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ORIGINAL ARTICLE

The Association Between IL-4 Expression and Thrombus Events in Rheumatic Heart Disease Patients

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Abstract

Background: Rheumatic heart disease (RHD) is the leading cause of valve disease in developing countries, with intracardiac thrombus events as one of the frequent manifestations. Based on Trias Virchow, a thrombus occurs because of endothelial injury, blood flow abnormality, and hypercoagulability. In RHD, this condition is caused by inflammation, mitral stenosis (MS), and atrial fibrillation (AF). Pro-inflammatory cytokines promote thrombus formation, and anti-inflammatory cytokines inhibit it. IL-4 is an anti-inflammatory cytokine that can potentially inhibit thrombus formation because it can inhibit other pro-inflammatory cytokines and polarize M2 macrophages.

Objectives: This study aims to know the association of IL-4 expression with thrombus events in RHD patients.

Methods: Twenty-five (25) patients enrolled in this study were diagnosed with RHD and underwent mitral valve replacement surgery. Valve tissues were collected for histopathological and immunohistochemical examination using IL-4. Thrombus event obtained from echocardiography result. The Mann-Whitney test was used to compare IL-4 expression in thrombus and non-thrombus patients. A p-value of less than 0.05 ($p<0.05$) was considered the significance level.

Results: Of the 25 patients, 88% were older than 30 years. Thrombus occurs in 28% of RHD patients and is associated with AF (p 0.001). Patients with moderate and high IL-4 expression did not have less thrombus than patients with low IL-4 expression.

Conclusions: Intracardiac thrombus formation in RHD patients is not associated with IL-4 expression but is strongly associated with AF. Further research that identifies other cytokines coexisting with IL-4 at the site of injury may reveal better insight into the event.

Keywords: Interleukin-4; Thrombosis; Atrial Fibrillation; Rheumatic Heart Disease.

Introduction

In developing countries, Rheumatic heart disease (RHD) is the leading cause of heart valve disease. In 2015, India (13.17 million cases), China (7.07 million), Pakistan (2.25 million), and Indonesia (1.18 million) were the countries with the most significant estimated number of RHD cases.[1] In RHD patients, the thrombus is a common manifestation. Atrial thrombus was found in 38% of patients with severe mitral stenosis (MS) and atrial fibrillation (AF).[2] Intracardiac thrombus formation is associated with endothelial injury, abnormal blood flow, and hypercoagulability (Trias Vircow).[3] Those conditions are affected by pro-inflammatory cytokines produced by subendothelial inflammatory cells, such as TNF-α and IFN-ɣ.[4] To inhibit thrombus formation, it is necessary to have anti-inflammatory cytokines that oppose the pro-

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Central Illustration: The Association Between IL-4 Expression and Thrombus Events in Rheumatic Heart Disease Patients

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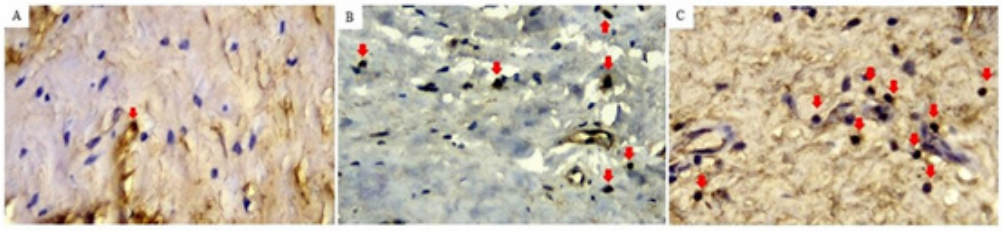
Comparison of IL-4 expression in thrombus and non-thrombus patients

		Thrombus (n (%))	Non-thrombus (n (%))	p
IL-4	+ (low)	1 (4)	1 (4)	
	++ (moderate)	5 (20)	12 (48)	0.376
	+++ (high)	1(4)	5 (20)	



Comparison of IL-4 expression in mitral valve. Red arrow indicate positive stained mononuclear cells (400x). (A) Low (+), (B) Moderate (++) , and (C) high (+++) expression.

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inflammatory action, such as IL-4. IL-4 is an anti-inflammatory cytokine that plays a role in reversing the inflammatory state and regulating autoimmunity.[5]

Currently, RHD therapy aims to prevent disease progression and optimize heart function. Secondary prophylactic antibiotics are the only therapy confirmed to reduce disease severity by preventing recurrent infections.[6] New research into the pathogenesis of the disease is being conducted to find alternative therapies. However, the role of cytokines at the site of inflammation still requires further investigation, as most studies have been conducted using peripheral blood.[7] This study aims to determine the role of IL-4 in the thrombus pathogenesis of RHD patients directly at the site of injury, serving as a basis for developing alternative therapies.

Methods

Samples and Data Collection

This research was a retrospective study conducted in 2022, taking the sample from RHD patients in 2013-2018. The sample size was calculated using the following formula:

$$n = \left(\frac{Z_\alpha \sqrt{2PQ} + Z_\beta \sqrt{P_1.Q_1. + P_2.Q_2.}}{P_1 - P_2} \right)^2$$

Type I error was 5% with a Zα value of 1.96. Type II error was 20% with a Zβ value of 0.84. The proportion of thrombus in RHD patients (P1) was obtained from the study by Farman et al.[2] The proportion of thrombus in nonrheumatic heart disease patients (P2) was obtained from the Tsai et al. study.[8] The minimum sample size required is 25 samples.

Thirty-three patients were initially enrolled in this study. However, eight patients had to be excluded because the slides were damaged during the immunohistochemistry (IHC) staining. Despite several re-staining attempts, the slides remained suboptimal, leading to the depletion of the sample block. As a result, these samples were not included in the study. Tissue samples from mitral valve replacement surgery were collected as formalin-fixed paraffin-embedded (FFPE). The 25 samples were stained with

Hematoxylin-eosin for histopathological examination and IHC of IL-4. Demographic and clinical data were obtained from registration data at the Medical Records Installation of Sardjito Hospital. A cardiologist in the Cardiac echocardiography division of the Department of Cardiology and Vascular Medicine interpreted echocardiographic images. This study was carried out by the Helsinki Declaration Principles and approved by the Institutional Review Board of the Faculty of Medicine, Public Health, and Nursing, Gadjah Mada University (KE/FK/0845/EC/2022).

IHC Staining

The paraffin blocks were cut into 3μm-thick sections and placed on charged slides (Superfrost Plus slides; Thermo Scientific (Newcastle upon Tyne, United Kingdom). They were dried at 60 °C for 30 minutes and then deparaffinized and rehydrated. Sections were pre-treated with Tris EDTA in pH 9.0 at 80 °C for 20 minutes using a pressure cooker, incubated at room temperature for 30 minutes, and washed with Phosphate Buffer Saline (PBS) for 4 minutes. Endogenous peroxidase blocking was done by adding 1–2 drops of 5% hydrogen peroxidase at 80 °C. Peroxidase blocking was performed for 20 minutes. The tissue specimen was incubated by using IL-4 (Mouse monoclonal antibody, Finetest, China, catalog no: FNab04279) that was diluted to 1:100 in PBS, followed with UltraTek Anti-Polyvalent and UltraTek HRP incubation each for 10 minutes. Immunoreactions were visualized using 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB) with subsequent counterstaining of Mayer's hematoxylin. The slides were then dehydrated, cleared, and mounted.

Interpretation of IL-4 Immunostaining

IL-4 expressions were evaluated independently and blindly by two pathologists with a cappa coefficient of 0.83. IL-4 expressions were assessed by counting the percentage of positive cells and the total number of infiltrating cells in 10 microscopic fields at the hot spot area and viewed under high magnification (400x). The percentage of positive cells was calculated by counting the number of positive cells divided by the number of total cells times 100, and the results were classified into + (low), ++ (moderate), and +++ (high) groups. They were classified into + (low) group if the percentage was <10%, ++ (moderate) group if 10 to 50%, and +++ (high) group if >50%.[5]

Data Analysis

All of the data were presented in the form of tables. Categorical variables were reported as frequency (percentage), and continuous variables were reported as mean ± standard deviation or median (interquartile range), as appropriate. The normality of the data was assessed through the Shapiro-Wilk test. The Mann-Whitney test was used to compare the age and gender of patients in the IL-4 group and compare left ventricular ejection fraction (LVEF), left atrium diameter (LAD), and IL-4 expression in thrombus and non-thrombus patients. The Fisher Exact test compared age, gender, and AF incidence in thrombus and non-thrombus patients. A *p-value* of less than 0.05 ($p<0.05$) was considered the significance level. The data was analyzed using IBM Statistical Package for Social Sciences (SPSS) software version 26.0.

Results

The result showed that 16 patients (64%) were female and were dominated by patients older than 30 years (88%) ($p = 0.001$) with echocardiographic findings listed (Table 1 and 2). The normality test revealed that the data for LVEF was normally distributed, and the data for LAD was not normally distributed. The distribution of LVEF data in the patient with a thrombus was abnormal, while without a thrombus, it was normal. The distribution of LAD data in patients with thrombus was normal, while those without thrombus were abnormal. There were seven patients (28%) with intracardiac thrombus, and there was no significant difference based on age ($p = 1.000$), sex ($p = 0.673$), LVEV ($p = 0.317$), and LAD ($p = 0.739$) in thrombus and non-thrombus patients. All patients had mitral valve calcification, and patients with thrombus significantly correlated with AF ($p = 0.001$) (Table 3).

Patients with moderate and high IL-4 expression did not have less thrombus than patients with low IL-4 expression (Table 5/ central figure). IL-4 expressions are shown in Figure 1 (Central Illustration), and intracardiac thrombus events are displayed by echocardiography in Figure 2.

Discussion

RHD is an autoimmune disease that can affect both males and females, with a higher prevalence in females.[9,10] This study showed similar results, with female patients comprising the majority of subjects (65%). Sex-linked hormones are known to regulate adaptive and acquired immune responses,

Table 1 - Characteristics of the patients

Variable	n (%)	p
Gender		
Male	9 (36)	0.230
Female	16 (64)	
Age (years)		
≤30	3 (12)	0.001
>30	22 (88)	

Table 2 - Echocardiographic findings of the study

Echocardiographic parameter	
LVEF (%, mean ± SD)	59.24 ± 10.20
LVEF <50% (n (%))	4 (16)
LAD (cm, median (Q1-Q3))	5.40 (4.90-59.50)
LA enlargement (n (%))	25 (100)
LAT/ LAAT (n (%))	7 (28)
SEC LA (n (%))	12 (48)

LA: left atrial; LA: left atrial thrombus; LAAT: left atrial appendage thrombus; LAD: left atrium diameter; LVEF: left ventricular ejection fraction; SEC LA: spontaneous echocardiographic contrast in the left atrial.

explaining the higher incidence of autoimmune diseases in females.[9]

The research subjects were 27-68 years old, and the majority (88%) were over 30, averaging 43.4 years. Patients in this study were younger than those in the previous survey by Jiao et al. in 2022, which showed mitral valve surgery caused by rheumatic disease occurring at 40-70 years old.[11] This finding may be related to limited access to secondary prophylaxis antibiotics in Indonesia, which means that heart valve damage requiring surgery occurs earlier.[12] A study from Huntley in 2019 also stated that RHD manifested at younger ages in developing countries. This condition is due to limited access to health services and the use of prophylactic antibiotics, poverty, and overcrowding environments.[13]

The results showed that 28% of patients had intracardiac thrombus in LA and LAA without significant differences in sex and age. Previous studies have shown different prevalence rates, ranging from 24.9-38%.[2,13] Our study is consistent with a previous study from Ozal et al. in 2016, which showed that 24.9% of RHD patients had thrombus in the left atrial without significant differences in sex and age compared to those without thrombus.[14]

The results showed that patients with moderate (++) and high (+++) IL-4 expression did not have less intracardiac thrombus than those with low IL-4 expression. This result is discordant with previous studies, which showed that IL-4 can potentially prevent thrombus formation by inhibiting TNF-α formation[15] and polarizing M2 macrophage.[16] It was stated that TNF-α is directly associated with endothelial dysfunction by mediating NF-kB translocation, increasing Reactive Oxygen Species production, and influencing endothelial nitric oxide synthase expression. TNF-α also induces tissue factor expression and suppresses thrombomodulin and endothelial cell protein C receptors.[4] By inhibiting this TNF-α, thrombus formation can be prevented. The study by Celik et al. in 2020 showed that IL-4 has a role in

Table 3 - Patient characteristics based on thrombus events

	Thrombus	Non-thrombus	p
Total	7 (28)	18 (72)	
Sex (n (%))			
Male	3 (12)	6 (24)	0.673
Female	4 (16)	12 (48)	
Age (years) (n (%))			
≤30	1 (4)	2 (8)	
>30	6 (24)	16 (64)	1.000
AF (n (%))			
Present	7 (28)	0 (0)	
Absent	0 (0)	18 (72)	0.001
Echocardiographic parameter			
LVEF (%, median(Q1-Q3))	65.0 (58.0-67.0)	58.50 (52.75-66.0)	0.317
LAD (cm, median (Q1-Q3))	5.50 (5.20-5.70)	5.30 (4.67-6.02)	0.739
Valve calcification (n (%))	7 (28)	18 (72)	
Treatment with warfarin (n (%))			
Yes	5 (20)	9 (36)	
No	0 (0)	7 (28)	
N/A	2 (8)	2 (8)	

This study showed that IL-4 expression was dominated by ++ (moderate) and +++ (high) groups and showed no significant difference in sex (p 0.654) and age (p 0.541). (Table 4)
AF: atrial fibrillation; LVEF: left ventricular ejection fraction; LAD: left atrium diameter.

Table 4 - Patient characteristics based on IL-4 expression

	IL-4			p
	+ (low)	++ (moderate)	+++ (high)	
Total	2 (8)	17 (68)	6 (24)	
Sex				
Male	0 (0)	8 (32)	1 (4)	0.654
Female	2 (8)	9 (36)	5 (20)	
Age (years)				
≤30	0 (4)	3 (12)	0 (0)	
>30	2 (8)	14 (56)	6 (24)	0.541

Table 5 - Comparison of IL-4 expression in thrombus and non-thrombus patients

		Thrombus (n (%))	Non-thrombus (n (%))	p
IL-4	+ (low)	1 (4)	1 (4)	0.376
	++ (moderate)	5 (20)	12 (48)	
	+++ (high)	1(4)	5 (20)	

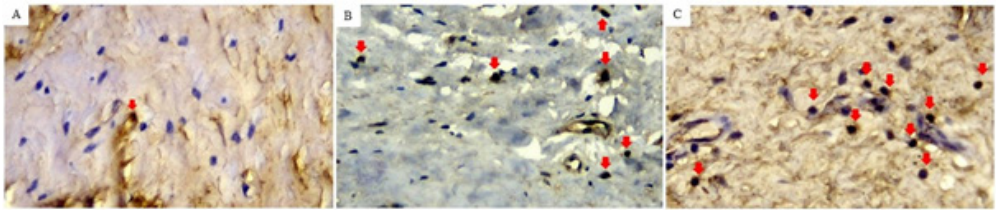

Figure 1 - IL-4 expression in the mitral valve. The red arrow indicates positive stained mononuclear cells (400x). (A) Low (+), (B) Moderate (++), and (C) high (+++) expression.

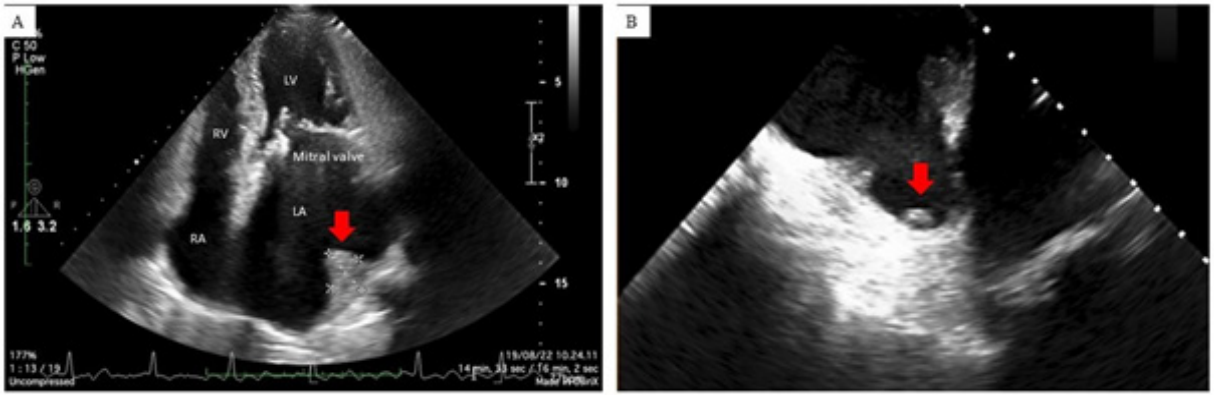

Figure 2 - Echocardiography examination. (A) Thrombus in the left atrium (red arrow). (B) Thrombus in left atrial appendage (LAA) (red arrow). RV: Right ventricle, RA: Right atrium, LV: Left ventricle.

increasing the number of macrophages and polarizing them toward M2 macrophages.[17] The role of macrophages in thrombus pathogenesis depends on the phenotype. M1 proinflammatory macrophages promote thrombus formation, and M2 anti-inflammatory macrophages have the opposite effect. M2 macrophages inhibit thrombus formation by producing IL-10, TGF-β, and IL-1Ra15. The shifting of M2 macrophages will cause the amount of M1 macrophages to decrease so that the production of pro-inflammatory cytokines also decreases. While performing, IL-4 is influenced by the amount, time, and location of the expression and other signals received by cells together with IL-4. These factors affect whether these cytokines have a protective or harmful effect.[15]

This difference in results in our study may be due to a condition that thrombus formation in RHD is not only associated with inflammation but also AF and MS. Our study results confirm that AF is strongly associated with thrombus events. AF triggers thrombus formation, especially in LA and

LAA. The pathogenesis of thrombus in AF conditions involves three aspects: abnormalities in the heart wall (endothelial dysfunction), stasis (disturbances in blood flow), and abnormalities in blood components.[18] There are two opinions regarding the relationship between AF and endothelial dysfunction. The first opinion states that AF causes endothelial dysfunction, and the second opinion states that endothelial dysfunction increases atrial arrhythmic substrates, thereby increasing the incidence of AF.[19] In AF condition, there are also abnormal changes in platelets and blood clotting components such as von Willebrand factor (vWF), tissue factors, β-thrombomodulin, and VEGF. The increase of platelets and VEGF play a role in hypercoagulable status, and the vWF elevates the stasis condition. The accumulation of tissue factors in significant amounts causes thrombin formation and platelet activation. The increase of β-thrombomodulin also activates platelets.[18]

Our study also indicates that thrombus formation in RHD patients is associated with MS. All of the patients with intracardiac thrombus had mitral valve calcification and stenosis. Valve calcification causes stiffness resulting in MS. MS can cause left atrial enlargement, blood stasis in the cardiac chambers, and increased risk of thrombus formation in the LA and LAA.[3] However, this study also showed that not all patients with mitral calcification and stenosis had intracardiac thrombus because several factors, including AF, influence thrombus formation in patients with MS, left atrial size, duration of symptoms, advanced age, the severity of MS, inflammation, oxidative stress, and platelet levels.[20,21] This study exhibited that all patients with thrombus had AF, left atrial dilatation, and severe MS.

In addition, the results of the study that did not show a protective function of IL-4 against thrombus incidence may also be related to the fact that while performing IL-4 is influenced not only by the amount but also by time, location of the expression and other signals received by cells together with IL-4. These factors affect whether these cytokines have a protective or harmful effect.[15]

This study's limitation arises from the necessity to exclude specific samples due to technical difficulties encountered during the IHC staining process. These technical challenges resulted in the depletion of the block material, rendering these samples unsuitable for analysis and thereby necessitating their exclusion from the study. As a consequence, the sample size is small. Another area for improvement is that this study was single center.

Conclusion

In conclusion, intracardiac thrombus formation in RHD patients is not associated with IL-4 expression but is strongly associated with AF. Further research that identifies other cytokines co-existing with IL-4 at the injury site may reveal better insight into the event.

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Author Contributions

Conception and design of the research, statistical analysis and obtaining financing: Wijayanti AR, Mayasari DS, Dwianingsih EK; acquisition of data and analysis and interpretation of the data: Wijayanti AR, Mayasari DS, Pranacipta S, Theresia E, Dwianingsih EK; writing of the manuscript and critical revision of the manuscript for intellectual content: Wijayanti AR, Mayasari DS, Maharani E, Mumpuni H, Anggorowati N, Irianiwati, Dwianingsih EK

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Sources of Funding

There were no external funding sources for this study.

Study Association

This article is part of the thesis of Anatomical Pathology submitted by Arini Rizky Wijayanti, from Gadjah Mada University.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of the Gadjah Mada University under the protocol number KE/FIC/0845/EC/2022. All the procedures in this study were in accordance with the 1975 Helsinki Declaration, updated in 2013. Informed consent was obtained from all participants included in the study.

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