

 **Original Article** 

STAT3 Activation Correlates with Adventitial Neutrophil Infiltration in Human Aortic Dissection

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Objective: Aortic dissection (AD) is a fatal disease that is caused by the rapid destruction of the aortic wall. Although recent studies in animal models indicate an important relationship between inflammation and tissue destruction, activation status of inflammatory signaling and its relation to the inflammatory cell infiltration are poorly characterized in human AD.

Materials and Methods: We examined the activation of inflammatory signaling molecules NFκB and STAT3, and neutrophil infiltration in AD tissue samples that were obtained during the surgical repair within 24 h after AD onset.

Results: Activation of NFκB was observed mainly in the intima both in AD samples and in aortic samples without AD. Activation of STAT3 was observed in AD samples, but not in the aortic sample without AD. Neutrophil infiltration was observed predominantly in the adventitial layer of AD samples. Histological analysis revealed that STAT3 was activated in cells other than neutrophils. Notably, STAT3 activation and neutrophil infiltration showed positive correlation in adventitial layer of AD tissue.

Conclusion: These findings demonstrated that adventitial STAT3 activation was associated with neutrophil infiltration,

suggesting their importance in AD pathogenesis.

Keywords: aortic dissection, inflammation, STAT3, NFκB, neutrophil

Introduction

Aortic dissection (AD) is a fatal medical condition in which the aortic wall is detached into two layers at the medial level, resulting in two lumens having a certain length along the arterial course.¹⁾ In the acute phase, it poses a high mortality rate due to rapid progression of tissue destruction, and in the chronic phase, various complications occur, including aneurysm formation, recurrent dissection and peripheral organ ischemia due to aortic tissue fragility.^{1,2)} The mortality of type A dissection increases 1–2% per hour early after onset, and reaches about 25% at 6 h and 50% by 24 h, due to the aortic rupture, cardiac tamponade and distal ischemia.³⁾ Therefore, tissue destruction in the early phase of the disease time course has an important impact on the clinical outcome. In the Stanford classification, dissections that affect the ascending aorta are defined as type A, and those that do not are defined as type B. In type A, surgery in the acute phase is a class I recommendation.⁴⁾ Despite advances in diagnostic imaging and treatment,⁵⁾ AD remains a life-threatening condition because of its unpredictable nature, and the mechanisms of tissue destruction have not yet been clarified.⁶⁾


Recently, the inflammatory response in aortic walls has drawn much attention as a mechanism of tissue destruction both in human AD^{7,8)} and in animal models of AD.^{9–11)} Specifically, neutrophils and macrophages play a critical role in tissue destruction during AD development in mice.^{10–12)} Neutrophils may also participate in tissue destruction in human AD, as the increase in neutrophil population in peripheral blood predicts in-hospital mortality in patients with type A dissection.¹³⁾ Histological analysis of human AD revealed that neutrophils are the predominant inflammatory cells, peaking between 12 and 24 h, whereas macrophages peaked between 2 and

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Received: January 8, 2019; Accepted: February 27, 2019
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7 days after onset.⁸⁾ At the molecular level, a proinflammatory cytokine, IL-6, and its downstream signal mediators, JAK2 and STAT3, play critical roles in murine models of AD.^{9,11,14)} Transcriptome analysis of human AD samples also suggested the importance of JAK2.⁷⁾ NFκB, another inflammatory signal mediator for TNFα, IL-1β, and IL-17, may also be important in vascular inflammation. In addition, it has been proposed that STAT3 and NFκB may make an amplification loop for the inflammatory signaling.¹⁵⁾ However, the involvement of NFκB in AD pathogenesis has not been well characterized.¹⁶⁾ To translate these findings to clinical practice, it is essential to characterize these proinflammatory molecules in relation to the cellular infiltration in human AD. Accordingly, we analyzed the activation status of STAT3 and NFκB, and cellular infiltration in human AD tissue samples to gain insight into their involvement in AD pathogenesis.

Methods

Curation of human aortic samples

All protocols that involved human specimens were approved by the Institutional Review Board at Kurume University Hospital, and all samples were obtained with the written, informed consent of the patients. Human AD tissue was obtained from a set of six randomly chosen patients during the surgical repair of type A dissection within 24h after the onset (Table 1). AD patients with an obvious family history or syndromic AD as judged by the physical phenotypes were excluded. A control aortic sample without AD was obtained from a patient with hypertrophic cardiomyopathy who died of fatal arrhythmia. After obtaining the samples of ascending aorta, the entry site was located, and the circumferential extent of the pseudolumen at the level of the entry site was determined by visual observation. Aortic wall samples were obtained at the border zone between the regions with and without visible medial dissection, ap-

proximately 1.5cm in circumferential length and the full thickness of the aortic wall including the dissection flap.

Histological analysis

For histological analyses, AD tissues were fixed in 4% paraformaldehyde, paraffin-embedded, and sliced into 5-μm-thick tissue sections with circumferential orientation. Sections of aortic tissue were stained with hematoxylin and eosin (H&E), and elastica van Gieson (EVG) staining.

Immunofluorescence staining of the aortic tissue was performed using antibodies for STAT3 phosphorylated at Tyr705 (P-STAT3, Cell Signaling Technologies #9145, Danvers, MA, USA) with a TSA labeling kit with AlexaFluor 488 tyramide (Thermo Fisher Scientific #T-20922, Waltham, MA, USA), NFκB (Abcam, #ab13594, Cambridge, UK) with Cy3-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories #111-115-144, West Grove, PA, USA), and TOPRO-3 (Thermo Fisher Scientific #T3605, Waltham, MA, USA) for nuclear staining. Immunohistochemical staining of AD tissue for P-STAT3 (Cell Signaling Technologies #9145), and neutrophils (neutrophil elastase, Dako #M0752, Santa Clara, CA, USA) were also performed using avidin-biotin complex staining kits (Vectastain #PK-4001 and #PK-4002, Vector Labs, Burlingame, CA, USA) according to the instructions from the manufacturer.

Imaging cytometry

Microscopic images were obtained using an automated microscope (BZ-9000, Keyence, Osaka, Japan). The specificity of the fluorescence signal was validated by the comparable level of background signals among the samples with multicolor staining and without staining. Fluorescence images were obtained from intimal, medial, and adventitial layers of the aortic walls. The medial layer was further divided into three layers with approximately the same thickness, namely the inner, middle, and outer medial layers, and images were

Table 1 Clinical characteristics of patients

Patient	Age (years)/sex	Entry	Surgery	Timing of surgery	Comorbidity	Current smoking	Medication
Control	45/M	N/A	N/A	N/A	HCM fatal arrhythmia	No	Bisoprolol
Case #1	73/F	Ascending	HAR	12h	HT	No	Benidipine, Bazedoxifene acetate
Case #2	89/F	Arch	PAR	9h	None	No	None
Case #3	78/F	Ascending	HAR	4h	HT	No	None
Case #4	75/F	Ascending	HAR	14h	HT, DL	No	Bisoprolol, Azilsartan, Azosemide
Case #5	67/M	Descending	TAR	16h	HT	Yes	None
Case #6	64/M	Ascending	HAR	4h	HT, CI	No	None

The control sample was obtained at the autopsy of a patient with hypertrophic cardiomyopathy who died a sudden death due to the fatal arrhythmia.

M: male; F: female; PAR: partial aortic replacement; TAR: total arch replacement; HAR: hemiarch replacement; HT: hypertension; DL: dyslipidemia; CI: cerebral infarction; HCM: hypertrophic cardiomyopathy; N/A: not applicable

obtained at the center of the thickness of each part of the medial layer. Twenty fields of microscopic images with a 40× objective lens were obtained from each of these 5 layers in a single sample. In total, 100 microscopic images, [5 layers: intima, inner media, middle media, outer media, and adventitia]×20 fields, were obtained from a single patient and subjected to the imaging cytometric analysis using ArrayScan XTI (Thermo Fisher Scientific, Waltham, MA, USA). Because both STAT3 and NFκB are transcriptional factors that work within nuclei, their nuclear localization at the single cell level was determined by subtracting the cytosolic staining intensity from the nuclear staining intensities. The cytometric data obtained by ArrayScan XTI were analyzed and visualized by FlowJo ver. 10 software (FlowJo, Ashland, OR, USA). Immunohistochemical staining images were obtained by a 20× objective lens, and the stained area was quantitated using ImagePro Plus ver. 7 software (Media Cybernetics, Rockville, MD, USA).

Statistical analysis

Statistical analyses were performed with GraphPad PRISM 5 (GraphPad Software, San Diego, CA, USA).

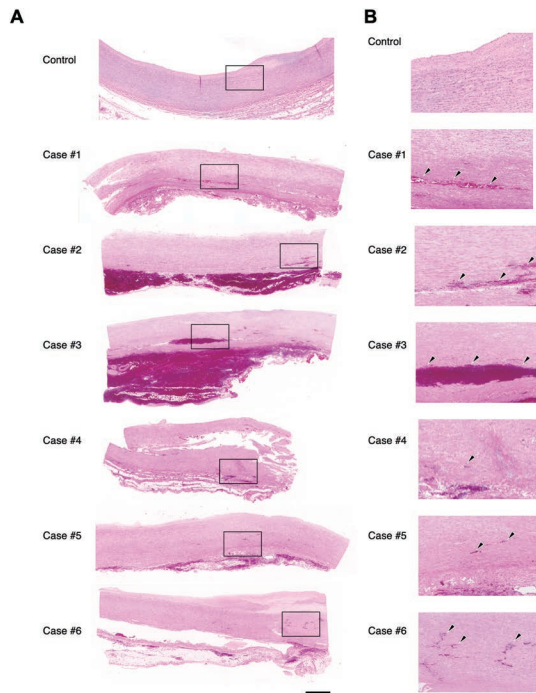


Fig. 1 Pathohistological analysis of aortic samples. (A) Aortic samples from control aorta without aortic dissection (AD) (Control) and those from the border zone of AD tissue (Cases #1–#6) are shown by hematoxylin and eosin (H&E) staining. All samples are shown with the intimal side up and the adventitial side down. Scale bar 1 mm. (B) Enlarged histological images are shown by H&E staining that correspond to the rectangles in panel (A). Arrowheads indicate the intramural bleeding in AD samples.

Linear regression analysis was performed to test the significance of correlation between two parameters. $P < 0.05$ was accepted as statistical significance.

Results

Activation of STAT3 and NFκB in border zone of acute AD

We collected AD samples from 6 AD patients within 24 h

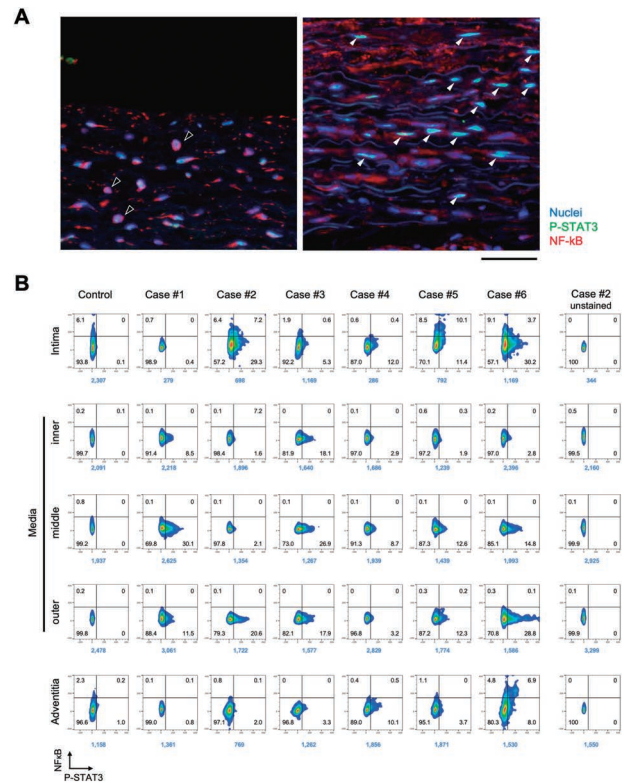


Fig. 2 Activation status of STAT3 and NFκB. (A) Immunofluorescence staining was performed for NFκB (red), phosphorylated (activated) STAT3 (P-STAT3, green), and nuclei (blue). Representative images are shown for the intima of Case #6 and the outer media of Case #2 to show the nuclear NFκB-positive (black arrowheads) and P-STAT3-positive (white arrowheads) cells. Scale bar 50 μm. (B) Scattergrams of the imaging cytometric analysis are shown for immunofluorescence staining for nuclei, P-STAT3 (x-axis) and NFκB (y-axis). Aortic samples without aortic dissection (AD) (Control) and those at the border zone of AD (Cases #1–#6) were segmented into the layers of intima, inner media, middle media, outer media, and adventitia. The rightmost panels show the samples from Case #2 that were processed in the same way as other samples but without the primary antibodies for NFκB or P-STAT3, which served as a negative control. The gates for nuclear NFκB- and P-STAT3-positive cells were arbitrarily determined, and applied to all of the samples. The percentages of cell populations are shown in the corresponding quadrants in black letters. The numbers of counted cells are shown under the corresponding panels in blue letters.

after the onset (Table 1), and analyzed the tissue focusing on the border zone between the entry site and the aortic wall without visible pseudolumen. We also obtained an ascending aortic sample as a control without dissection from a patient of hypertrophic cardiomyopathy who died of fatal arrhythmia. Histological analysis revealed various degree of tissue destruction with intramural hematoma in AD samples, whereas the control sample showed ordered tissue architecture (Fig. 1). Immunofluorescence staining was performed for NFκB and activated (phosphorylated) STAT3 (P-STAT3) (Fig. 2A). We assessed the activation status of NFκB and STAT3 in the aortic samples by imaging cytometric analysis (Fig. 2B). NFκB activation was observed mainly in the intimal layer to various extents, re-

gardless of the presence of AD. STAT3 activation was negligible in the control aorta, whereas AD samples showed STAT3 activation in intima, media, and adventitia. The cell population with simultaneous activations of NFκB and STAT3 at the single cell level was low in any layer of any sample. These findings indicated that although both NFκB and STAT3 are signaling molecules and transcription factors in the inflammatory response, their roles might be different in the context of AD pathogenesis, and STAT3 was specifically activated in AD.

Infiltration of neutrophils in the border zone of acute AD

To further investigate the inflammatory response in AD, we stained the aortic tissue for neutrophils, because this type of cell has been reported to be predominant in the acute phase in human AD⁸⁾ and play an important

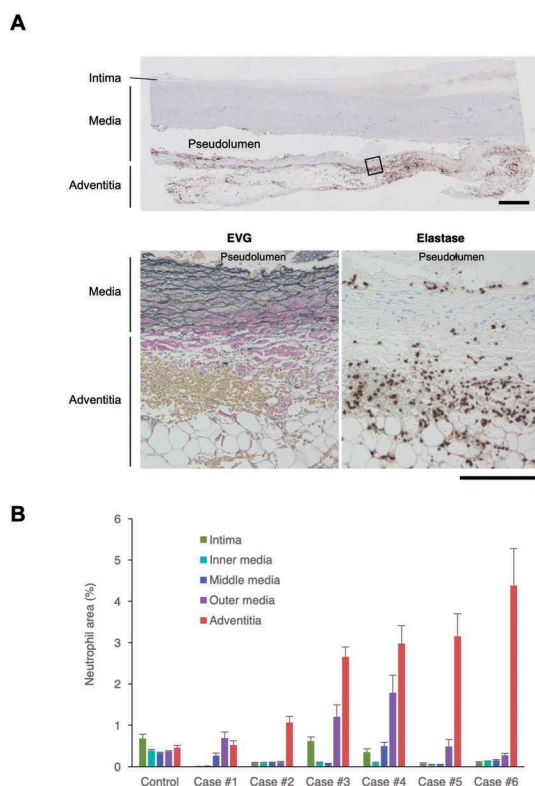


Fig. 3 Immunohistochemical staining for neutrophils. (A) Representative images are shown for immunohistochemical staining for neutrophil elastase (brown staining), a marker of neutrophils, and elastica van Gieson (EVG) staining for aortic dissection (AD) samples at the border zone. The top panel indicates the low magnification image. Scale bar 1 mm. The bottom panels indicate the magnified view of the area, as shown by a rectangle in the top panel, for EVG (left panel) and elastase staining (right panel, brown color) of the serial sections. Scale bar 200µm. (B) Quantitative analysis is shown for the percentages of the elastase-positive area in the tissue area of the aortic samples. The layers of intima, inner media, middle media, outer media, and adventitia are color-coded as shown. Data are expressed as means±standard errors of 20 observational areas in each group.

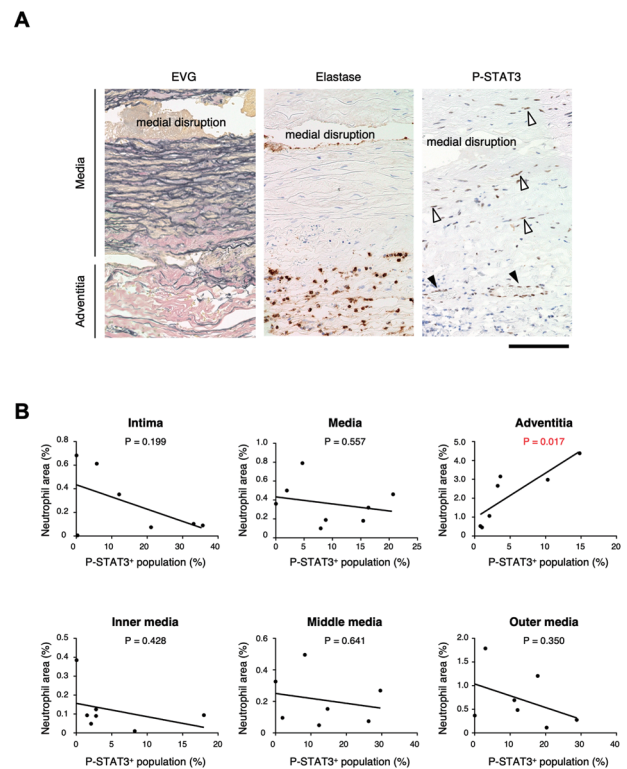


Fig. 4 Immunohistochemical staining for neutrophils and phosphorylated STAT3 (P-STAT3). (A) Representative images are shown for the outer media and the adventitia of the aortic dissection (AD) sample with elastica van Gieson (EVG), elastase (neutrophil) and P-STAT3 stainings of the serial sections. White and black arrowheads indicate the P-STAT3-positive nuclei in the media and in the adventitia, respectively. Scale bar 200µm. (B) Correlations are shown for the nuclear P-STAT3-positive cell (P-STAT3⁺) population and the elastase-positive area in the corresponding area in AD samples. P values are shown for the statistical analysis of the correlation by the linear regression analysis.

role in AD tissue destruction in mouse AD models.^{10,12} Neutrophils were observed in the adventitial layer and part of the surface of the pseudolumen (Fig. 3A). Quantitative analysis demonstrated that neutrophils infiltrated predominantly in the adventitial layer, and in the outer zone of the medial layer in some cases (Fig. 3B). To better understand the potential role of STAT3 in the inflammatory response in AD, we examined the STAT3 activation and neutrophil infiltration in the same samples (Fig. 4A). Elastase-positive neutrophils and P-STAT3-positive cells were observed abundantly in the AD tissue adjacent to the medial disruption. However, the pattern of cellular distributions was different between neutrophils and P-STAT3-positive cells. While elastase-positive neutrophils were observed predominantly in the adventitial layer adjacent to the disrupted media, P-STAT3-positive cells were observed both in the medial layer and in the adventitial layer. Elastase staining was localized to the cells with round shaped nuclei in the adventitial layer, whereas P-STAT3 staining was localized to the elongated nuclei in the medial layer that were packed between the elastic lamellae, and to the oval shape nuclei surrounding small vessels, but not in the round shape nuclei, suggesting that STAT3 activation may not be high neutrophils. We further compared the elastase staining-positive area and P-STAT3-positive cell population in each layer of the AD tissue (Fig. 4B). Interestingly, STAT3 activities showed significant correlation with the neutrophil infiltration only in the adventitial layer, suggesting that although STAT3 seemed to be active in non-neutrophils, STAT3 activities may be related to the neutrophil infiltration.

Discussion

In this study, we found that STAT3 was activated in all of the aortic wall layers in AD tissue; intima, media, and adventitia to various extents, whereas NF κ B was predominantly activated in the intimal layer regardless of the presence of AD. Infiltration of neutrophils, the dominant inflammatory cells in acute inflammation, was observed mainly in the adventitia of AD tissue, indicating that the inflammatory response is predominantly in the adventitial layer. Notably, the extent of neutrophil infiltration showed significant correlation with STAT3 activation in the adventitial layer, suggesting that STAT3 may be involved in the adventitial inflammation in AD.

Regarding the inflammatory response and tissue destruction in AD, it has been demonstrated in a murine model that adventitial IL-6 and STAT3 activation are causally involved in AD pathogenesis.¹¹ STAT3-activating cytokines including IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte-colony stimulating factor (G-CSF) cause recruitment of

neutrophils and rupture of the aorta.^{12,17} It has also been reported that neutrophils are directly involved in the tissue destruction in mouse AD by secreting matrix metalloproteinases.¹⁰ The central role of the JAK2/STAT3 pathway was demonstrated by transcriptome and network analyses in human Stanford type A dissection.⁷ Positive correlation between STAT3 activity and neutrophil infiltration in human AD tissue as shown in this study is consistent with these previous reports, supporting the notion that STAT3 signaling is involved in AD pathogenesis.

Currently, it is unclear whether neutrophil infiltration and STAT3 activation are the cause or the result of AD onset. A previous report showed that neutrophils appear in the adventitia of the human AD tissue within the first 12 h, peaked between 12 and 24 h after onset, and were then rare after 2 days.⁸ Therefore, neutrophil infiltration is likely to occur after AD onset, which in turn may participate in the adverse remodeling of aortic walls, as suggested by mouse studies.^{10,12} It is also unclear whether the activation of STAT3 is the cause, the consequence or a parallel phenomenon to neutrophil infiltration. It should be noted that these possibilities are not mutually exclusive. Indeed, it is possible that STAT3 activation and cellular infiltration constitutes a positive feedback loop as reported in the mouse model.¹¹ Mechanistically, neutrophils and other inflammatory cells secrete various cytokines to activate STAT3,^{7,12} which induce the expression of chemokines that promote cellular infiltration.¹⁸ Consistent with this notion, deletion of IL-6 gene or inhibition of STAT3 in murine model of AD resulted in the blunted activation of local Th17 cells and production of IL-17,⁹ which is critically involved in the recruitment of neutrophils in aortopathies.¹⁹ However, the proof of direct link between STAT3 activation and neutrophil infiltration awaits further research in the context of human AD.

The inflammatory network for AD pathogenesis may be formed by intercellular interactions involving inflammatory cells and interstitial cells.¹¹ In this regard, it is noteworthy that STAT3 activation was observed in non-neutrophils in our study. Such an inflammatory network may represent a diagnostic marker, potentially by molecular imaging or circulating biomarkers. Of note, it has recently been reported that aortic wall inflammation, as detected by F-18 fluorodeoxyglucose positron emission tomography (FDG-PET), may precede AD onset.^{20,21} Also, high uptake of FDG in the aortic wall after AD is associated with higher risk for rupture and progression.²² If these observations are generally applicable, detection of such local inflammation may represent the high risk status of aortic wall destruction before and after AD onset. The inflammatory network involving STAT3^{9,11} and neutrophils¹⁰ may also be a therapeutic target, as previously proposed. These possibilities need to be pursued in future

research.

Limitations

This study has clear limitations. Since the number of specimens was small, the conclusions remain speculative. Due to the limited number of observations, the background characteristics of patients were not well-controlled, excepting that we selected samples that were obtained within 24 h after AD onset. This timing of tissue curation is rationalized by a previous report of the acute infiltration of neutrophils in the adventitia.⁸⁾ However, chronological dynamics was beyond the scope of the current study. Also, the causal relationship between STAT3 activation and neutrophil infiltration remains speculative because of the observational nature of the current study, although our finding is consistent with previous interventional studies in mouse models demonstrating the presence of feedback loop of STAT3 and cellular infiltration in pathogenesis murine AD models.¹⁰⁻¹²⁾ Because inflammation involves a complex network of inflammatory cells, cytokines, and signaling molecules, the mechanistic aspects of STAT3 and neutrophils in tissue destruction in the context of human AD require further study.

Conclusion

In conclusion, STAT3 activity was seen in AD tissue that correlated with the adventitial neutrophil infiltration, suggesting that STAT3 and neutrophil infiltration are important parts of the tissue destructive inflammation in human AD. Further studies are needed to establish how and when the STAT3 pathway is activated, and how it is involved in the pathogenesis of AD.

Acknowledgments

This work was funded in part by grants JSPS KAKENHI 21390367, 24390334, 24659640, 26670621, and 16H05428 (to HAO); grants from the Daiichi Sankyo Foundation of Life Science, Uehara Memorial Foundation (to HAO), and Vehicle Racing Commemorative Foundation (to HAO). We would like to thank Ms. Kiyohiro, Ms. Nishigata, Ms. Nakao, Ms. Shiramizu for their expertise.

Disclosure Statement

The authors declare no conflict of financial interest concerning this manuscript.

Author Contributions

Study conception: SY, MY, HAO, HT

Data collection: SY, MY, HAO, HF, KA, KT, TS, HAK, HT

Analysis: SY, MY, HAO

Writing: SY, MY, HAO

Funding acquisition: HAO, YF, HT

Critical review and revision: all authors

Final approval of the article: all authors

Accountability for all aspects of the work: all authors

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