Effect of lymphopenia on thrombus formation and resolution in $FeCl_3$-induced carotid artery thrombosis in mice

Sungeun Kim

The Graduate School
Yonsei University
Department of Biomedical Engineering

Effect of lymphopenia on thrombus formation and resolution in $FeCl_3$-induced carotid artery thrombosis in mice

Directed by Professor Ji Hoe Heo

A Master's Thesis Submitted
to the Department of Biomedical Engineering
and the Graduate School of Yonsei University
in partial fulfillment of the
requirements for the degree of
Master of Biomedical Engineering

Sungeun Kim

June 2024

This certifies that the Master's Thesis of Sungeun Kim is approved.

Thesis Supervisor Ji Hoe Heo

Thesis Committee Member Young Dae Kim

Thesis Committee Member Il Kwon

The Graduate School
Yonsei University
June 2024

Acknowledgments

I would like to thank everyone who have contributed and supported me directly or indirectly in the completion of this thesis.

Words cannot express my gratitude to my supervisor, Dr. Ji Hoe Heo, for his invaluable guidance, support, and encouragement throughout the entire process of completing this thesis. His expertise, patience, and insightful feedback have been instrumental in shaping this work. I am truly and sincerely thankful to him for providing the resources and facilities required to conduct this study. Without his guidance and persistent help, this would not have been possible.

I would also like to extend my sincere thanks to my other thesis advisors, Dr. Young Dae Kim and Dr. Il Kwon, for their endless discussions and inspiring advice. Their insightful feedback and unwavering support have truly enriched this study. My appreciation extends to Dr. Hyo Suk Nam and the lab members, whose knowledge and assistance have been essential to my academic journey.

Last but not least, I would like to thank my beloved family and friends for supporting me and loving me no matter what. Without their unconditional love and support, I would not have made until this far. Their belief in me has been a driving force throughout this challenging yet rewarding endeavor.

I can’t express how deeply grateful I am to have each and every one of you in my life. Thank you all for your contribution to my academic growth. Your support has been invaluable, and I am SUPER DUPER grateful for all the assistance and kindness I have received.

감사합니다.
With LOTS and LOTS of love,
Sungeun Kim
June 2024

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Effect of lymphopenia on thrombus formation and resolution in $FeCl_3$- induced carotid artery thrombosis in mice

Sungeun Kim
Department of Biomedical Engineering
The Graduate school, Yonsei University

Thrombosis involves the development of blood clots within arteries or veins, with its occurrence in the arterial system being a primary cause of stroke and myocardial infarction, both of which significantly contribute to global mortality and morbidity. Thrombus formation is basically depending on platelets and early infiltrating cells which includes neutrophils and monocytes, while thrombus resolution relies heavily on macrophages and monocytes. We investigated the role of lymphocytes in thrombosis by examining the effects of lymphopenia, induced by administering FTY720, on the formation and resolution of thrombi using a $FeCl_3$-induced carotid artery thrombosis model in mice.

This was a double-blind, randomized, placebo-controlled study designed to compare the effects of FTY720 and placebo on thrombosis and subsequent recanalization using a $FeCl_3$-induced carotid arterial thrombosis model. The blood samples collected at different time points were analyzed using a hematology analyzer to conduct a complete blood count test. These tests provide counts of various blood cell types, as well as parameters such as hemoglobin concentration and hematocrit. The results of the complete blood count were used to determine various ratios, including the neutrophil-to-lymphocyte ratio (NLR), monocyte-to-lymphocyte ratio (MLR), and platelet-to-lymphocyte ratio (PLR). These ratios were used to explore potential correlations with the time to occlusion and the time to recanalization.

The time to occlusion did not significantly differ between the lymphopenia group (median 3.56 min, interquartile range 3.03-5.32 min) and the control group (median 3.98 min, interquartile range 3.47-4.74 min) (p = 0.484). There was no significant correlation between the time to occlusion and NLR (r = -0.108, p = 0.379), MLR (r = -0.032, p = 0.796), or PLR (r = -0.002, p = 0.985). During the 2 hr monitoring period after inducing thrombotic occlusion, blood flow did not restore to 75% of the mean baseline blood flow in 34 mice subjected to the $FeCl_3$-induced carotid artery thrombosis experiment. Among the 38 mice that experienced recanalization, the time to recanalization was significantly shorter in the lymphopenia group (median 35.87 min, interquartile range 17.44-68.66 min) compared to the control group (median 72.33 min, interquartile range 62.63-88.92 min) (p = 0.006). There was a significant inverse correlation between the time to recanalization and NLR (r = -0.351, p = 0.031), MLR (r = -0.412, p = 0.010), and PLR (r = -0.430, p = 0.007).

FTY720 successfully induced significant lymphopenia in mice. While lymphopenia did not affect the time to occlusion in a $FeCl_3$-induced carotid artery thrombosis model in mice, it was associated with a faster resolution of the thrombus. These findings suggest that lymphocytes are involved in the stability of the thrombus rather than the early stages of thrombosis.

Key words : leukocytes, neutrophils, lymphocytes, monocytes, platelets, Fingolimod Hydrochloride, carotid artery thrombosis

1. INTRODUCTION

Thrombosis involves the development of blood clots within arteries or veins, with its occurrence in the arterial system being a primary cause of stroke and myocardial infarction, both of which significantly contribute to global mortality and morbidity[1,2]. The thrombus is composed of various compositions, including erythrocytes, tightly packed platelets with fibrin, and leukocytes. Preventing and resolving thrombus formation are primary treatment goals in thrombotic conditions such as stroke and myocardial infarction. Current antithrombotic therapies mainly target platelet, fibrin, and coagulating factors[3,4]. Despite the use of antiplatelet agents and anticoagulants, many patients still develop stroke or myocardial infarctions, and thrombi remain unresolved even with fibrinolytic agents such as alteplase and tenecteplase. Hence, the development of novel drugs using new molecular targets of thrombosis is imperative[5].

Leukocytes involve neutrophils, monocytes, lymphocyte, basophils, and eosinophils[6]. These immune cells primarily participate in inflammatory cellular responses to injury or pathogens and have been suggested to contribute to thrombosis. In fact, cyclophosphamide-induced leukopenia attenuated thrombosis in $FeCl_3$-induced carotid thrombosis of mice[7]. Leukocytes, specifically neutrophils, macrophages, and monocytes, interact with the hemostatic system through innate immunological processes. They express and release coagulation and fibrinolytic factors, interacting with the hemostatic system via innate immunity[6]. Leukocytes generate cytokines on the vascular endothelial surface that affect the expression of adhesion molecules and pro-coagulant factors[6]. Leukocytes also have the potential to enhance fibrin deposition and platelet activation[8-10]. Platelets and the coagulation cascade are directly activated by antimicrobial compounds produced during leukocyte degranulation and extracellular trap formation.

Thrombus resolution is also regulated by phagocytic activity and leukocyte chemotaxis[6]. Thrombus formation is largely dependent on platelets and early infiltrating cells such as neutrophils and monocytes, whereas thrombus resolution is largely dependent on macrophages and monocytes[11,12]. The early neutrophil influx and the later migration of monocytes and macrophages into a resolving thrombus have been documented in venous thrombus resolution[11]. During thrombus resolution, these inflammatory cells are a major source of cytokines and proteases[11]. On the other hand, the role of immune cells, specifically T cells, in the process of thrombus resolution is less clear[11].

A high platelet-to-lymphocyte ratio (PLR) and neutrophil-to-lymphocyte ratio (NLR) are linked to a higher risk of arterial thrombosis[13]. PLR and NLR are simple and straightforward laboratory measurements that can be calculated with the complete blood count test and have the benefit of combining data of hemostasis and inflammation[13,14]. The involvement of lymphocytes in these biomarkers suggests that lymphocytes may contribute to the thrombotic process. Despite early studies identifying lymphocytes in venous thrombi, their role in the process of thrombus resolution has only recently been investigated[11]. However, little is known on the role of lymphocytes in both thrombus formation and resolution.

FTY720 (2-Amino-2-[2-(4-octyl-phenyl)-ethyl]-propane-1,3-diol hydrochloride) is an immunosuppressant currently used in the treatment of multiple sclerosis[15,16]. As an agonist of 4 of sphingosine-1-phosphate (S1P) receptors[17], FTY720 primarily reduces peripheral lymphocyte counts by sequestering lymphocytes into secondary lymphoid organs and altering their migration into damaged tissues[18,19]. FTY720 works by preventing lymphocyte migration and circulation, making it effective in treating primary neuro-inflammatory disorders[20-24]. Therefore, we investigated the role of lymphocytes in thrombosis by inducing lymphopenia with FTY720 and examining its effects on the formation and resolution of thrombus using a $FeCl_3$-induced carotid artery thrombosis model of mice.

2. MATERIALS AND METHODS

2.1. Animals and ethical statements

Six to eight-week-old Institute of Cancer Research (ICR) mice weighing 32–34 g for male and 27–29 g for female (Orient Bio Inc., Seongnam, South Korea) were used in these experiments. All mice were kept in a temperature-controlled (23.0 ± 1.0 °C) and light-controlled (a 12-hr light/dark cycle) cage with free access to food and water. The weight of the mice was recorded every day before the experimental procedures. All animal procedures were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine and performed in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (approval number: 2022-0128).

2.2. Randomization and blinding

This was a double-blind, randomized, placebo-controlled study to compare the effects of FTY720 and placebo on thrombosis and subsequent recanalization using a $FeCl_3$-induced carotid arterial thrombosis model. A computer-generated randomization list with permuted blocks of four was created by a researcher to establish the study's double-blind setup. Using this list, another researcher prepared and sequentially numbered the study drugs, thus maintaining the integrity of the blinding. Subsequently, a third researcher administered the drugs and conducted the experiments and analyses without knowledge of the treatment allocations.

2.3. Induction of lymphopenia

Lymphopenia was induced in mice by administering FTY720 (Sigma-Aldrich, SML0700, St Lois, MO, USA). The compound was dissolved in 0.9% NaCl and diluted to appropriate concentrations immediately prior to administration to ensure stability and

efficacy. The dosage administered ranged from 0 to 2.0 mg/kg. Mice received the solutions intraperitoneally, with the volume calculated based on their body weight per injection. The injections were administered twice, with a 24 hr interval between each dose, across a total of 96 mice evenly distributed among the different dosage groups.

2.4. Determination of FTY720 dose

A total of 40 ICR mice (20 males and 20 females) were assigned to receive intraperitoneal injections of either normal saline or FTY720 at doses of 0.5 mg/kg, 1.0 mg/kg, or 2.0 mg/kg of FTY720 (5 males and 5 females in each group). The administrations were repeated twice, with a 24 hr interval between doses. Blood samples (~50 μL) were collected from the jugular vein into ethylenediaminetetraacetic acid tubes at three time points: just before drug administration (0 hr), 24 hr after the first dose (24 hr), and 24 hr after the second dose (48 hr). White blood cells with differential counts were assessed using a Mindray BC-5000 Vet hematology analyzer (Mindray, Shenzhen, China).

2.5. Study design and sample size determination

This study was organized into two experimental phases. The sample size for the first experimental phase was determined using time to occlusion data from a previously conducted preliminary study with FTY720 in ICR mice. We calculated that 18 mice per group, totaling 36 mice, would be necessary to detect significant differences in time to occlusion with an effect size of 0.96, a significance level of 0.05 (two-sided), and 80% power.

Data monitoring investigators, blinded to group assignments, monitored data and laboratory abnormalities in accordance with established guidelines throughout the study. Significant indications of differences in time to recanalization during the first phase led to the recommendation for a second phase. The sample size for the second experimental phase

was recalculated specifically to detect differences in time to recanalization, targeting an effect size of 0.76 with a significance level of 0.05 (two-sided) and 80% power. Initial calculations suggested that a total of 58 mice would be needed. However, considering the 36% dropout rate observed in the first phase, where only 23 successful recanalizations were recorded from the initial cohort of 36 mice, we recalculated the necessary sample size to account for potential losses. The required sample size was adjusted to 72 mice. Since 36 mice were already used in the first phase, we used an additional 36 mice in the second experimental phase to fulfill this requirement.

In both experimental phases, each group consistently included an equal number of males and females. All experimental methods were uniformly applied across both phases of the study.

2.6. Drug treatment and blood sampling

The lymphopenia group received 1.0 mg/kg of FTY720, while the control group received an equivalent volume of normal saline. Blood samples were collected from the jugular vein into tubes containing ethylenediaminetetraacetic acid at three time points: immediately before the initial drug administration (0 hr), 24 hr after the second dose (48 hr), and just prior to the induction of the $FeCl_3$-induced carotid arterial thrombosis model.

2.7. Induction and $FeCl_3$-induced carotid artery thrombosis and monitoring of blood flow

The $FeCl_3$-induced carotid artery thrombosis procedure was performed on the subjects 24 hr after the administration of the second dose of FTY720.

Thrombosis was induced in the carotid artery by using $FeCl_3$. Briefly, anesthesia was induced by inhalation of 5% isoflurane in a mixture of 70% nitrous oxide and 30% oxygen and maintained with 2% isoflurane throughout the whole operative procedure. Body temperature was monitored and maintained at 37.0 ± 0.5 °C with homoeothermic blanket control unit and a heating pad (Harvard Apparatus, Holliston, MA, USA) throughout. The cervical midline incision was made and the left common carotid artery was isolated. Once the left common artery was isolated, the carotid blood flow was monitored with ultrasound Doppler flow probe (0.5 PSB; Transonic Instruments, Ithaca, NY, USA) which connected to Transonic TS420 blood flow meter (Transonic Instruments) and an iWorx IX-304T data acquisition system (iWorx Systems, Inc., Dover, NH, USA). The baseline blood flow was measured for 5 min to establish an average baseline blood flow. Then, a strip of saturated 0.5 × 1.0 mm filter paper (Grade 2, 150 mm Qualitative Filter Paper, Tokyo Roshi Kaisha Ltd, Japan) soaked in 0.5 μL of 10% $FeCl_3$ was placed on the left common carotid artery for 5 min. After 5 min of the exposure to $FeCl_3$, the filter paper was removed and rinsed with normal saline. The doppler flow probe then placed around the artery again to measure the blood flow. The time taken in between removing the filter paper and the recording started was recorded separately and these data later used as 'loss time' to be added in the time to occlusion. The carotid blood flow continuously monitored for 2 hr. Measured carotid blood flow was standardized by subtracting the minimum blood flow.

2.8. Primary outcomes and hematological parameters

The primary outcomes were the time to occlusion and time to recanalization. The time to occlusion was defined as the duration from the removal of the $FeCl_3$-soaked filter paper to the cessation of blood flow. Time to recanalization was defined as the duration from the onset of occlusion to the restoration of blood flow to at least 75% of the mean baseline flow.

The blood samples collected from the jugular vein were treated with an anticoagulant and then analyzed using the Mindray BC-5000 Vet hematology analyzer to conduct a complete blood count test. Each blood sample was loaded into the cartridge for analysis.

The test processes the samples and provides results including counts of various blood cell types, as well as parameters such as hemoglobin concentration and hematocrit. These results were recorded, and specific parameters relevant to this study were examined.

Additionally, ratios such as the NLR, monocyte-to-lymphocyte ratio (MLR), and PLR were calculated based on results from complete blood count tests. These ratios were used to explore potential correlations with the time to occlusion and the time to recanalization, aiming to assess the significance of leukocyte subset proportions in thrombosis.

2.9. Statistical analysis

Statistical analysis was performed using SPSS statistics (version 26.0, IBM Corp., Armonk, NY, USA). Data were presented as median (interquartile range [IQR]) based on the results of the normality test. The normality of data was assessed using the Shapiro-Wilk test. Significant differences between the groups were analyzed using the Kruskal-Wallis test, followed by Mann-Whitney U test for post-hoc comparisons. Correlations between the time to occlusion or the time to recanalization and leukocyte subsets were investigated using Pearson's correlation analysis for data meeting normality assumptions, and Spearman's rank correlation was used otherwise. Significance was set at $p < 0.05$.

3. RESULTS

3.1. Dose determination and FTY720

In the dose-finding study comparing leukocyte counts among 40 mice treated with normal saline or FTY720 (doses of 0.5, 1.0, or 2.0 mg/kg), FTY720 significantly reduced total blood leukocyte and lymphocyte counts at 48 hr after the initial administration of the study drugs ($p < 0.001$) (Table 1). The lymphocyte counts decreased by 90.70 ± 5.99 % for 0.5 mg/kg, 92.30 ± 2.88 % for 1.0 mg/kg, and 92.56 ± 3.86 % for 2.0 mg/kg, 48 hr after treatment with FTY720 (Fig. 1). Based on these findings, the dose of FTY720 at 1.0 mg/kg, administered twice with a 24 hr interval, was chosen for the randomized study, as it effectively induced lymphopenia with minimal variability.

Table 1. Complete blood counts and white blood cell differential counts for the dose test groups.

	Baseline (0 hr)			
	Control	0.5 mg/kg	1.0 mg/kg	2.0 mg/kg
Red blood cell, 10^6 cells/μL	8.67 [8.55-8.82]	8.63 [8.34-9.06]	8.94 [8.41-9.14]	8.27 [7.95-8.78]
Platelet, 10^3 cells/μL	1393 [1239-1535]	1307 [1156-1413]	1353 [1320-1453]	1264 [1138-1361]
White blood cell, 10^3 cells/μL	5.30 [4.03-7.15]	4.99 [4.44-6.54]	5.52 [4.65-5.78]	5.30 [4.20-6.54]
Neutrophil	0.85 [0.71-0.92]	0.88 [0.74-0.92]	0.80 [0.57-0.94]	0.82 [0.65-0.89]
Lymphocyte	4.16 [2.90-6.09]	3.88 [3.52-4.95]	4.44 [3.87-4.75]	4.20 [3.21-5.51]
Monocyte	0.13 [0.10-0.17]	0.13 [0.09-0.14]	0.13 [0.10-0.15]	0.10 [0.08-0.13]
Eosinophil	0.13 [0.10-0.15]	0.10 [0.07-0.11]	0.11 [0.08-0.16]	0.09 [0.06-0.13]
Basophil	0.00 [0.00-0.00]	0.00 [0.00-0.00]	0.00 [0.00-0.00]	0.00 [0.00-0.00]
Neutrophil, %	14.1 [10.8-21.3]	17.5 [16.3-17.9]	14.7 [12.8-17.1]	15.5 [10.4-17.7]
Lymphocyte, %	81.3 [71.8-84.7]	78.1 [77.8-79.8]	80.2 [76.0-83.4]	80.5 [77.6-86.1]
Monocyte, %	2.6 [2.2-3.0]	2.4 [2.1-2.6]	2.4 [2.2-2.8]	1.8 [1.7-2.5]
Eosinophil, %	2.6 [1.6-3.2]	1.8 [1.4-2.2]	2.4 [1.5-3.6]	1.8 [1.1-2.4]
Basophil, %	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]

	24 hr			
	Control	0.5 mg/kg	1.0 mg/kg	2.0 mg/kg
Red blood cell, 10^6 cells/μL	7.90 [7.60-8.42]	8.18 [7.72-9.05]	8.18 [7.88-8.59]	7.50 [7.25-7.80]
Platelet, 10^3 cells/μL	1463 [1259-1535]	1381 [1239-1465]	1489 [1424-1584]	1318 [1159-1401]
White blood cell, 10^3 cells/μL	4.60 [4.19-5.52]	1.73 [1.16-2.26]	1.31 [1.12-1.84]	1.33 [1.25-1.57]
Neutrophil	0.85 [0.61-0.93]	0.72 [0.65-1.28]	0.82 [0.57-0.91]	0.74 [0.63-0.88]
Lymphocyte	3.72 [2.73-4.72]	0.53 [0.32-0.73]	0.38 [0.33-0.44]	0.41 [0.34-0.49]
Monocyte	0.06 [0.03-0.12]	0.10 [0.08-0.11]	0.09 [0.06-0.11]	0.17 [0.12-0.20]
Eosinophil	0.11 [0.09-0.13]	0.08 [0.05-0.11]	0.10 [0.06-0.11]	0.05 [0.03-0.07]
Basophil	0.00 [0.00-0.00]	0.00 [0.00-0.00]	0.00 [0.00-0.00]	0.00 [0.00-0.00]
Neutrophil, %	17.9 [13.0-19.7]	54.2 [45.4-59.7]	54.2 [50.8-58.9]	56.7 [49.8-59.7]
Lymphocyte, %	78.7 [74.5-84.3]	28.9 [23.9-434]	29.3 [26.0-34.5]	27.8 [26.0-34.2]
Monocyte, %	1.4 [1.1-1.6]	5.9 [5.0-9.3]	7.4 [5.5-7.9]	9.8 [9.1-13.9]
Eosinophil, %	2.0 [1.4-3.1]	5.7 [3.4-7.2]	6.4 [4.0-9.4]	3.7 [2.7-5.1]
Basophil, %	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]

	48 hr			
	Control	0.5 mg/kg	1.0 mg/kg	2.0 mg/kg
Red blood cell, 10^6 cells/μL	7.59 [7.30-7.92]	7.88 [7.73-8.40]	8.05 [7.55-8.44]	7.18 [6.93-7.72]
Platelet, 10^3 cells/μL	1470 [1352-1520]	1393 [1251-1590]	1384 [1312-1462]	1240 [1180-1346]
White blood cell, 10^3 cells/μL	5.15 [3.74-6.01]	1.24 [1.01-1.90]	1.19 [0.86-1.70]	1.40 [1.25-1.71]
Neutrophil	0.77 [0.69-0.97]	0.72 [0.59-1.17]	0.75 [0.52-0.96]	0.87 [0.83-1.19]
Lymphocyte	3.97 [2.93-4.78]	0.33 [0.22-0.43]	0.27 [0.19-0.40]	0.31 [0.23-0.36]
Monocyte	0.08 [0.08-0.11]	0.10 [0.07-0.14]	0.12 [0.07-0.17]	0.15 [0.11-0.16]
Eosinophil	0.07 [0.06-0.10]	0.04 [0.03-0.07]	0.05 [0.04-0.09]	0.05 [0.03-0.06]
Basophil	0.00 [0.00-0.00]	0.00 [0.00-0.00]	0.00 [0.00-0.00]	0.00 [0.00-0.00]
Neutrophil, %	17.4 [12.7-22.8]	60.0 [51.0-62.1]	60.2 [59.5-61.0]	66.2 [56.9-68.6]
Lymphocyte, %	79.3 [72.9-82.8]	25.1 [21.5-35.6]	24.1 [20.6-25.9]	23.1 [16.4-26.4]
Monocyte, %	2.1 [1.7-2.3]	7.1 [5.7-9.4]	10.9 [8.4-13.2]	10.4 [9.0-12.4]
Eosinophil, %	1.8 [1.5-2.1]	4.3 [3.7-4.9]	5.7 [3.6-6.8]	3.0 [2.8-4.5]
Basophil, %	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]

Complete blood count results at baseline (0 hr), 24 hr, and 48 hr post-first injection for the control group and FTY720-treated groups (0.5, 1.0, and 2.0 mg/kg), with 10 mice in each group. Values are median [interquartile range].

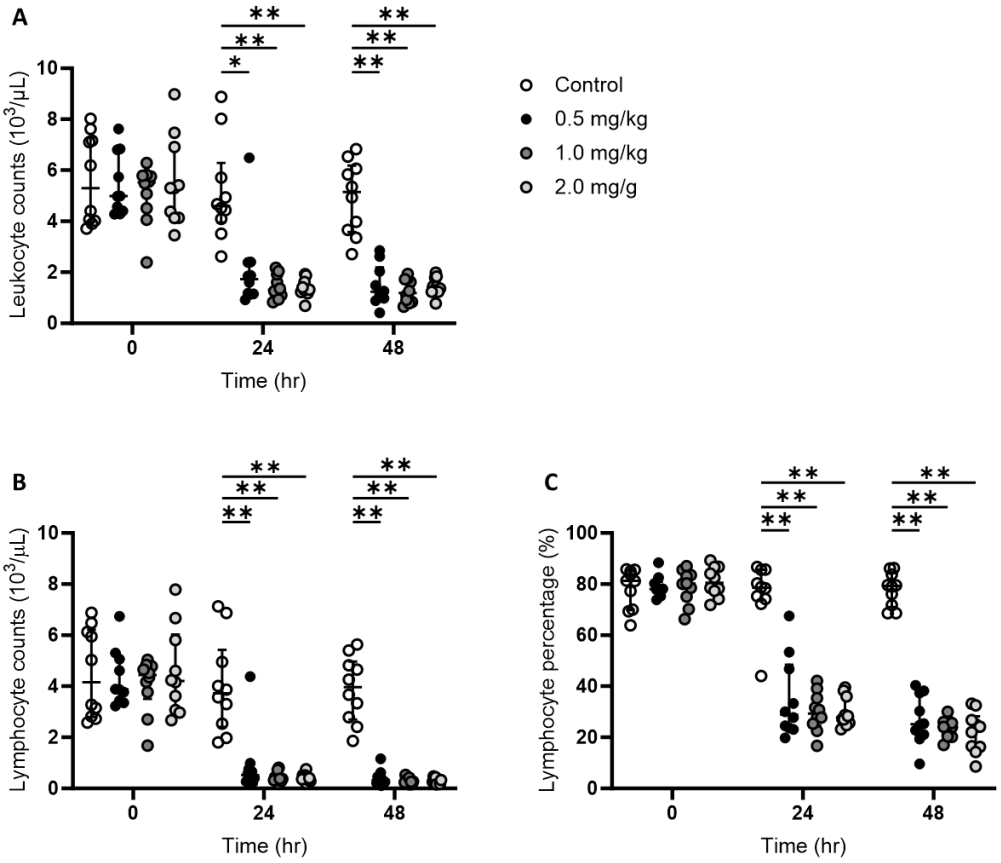

Figure 1. Complete blood counts and white blood cell differential counts for the dose test groups.

Total leukocyte counts (A), lymphocyte counts (B), and lymphocyte percentages (C) after 0, 24, and 48 hr after administration of normal saline or different doses of FTY720 (0.5 mg/kg, 1.0 mg/kg, or 2.0 mg/kg). Median [IQR] for all of the graph. *$p < 0.05$,**$p < 0.001$.

3.2. Study group and blood cell counts

A total of 72 ICR mice (36 males and 36 females) were randomized to evaluate the difference in the time to occlusion and time to recanalization between the lymphopenia group and the control group. Compared to the normal saline-treated mice, FTY720-treated mice showed significantly lower counts for total leukocytes (2.08 x 10^3 cells/μL vs. 7.30 x 10^3 cells/μL, $p < 0.001$) and lymphocytes (0.52 x 10^3 cells/μL vs. 5.74 x 10^3 cells/μL, $p < 0.001$), and higher counts for monocytes (0.24 x 10^3 cells/μL vs. 0.16 x 10^3 cells/μL, $p = 0.005$) (Table 2, and Fig. 2) at 48 hr after administration of the study drug.

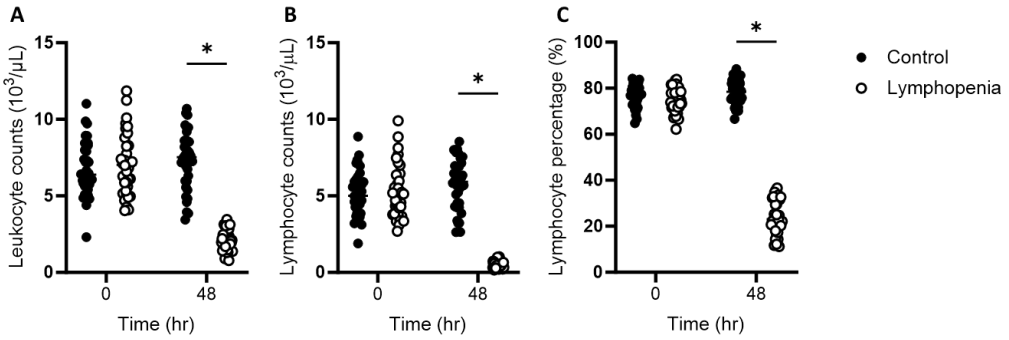

Figure 2. Comparison of complete blood counts between the control group and the lymphopenia group.

Total leukocyte counts (A), lymphocyte counts (B), and lymphocyte percentages (C) were significantly decreased in the lymphopenia group 48 hr after administration of the study drug. *$p < 0.001$.

Table 2. Comparison of complete blood counts and white blood cell differential counts between the groups.

	Baseline (0 hr)			After injection (48 hr)		
	Control (n=36)	Lymphopenia (n=36)	*p*	Control (n=36)	Lymphopenia (n=36)	*p*
Red blood cell, 10^6 cells/μL	9.03 [8.25-9.95]	8.54 [8.18-9.45]	0.169	8.01 [7.66-8.98]	8.49 [7.92-9.24]	0.094
Platelet, 10^3 cells/μL	1287 [1086-1347]	1251 [1118-1408]	0.612	1244 [1082-1339]	1232 [1067-1447]	0.857
White blood cell, 10^3 cells/μL	6.38 [5.70-8.07]	7.02 [5.55-8.25]	0.710	7.52 [5.97-8.55]	2.07 [1.61-2.47]	<0.001
Neutrophil	1.28 [1.09-1.53]	1.48 [1.19-1.61]	0.110	1.23 [0.98-1.44]	1.16 [0.90-1.53]	0.539
Lymphocyte	5.00 [4.18-5.97]	5.13 [4.12-6.38]	1.000	5.92 [4.47-6.93]	0.47 [0.32-0.70]	<0.001
Monocyte	0.14 [0.12-0.17]	0.17 [0.14-0.22]	0.062	0.15 [0.12-0.21]	0.20 [0.16-0.31]	0.005
Eosinophil	0.12 [0.05-0.13]	0.10 [0.06-0.14]	0.839	0.10 [0.07-0.18]	0.09 [0.06-0.11]	0.054
Basophil	0.00 [0.00-0.00]	0.00 [0.00-0.00]	0.317	0.00 [0.00-0.00]	0.00 [0.00-0.00]	0.317
Neutrophil, %	18.6 [16.18-22.23]	20.8 [16.40-24.05]	0.239	17.5 [14.15-21.38]	58.8 [52.83-61.85]	<0.001
Lymphocyte, %	77.3 [73.10-80.35]	74.5 [71.88-78.80]	0.244	78.5 [75.13-82.23]	23.1 [20.60-31.80]	<0.001
Monocyte, %	2.2 [1.80-2.60]	2.5 [2.08-2.98]	0.057	2.1 [1.68-2.70]	10.8 [8.18-13.48]	<0.001
Eosinophil, %	1.6 [0.80-2.08]	1.5 [0.90-1.95]	0.571	1.5 [0.98-2.13]	4.1 [3.18-5.68]	<0.001
Basophil, %	0.00 [0.00-0.00]	0.00 [0.00-0.00]	0.317	0.00 [0.00-0.00]	0.00 [0.00-0.00]	0.160

Complete blood count results at baseline (0 hr), and 48 hr post-first injection for the control group and the lymphopenia group, with 36 mice in each group. Values are median [interquartile range].

In comparison to the control group, the lymphopenia group exhibited significantly higher neutrophil-to-lymphocyte ratio (2.77 vs. 0.23, $p < 0.001$), monocyte-to- lymphocyte ratio (0.55 vs. 0.03, $p < 0.001$), and platelet-to-lymphocyte ratio (2942.06 vs. 235.97, $p < 0.001$) (Fig. 3).

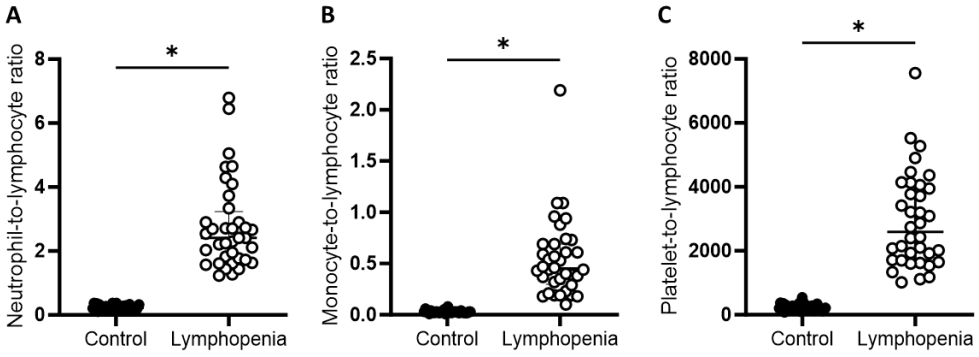

Figure 3. Comparison of blood cell ratios between the groups.
Neutrophil-to-lymphocyte ratio (A), monocyte-to-lymphocyte ratio (B), and platelet-to-lymphocyte ratio (C) were significantly higher in the lymphopenia group 48 hr after administration of the study drug. *$p < 0.001$.

3.3. Time to occlusion and time to recanalization

Out of the 72 mice subjected to $FeCl_3$-induced carotid arterial thrombosis, 4 mice (1 male and 3 females) failed to achieve thrombotic occlusion. After excluding these 4 mice, 68 mice (35 of the lymphopenia group and 33 of the control group) were included in the analysis of time to occlusion and time to recanalization. The time to occlusion did not significantly differ between the lymphopenia group (median 3.56 min, interquartile range 3.03-5.32 min) and the control group (median 3.98 min, interquartile range 3.47-4.74 min) ($p = 0.484$; Fig. 4A). The time to occlusion, defined as the point when the blood flow drops to 25% of the mean baseline blood flow after the removal of $FeCl_3$-soaked filter paper, was also compared between the groups (Fig. 4B). There was no significant difference between the groups ($p = 0.473$), consistent with previous calculations. The time to occlusion between the groups was further analyzed using a chi-square to assess the distribution of the time to occlusion data in each group (Fig. 4C). There was no significant correlation found between the time to occlusion and NLR (r = -0.108, $p = 0.379$; Fig. 5A), between the time to occlusion and MLR (r = -0.032, $p = 0.796$; Fig. 5B), or between the time to occlusion and PLR (r = -0.002, $p = 0.985$; Fig. 5C).

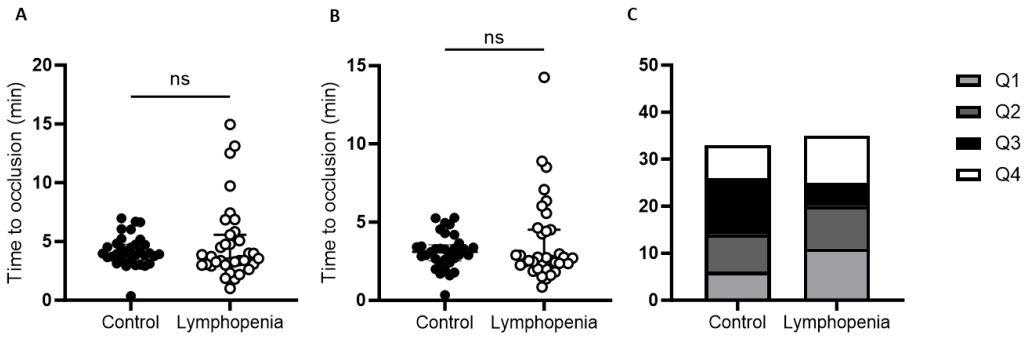

Figure 4. Comparison of the time to occlusion between the groups.

The time to occlusion after inducing $FeCl_3$-induced carotid artery thrombosis did not differ between the groups (A). The time to occlusion which was calculated as when the blood flow drops to 25% of the mean baseline blood flow after removal of $FeCl_3$-soaked filter paper (B). The distribution of time to occlusion data across two different groups is shown, with the x-axis representing the number of mice (C).

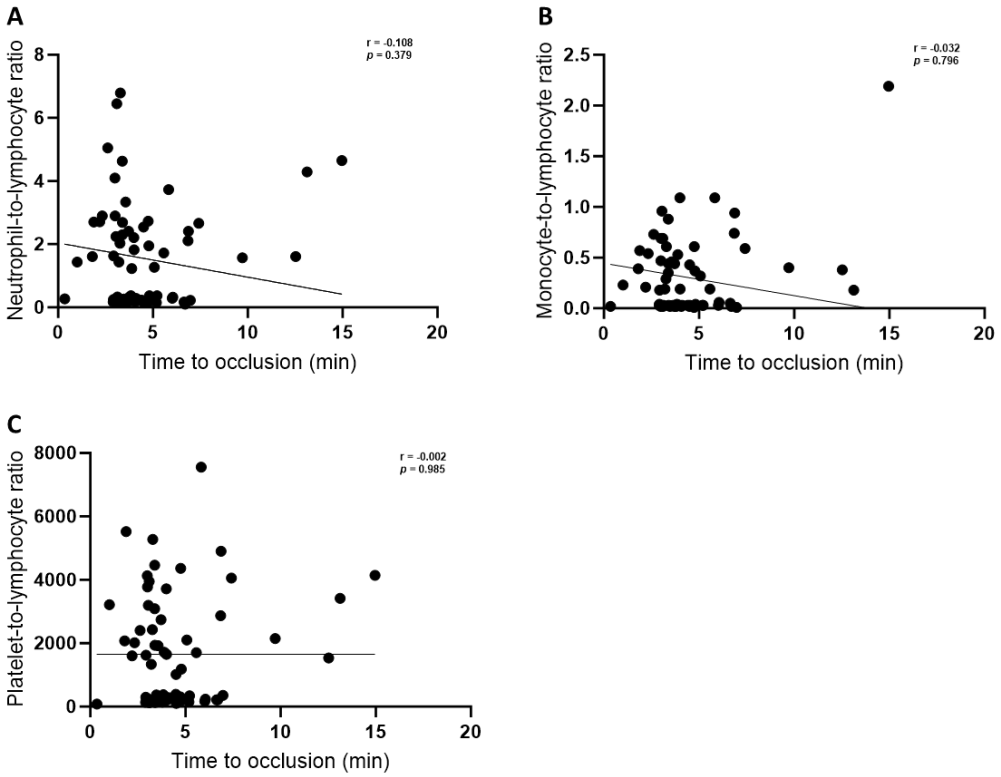

Figure 5. Correlation between the time to occlusion and blood cell ratios.
No correlation was observed between the time to occlusion and neutrophil-to-lymphocyte ratio (A), monocyte-to-lymphocyte ratio (B), and platelet-to-lymphocyte ratio (C).

During the 2 hr monitoring period after induction of thrombotic occlusion, blood flow was not restored to 75% of the mean baseline blood flow in 34 ICR mice (17 of the lymphopenia group and 17 of the control group). Among 38 mice that achieved recanalization, the time to recanalization was significantly shorter in the lymphopenia group (median 35.87 min, interquartile range 17.44-68.66 min) compared to the control group (median 72.33 min, interquartile range 62.63-88.92 min) (p = 0.006; Fig. 6). There was a significant inverse correlation between the time to recanalization and NLR (r = -0.351, p = 0.031; Fig. 7A), MLR (r = -0.412, p = 0.010; Fig. 7B), or PLR (r = -0.430, p = 0.007; Fig. 7C).

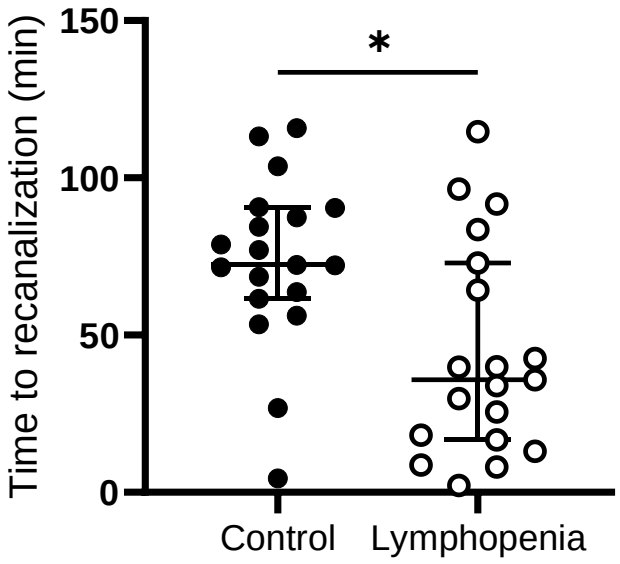

Figure 6. Comparison of the time to recanalization between the groups.
The time to recanalization after inducing $FeCl_3$-induced carotid artery thrombosis significantly different between the groups.

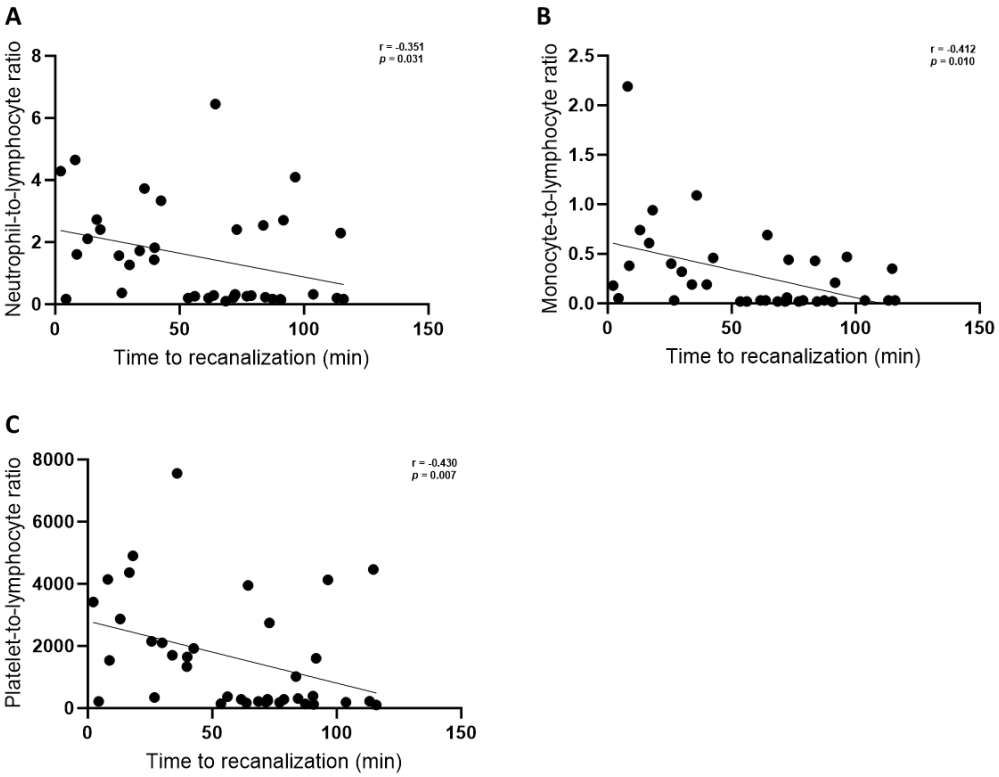

Figure 7. Correlation between the time to recanalization and blood cell ratios.
Significant negative correlation was observed between the time to recanalization and neutrophil-to-lymphocyte ratio (A), monocyte-to-lymphocyte ratio (B), and platelet-to-lymphocyte ratio (C).

4. DISCUSSION

In this study, FTY720 successfully induced significant lymphopenia. This study showed that there were no significant changes in the time to occlusion after $FeCl_3$-induced thrombosis in mice with FTY720-induced lymphopenia. However, the time to recanalization was significantly shortened in the lymphopenia group.

Previous studies have suggested the role of lymphocytes in thrombosis. In a transient middle cerebral artery occlusion model in mice, FTY720 treatment significantly reduced infarct sizes[17,18]. This protective effect against ischemia was suggested to be associated with lymphopenia-induced reduction of microvascular thrombosis and fibrin amounts[18,25]. However, in this study, the time to occlusion after the induction of thrombosis was not changed in the lymphopenia group. Our findings are consistent with those of a previous study that showed no difference in the time to the initial appearance of thrombi between FTY720-treated mice and controls after $FeCl_3$-induced mesenteric artery thrombosis[18]. These findings suggest that lymphocytes may not play a significant role in the initial process of thrombus formation.

Although time to occlusion was not different between the groups, the time to recanalization was significantly reduced in the lymphopenia group. This suggests that lymphocytes may contribute to the stabilization of thrombi. Thrombi formed within the vasculature may undergo spontaneous resolution by the degradation of fibrin[26]. Plasminogen activators play a key role in the degradation of fibrin by proteolytic activation of plasminogen to form plasmin. Several studies have suggested that the depletion of T cells may selectively impair thrombus resolution, as T cell depletion leads to a decrease in the number of macrophages, which then reduces the expression of urokinase plasminogen activator[11,27,28].

Despite early research confirming the presence of lymphocytes in venous thrombi, their functional significance in the resolution of venous thrombosis has only recently been studied[29]. The early influx of neutrophils, followed by the subsequent migration of monocytes and macrophages into thrombus, has been documented[30]. Inflammatory cells are a primary source of cytokines and proteases, which play a role in the resolution of venous thrombi[11]. B cells can produce both pro- and anti-inflammatory cytokines[31]. Several studies have shown that FTY720 treatment may decrease the proportion of memory B cells[32-34]. B cell depletion in a venous thrombosis model in mice resulted in impaired thrombus resolution[35]. A recent study examined the involvement of effector memory T cells in a mouse model of venous thrombosis induced by stenosis. The specific depletion of the T_{EM} population by antibody-mediated ablation of $CD4^+$ and $CD8^+$ T cells expedited the resolution of venous thrombus[35,36]. These findings regarding the association between T-cells or B-cells and thrombus resolution support our findings.

The intricate interplay among immune system cells, including neutrophils, lymphocytes, monocytes, and macrophages, contributes to the onset of inflammation and thrombosis[37]. NLR, MLR, and PLR are well-known biomarkers for predicting outcomes and recurrence in patients with stroke or coronary artery disease[38]. Therefore, we further analyzed the relationships between these biomarkers and the time to recanalization. NLR, MLR, and PLR were all negatively correlated with time to recanalization. The higher the NLR, MLR, and PLR, the faster the thrombus resolved in our study. This association with outcomes may be ascribed to the role of neutrophils, monocytes, and platelets in inflammation and thrombosis[14,39]. However, our findings indicate that lymphocytes may also contribute to poor outcomes by impeding thrombus resolution.

This study has various limitations. First, it used a vascular injury-mediated thrombosis model with $FeCl_3$. However, this model does not represent the diverse mechanisms of thrombosis in humans. Second, mice have different leukocyte subset profiles compared to

humans, with higher lymphocyte and lower neutrophil counts. Therefore, these model-related factors should be considered when interpreting the findings. Finally, while the study suggested the potential role of lymphocytes in thrombus resolution, the exact mechanism remains unknown. Additional studies are required to ascertain the mechanism how lymphocytes contribute to thrombus resolution.

5. CONCLUSION

Lymphopenia was not associated with the time to occlusion but was associated with faster resolution of thrombus in a $FeCl_3$-induced carotid thrombosis model in mice. These findings suggest that lymphocytes do not play a role in initial stage of thrombosis, but rather in the stability of thrombus.

REFERENCES

1 Wendelboe, A. M. & Raskob, G. E. Global Burden of Thrombosis: Epidemiologic Aspects. *Circ Res* **118**, 1340-1347, doi:10.1161/CIRCRESAHA.115.306841 (2016).

2 Raskob, G. E. *et al.* Thrombosis: a major contributor to global disease burden. *Arterioscler Thromb Vasc Biol* **34**, 2363-2371, doi:10.1161/ATVBAHA.114.304488 (2014).

3 Jang, H. J., Schellingerhout, D., Kim, J., Chung, J. & Kim, D. E. Towards a biomarker for acute arterial thrombosis using complete blood count and white blood cell differential parameters in mice. *Sci Rep* **13**, 4043, doi:10.1038/s41598-023-31122-9 (2023).

4 Ming, L. *et al.* Platelet-to-lymphocyte ratio, neutrophil-to-lymphocyte ratio, and platelet indices in patients with acute deep vein thrombosis. *Vasa* **47**, 143-147, doi:10.1024/0301-1526/a000683 (2018).

5 Xue, J., Ma, D., Jiang, J. & Liu, Y. Diagnostic and Prognostic Value of Immune/Inflammation Biomarkers for Venous Thromboembolism: Is It Reliable for Clinical Practice? *J Inflamm Res* **14**, 5059-5077, doi:10.2147/JIR.S327014 (2021).

6 Swystun, L. L. & Liaw, P. C. The role of leukocytes in thrombosis. *Blood* **128**, 753-762, doi:10.1182/blood-2016-05-718114 (2016).

7 Lee, K. O. *et al.* Effect of leukopenia induced by cyclophosphamide on the initial stage of arterial thrombosis in mice. *Thromb Res* **206**, 111-119, doi:10.1016/j.thromres.2021.08.017 (2021).

8 Cerletti, C., de Gaetano, G. & Lorenzet, R. Platelet - leukocyte interactions: multiple links between inflammation, blood coagulation and vascular risk. *Mediterr J Hematol Infect Dis* **2**, e2010023, doi:10.4084/MJHID.2010.023 (2010).

9 Palabrica, T. *et al.* Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets. *Nature* **359**, 848-851, doi:10.1038/359848a0 (1992).

10 de Gaetano, G., Donati, M. B. & Cerletti, C. Prevention of thrombosis and vascular

inflammation: benefits and limitations of selective or combined COX-1, COX-2 and 5-LOX inhibitors. *Trends Pharmacol Sci* **24**, 245-252, doi:10.1016/S0165-6147(03)00077-4 (2003).

11 Mukhopadhyay, S. *et al.* Depletion of CD4 and CD8 Positive T Cells Impairs Venous Thrombus Resolution in Mice. *Int J Mol Sci* **21**, doi:10.3390/ijms21051650 (2020).

12 Hasselwander, S. *et al.* B lymphocyte-deficiency in mice promotes venous thrombosis. *Heliyon* **8**, e11740, doi:10.1016/j.heliyon.2022.e11740 (2022).

13 Artoni, A. *et al.* Platelet to Lymphocyte Ratio and Neutrophil to Lymphocyte Ratio as Risk Factors for Venous Thrombosis. *Clinical and Applied Thrombosis/Hemostasis* **24**, 808-814, doi:10.1177/1076029617733039 (2017).

14 Duygulu, M. E. *et al.* Relationship between Neutrophil/Lymphocyte Ratio (NLR), Platelet/Lymphocyte Ratio (PLR) and Thrombosis in Patients with Portal Vein Thrombosis (PVT) without Acquired Risk Factor for Thrombosis. *Niger J Clin Pract* **27**, 89-94, doi:10.4103/njcp.njcp_464_23 (2024).

15 Kihara, A. & Igarashi, Y. Production and release of sphingosine 1-phosphate and the phosphorylated form of the immunomodulator FTY720. *Biochim Biophys Acta* **1781**, 496-502, doi:10.1016/j.bbalip.2008.05.003 (2008).

16 Pelayo, C. *et al.* Multiple sclerosis and related disorders: Short report peripheral vascular events in a real-world cohort of multiple sclerosis patients using Fingolimod. *Mult Scler Relat Disord* **45**, 102411, doi:10.1016/j.msard.2020.102411 (2020).

17 Brait, V. H., Tarrason, G., Gavalda, A., Godessart, N. & Planas, A. M. Selective Sphingosine 1-Phosphate Receptor 1 Agonist Is Protective Against Ischemia/Reperfusion in Mice. *Stroke* **47**, 3053-3056, doi:10.1161/STROKEAHA.116.015371 (2016).

18 Kraft, P. *et al.* FTY720 ameliorates acute ischemic stroke in mice by reducing thrombo-inflammation but not by direct neuroprotection. *Stroke* **44**, 3202-3210, doi:10.1161/STROKEAHA.113.002880 (2013).

19 Kurtuncu, M. *et al.* Impact of fingolimod on CD4+ T cell subset and cytokine profile of relapsing remitting multiple sclerosis patients. *J Neuroimmunol* **337**,

577065, doi:10.1016/j.jneuroim.2019.577065 (2019).

20 Liesz, A. *et al.* FTY720 reduces post-ischemic brain lymphocyte influx but does not improve outcome in permanent murine cerebral ischemia. *PLoS One* **6**, e21312, doi:10.1371/journal.pone.0021312 (2011).

21 Keul, P. *et al.* The sphingosine-1-phosphate analogue FTY720 reduces atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* **27**, 607-613, doi:10.1161/01.ATV.0000254679.42583.88 (2007).

22 Wang, L. Y. *et al.* Sphingosine-1-phosphate receptor modulator FTY720 attenuates experimental myeloperoxidase-ANCA vasculitis in a T cell-dependent manner. *Clin Sci (Lond)* **134**, 1475-1489, doi:10.1042/CS20200497 (2020).

23 Bail, K. *et al.* Differential effects of FTY720 on the B cell compartment in a mouse model of multiple sclerosis. *J Neuroinflammation* **14**, 148, doi:10.1186/s12974-017-0924-4 (2017).

24 Klingenberg, R. *et al.* Sphingosine-1-phosphate analogue FTY720 causes lymphocyte redistribution and hypercholesterolemia in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* **27**, 2392-2399, doi:10.1161/ATVBAHA.107.149476 (2007).

25 Schuhmann, M. K., Krstic, M., Kleinschnitz, C. & Fluri, F. Fingolimod (FTY720) Reduces Cortical Infarction and Neurological Deficits During Ischemic Stroke Through Potential Maintenance of Microvascular Patency. *Curr Neurovasc Res* **13**, 277-282, doi:10.2174/1567202613666160823152446 (2016).

26 Shim, Y. *et al.* Characterization of Ferric Chloride-Induced Arterial Thrombosis Model of Mice and the Role of Red Blood Cells in Thrombosis Acceleration. *Yonsei Med J* **62**, 1032-1041, doi:10.3349/ymj.2021.62.11.1032 (2021).

27 Gabre, J. *et al.* Activated protein C accelerates venous thrombus resolution through heme oxygenase-1 induction. *J Thromb Haemost* **12**, 93-102, doi:10.1111/jth.12424 (2014).

28 Mukhopadhyay, S., Antalis, T. M., Nguyen, K. P., Hoofnagle, M. H. & Sarkar, R. Myeloid p53 regulates macrophage polarization and venous thrombus resolution by inflammatory vascular remodeling in mice. *Blood* **129**, 3245-3255, doi:10.1182/blood-2016-07-727180 (2017).

29 Henke, P. K., Nicklas, J. M. & Obi, A. Immune cell-mediated venous thrombus resolution. *Res Pract Thromb Haemost* **7**, 102268, doi:10.1016/j.rpth.2023.102268 (2023).

30 Deatrick, K. B. *et al.* The effect of matrix metalloproteinase 2 and matrix metalloproteinase 2/9 deletion in experimental post-thrombotic vein wall remodeling. *J Vasc Surg* **58**, 1375-1384 e1372, doi:10.1016/j.jvs.2012.11.088 (2013).

31 de Gruijter, N. M., Jebson, B. & Rosser, E. C. Cytokine production by human B cells: role in health and autoimmune disease. *Clin Exp Immunol* **210**, 253-262, doi:10.1093/cei/uxac090 (2022).

32 Blumenfeld, S., Staun-Ram, E. & Miller, A. Fingolimod therapy modulates circulating B cell composition, increases B regulatory subsets and production of IL-10 and TGFbeta in patients with Multiple Sclerosis. *J Autoimmun* **70**, 40-51, doi:10.1016/j.jaut.2016.03.012 (2016).

33 Claes, N. *et al.* Compositional changes of B and T cell subtypes during fingolimod treatment in multiple sclerosis patients: a 12-month follow-up study. *PLoS One* **9**, e111115, doi:10.1371/journal.pone.0111115 (2014).

34 Cinamon, G. *et al.* Sphingosine 1-phosphate receptor 1 promotes B cell localization in the splenic marginal zone. *Nat Immunol* **5**, 713-720, doi:10.1038/ni1083 (2004).

35 Luther, N. *et al.* Innate Effector-Memory T-Cell Activation Regulates Post-Thrombotic Vein Wall Inflammation and Thrombus Resolution. *Circ Res* **119**, 1286-1295, doi:10.1161/CIRCRESAHA.116.309301 (2016).

36 Siefert, S. A. *et al.* Enhanced venous thrombus resolution in plasminogen activator inhibitor type-2 deficient mice. *J Thromb Haemost* **12**, 1706-1716, doi:10.1111/jth.12657 (2014).

37 Chen, L. *et al.* Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget* **9**, 7204-7218, doi:10.18632/oncotarget.23208 (2018).

38 Tudurachi, B. S., Anghel, L., Tudurachi, A., Sascau, R. A. & Statescu, C. Assessment of Inflammatory Hematological Ratios (NLR, PLR, MLR, LMR and Monocyte/HDL-Cholesterol Ratio) in Acute Myocardial Infarction and

Particularities in Young Patients. *Int J Mol Sci* **24**, doi:10.3390/ijms241814378 (2023).

39 Muresan, A. V. *et al.* The Predictive Value of NLR, MLR, and PLR in the Outcome of End-Stage Kidney Disease Patients. *Biomedicines* **10**, doi:10.3390/biomedicines10061272 (2022).

국문요약

마우스의 $FeCl_3$ 유도 총경동맥 혈전증에서 림프구 감소증이 혈전 형성 및 용해에 미치는 영향

혈전증은 동맥이나 정맥 내에서 혈전이 형성되는 것을 의미하며, 동맥계에서의 발생은 뇌졸중과 심근경색의 주요 원인 중 하나다. 혈전 형성은 주로 혈소판과 호중구 및 단핵구와 같은 초기 침윤 세포들에 의해 발생하게 되며, 혈전 용해는 주로 대식세포와 단핵구에 의해 발생하게 된다. 본 연구에서는 FTY720을 투여하여 림프구 감소를 유발하고 림프구의 역할을 탐구하며 마우스의 $FeCl_3$ 유도 경동맥 혈전증 모델을 사용하여 혈전 형성 및 해결에 미치는 영향을 조사하고자 하였다.

$FeCl_3$로 유발된 경동맥 혈전증 모델을 사용하여 FTY720 투여 및 식염수 투여 후 혈전 및 이후 재관류에 미치는 영향을 비교하기 위한 이중맹검, 무작위 대조 연구를 진행하였다. 다양한 시간대에 수집된 혈액 샘플을 혈액학 분석기를 사용하여 혈액 검사를 실시하였다. 이 검사를 통해 다양한 혈액 세포 유형의 수와 헤모글로빈 농도 및 혈액 용적 비율과 같은 매개 변수를 포함한 결과를 얻을 수 있었다. 또한, neutrophil-to-lymphocyte ratio (NLR), monocyte-to-lymphocyte ratio (MLR), and platelet-to-lymphocyte ratio (PLR) 같은 다양한 비율은 혈액 검사 결과를 기반으로 계산하였으며, 폐색 시간 및 재관류 시간과의 잠재적 상관 관계를 탐색하는 데 사용되었다.

혈전으로 인한 폐색 시간은 림프구 감소 그룹 (3.56 [3.03-5.32] 분)과 대조군 그룹 (3.98 [3.47-4.74] 분) 간 유의적으로 차이가 나지 않았다 (p = 0.484). 폐색 시간과 NLR 간에는 유의한 상관관계가 없었다 (r = −0.108, p = 0.379), 폐색 시간과 MLR 간에도 유의한 상관관계가 없었다 (r = −0.032, p = 0.796), 또한 폐색 시간과 PLR 간에도 유의한 상관관계가 없었다(r = −0.002, p = 0.985). 혈전증 유도 후 2시간 모니터링 중에, 총 72마리중 38마리의 마우스에서는 기준 혈류의 평균치의

75%로의 혈류가 회복되지 않았다. 재관류를 경험한 마우스 중에서는, 림프구 감소 그룹의 재관류 시간이 대조군 그룹보다 유의적으로 짧았다 (35.87 분 대 72.33 분, $p = 0.006$). 재관류 시간과 NLR ($r = -0.351$, $p = 0.031$), MLR ($r = -0.412$, $p = 0.010$), 또는 PLR ($r = -0.430$, $p = 0.007$) 간에는 유의한 상관관계가 있었다.

FTY720은 마우스에서 유의적인 림프구 감소를 유발했다. 림프구 감소는 혈전증 폐색 시간과 관련이 없었지만, 혈전의 빠른 해결과 관련이 있었다. 이러한 결과는 림프구가 혈전증의 초기 단계에는 역할을 하지 않고 혈전의 안정성에 영향을 줄 수 있음을 암시할 수 있다.

핵심되는 말 : 백혈구, 호중구, 림프구, 단핵구, 혈소판, 핑골리모드 염산염, 경동맥 혈전증

PUBLICATION LIST

1. Heo, J., Y. Seog, H. Lee, I. H. Lee, S. Kim, J. H. Baek, H. Park, K. D. Seo, G. S. Kim, H. J. Cho, M. Baik, J. Yoo, J. Kim, J. Lee, Y. K. Chang, T. J. Song, J. H. Seo, S. H. Ahn, H. W. Lee, I. Kwon, E. Park, Y. D. Kim and H. S. Nam (2022). "Automated Composition Analysis of Thrombus from Endovascular Treatment in Acute Ischemic Stroke Using Computer Vision." J Stroke **24**(3): 433-435.

2. Cha, M. J., J. Ha, H. Lee, I. Kwon, S. Kim, Y. D. Kim, H. S. Nam, H. S. Lee, T. J. Song, H. J. Choi and J. H. Heo (2022). "Neutrophil Recruitment in Arterial Thrombus and Characteristics of Stroke Patients with Neutrophil-Rich Thrombus." Yonsei Med J **63**(11): 1016-1026.

3. Heo, J., H. Lee, Y. Seog, S. Kim, J. H. Baek, H. Park, K. D. Seo, G. S. Kim, H. J. Cho, M. Baik, J. Yoo, J. Kim, J. Lee, Y. Chang, T. J. Song, J. H. Seo, S. H. Ahn, H. W. Lee, I. Kwon, E. Park, B. M. Kim, D. J. Kim, Y. D. Kim and H. S. Nam (2023). "Cancer Prediction With Machine Learning of Thrombi From Thrombectomy in Stroke: Multicenter Development and Validation." Stroke **54**(8): 2105-2113.

4. Yoo, J., I. Kwon, S. Kim, H. M. Kim, Y. D. Kim, H. S. Nam and J. H. Heo (2023). "Coagulation Factor Expression and Composition of Arterial Thrombi in Cancer-Associated Stroke." Stroke **54**(12): 2981-2989

.