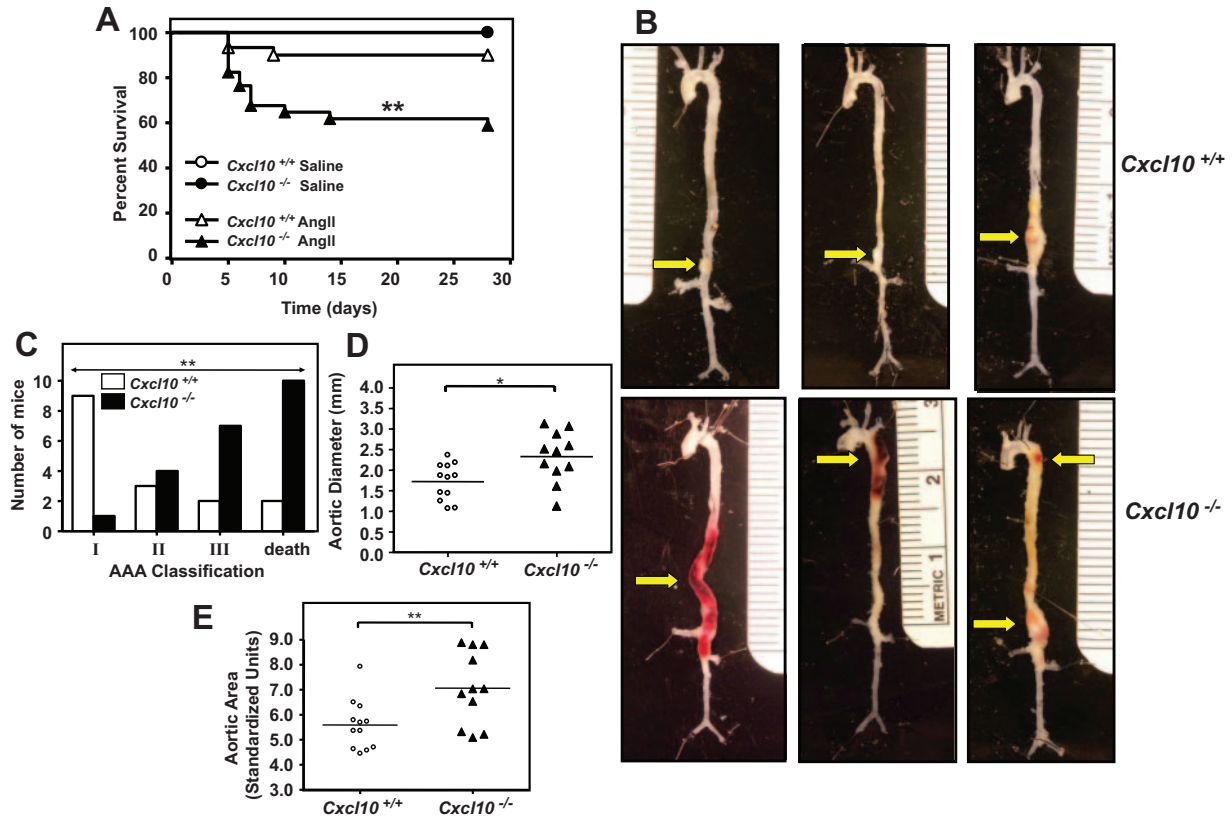


Figure 6. Increased mortality and severity of AAAs in CXCL10-deficient mice. A, Ang II ($1000 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) or saline was infused for 28 days into $Apoe^{-/-}/Cxcl10^{+/+}$ or $Apoe^{-/-}/Cxcl10^{-/-}$ mice. Data represent percentage survival in $Apoe^{-/-}/Cxcl10^{+/+}$ (open symbols, $n=30$) or $Apoe^{-/-}/Cxcl10^{-/-}$ (closed symbols, $n=36$) mice after infusion with saline (circles, $n=6$ mice per genotype) or Ang II (triangles, $Apoe^{-/-}/Cxcl10^{+/+}$, $n=18$; $Apoe^{-/-}/Cxcl10^{-/-}$, $n=24$). $**P<0.01$. B, Representative aortas from $Apoe^{-/-}/Cxcl10^{+/+}$ mice (upper row) and $Apoe^{-/-}/Cxcl10^{-/-}$ mice (bottom row), with arrows pointing to abdominal and thoracic aneurysms, with an example of a spiral dissection (lower left). C, Histograms represent severity of AAAs in $Apoe^{-/-}/Cxcl10^{+/+}$ (open bars) and $Apoe^{-/-}/Cxcl10^{-/-}$ (solid bars) mice based on morphological classification (see Methods; $Apoe^{-/-}/Cxcl10^{+/+}$, $n=16$; $Apoe^{-/-}/Cxcl10^{-/-}$, $n=22$ mice). $**P<0.01$ by χ^2 analysis. D, Circles ($Apoe^{-/-}/Cxcl10^{+/+}$, $n=12$) and triangles ($Apoe^{-/-}/Cxcl10^{-/-}$, $n=11$) represent suprarenal aortic diameters in individual mice infused with Ang II ($1000 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) for 28 days. Data were analyzed by Mann-Whitney rank sum test. $*P<0.05$. E, Circles ($Apoe^{-/-}/Cxcl10^{+/+}$, $n=12$) and triangles ($Apoe^{-/-}/Cxcl10^{-/-}$, $n=11$) represent ratios of suprarenal/thoracic to infrarenal aortic areas in individual mice infused with Ang II ($1000 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$). Data were analyzed by Mann-Whitney rank sum test. $**P<0.01$.

elements of remodeling, dilation, and rupture, which involved more of the aorta than previously reported with this model. Thus, deficiency of either IFN- γ or the IFN- γ -inducible chemokine, CXCL10, yielded exacerbation of AAA pathology, despite there being diminished plaque formation in the absence of either of these cytokines.

Reduction of T-Cell Accumulation in CXCL10-Deficient Aneurysms

We next performed immunohistochemical studies of aortic vessel-wall constituents to further delineate the effects of CXCL10 deletion on AAA formation. As previously reported, cross-sectional histology demonstrated lumen dilation, breaks in medial elastin, and thrombus formation.²⁸ Consistent with deletion of the effector T-lymphocyte chemokine, CXCL10, we documented a significant decrease in CD4⁺ T-lymphocyte accumulation in the suprarenal AAAs of $Apoe^{-/-}/Cxcl10^{-/-}$ mice compared with $Apoe^{-/-}$ controls, as assessed both by quantitative PCR and by immunohistochemical analysis (Figure 7A through 7C). A concordant reduction was found in mRNA for the CXCL10 receptor, CXCR3, consistent with decreased infiltration of effector T cells

($P=0.004$; Figure 7D).^{7,35} Also consistent with the decreased accumulation of activated T lymphocytes in particular, we observed a concomitant reduction in IFN- γ production within the vessel wall, as assessed by quantitative real-time PCR (Figure 7E). Interestingly, a significant decrease was also found in macrophage accumulation in the arterial wall of the $Apoe^{-/-}/Cxcl10^{-/-}$ double-knockout mice compared with $Apoe^{-/-}$ controls (data not shown). Because CD4 is present at very low levels in macrophages, we also performed immunostaining with an anti-Thy-1.2 antibody, which confirmed the reduction in T cells in the lesions (online-only Data Supplement Figure IV).

TGF- β 1 Blockade Inhibits AAA Size in CXCL10-Deficient Mice

We hypothesized that in the absence of IFN- γ and CXCL10, the lesional cytokine milieu would be enriched for non-Th1-related signals, such as TGF- β 1. Recent studies have also demonstrated that TGF- β 1 activation appears to potentiate aortic root aneurysm formation in murine models of Marfan syndrome.²⁴ In this context, we found that $Apoe^{-/-}/Cxcl10^{-/-}$ aneurysmal sections contained significantly greater amounts

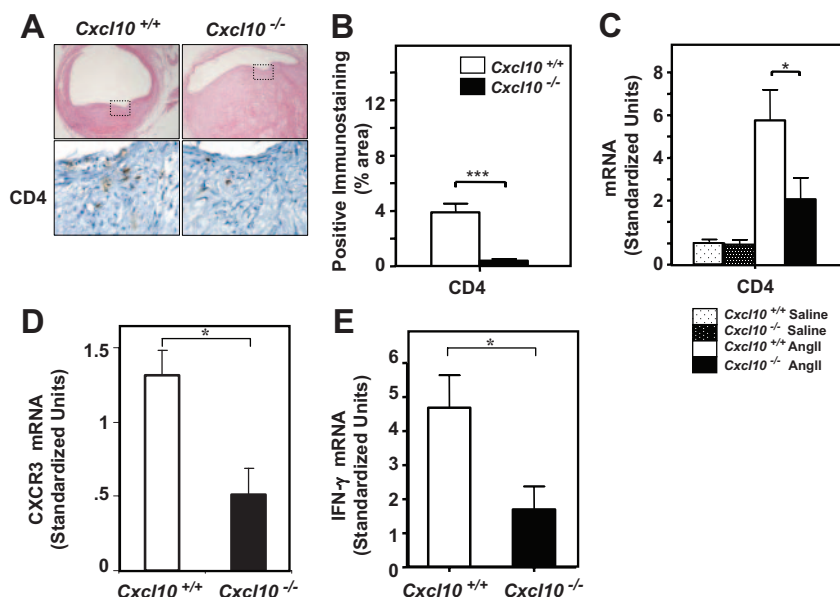


Figure 7. CXCL10 deficiency attenuates Ang II-induced T-cell infiltration into the vascular wall. Immunostaining analysis of aneurysms from *Apoe*^{-/-}/*Cxcl10*^{+/+} or *Apoe*^{-/-}/*Cxcl10*^{-/-} mice infused with Ang II (1000 ng · kg⁻¹ · min⁻¹) or saline for 28 days. A, Representative cross sections (40×) of the suprarenal aorta from *Apoe*^{-/-}/*Cxcl10*^{+/+} (left top) or *Apoe*^{-/-}/*Cxcl10*^{-/-} (right top) mice. Boxed area is expanded to show representative high-power fields with immunostaining for T lymphocytes in serial sections (CD4, lower panels). B, Quantification of immunostaining (5 or more fields per slide) from *Apoe*^{-/-}/*Cxcl10*^{+/+} (open bar) or *Apoe*^{-/-}/*Cxcl10*^{-/-} (solid bar) mice (n≥3 mice per group). Data are normalized to lesion area (***P<0.0001). C, Quantitative real-time PCR analysis of CD4 mRNA expression in suprarenal aortas from *Apoe*^{-/-}/*Cxcl10*^{+/+} or *Apoe*^{-/-}/*Cxcl10*^{-/-} mice (n≥6 mice/group; *P<0.05). D, Quantitative real-time PCR analysis of CXCR3 mRNA expression in suprarenal aorta from *Apoe*^{-/-}/*Cxcl10*^{+/+} and *Apoe*^{-/-}/*Cxcl10*^{-/-} mice (n≥10 mice per group; *P<0.05). E, Quantitative real-time PCR analysis of IFN-γ mRNA expression in suprarenal aorta from *Apoe*^{-/-}/*Cxcl10*^{+/+} and *Apoe*^{-/-}/*Cxcl10*^{-/-} mice (n≥10 mice per group; *P<0.05).

of activated TGF-β, as assessed by immunohistochemical analysis with an activation-specific TGF-β antibody³⁶ (Figure 8A and 8B). Furthermore, inhibition of TGF-β activity with a neutralizing antibody²⁴ significantly diminished aortic area in CXCL10-deficient mice treated with Ang II for 2 weeks (Figure 8C).

Discussion

Here, we specifically explored the roles of IFN-γ and CXCL10 in the formation of AAAs. Although Ang II-induced atherosclerotic lesion formation was attenuated in IFN-γ-deficient mice, an unexpected increase was found in suprarenal aortic diameter and AAA incidence. The IFN-γ-inducible effector T-cell chemokine CXCL10, which is highly upregulated by Ang II infusion in *Apoe*^{-/-} mice and downregulated in the setting of IFN-γ deficiency, also conferred protection from AAA formation. Compared with *Apoe*^{-/-} control mice, compound-deficient *Apoe*^{-/-}/*Cxcl10*^{-/-} mice had increased aortic size, worse morphological grades of aneurysms, and a higher incidence of death due to aortic rupture. The aortas of *Apoe*^{-/-}/*Cxcl10*^{-/-} mice were characterized by downregulation of IFN-γ and upregulation of the proaneurysmal growth factor TGF-β1. Furthermore, inhibition of TGF-β with a neutralizing antibody diminished aortic area in the Ang II-treated *Apoe*^{-/-}/*Cxcl10*^{-/-} mice.

Although clinical evidence suggests that coronary atherosclerosis and AAA formation share some common features, important differences exist, such as the discordant effect of diabetes on the prevalence of these 2 disease manifestations. A prominent inflammatory component is common to both

vascular pathologies, although histological analyses also show differences. Coronary atherosclerosis is marked specifically by the accumulation of a Th1-type immune response.⁷ In contrast, studies of AAA have found evidence for both Th1- and Th2-type responses.^{8,12} Prior studies have hinted at molecular signals that differ in aneurysmal versus stenotic vascular disease. For example, genetic deficiency of matrix metalloproteinase-3, tissue inhibitor of metalloproteinase-1, or 5-lipoxygenase and pharmacological inhibition of matrix metalloproteinases in hyperlipidemic mice have had variable effects on atherosclerosis compared with AAA formation.^{37–40} The present study extends prior work by defining a novel pathway in which IFN-γ and its effector, CXCL10, lead to discordant effects in the 2 vascular disease processes. Thus, although multiple lines of investigation have shown that Th1 cells and their related proinflammatory cytokines promote atherogenesis, we show that these same cytokines protect against the formation of AAAs in a well-characterized murine system.

An extensive literature documents the function of IFN-γ in potentiating the inflammatory response; however, more recent evidence suggests that IFN-γ also plays a role in the resolution of inflammatory processes. Data supporting the complex role of IFN-γ have come both from antibody-blocking experiments and from attempts to induce autoimmune inflammation in IFN-γ and IFN-γ-receptor knockout mice. For example, increased disease severity was documented in mouse models of multiple sclerosis and collagen-induced arthritis with animals deficient for IFN-γ or the IFN-γ receptor.^{13–15} The present data are therefore consistent

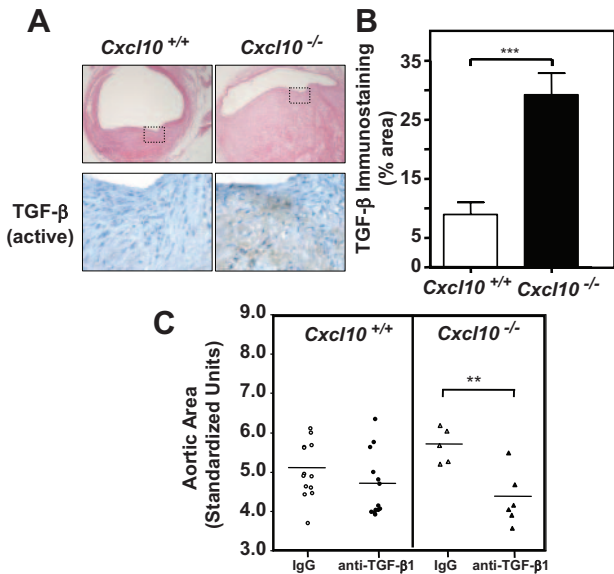


Figure 8. Enhanced TGF- β activation contributes to worse AAA in CXCL10-deficient mice. A, Representative cross sections of suprarenal aorta from Ang II-infused *Apoe*^{-/-}/*Cxcl10*^{+/+} (left) and *Apoe*^{-/-}/*Cxcl10*^{-/-} (right) mice were immunostained with activation-specific TGF- β antibody (bottom). B, Quantification of active TGF- β immunostaining from *Apoe*^{-/-}/*Cxcl10*^{+/+} (open bar) or *Apoe*^{-/-}/*Cxcl10*^{-/-} (solid bar) mice ($n \geq 3$ mice per group; $***P < 0.0001$). C, *Apoe*^{-/-}/*Cxcl10*^{+/+} and *Apoe*^{-/-}/*Cxcl10*^{-/-} mice were infused with Ang II ($1000 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) for 14 days. Anti-TGF- β neutralizing antibody (1 mg/kg; closed symbols) or IgG isotype control (1 mg/kg; open symbols) was administered by intraperitoneal injection on the day before pump placement and the day after. Circles (*Apoe*^{-/-}/*Cxcl10*^{+/+}, $n=24$) and triangles (*Apoe*^{-/-}/*Cxcl10*^{-/-}, $n=11$) represent the ratios of the suprarenal/thoracic to infrarenal aortic areas in individual mice. Data were analyzed by Mann-Whitney rank sum. $**P < 0.01$.

with the emerging notion of IFN- γ as a master regulator, upstream of multiple pathways that evolve during the disease process. With regard specifically to vascular biology, the contrasting effects of IFN- γ deficiency on AAA and stenotic vascular disease may be attributed to localization of these diseases to different layers of the aorta. Additionally, differences in the temporal accumulation of different cell types and the presence of different cytokines and growth factors might account for the opposing effect of IFN- γ deficiency on atherosclerosis and aneurysms.

We observed significantly increased levels of TGF- β 1 in the Ang II-induced AAAs of *Apoe*^{-/-}/*Cxcl10*^{-/-} double-knockout mice compared with *Apoe*^{-/-} controls. This finding is consistent with our prior studies in which we found enhanced expression of non-Th1 cytokines, including TGF- β 1, in the diet-induced atherosclerotic plaques of CXCL10-deficient mice, despite overall diminished T-cell accumulation.³³ We were particularly interested to find increases in TGF- β 1 activation in Ang II-infused *Apoe*^{-/-}/*Cxcl10*^{-/-} double-knockout mice in light of recent studies demonstrating the role of TGF- β 1 in aortic root aneurysm formation in murine models of Marfan syndrome.²⁴ Investigators now postulate that fibrillin-1 tonically inhibits TGF- β 1 signaling in the vessel wall. In Marfan syndrome, mutations in fibrillin-1 lead to enhanced TGF- β 1 activation and ultimately vascular dilation. Furthermore, aneurysm formation in mice

expressing a fibrillin mutation characteristic of human Marfan syndrome is inhibited by a TGF- β -blocking antibody or by the Ang II type 1 receptor blocker losartan,²⁴ although there are likely to be important differences in pathways triggered in the hyperlipidemia and Ang II-induced aneurysm model as opposed to the genetically induced Marfan model. Our working model is that CXCL10, an IFN- γ -dependent chemokine, modulates the recruitment of effector T cells. The recruitment of T cells influences the local T-cell cytokine profile in the vessel wall, including the expression of additional IFN- γ . We have demonstrated that when CXCL10 is deleted, the recruitment of effector T cells is diminished, and the local cytokine milieu shifts away from a Th1 profile, which leads to an enrichment of signals including TGF- β 1. Growth factors such as TGF- β 1 activate fibroblasts and other cell types and elicit further TGF- β 1 and cytokine generation,⁴¹ which may serve to amplify the initial changes in the cytokine profile. In atherosclerosis, shifting the balance away from Th1 cytokines with upregulation of TGF- β 1 inhibits luminal plaque formation; however, TGF- β 1 induction has been demonstrated to be a critical mechanism in aneurysmal dilation. Characterization of the downstream targets of TGF- β 1 responsible for these discordant effects in the vasculature merits future investigation. Of note, precedent exists for TGF- β 1 activity having dramatically different effects on specific aspects of disease pathology, such as mitigating inflammation but contributing to dysregulated tissue repair.⁴²

Several findings of the present study potentially contrast with previous work. Although studies defining the effects of IFN- γ on atherosclerosis have been uniform, there are conflicting reports of the role of IFN- γ in aneurysm-associated disease models. Blockade of IFN- γ signaling with IFN- γ receptor-deficient mice increased AAA formation in an aortic allograft model of the disease,¹² which is in agreement with the present study. However, investigators have demonstrated that increases in the abdominal aortic diameter of C57BL/6J mice triggered by intraperitoneal administration of calcium chloride were attenuated by IFN- γ deficiency.¹⁰ One other study has found that adenoviral mediated overexpression of TGF- β 1 attenuated aortic dilation of SDS-treated guinea pig xenografts transplanted into Lewis rats.⁴³ Finally, a recent report suggests that CXCR3 (the receptor for CXCL10) deficiency has no significant effect on calcium chloride-triggered aortic dilation.⁴⁴ We would postulate that the marked differences between our model and prior systems account for the contrasting findings. The present studies used an Ang II-treated hyperlipidemic murine model on the C57BL/6J background, which differs significantly from the prior studies, which used either surgical intervention to apply calcium chloride to the external surface of the vessel or aortic transplantation to trigger AAA formation. We note that although the present data highlight a role for IFN- γ and effector T cells in AAA formation, recent findings have also suggested a role for other types of IFN- γ -producing cells, such as mast cells, in AAA generation.⁴⁵

Limitations to the present study must also be considered. Debate about the fidelity of the most commonly used AAA mouse models (adventitial calcium chloride, intraluminal porcine elastase, and subcutaneous Ang II infusion) for human

disease is considerable. The task of assessing the relevance of any of these commonly used mouse models is made difficult by our lack of knowledge of the initiation and formative stages of human AAAs. Unfortunately, the most accessible human AAA tissue is from advanced lesions that have been resected during open surgical repair, which provides limited insight into the earlier stages. Despite these formidable barriers to validating any of the murine models, there are some indications that the renin-angiotensin system in particular is involved in the formation of human AAAs. Components of the renin-angiotensin system are highly expressed in human AAA tissue, particularly angiotensin-converting enzyme and chymase-3.⁴⁶ Retrospective clinical analyses have revealed that angiotensin-converting enzyme inhibition is associated with reduced AAA rupture.⁴⁷ Finally, genetic association studies are emerging that link specific Ang II type 1 receptor and angiotensin-converting enzyme polymorphisms with AAA as well.^{48,49}

Experimental studies are only beginning to clarify the functional role of adaptive immunity in stenotic vascular occlusive disease and AAA formation. The prevailing dogma is that Th1 immune responses contribute in a causal manner to atherosclerosis in general, but particularly with regard to luminal atherosclerotic plaque buildup. In striking contrast, the present findings clearly demonstrate that 2 major Th1-associated cytokines, IFN- γ and CXCL10, play a protective role in AAA formation. The present data suggest that local modulation of CXCL10 represents a potential therapeutic strategy for AAA. Most importantly, the present study also suggests that efforts to develop antiinflammatory strategies for atherosclerosis must carefully consider the potential effects on all types of vascular disease manifestations and must consider both salutary and harmful aspects of the immune system.

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Disclosures

None.

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CLINICAL PERSPECTIVE

A wealth of recent findings suggest that inflammation contributes to vascular disease; however, our understanding of the precise mechanisms involved remains incomplete. The present study defines a novel pathway in which 2 related inflammatory mediators, interferon- γ and CXCL10, contribute to plaque formation in arteries but protect against the formation of vessel aneurysms. Thus, efforts to develop antiinflammatory strategies for atherosclerosis must carefully consider potential effects on all types of vascular disease manifestations and must consider both the salutary and harmful aspects of the immune system.