Overexpression of SOCS3 in T Lymphocytes Leads to Impaired Interleukin-17 Production and Severe Aortic Aneurysm Formation in Mice—Brief Report

Mélissa Romain,* Soraya Taleb,* Marion Dalloz, Padmapriya Ponnuswamy, Bruno Esposito, Nicolas Pérez, Yu Wang, Akihiko Yoshimura, Alain Tedgui, Ziad Mallat

Objective—Mutations of signal transducer and activator of transcription 3 (STAT3) are responsible for autosomal dominant hyperimmunoglobulin E syndrome. Recently, we reported frequent vascular abnormalities, including aneurysms in these patients, and demonstrated that STAT3 inhibition promoted aneurysm in mice. The purpose of this study was to investigate the role of cell-specific STAT3 signaling in the susceptibility to aneurysm.

Methods and Results—C57BL/6 wild-type mice were irradiated and repopulated with bone marrow cells isolated from either wild-type mice or from mice with defective STAT3 signaling as a result of overexpression of suppressor of cytokine signaling 3 (SOCS3-Tg mice). Mice were then subjected to a validated model of abdominal aortic aneurysm induced by angiotensin II infusion for 28 days, along with repetitive injections of a neutralizing antitransforming growth factor-β antibody. We found that overexpression of SOCS3 in bone marrow–derived cells significantly increased aneurysm severity (P=0.04). In contrast, overexpression of SOCS3 in the vessel wall had no effect on the disease process. Surprisingly, deletion of STAT3 signaling in macrophages did not affect aneurysm development. Interestingly, however, defective STAT3 signaling in SOCS3-Tg T cells markedly increased aneurysm severity (P=0.01) and mortality from aneurysm rupture (P=0.008). Overexpression of SOCS3 in T cells significantly decreased interleukin-17 production (P<0.0001) and was associated with a reduction of its plasma levels (P=0.02).

Conclusion—These findings clearly identify a central role for T cell-specific STAT3 signaling in the promotion of vascular aneurysm and support previous work on interleukin-17 protective role in this process. (Arterioscler Thromb Vasc Biol. 2013;33:581-584.)

Key Words: aneurysm immunity leukocytes

Abdominal aortic aneurysm (AAA) is an age-associated disease with increasing incidence and considerable consequences on morbidity and mortality.1 Deficiency of signal transducer and activator of transcription 3 (STAT3) is a rare primary immunodeficiency that is referred to as an autosomal dominant hyperimmunoglobulin E syndrome, caused by mutations in several domains of STAT3.2 These patients display defective STAT3 signaling, characterized by recurrent bacterial and fungal infections, connective tissue abnormalities, hyperimmunoglobulin E, and Th17 lymphopenia.3,4 We recently studied 36 autosomal dominant hyperimmunoglobulin E syndrome STAT3-deficient patients and reported the presence of peripheral and brain arterial abnormalities, mainly ectasia and aneurysms in 84% of the patients, suggesting involvement of STAT3 signaling in aneurysm formation.5 We also showed that mice with overexpression of suppressor of cytokine signaling 3 (SOCS3), which exhibit reduced STAT3 phosphorylation and activation, were highly susceptible to aneurysm formation and rupture in response to angiotensin II (Ang II) infusion and administration of a neutralizing antitransforming growth factor (TGF)-β antibody.6 However, the mechanisms and the specific cell type(s) responsible for the vascular phenotype remained to be identified. In this study, we examined the consequences of defective STAT3 signaling in specific cell types on the susceptibility to aneurysm formation and rupture.

Materials and Methods
Details of Methods can be found in the online-only Data Supplement. We used mice with genetic overexpression of SOCS3 or cell-specific deletion of STAT3. AAA was induced by subcutaneous infusion of Ang II along with systemic neutralization of TGF-β.
**Results**

**Disruption of STAT3 Signaling in Bone Marrow–Derived Cells Aggravates AAA**

To induce severe AAA, mice were infused with Ang II and were repetitively injected with anti–TGF-β antibody for 28 days, as previously described. As depicted in Figure 1A, repopulation of C57BL/6 mice with SOCS3-Tg bone marrow increased the severity of AAA (P=0.04). Aneurysm formation was associated with accumulation of macrophages and T lymphocytes within the adventitia and their infiltration within the media (Figure 1A and 1B in the online-only Data Supplement). Also, elastin elongation and degradation were observed in advanced aneurysms (Figure 1C in the online-only Data Supplement). Because STAT3 signaling is known to control Th17 development, we examined cytokine production by spleen lymphocytes and observed a marked reduction of intracellular interleukin (IL)-17 staining (P=0.04; Figure 1B), but no change in interferon-γ expression (P=0.34; Figure 1C) in mice reconstituted with SOCS3-Tg bone marrow. In contrast, Th17 development and aneurysm severity were not altered in SOCS3-Tg mice reconstituted with wild-type bone marrow.

![Figure 1](https://www.ahajournals.org/doi/fig/10.1161/ATVAB.113.302965)

**Figure 1.** Overexpression of suppressor of cytokine signaling 3 (SOCS3) in bone marrow–derived cells increases aneurysm severity. **A**–**C**, Lethally irradiated (9.5 Gy) wild-type (WT) mice were reconstituted with bone marrow from either WT (WT→WT) or SOCS3-Tg (SOCS3-Tg→WT) mice. After 3 weeks of recovery, mice were treated with angiotensin (Ang) II + antitransforming growth factor (TGF)-β and euthanized after 28 days (Methods in the online-only Data Supplement). **A**, Classification of aortic aneurysms in the 2 groups of mice as described in the Methods in the online-only Data Supplement (increasing severity from stage 0= no aneurysm to stage IV= aneurysm rupture). **B** and **C**, Representative examples and quantitative analysis of flow cytometry-based intracellular staining of interleukin (IL)-17 (B) and interferon (IFN)-γ (C) gated on lymphocytes T in the 2 groups of mice (WT→WT, n=7; SOCS3-Tg→WT, n=5). **D** and **E**, Lethally irradiated WT (WT→WT) or SOCS3-Tg (WT→SOCS3-Tg) mice were reconstituted with bone marrow from WT mice. After 3 weeks of recovery, mice were treated with Ang II + anti-TGF-β and euthanized after 28 days. **D**, Quantitative analysis of intracellular staining of IFN-γ and IL-17 gated on lymphocytes (WT→WT, n=5; WT→SOCS3-Tg, n=5). **E**, Classification of aortic aneurysms in the 2 groups of mice as described in the Methods in the online-only Data Supplement. Results are mean±SEM. **P<0.01.**
(Figure 1D and 1E). These results suggest that SOCS3/STAT3 signaling in vascular cells is dispensable for AAA and identify an important contribution of STAT3/SOCS3 signaling in bone marrow–derived cells to AAA.

STAT3 Signaling in Macrophages and Neutrophils Is dispensable for AAA Formation

Macrophages and neutrophils contribute to AAA. However, to our surprise, mice with selective deletion of STAT3 in macrophages and neutrophils (STAT3\(^{flox/flox}\)/LysM-Cre) did not show significant alteration of disease severity in comparison with control STAT3\(^{flox/flox}\) mice (Figure II in the online-only Data Supplement). This result clearly indicates that STAT3 signaling in macrophages and neutrophils is dispensable for AAA development.

Alteration of STAT3 Signaling in T Cells Impacts AAA Formation and Severity

T helper cell responses have been shown to contribute to aneurysm development. We therefore addressed the contribution of SOCS3/STAT3 signaling in T cells to AAA formation. Spleen CD4+ T cells were isolated from either wild-type or SOCS3-Tg mice and were used in an adoptive transfer model to reconstitute Rag1\(^{-/-}\) mice. SOCS3-Tg CD4+ T cells showed altered STAT3 phosphorylation (Figure 2A), no change in IL-4 (Figure 2B) but reduced interferon-\(\gamma\) production (\(P=0.01\)), and an inability to produce IL-17 (Figure 2B). In addition, reconstitution of Rag1\(^{-/-}\) mice with SOCS3-Tg CD4+ T cells was associated with reduced plasma IL-17 levels (\(P=0.02\)) compared with the transfer of SOCS3\(^{+/+}\) CD4+ T cells (Figure 2C). This decrease of IL-17 production is specific to the model because Th17 differentiation at basal state in SOCS3-Tg CD4+ cells is not altered compared with WT cells (Figure III in the online-only Data Supplement). Interestingly, adoptive transfer of SOCS3-Tg CD4+ T cells in Rag1\(^{-/-}\) mice markedly increased the susceptibility of these mice to AAA compared with the transfer of SOCS3\(^{+/+}\) CD4+ T cells as shown by increased aneurysm incidence, severity (\(P=0.01\); Figure 2D and 2E), and increased mortality from aneurysm rupture (\(P=0.008\); Figure 2F). Of note, repopulation of C57BL/6 mice with IL-17\(^{-/-}\) bone marrow increased the severity of AAA (\(P=0.08\); Figure IV in the online-only Data Supplement). These results clearly indicate an important role for SOCS3/STAT3-dependent Th17 in the development and progression of AAA. Finally, overexpression of SOCS3 also promoted Ang II–induced AAA in the absence of anti–TGF-\(\beta\) antibody (Figure V in the online-only Data Supplement), ruling out the possibility of a specific interaction with TGF-\(\beta\) signaling.

Discussion

In this study, we investigated the role of cell type–specific SOCS3/STAT3 signaling in aneurysm development. Unexpectedly, inhibition of STAT3 signaling in vascular and myeloid cells did not alter mice susceptibility to AAA. We therefore considered a role for T cells. A review of the literature indicated that their role in vascular aneurysm formation has been shown to contribute to aneurysm development. We therefore addressed the contribution of SOCS3/STAT3 signaling in T cells to AAA formation. Spleen CD4+ T cells were isolated from either wild-type or SOCS3-Tg mice and were used in an adoptive transfer model to reconstitute Rag1\(^{-/-}\) mice. SOCS3-Tg CD4+ T cells showed altered STAT3 phosphorylation (Figure 2A), no change in IL-4 (Figure 2B) but reduced interferon-\(\gamma\) production (\(P=0.01\)), and an inability to produce IL-17 (Figure 2B). In addition, reconstitution of Rag1\(^{-/-}\) mice with SOCS3-Tg CD4+ T cells was associated with reduced plasma IL-17 levels (\(P=0.02\)) compared with the transfer of SOCS3\(^{+/+}\) CD4+ T cells (Figure 2C). This decrease of IL-17 production is specific to the model because Th17 differentiation at basal state in SOCS3-Tg CD4+ cells is not altered compared with WT cells (Figure III in the online-only Data Supplement). Interestingly, adoptive transfer of SOCS3-Tg CD4+ T cells in Rag1\(^{-/-}\) mice markedly increased the susceptibility of these mice to AAA compared with the transfer of SOCS3\(^{+/+}\) CD4+ T cells as shown by increased aneurysm incidence, severity (\(P=0.01\); Figure 2D and 2E), and increased mortality from aneurysm rupture (\(P=0.008\); Figure 2F). Of note, repopulation of C57BL/6 mice with IL-17\(^{-/-}\) bone marrow increased the severity of AAA (\(P=0.08\); Figure IV in the online-only Data Supplement). These results clearly indicate an important role for SOCS3/STAT3-dependent Th17 in the development and progression of AAA. Finally, overexpression of SOCS3 also promoted Ang II–induced AAA in the absence of anti–TGF-\(\beta\) antibody (Figure V in the online-only Data Supplement), ruling out the possibility of a specific interaction with TGF-\(\beta\) signaling.
was still debated. 

Th1 responses have been shown to be pathogenic in calcium chloride-induced aneurysm formation, whereas Th2-mediated immunity contributed to aneurysms in allografted aortas. In the Ang II model, lymphocyte deficiency under a hypercholesterolemic Apoe−/−/Rag−/− background did not significantly alter aneurysm development. In contrast, we have previously observed a significant protection from the disease in normcholesteromic Rag−/− mice using a more severe aneurysm model involving neutralization of TGF-β activity on top of Ang II infusion. It was therefore remarkable that the selective transfer of CD4+ T cells exhibiting defect in STAT3 signaling in immunodeficient mice markedly aggravated aneurysms and led to fatal vascular rupture, emphasizing the critical role of T cell–specific STAT3 signaling in this disease. It should be acknowledged that although deficient STAT3 signaling is a main consequence of SOCS3 overexpression, it is not the only process controlled by SOCS3. It will be interesting to confirm our results using specific deletion of STAT3 in T cells.

The mechanisms by which STAT3 signaling in T cells play a role in aneurysm development are unknown. Collectively, our results suggest that reduced IL-17 production may contribute to enhanced vascular inflammation and increased susceptibility to aneurysms. These data are supported by results from Xiong et al that reported (in abstract form) a protective role of IL-23–IL17 axis in calcium chloride-induced aneurysm formation (Xiong, Baxter, Experimental Biology 2010, abstract no. 751.6). Other investigators reported either a detrimental role of IL-17 deficiency (Madhur, Harrison, Experimental Biology 2010, abstract no. 589.8) or no effect in Ang II–induced AAA under hypercholesterolemic Apoe−/− background without mechanistic details. Future studies should delineate more precisely the role of IL-17 and the different STAT3 signaling targets in aneurysm progression and identify the critical STAT3–related factors responsible for destructive vessel remodeling.

Acknowledgments

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Disclosures

None.

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Methods online

Mice

All experiments were conducted on male mice between 8 and 12 weeks of age. Mice with genetic overexpression of SOCS3 or cell-specific deletion of STAT3 were used. Mice with a macrophage/neutrophil–specific deletion of the STAT3 gene (STAT3\(^{floxed}/\)LysM-Cre; background C57BL/6 x 129\(\text{Ola}\)) and STAT3\(^{floxed}\) mice were provided by Irmgard Förster (Institute for Genetics, University of Cologne, Germany). The IL-17A deficient mice were provided by Y. Iwakura (University of Tokyo, Tokyo, Japan). We subjected C57BL/6 or C57BL/6 \(\text{SOCS3-Tg}\) mice to medullar aplasia by 9.5 Gray of lethal total body irradiation. We repopulated the C57BL/6 or \(\text{SOCS3-Tg}\) mice with an intravenous injection of 5. 10\(^6\) bone marrow-derived cells isolated from femurs and tibias of C57BL/6 or \(\text{SOCS3-Tg}\) mice. Besides, C57BL/6 \(\text{Rag-1}^{−/−}\) mice (from Charles River) were transferred intravenously with 2. 10\(^6\) CD4\(^+\) cells isolated from the spleen of either C57BL/6 or \(\text{SOCS3-Tg}\) mice. After 2 weeks of recovery, AAA was induced. Experiments were conducted according to the guidelines formulated by the European Community for experimental animal use (L358-86/609EEC) and were approved by the Ethical Committee of INSERM and by the French Ministry of Agriculture (agreement no. A75-15-32).

Aneurysm model

AAAs were induced in normocholesterolemic mice by subcutaneous infusion of Angiotensin II (Sigma-Aldrich) at 1,000 ng/kg/min for 5 days or 28 days (mini-pumps Alzet, model 2001 and 2004, Charles River), along with systemic neutralization of TGF-\(\beta\) (i.p. injection of 500 \(\mu\)g, 3 times per week; Biotem) as previously described. A 5-point grading system was used to classify aneurysms based on Daugherty et al classification.

Flow cytometry

Peripheral blood cells were stained with an anti-CD4 (clone RM4-5, eBioscience) antibody. For intracellular cytokine determination, splenocytes were stimulated with leukocyte activation cocktail (BD Biosciences) for 4 to 6 hrs prior to staining. Intracellular staining of IFN\(\gamma\) (clone XMG1.2; BD Biosciences) and IL-17A (clone TC11-18H10.1; BioLegend) was performed according to the manufacturer's instructions (eBioscience). Phospho-stat3 (Tyr705) was used to assess P-STAT3 (clone D3A7; Cell Signalling). Labeled cells were analyzed by a flow cytometer (Fortessa; BD Biosciences).

Western blot analysis

Splenocytes or macrophages were lysed in detergent buffer (RIPA) containing protease inhibitors mixture (Roche) and sodium orthovanadate. Proteins were separated on 4–12% NuPage Tris-Bis gels using NuPage MES-SDS running buffer (Invitrogen) and were transferred onto nitrocellulose membranes. The filters were probed with antibodies directed against phospho (P)-Stat3, Stat3, P-Stat1 (Cell Signaling).

Cell purification, culture and cytokine assays

CD4\(^+\) cells were differentiated toward Th17 enriched cells in IMDM medium supplemented with TGF-\(\beta\) (20 ng/ml), IL-6 (20 ng/ml) and cells were stimulated by coated anti-CD3 (5 \(\mu\)g/ml) and soluble anti-CD28 (1 \(\mu\)g/ml) during 72 h.
CD4$^+$ cells were negatively selected from the splenocytes by the use of a cocktail of antibody-coated magnetic beads (Miltenyi Biotech). Cells were then cultured in RPMI 1640 supplemented with Glutamax, 10% FCS, 0.02 mmol/L 2β-mercaptoethanol and antibiotics. For cytokine measurements, CD4$^+$ T cells were cultured at 2×10$^5$ cells per well for 48 hours in anti-CD3–coated (2 µg/ml) and anti-CD28–coated (2 µg/ml) microplates. IFN-γ, IL-4, and IL-17A productions in the supernatants or plasma were measured using specific ELISAs (BD Biosciences and R&D Systems).

Statistics
Values are expressed as percentage or mean ± SEM when appropriate. Statistical tests included unpaired t test, Mann–Whitney test, $\chi^2$ test for a trend, and Fisher’s exact tests. Kaplan-Meier survival curves were constructed and analyzed using log-rank (Mantel-Cox) test. $P$ values of less than 0.05 were considered significant.

References

Supplementary Figure legends

**Supplementary Figure I. Characterization of aneurysms.** Lethally irradiated (9.5Gy) WT mice were reconstituted with bone marrow from SOCS3-Tg (SOCS3-Tg→WT; right) mice. After 3 weeks of recovery, mice were treated with Ang II + anti-TGFβ and sacrificed after 28 days (see Methods online). A and B, Representative photographs of CD68 (A) and CD3 (B) (x40) staining showing infiltrates of macrophages and T cells. C, Representative photographs of orcein staining (x40) showing elastin elongation and degradation.

**Supplementary Figure II. Alteration of STAT3 signaling in macrophages/neutrophils has no effect on aneurysm development.** A, STAT3 staining in bone marrow-derived macrophages from STAT3<sup>flox/flox</sup> and STAT3<sup>flox/flox</sup>/LysM-cre mice. B and C, STAT3<sup>flox/flox</sup> (n=9) and STAT3<sup>flox/flox</sup>/LysM-cre (n=9) mice were treated with Ang II + anti-TGFβ and sacrificed after 28 days. Classification of aortic aneurysms (B) and Kaplan-Meier curves of survival free from aneurysm rupture (C) in the two groups of mice.

**Supplementary Figure III. Overexpression of SOCS3 in CD4<sup>+</sup> cells has no effect on Th17 differentiation at basal state.** CD4<sup>+</sup> cells were differentiated into Th17 in IMDM medium supplemented with TGF-β and IL-6 during 72 h. A, Quantitative analysis of intracellular staining of IL-17. B, IL-17 production in supernatants measured by ELISA (n=3).

**Supplementary Figure IV. Absence of IL-17 in bone marrow cells lead to increased aneurysm severity.** Lethally irradiated (9.5Gy) WT mice were reconstituted with bone marrow from either WT (WT→WT) or IL-17<sup>−/−</sup> (IL-17<sup>−/−</sup>→WT) mice. After 3 weeks of recovery, mice were treated with Ang II + anti-TGFβ and sacrificed after 28 days (see Methods online). Photographs (A), classification of aortic aneurysms (B).

**Supplementary Figure V. Overexpression of SOCS3 in mice increases aneurysm severity without the need for TGFβ neutralization.** A, P-STAT1, P-STAT3 and STAT3 in splenocytes isolated from WT and SOCS3-Tg mice and stimulated by IL-6 in vitro during 30 min. Ubiquitous overexpression of SOCS3 decreases P-STAT3 but does not alter P-STAT1. B, WT (n=19) and SOCS3-Tg (n=13) Mice were treated with Ang II and sacrificed after 28 days (see Methods online). Classification of aortic aneurysms in the two groups of mice as described in Methods online.
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