Abdominal Aortic Aneurysm

Foxp3+ Regulatory T Cells Play a Protective Role in Angiotensin II–Induced Aortic Aneurysm Formation in Mice


Abstract—Although regulatory T cells (Tregs) have been shown to play a protective role in abdominal aortic aneurysm (AAA) formation, it remains unclear whether expansion of endogenous Foxp3+ Tregs prevents AAA. In the current study, we determined the effects of endogenous Foxp3+ Treg expansion or depletion in an experimental model of AAA. We continuously infused 12-week-old apolipoprotein E–deficient mice fed a high-cholesterol diet with angiotensin II (n=60) or normal saline (n=12) by implanting osmotic mini-pumps and evaluated AAA formation at 16 weeks. The angiotensin II–infused mice received interleukin-2/anti–interleukin-2 monoclonal antibody complex (interleukin-2 complex; n=31) or PBS (n=29). Eighty-one percent of angiotensin II–infused mice developed AAA, with 42% mortality possibly because of aneurysm rupture. Interleukin-2 complex treatment systemically increased the number of Foxp3+ Tregs and significantly decreased the incidence (52%) and mortality (17%) of AAA. Immunohistochemical analysis showed reduced accumulation of macrophages and increased numbers of Foxp3+ Tregs in aneurysmal tissues, suggesting that expansion of Tregs may suppress local inflammation in the vessel wall and provide protection against AAA formation. Furthermore, genetic depletion of Foxp3+ Tregs led to a significant increase in the mortality of AAA, suggesting the protective role of Foxp3+ Tregs against AAA. Our findings suggest that Foxp3+ Tregs may play a protective role in AAA formation and that promotion of an endogenous regulatory immune response may be potentially a valuable therapeutic approach for preventing AAA. (Hypertension. 2015;65:889-895. DOI: 10.1161/HYPERTENSIONAHA.114.04934.)

Key Words: aortic aneurysm, abdominal □ immunology □ inflammation □ interleukin-2 □ T-lymphocytes, regulatory

Abdominal aortic aneurysm (AAA) is an important cause of mortality, which is principally managed by surgical interventions.1 Although patients with AAA and coronary heart diseases have similar risk factors, typical medical therapies for coronary heart disease are not effective for preventing AAA.2 Notably, recent evidence suggests that vascular inflammation is a key feature shared between AAA and atherosclerotic disease, indicating that regulation of pathogenic inflammatory reactions could be a possible therapeutic approach for preventing AAA.3 A thorough understanding of the mechanisms underlying AAA formation could lead to effective medical methods to prevent AAA.

Previous studies using various mouse AAA models have provided important clues to the pathogenesis of AAA including the roles of immune cells such as macrophages and T cells. Although AAA shares many characteristics of atherosclerotic diseases, recent animal studies have shown that completely different immune responses including effector T cell (Teff)–mediated immune responses are involved in the mechanisms of AAA as compared with atherosclerosis. T-helper type 1 (Th1) cells are known to contribute to the development of atherosclerosis,4 whereas several studies demonstrate that a shift toward Th2 immune response is important for AAA formation in mice and humans.5,6 suggesting that promotion of Th1 immune responses to prevent AAA may cause detrimental effects on atherosclerotic disease.7 However, the role of each helper T-cell subset in AAA has not been elucidated fully. Compelling data suggest that CD4+CD25+Foxp3+ regulatory T cells (Tregs), which are responsible for maintaining immunologic tolerance and suppressing excessive immune responses,8 inhibit the development or progression of atherosclerosis by dampening Teff-mediated immune responses in atherosclerosis-prone mice.9-11 We and others have shown the contribution of reduced Treg levels to the progression of human coronary artery disease.12,13 Recently, CD4+CD25+ Treg deficiency caused by genetic disruption of CD80/CD86 or CD28 costimulatory molecules has been shown to promote the development and rupture of angiotensin II–induced AAA in C57BL/6 wild-type mice,14 suggesting a crucial role for endogenous CD4+CD25+ Tregs in controlling AAA formation.

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and rupture. A recent report has demonstrated that adoptive transfer of CD4+CD25+ Tregs prevents angiotensin II–induced AAA in atherosclerosis-prone apolipoprotein E–deficient (ApoE−/−) mice.15 Notably, a clinical report has shown a decrease in CD4+CD25+Foxp3+ cell numbers and reduced Foxp3 expression levels in CD4+CD25+ cells in the peripheral blood from patients with AAA, implying that impaired immunoregulation by Tregs may be involved in the pathogenesis of human AAA.16 Collectively, these data suggest that promotion of endogenous regulatory immune responses could be a possible therapeutic approach to prevent AAA as well as atherosclerotic disease. Interleukin (IL)-2–2 is known to be a critical cytokine for proliferation and differentiation of CD4+CD25+Foxp3+ Tregs because CD25 molecule is a component of the high-affinity IL-2 receptor.8 Recent experimental studies have shown that injection of a recombinant mouse IL-2/anti–IL-2 monoclonal antibody complex (IL-2 complex) selectively expanded CD4+CD25+Foxp3+ Tregs and effectively inhibited atherosclerosis in ApoE−/− mice without adverse effects.17 These data suggest that expansion of endogenous CD4+CD25+Foxp3+ Tregs by IL-2 complex may be effective for preventing AAA.

Although previous studies demonstrated a critical role of CD4+CD25+ Tregs in the protection against AAA,14,15 the role of Tregs expressing transcriptional factor Foxp3, which is considered to be the most specific molecular marker for natural Tregs,8 has not been examined yet. Depletion of regulatory T cells (DEREG) mice, which express a diphtheria toxin receptor under the control of the foxp3 gene locus, allow selective and efficient depletion of Foxp3+ Tregs by DT injection.18 We crossed DEREG mice with ApoE−/− mice and established DEREG/ApoE−/− mice, allowing us to evaluate the exact effect of endogenous Foxp3+ Treg deficiency on AAA formation. We tested the hypothesis that induction of Foxp3+ Tregs would inhibit the development of AAA in angiotensin II–infused ApoE−/− mice. Moreover, as a novel approach to unravel the impact of Foxp3+ Treg depletion on AAA formation, we took advantage of newly established DEREG/ApoE−/− mice and determined if genetic depletion of Foxp3+ Tregs would aggravate AAA formation in angiotensin II–infused ApoE−/− mice. We demonstrate that endogenous Foxp3+ Tregs play a protective role against AAA and that induction of Foxp3+ Tregs with IL-2 complex treatment may be a potentially valuable therapeutic approach for preventing AAA.

Methods

Six-week-old male mice were fed a high-cholesterol diet containing 0.2% cholesterol and 21% fat (CLEA, Tokyo, Japan) and water ad libitum. At 12 weeks of age, mice were infused with 1000 ng/kg per minute angiotensin II or saline for 28 days by implanting ALZET mini-osmotic pumps. For in vivo Foxp3+ Treg expansion, 12-week-old ApoE−/− mice were intraperitoneally injected with IL-2 complex (1 μg recombinant mouse IL-2 plus 5 μg anti–IL-2 mAb [JES6-1] incubated at 37°C for 30 minutes) for 3 consecutive days and thereafter once weekly for 3 weeks. Control mice received PBS. For selective depletion of Foxp3+ Tregs, we used DEREG mice on a C57BL/6 background previously generated18 and crossed them with ApoE−/− mice to obtain DEREG/ApoE−/− mice. We produced angiotensin II–induced AAA, and Reduces the Mortality in ApoE−/− Mice

Results

IL-2 Complex Therapy Induces Foxp3+ Tregs, Inhibits the Development of Angiotensin II–Induced AAA, and Reduces the Mortality in ApoE−/− Mice

To investigate the role of Tregs in aortic aneurysm formation, we used angiotensin II–induced AAA model and treated 12-week-old ApoE−/− mice fed a high-cholesterol diet with angiotensin II infusion for 28 days. The mice received injections with either IL-2 complex for in vivo Treg expansion or PBS as a control, for 3 consecutive days and thereafter once a week for 3 weeks and were euthanized at 16 weeks of age for evaluation of AAA formation (Figure 1A). Knowing the fact that IL-2 complex treatment leads to systemic expansion of Foxp3+ Tregs,17,18 we first investigated the effect of IL-2 complex on these cells by flow cytometry. Consistent with

Figure 1. Interleukin (IL)-2 complex therapy induces Foxp3+ regulatory T cells (Tregs), inhibits the development of angiotensin II–induced abdominal aortic aneurysm (AAA), and reduces the mortality in ApoE−/− mice. A, Experimental design. Arrows with circle represent intraperitoneal injection of IL-2/anti-IL-2 mAb (IL-2 complex). B to D, For analysis of systemic immune responses, lymphocytes were isolated from spleen at 5 days after the first injection of IL-2 complex or PBS. B, Representative results of the proportion of CD4+ and Foxp3+ cells in the spleen assessed by flow cytometry. The percentage of (C) Foxp3+ Tregs within the CD4+ T-cell population were determined by flow cytometry. D, The ratio of CD4+Foxp3+ Tregs to CD4+Foxp3− Teffs was also determined (Treg/Teff ratio). n=4 mice per group. E, Survival rate in control (n=31), IL-2 complex–treated (n=29), and sham mice (n=12). F, Mortality of AAA in control (n=31) and IL-2 complex–treated (n=29) mice. *P<0.05, †P<0.0001.
previous reports, on day 5 after the 3-day injection of IL-2 complex, we found a dramatic increase in Foxp3+ Tregs in the CD4+ T-cell population in the spleen and LNs (lymph nodes) of angiotensin II–infused mice (Figure 1B–1D and data not shown).

Eighty-one percent of angiotensin II–infused ApoE−/− mice treated with PBS developed AAA with 42% mortality possibly because of aneurysm rupture (Figures 1E and 1F and 2A and 2B). However, a significant decrease in incidence of AAA (52%) and mortality (17%) was seen on IL-2 complex treatment (Figures 1E and 1F and 2A and 2B). No statistically significant difference in body weight and plasma lipid profile was detected between the 2 groups (Table S1 in the online-only Data Supplement). Angiotensin II infusion significantly increased systolic blood pressure in IL-2 complex–treated and control mice, whereas there was no significant difference in systolic blood pressure between the 2 groups (Table S1).

These results indicate that IL-2 complex therapy can increase the number of Tregs, efficiently shift the Treg/Teff balance toward Tregs, and reduce the incidence and mortality of AAA possibly via suppressing inflammatory responses.

**IL-2 Complex Therapy Suppresses Inflammatory Responses in the Aneurysmal Lesions and Reduces the Severity of Angiotensin II–Induced AAA**

In parallel with the evaluation of AAA formation and survival, histological analysis of the aortic aneurysm tissue was performed. Notably, along with a reduction in incidence and mortality (Figures 1E and 1F and 2B), IL-2 complex therapy markedly decreased the severity of AAA in angiotensin II–infused mice (Figure 2C). IL-2 complex–treated mice had significantly smaller diameter of the abdominal aneurysm (Figure 2D) and more preserved elastin content in the aortic aneurysm tissue than control mice (Figure S1A and S1B).

Because vascular inflammation is reported to be involved in the mechanisms of angiotensin II–induced AAA development in ApoE−/− mice, we investigated the effects of IL-2 complex therapy on inflammatory cell infiltration to the aortic wall. Immunohistochemical studies showed massive accumulation of macrophages within the aneurysmal lesions, which are known to be the main inflammatory cells in this AAA model after angiotensin II infusion. In contrast, the aneurysmal lesions of IL-2 complex–treated mice showed a marked reduction in the infiltration of these cells compared with control mice (Figure S1C and S1D).

Recent studies using atherosclerosis-prone mice have shown that Tregs may migrate into atherosclerotic plaques and potentially suppress lesional immune reactions. Therefore, we determined whether increased accumulation of Tregs in aneurysmal lesions is involved in the inhibition of AAA formation. Immunohistochemical analysis using anti-Foxp3 antibody revealed a marked increase in the numbers of Foxp3+ Tregs and the Treg/CD4+ T-cell ratio within the aneurysmal lesions in IL-2 complex–treated mice compared with control mice, although lesional CD4+ T cells were also significantly increased in IL-2 complex–treated mice (Figure 3A–3C).

Collectively, these results suggest that IL-2 complex therapy promotes migration of systemically expanded Foxp3+ Tregs into the aneurysmal lesions of angiotensin II–induced ApoE−/− mice and subsequently increases the Treg/Teff ratio in both the aneurysmal lesions and lymphoid tissues, leading to preserved vessel integrity and decreased susceptibility.
Effects of IL-2 Complex Therapy on Immune Responses in Lymphoid Organs

The effects of IL-2 complex therapy on the expressions of Treg-associated molecules in splenic Foxp3+ Tregs were determined by flow cytometry at 5 days after the first injection of IL-2 complex or PBS. Consistent with a previous report, Foxp3+ Tregs from IL-2 complex–treated mice expressed higher levels of CD25, CD103, glucocorticoid-induced tumor necrosis factor receptor family–related gene/protein, and cytotoxic T lymphocyte–associated antigen-4 compared with those from control mice (Figure S2A), implying an activated phenotype of Foxp3+ Tregs after the IL-2 complex therapy.

To determine whether IL-2 complex therapy changes Teff responses and polarization, we isolated splenic CD4+ T cells at 5 days after the first injection of IL-2 complex or PBS and examined cytokine secretion by intracellular cytokine staining. We found that IL-2 complex therapy markedly increased the percentage of splenic IL-10–producing CD4+ T cells compared with control mice, whereas the percentage of interferon-γ–producing Th1 cells, IL-4–producing Th2 cells, or IL-17–producing Th17 cells was not changed by this therapy (Figure S2B). The Th1/Th2 ratio was similar between the 2 groups (Figure S2C). IL-2 complex did not affect the expressions of maturation markers CD80 and CD86 on splenic CD11c+ dendritic cells (Figure S2D).

These results indicate that IL-2 complex therapy increases the proportion and suppressive function of Foxp3+ Tregs, which may affect the protective effects of IL-2 complex on AAA development.

Selective Depletion of Foxp3+ Tregs Aggravates Inflammatory Responses in the Aneurysmal Lesions and Increases the Mortality of Angiotensin II–Induced AAA

To address the issue of whether endogenous Foxp3+ Tregs play a protective role in AAA development, we used DEREG mice on an ApoE−/− background (DEREG/ApoE−/− mice). Twelve-week-old DEREG/ApoE−/− mice or control ApoE−/− mice fed a high-cholesterol diet were infused with angiotensin II by osmotic pumps, injected with DT once a week for 4 weeks and euthanized at 16 weeks of age for quantitative analysis of aortic aneurysmal lesions (Figure 4A). Consistent with a previous study, the transgene coding a fusion protein of diphtheria toxin receptor and enhanced green fluorescent protein was mainly expressed in Foxp3+ Tregs, but not in other immune cells (data not shown). We injected DT to DEREG/ApoE−/− mice and observed effective elimination of Foxp3+ Tregs on the following day in spleen and peripheral LNs (Figure S3A and S3B and data not shown), which was associated with marked upregulation of Teff immune responses (data not shown). Depletion of Foxp3+ Tregs in angiotensin II–infused mice resulted in a significant increase in the mortality (53%), although we found no significant differences in the incidence of AAA (81% in control ApoE−/− mice versus 94% in DEREG/ApoE−/− mice), and the diameter and severity of AAA between Treg-depleted and nondepleted controls (Figure 4B and 4C; Figure S4A and S4B). Depletion of Foxp3+ Tregs did not affect plasma lipid profile or systolic blood pressure, but decreased body weight in angiotensin II–infused mice without sign of autoimmune disease (Table S2).

Histological examination revealed a significant increase in infiltration of CD4+ T cells in the aneurysmal lesions of Treg-depleted mice (Figure 4D). Notably, Treg-depleted mice have low numbers of Foxp3+ Tregs in the aneurysmal lesions (Figure 4E), resulting in a decreased Treg/CD4+ T-cell ratio in the lesions (Figure 4F). However, we found no differences either in the macrophage accumulation or relative collagen content in the aneurysmal lesions between Treg-depleted and nondepleted controls (data not shown), implying that Treg-depleted mice without aneurysm rupture might not show characteristics of severe AAA. These results suggest that Foxp3+ Treg depletion aggravates lesional inflammation and vessel integrity, which may lead to the increased development and rupture of AAA.

Discussion

In this study, we examined the effects of endogenous Foxp3+ Treg expansion or depletion on AAA formation in angiotensin II–infused ApoE−/− mice. Expansion of Foxp3+ Tregs by IL-2 complex treatment resulted in a marked decrease in the incidence and mortality of AAA. The protective effects of this therapy were associated with a dramatic increase in the Treg/Teff ratio and IL-10–producing CD4+ T cells, and upregulation of Treg activation markers, but no change in the Th1/Th2 balance in lymphoid organs. Similar to these findings in lymphoid organs, we observed a significant increase in the Treg/Teff ratio in aneurysmal lesions, which may contribute...
to preserving vessel integrity and decreasing susceptibility to AAA and aortic rupture by regulating local inflammatory responses. Moreover, we newly established a mouse AAA model, which permits conditional depletion of Foxp3+ Tregs and demonstrated that genetic depletion of Foxp3+ Tregs aggravated AAA formation and rupture. Collectively, these data indicated a critical role of Foxp3+ Tregs in the protection against AAA and suggest that induction of endogenous Foxp3+ Tregs by IL-2 complex treatment may be a potentially valuable therapeutic approach for preventing AAA.

Previous studies have demonstrated that adoptive transfer of CD4+CD25+ Tregs suppressed angiotensin II or aldosterone-mediated experimental hypertension through suppressing inflammatory responses, suggesting the possible protective role of Tregs in hypertension as well as atherosclerosis. Consistent with previous reports,15,22 we observed a marked elevation in systolic blood pressure in angiotensin II–infused ApoE−/− mice. Notably, we found that IL-2 complex treatment had no significant effect on systolic blood pressure in angiotensin II–infused ApoE−/− mice. These results indicate that the protective role of IL-2 complex in angiotensin II–induced AAA formation is independent of blood pressure, which is in line with previous findings showing that Tregs ameliorate angiotensin II–induced cardiac inflammation and damage independently of blood pressure–lowering effects.24

Accumulating evidence suggests that the balance between pathogenic Teffs and Tregs may play an important role in the control of atherosclerotic diseases.11 Several therapeutic strategies to suppress Teff responses and promote Treg responses have been shown to be effective for suppressing or regressing experimental atherosclerosis.19,25-27 Similarly, a recent clinical study has suggested that such an imbalance may also be involved in the pathogenesis of AAA and that expansion of Tregs could be a promising strategy to prevent AAA.16 However, obstacles still remain for clinical applications such as cell-based therapy involving transfer of ex vivo expanded Tregs because of difficulties in separating pure Tregs and maintaining them stable after expansion on antigenic stimulation. Although other therapeutic approaches should be considered, no study has uncovered the effects of endogenous Foxp3+ Treg expansion in experimental models of AAA to date. Previous studies have demonstrated that treatment with IL-2 complex selectively increases CD4+CD25+Foxp3+ Tregs without affecting other immune cells including CD4+ T cells, CD8+ T cells, or natural killer cells.17 Our recent study has shown that IL-2 complex treatment efficiently shifts the Treg/Teff balance toward Tregs, associated with its highly activated status, without affecting the Th1/Th2 balance.19 In the present study, we applied this method for the prevention of experimental AAA and found that IL-2 complex therapy selectively increased the proportion and anti-inflammatory capacity of Foxp3+ Tregs without affecting the Th1/Th2 balance and that induction of Foxp3+ Tregs specifically led to protection against AAA formation in angiotensin II–infused ApoE−/− mice. As described before, completely different T-cell immune responses between atherosclerotic disease and AAA make direct modulation of these responses challenging as a therapeutic approach for AAA under atherosclerotic conditions.3 Because Foxp3+ Tregs specifically suppress excessive inflammation associated with helper T-cell–mediated immune responses,8 we think that this regulatory cell population could be a novel therapeutic target to treat AAA.

It was shown that in addition to Foxp3+ Tregs, several kinds of inducible Tregs including T regulatory type 1 also have regulatory properties by producing anti-inflammatory cytokine IL-10 and play a protective role in the development of atherosclerosis in mice.28 A recent study identified a critical role for IL-10 in suppressing vascular inflammation and AAA formation and rupture in angiotensin II–infused mice.14 In the present study, we found that IL-2 complex therapy markedly increased the percentage of not only Foxp3+ Tregs but also IL-10–producing T regulatory type 1 cells compared with control mice, suggesting the contribution of this subset to the prevention of AAA after IL-2 complex therapy.

It has been reported that patients with AAA have markedly decreased numbers of CD4+CD25+Foxp3+ cells and reduced Foxp3 expression levels in peripheral CD4+CD25+ cells compared with healthy control subjects.19 Moreover, Foxp3 mRNA and protein expression levels in human atherosclerotic tissues were shown to be significantly lower than in normal thoracic aortic tissues.15 These findings suggest that there might be a close association between impairment of Treg-mediated immune responses and AAA formation, and that inhibition of Treg accumulation within atherosclerotic lesions may lead to augmented local inflammation and contribute to the development of AAA. In the present study, we found a significant increase in the frequency of Foxp3+ Tregs in both the atherosclerotic lesions and lymphoid organs of IL-2 complex–treated mice, implying that the beneficial effects of IL-2 complex treatment may partly be because of the local induction of Foxp3+ Tregs in abdominal aortic tissues. Further studies will be required to identify the exact role and characteristics of Foxp3+ Tregs in atherosclerotic tissues in this disease development.

Although endogenous CD4+CD25+ Tregs are shown to be involved in the control of AAA formation, there is no direct evidence demonstrating the protective role of endogenous natural Foxp3+ Tregs in AAA. Previous work has used CD25 (IL-2 receptor α-chain) molecule to identify Tregs, but this molecule is also upregulated on activation of Teffs.8 Furthermore, deficiency of costimulatory molecules such as CD80/CD86 or CD28 may affect the function of not only Tregs but also Teffs. Foxp3 is known to be specifically expressed in natural Tregs and a master regulator of Treg development and function.3 By inducing genetic depletion of Foxp3+ Tregs under hypercholesterolemic conditions, we evaluated the exact effects of Foxp3– Treg deficiency on the development of AAA and provided direct evidence that Foxp3+ Tregs prevent AAA formation and rupture by dampening inflammation. Notably, by performing bone marrow transplantation from DEREG mice into lethally irradiated, atherosclerosis-prone low-density lipoprotein receptor–deficient mice, Klingenberg et al5 showed that depletion of Foxp3+ Tregs aggravated hypercholesterolemia and led to a consequent increase in atherosclerotic lesions, indicating Foxp3+ Tregs as powerful inhibitors of atherosclerosis. When it is taken into consideration that patients with AAA have high incidence of atherosclerotic disease, these results suggest that immunomodulatory approaches such as enhancing Treg-mediated immune responses may be effective
for preventing both AAA and atherosclerosis. Importantly, unlike the results of atherosclerosis experiments reported by another group, we observed no changes in plasma lipid profiles after Foxp3+ Treg depletion, indicating that Foxp3+ Tregs may prevent AAA formation by directly suppressing inflammatory responses but not by modulating lipid metabolism. Discrepancy between our findings and previous work with inflammatory responses but not by modulating lipid metabolism. Further studies are needed to identify the role of Foxp3+ Tregs in the modulation of lipoprotein metabolism.

**Perspectives**

Our study establishes Foxp3+ Treg as an attractive therapeutic target for preventing AAA under atherosclerotic conditions. We provide evidence that expansion of endogenous Foxp3+ Tregs with IL-2 complex treatment results in significantly decreased incidence and mortality of AAA, which may be mediated by shifting the Treg/Teff balance toward Tregs in lymphoid organs and aortic aneurysmal lesions followed by subsequent attenuation of local inflammatory responses. Our data imply that therapeutic intervention aimed at enhancing a Treg-mediated immune response may be a potentially valuable approach for preventing AAA.

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**Disclosures**

None.

**References**


What Is New?

- Expansion of Foxp3+ regulatory T cells (Tregs) by a recombinant mouse interleukin-2/anti–interleukin-2 monoclonal antibody complex (interleukin-2 complex) treatment resulted in a marked decrease in the incidence and mortality of angiotensin II–induced abdominal aortic aneurysm (AAA). Genetic depletion of Foxp3- Tregs aggravates AAA formation and rupture.

What Is Relevant?

- Expansion of Foxp3+ Tregs results in significantly decreased incidence and mortality of AAA, which may be mediated by shifting the Treg/Teff balance toward Tregs in lymphoid organs and aortic aneurysmal lesions followed by subsequent attenuation of local inflammatory responses.
- Selective depletion of Foxp3+ Tregs aggravates inflammatory responses in the aneurysmal lesions and increases the mortality of AAA.

Summary

Therapeutic intervention aimed at enhancing a Treg-mediated immune response may be a potentially valuable approach for preventing AAA.
Foxp3+ Regulatory T Cells Play a Protective Role in Angiotensin II–Induced Aortic Aneurysm Formation in Mice

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Foxp3⁺ Regulatory T Cells Play a Protective Role in Angiotensin II-Induced Aortic Aneurysm Formation in Mice

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Supplemental Methods

Animals and Experimental Design

Male apolipoprotein E-deficient (ApoE\textsuperscript{-/-}) mice (offspring of homozygous ApoE-KO mice, backcrossed onto the C57BL/6 background)\textsuperscript{1} were housed in a specific pathogen-free animal facility at Kobe University. Ninety 6-week-old male mice were fed a high-cholesterol diet containing 0.2\% cholesterol and 21\% fat (CLEA, Tokyo, Japan) and water ad libitum. At 12 weeks of age, mice were infused with angiotensin II (1000 ng/kg/min, Sigma, St Louis, Mo, n=78) or normal saline (n=12, sham group) for 28 days by implanting ALZET mini-osmotic pumps (Model 2004; DURECT Corp, Cupertino, Calif) as described previously.\textsuperscript{2} IL-2 complex was prepared by mixing 1\µg mouse IL-2 recombinant protein (eBioscience) with 5\µg anti-IL-2 mAb (clone JES6-1; eBioscience) in sterile PBS and incubated at 37ºC for 30 min. For in vivo Foxp3\textsuperscript{+} Treg expansion, 12-week-old ApoE\textsuperscript{-/-} mice were intraperitoneally injected with IL-2 complex for 3 consecutive days and thereafter once weekly for 3 weeks (n=40). Control mice were injected with phosphate buffered saline (PBS) instead of IL-2 complex (n=38). For selective depletion of Foxp3\textsuperscript{+} Tregs, we used Depletion of regulatory T cell (DEREG) mice on a C57BL/6 background previously generated,\textsuperscript{3} and crossed them with ApoE\textsuperscript{-/-} mice to obtain DEREG/ApoE\textsuperscript{-/-} mice. We produced angiotensin II-induced AAA in DEREG/ApoE\textsuperscript{-/-} (n=45) and control ApoE\textsuperscript{-/-} mice (n=42) and under the same protocol described above, and treated these mice with 0.5 \µg DT (Sigma, St Louis, Mo) diluted in endotoxin-free PBS once weekly for 4 weeks. Mice were euthanized at 16 weeks of age for evaluation of abdominal aortic aneurysm (AAA) formation. All animal experiments were conducted according to the Guidelines for Animal Experiments at Kobe University School of Medicine.

Blood Pressure Measurement

Systolic blood pressure (SBP) was measured by noninvasive tail-cuff method (BP-98 Softron, Tokyo, Japan) as described previously.\textsuperscript{4} Conscious mice were placed on the warmed platform of the machine, which was maintained at 37ºC, and were acclimated to the apparatus for 5 minutes before the start of measurement. The SBP was measured at least five times at baseline and 4 weeks after angiotensin II pump implantation. The mean SBP for each group was determined by averaging the SBPs of each mouse included in that group.

Plasma Lipid Analysis

After overnight fasting, blood was collected by the cardiac puncture under anesthesia using 2,2,2-tribromoethanol (250 mg/kg intraperitoneal injection; Wako Pure Chemical Industries,
Osaka, Japan). Plasma was obtained through centrifugation and stored at -80°C until measurement. Concentrations of plasma total cholesterol, high density lipoprotein, and triglyceride were determined enzymatically using an automated chemistry analyzer (SRL, Tokyo, Japan).

**Morphological Analysis of AAA**

Aortic diameters and AAA incidence were determined as described previously. For morphological analyses, aortas were perfused with normal saline and fixed in 10% buffered formalin. The maximum external aortic diameters were measured using Image J (National Institutes of Health, Bethesda, MD). Aneurysm incidence was quantified on the basis of a definition of an external suprarenal aorta width that was increased by 50% or more compared to saline-infused mice. We used a previously described classification system to categorize the morphological severity of the aneurysms: no aneurysm, type I (a discernable dilation that is 1.5 to 2 times the diameter of a normal abdominal aorta), type II (a single large dilation that is more than 2 times the diameter of a normal abdominal aorta), type III (multiple dilations generally extending proximal to the suprarenal region), type IV (death due to aneurysmal rupture).

**Histological and Immunohistochemical Analysis of Aneurysmal Lesions**

Mice were anesthetized and the aorta was perfused with saline. The AAA lesions were cut and embedded in OCT compounds (Tissue-Tek; Sakura Finetek, Tokyo, Japan), and cross-sections (10 µm) were prepared. For the determination of elastin degradation, we performed Elastica van Gieson (EVG) staining and used a standard for the grades of elastin degradation as described previously. The grades were defined as follows: grade 1, no degradation; grade 2, mild elastin degradation; grade 3, severe elastin degradation; grade 4 aortic rupture. Immunohistochemistry was performed as described previously on acetone-fixed or formalin-fixed cryosections (10 µm) of the maximum aneurysmal lesions using antibodies to identify macrophages (MOMA-2, 1:400; BMA Biomedicals), CD4+ T cells (CD4, clone H129.19, 1:100; BD Biosciences) and Foxp3+ cells (Foxp3, clone FJK-16s, 1:100; eBioscience), followed by detection with biotinylated secondary antibodies and streptavidin-horseradish peroxidase. Stained sections were observed under an All-in-one Type Fluorescence Microscope (BZ-X700; Keyence, Osaka, Japan) using the BZ Analyzer Software (Keyence). Stained sections were digitally captured and the stained area was calculated. Quantitative analyses of CD4+ T cells and Foxp3+ cells of the aneurysm lesion was performed by counting the positive-stained cells, which was divided by total area of...
Flow Cytometric Analysis

For analyses of immune cells with flow cytometry, splenocytes and peripheral (inguinal and axillary) lymph node cells were isolated and stained in PBS containing 2% FCS. Flow cytometry analysis was performed by Attune Acoustic Focusing Cytometer (Life Technologies) using FlowJo software (Tree Star). For Intracellular cytokine staining, cells were stimulated with 20 ng/mL phorbol 12-myristate 13-acetate (Sigma) and 1 mmol/L ionomycin (Sigma) for 5 hours in the presence of a GolgiStop (BD Bioscience). The antibodies used were as follows; anti-CD16/CD32 (clone 2.4G2; BD Bioscience), anti-CD4 (clone H129.19; BD Bioscience), anti-CD25 (clone PC61; BD Bioscience), anti-CD103 (clone M290; BD Bioscience), anti-GITR (clone DTA1; BD Bioscience), anti-CTLA-4 (clone UC10; BD Bioscience), anti- Foxp3 (clone FJK-16s; eBioscience), anti-CD11c (clone HL3; BD Bioscience), anti-CD80 (clone 16-10A1; BD Bioscience), anti-CD86 (clone GL1; BD Bioscience), anti-IFN-γ (clone XMG1.2; eBioscience), anti-IL-4 (clone BVD4-1D11; eBioscience), anti-IL-10 (clone JES5-16E3; eBioscience), anti-IL-17 (clone 17-B7; BD Bioscience), and isotype-matched control antibodies.

Statistical Analysis

SBP data were compared with paired t-test. The other data were analyzed by unpaired t-test or if a t-test was not suitable, Mann-Whitney U-test to detect significant differences between 2 groups. Kaplan-Meier survival curves were constructed and analyzed using log-rank (Mantel-Cox) test. Incidence and mortality of AAA were analyzed by chi-square tests. A value of $P<0.05$ was considered statistically significant. For statistical analysis, GraphPad Prism version 6.0 (GraphPad Software) was used. All data were expressed as the mean ±SEM.

Supplemental References


Table S1. Body Weight, Systolic Blood Pressure and Plasma Lipid Profile in IL-2 complex-treated and control ApoE<sup>-/-</sup> mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n=7)</th>
<th>IL-2 complex (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>29.7 ± 1.0</td>
<td>29.3 ± 1.3</td>
</tr>
<tr>
<td>SBP, mmHg (pre)</td>
<td>106.7 ± 3.8</td>
<td>100.6 ± 6.1</td>
</tr>
<tr>
<td>SBP, mmHg (post)</td>
<td>125.0 ± 4.6*</td>
<td>116.3 ± 5.4*</td>
</tr>
<tr>
<td>Total-cholesterol, mg/dL</td>
<td>912.6 ± 60.3</td>
<td>1022.0 ± 110.6</td>
</tr>
<tr>
<td>HDL-cholesterol, mg/dL</td>
<td>13.2 ± 1.2</td>
<td>11.7 ± 1.6</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>58.7 ± 3.8</td>
<td>67.1 ± 6.9</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SEM.  *P<0.05 vs SBP (pre) in the same group.  SBP indicates systolic blood pressure; HDL, high-density lipoprotein; IL, interleukin.
Table S2. Body Weight, Systolic Blood Pressure and Plasma Lipid Profile in DEREG/ApoE⁻/⁻ and control ApoE⁻/⁻ mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ApoE⁻/⁻ (n=10-12)</th>
<th>DEREG/ApoE⁻/⁻ (n=7-8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>30.7 ± 0.6</td>
<td>24.6 ± 0.9†</td>
</tr>
<tr>
<td>SBP, mmHg (pre)</td>
<td>101.7 ± 2.5</td>
<td>103.8 ± 2.7</td>
</tr>
<tr>
<td>SBP, mmHg (post)</td>
<td>123.3 ± 4.0†</td>
<td>131.3 ± 8.5*</td>
</tr>
<tr>
<td>Total-cholesterol, mg/dL</td>
<td>846.0 ± 58.2</td>
<td>1051.0 ± 106.3</td>
</tr>
<tr>
<td>HDL-cholesterol, mg/dL</td>
<td>11.5 ± 1.2</td>
<td>9.5 ± 1.7</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>45.2 ± 7.3</td>
<td>26.6 ± 5.9</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SEM. ‡P<0.0001 vs ApoE⁻/⁻ mice, *P<0.05, †P<0.01 vs SBP (pre) in the same group. SBP indicates systolic blood pressure; HDL, high-density lipoprotein; IL, interleukin.
Figure S1. Effects of IL-2 complex therapy on elastin degradation and macrophage infiltration in aneurysmal lesions.
A and B, Representative photomicrographs (A) and categorical score (B) of elastin degradation in aneurysmal lesions (EVG staining; n=12 to 13 mice per group). Boxed area is expanded to show representative high-power fields in serial sections. C and D, Representative photomicrographs (C) and quantitative analysis (D) of macrophage infiltration in aneurysmal lesions (MOMA-2 staining; n=5 mice per group). *P<0.05. Scale bar, as shown in figures. Lm indicates lumen.
Figure S2. Effects of IL-2 complex therapy on Treg-associated molecules, T cell responses and DC maturation.

For analysis of immune responses in the lymphoid tissues, lymphocytes from spleen were prepared 5 days after the first injection of IL-2 complex or PBS.

A, The expression levels of Treg-associated markers were analyzed by flow cytometry gating on CD4+ Foxp3+ Tregs in the spleen. n=5 mice per group.

B, Splenocytes in each group were prepared and intracellular cytokine staining was performed. The graphs represent the frequencies of IFN-γ, IL-4, IL-10, and IL-17 producing CD4+ T cells.

C, The ratio of IFN-γ producing CD4+ T cells to IL-4 producing CD4+ T cells in the spleen was determined as Th1/Th2 ratio. n=3 mice per group.

D, The expression of CD80 and CD86 was determined gating on CD11c+ DCs in spleens. n=3 to 4 mice per group. *P<0.05, †P<0.01, ‡P<0.001, §P<0.0001. IFN indicates interferon; GITR, glucocorticoid-induced TNF receptor family-related gene/protein; CTLA4, cytotoxic T lymphocyte-associated protein 4; IL, interleukin; Th, T helper type.
Figure S3. Selective depletion of Foxp3⁺ Treg population.
Mice were treated with diphtheria toxin (DT) and sacrificed 24 hours after the first DT injection.
A, Representative results of CD4 and Foxp3 expression in the spleen assessed by flow cytometry. B. The percentage of Foxp3⁺ regulatory T cells within the CD4⁺ population in the total splenocytes. n= 3 mice per group. *P<0.05.
Figure S4. Effects of Foxp3+ Treg depletion on severity and the diameter of Ang II-induced AAA.
A, Severity of aneurysm in control ApoE−/− (n=10) and DREG/ApoE−/− mice (n=6). B, Maximal diameter of abdominal aorta in control ApoE−/− (n=14) and DREG/ApoE−/− mice (n=9).