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Effects of Interleukin-17A on the Early Stages of Arterial Thrombosis in Mice

Youngseon Park[1,2,3], Yeseul Shim[1,2,3], Il Kwon[2], Heow Won Lee[2], Hyo Suk Nam[1,2], Hyun-Jung Choi[2], and Ji Hoe Heo[1,2,3]

[1]Department of Neurology, Yonsei University College of Medicine, Seoul;
[2]Integrative Research Center for Cerebrovascular and Cardiovascular Diseases, Yonsei University College of Medicine, Seoul;
[3]Department of Neurology, Graduate School of Medical Science, Brain Korea 21 Project, Yonsei University College of Medicine, Seoul, Korea.

Purpose: Interleukin (IL)-17A has been suggested to play a role in the growth and organization of thrombi. We examined whether IL-17A plays a role in the early stages of thrombosis and whether there are sex differences in the effects of IL-17A.
Materials and Methods: We performed a blinded, randomized, placebo-controlled study to compare time to thrombotic occlusion and sex differences therein between mice treated with IL-17A and those treated with saline using a ferric chloride-induced model. We also assessed thrombus histology, blood coagulation, and plasma levels of coagulation factors.
Results: Time to occlusion values did not differ between the IL-17A group and the control group (94.6±86.9 sec vs. 121.0±84.4 sec, p=0.238). However, it was significantly shorter in the IL-17A group of female mice (74.6±57.2 sec vs. 130.0±76.2 sec, p=0.032). In rotational thromboelastometry, the IL-17A group exhibited increased maximum clot firmness (71.3±4.5 mm vs. 66.7±4.7 mm, p=0.038) and greater amplitude at 30 min (69.7±5.2 mm vs. 64.5±5.3 mm, p=0.040) than the control group. In Western blotting, the IL-17A group showed higher levels of coagulation factor XIII (2.2±1.5 vs. 1.0±0.9, p=0.008), monocyte chemoattractant protein-1 (1.6±0.6 vs. 1.0±0.4, p=0.023), and tissue factor (1.5±0.6 vs. 1.0±0.5, p=0.003).
Conclusion: IL-17A plays a role in the initial st ages of arterial thrombosis in mice. Coagulation factors and monocyte chemoattractant protein-1 may be associated with IL-17A-mediated thrombosis.

Key Words: Thrombosis, interleukin-17A, inflammation, monocyte chemoattractant protein-1, tissue factor, factor XIII

INTRODUCTION

Arterial thrombosis is a dynamic process in which the adhesion of platelets to the endothelium is believed to initiate thrombosis. Thrombi are formed by subsequent platelet aggregation and activation of the coagulation system.[1-3] The release of tissue factor (TF) from atherosclerotic plaques and turbulent blood flow in the poststenotic segment of the atherosclerotic artery further contributes to thrombus formation.[4] While previous studies have focused on the role of endothelium, platelets, and fibrin in thrombosis, recent studies have shown that leukocytes and inflammation also contribute to coagulation and thrombosis.[5,6] Many leukocytes are found in human thrombi.[7] Leukocytes including neutrophils and monocytes interact with platelets and are related to the generation of clotting factors, such as thrombin and TF.[8-10] Neutrophils release neutrophil extracellular traps (NETs), which serve as scaffolds for thrombi and contribute to thrombosis by interacting with blood cells.[6,11,12] In stroke patients, NET activity has been shown to be correlated with the number of neutrophils in thrombi.[13]

Previous studies have suggested that leukocytes in thrombosis primarily play a role in the organization and maturation of a thrombus.[6] However, in our recent study, time to thrombotic occlusion by ferric chloride ($FeCl_3$) treatment of carotid arteries was attenuated in mice with leukopenia. In addition,

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Corresponding author: Ji Hoe Heo, MD, PhD, Department of Neurology, Integrative Research Center for Cerebrovascular and Cardiovascular Diseases, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea.
Tel: 82-2-2228-1605, Fax: 82-2-393-0705, E-mail: jhheo@yuhs.ac

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the effect of leukopenia on the attenuation of thrombosis was more obvious in female mice.[14] These findings suggested that leukocytes may also play a role in the initial and early stages of thrombosis and that there may be sex difference in leukocyte-mediated thrombosis. However, little is known of how leukocytes are associated with initial stage of thrombosis and whether sex is associated with inflammation-mediated thrombosis.

Leukocytes release pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α. These cytokines have been suggested to enhance coagulation and inhibit fibrinolysis.[15] IL-17A is a potent pro-inflammatory cytokine that is primarily produced by helper T cells. IL-17A has been suggested to promote thrombosis: IL-17A enhanced the activation and aggregation of platelets and increased neutrophil infiltration into the thrombus.[16,17] The administration of recombinant IL-17A (rIL-17A) increased thrombus size at 24 h in a deep venous thrombosis model in mice.[16] IL-17A was associated with thrombus growth.[18] Moreover, IL-17A upregulated the expression of TF in human umbilical vein endothelial cells.[19] However, the role of IL-17A in the early stages of arterial thrombosis remains unclear.

Several studies have highlighted possible sex-related differences in thrombosis. A systematic review and meta-analysis showed that women with atrial fibrillation face a higher risk of stroke than men.[20,21] A study showed that women treated with new oral anticoagulants had a higher risk of embolism than men.[22] Moreover, among patients with coronary artery disease, women had worse outcomes and decreased fibrin clot lysability than men.[23,24] Additional sex-specific differences in platelet function and neutrophil responsiveness have been suggested.[25,26] These findings indicate that there may be sex-related differences in inflammation-mediated thrombosis.

We hypothesized that IL-17A would exhibit a role in the early stages of arterial thrombosis and that there may be sex-related differences in the effect of IL-17A. To test this hypothesis, we performed a blinded, randomized, placebo-controlled study using a mouse model of $FeCl_3$-induced thrombosis to compare time to thrombotic occlusion and whether the time was different between male and female mice treated with IL-17A and those treated with saline.

MATERIALS AND METHODS

Animals and ethical statements

We used 6 to 8-week-old Institute of Cancer Research (ICR) mice weighing 32–34 g for male and 26–28 g for female mice. The animals were kept in a temperature-controlled animal facility under a standard light and dark cycle (12 h:12 h), placed in a plastic cage with soft bedding, and allowed free access to food and water. All the animal procedures were approved by the Institutional Animal Care and Use Committee of the Yonsei University College of Medicine (Approval number: 2020-0074, 22 March 2020) and were performed in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care.

Study design, study groups, randomization, blinding, and outcomes

A blinded, randomized, placebo-controlled study was performed to compare the effect of IL-17A on arterial thrombosis in ICR mice. A total of 60 mice randomly received either rIL-17A (IL-17A group) or normal saline (control group) (15 male and 15 female mice in each group). A randomization list with random numbers in permuted blocks with a block size of 4 was computer-generated by a researcher who was not involved in the animal experiments. After preparing rIL-17A and the same volume of normal saline, a randomized number was assigned. Surgical procedures and outcome assessments were performed by an investigator who was blinded to study grouping. The primary outcome was time to occlusion after $FeCl_3$-induced thrombosis had been established in the left common carotid artery (CCA). The secondary outcome was thrombus size.

Determination of rIL-17A dose

The dose of murine rIL-17A (Peprotech, Cranbury, NJ, USA) was determined based on the peak increase in plasma IL-17A levels after treatment with rIL-17A. Four mice each received normal saline or three different doses (10, 25, and 50 μg/kg) of rIL-17A intraperitoneally. Blood (200 μL) was drawn from the tail vein of each mouse into tubes containing sodium citrate at 30 min, 1, 3, and 6 h after peritoneal injection. Plasma was immediately isolated by centrifuging blood at 3000 rpm for 20 min in a refrigerated centrifuge. The plasma was stored at -80°C until use. Plasma concentrations of IL-17 were determined using a commercially available enzyme-linked immunosorbent assay kit (Quantikine ELISA Kit, R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. All measurements were performed in duplicate. Plasma IL-17A levels increased in a dose-dependent manner. The level peaked at 30 min and decreased thereafter (Supplementary Fig. 1, only online). Based on these results, 25 μg/kg of rIL-17A was intraperitoneally administered in subsequent experiments, and arterial thrombosis was induced 30 min after rIL-17A administration.

Induction of $FeCl_3$-induced carotid thrombosis and measurement of blood flow

Arterial thrombosis was induced using $FeCl_3$. Briefly, anesthesia was induced with 5% isoflurane in a mixture of 70% N_2O and 30% O_2 and maintained with 2% isoflurane. Body temperature was monitored continuously with a rectal probe and maintained at 37.0±0.2°C using a heating pad (Harvard apparatus, Holliston, MA, USA) during the operative procedure. The left CCA was carefully isolated under a surgical microscope. Blood flow in the CCA was measured in the middle portion

using an ultrasonic Doppler flow probe (MAD.7PSB; Transonic Instruments, Ithaca, NY, USA) and an iWorx IX-304T data acquisition system (iWorx Systems, Inc., Dover, NH, USA). A piece of filter paper (F2877; Sigma-Aldrich, St. Louis, MO, USA, 0.5×0.7 mm) was soaked in 2 μL of 50% $FeCl_3$ solution and placed onto the CCA for 5 min. The $FeCl_3$ concentration was determined based on our previous study that induced complete and stable occlusion in ICR mice.[27] After removing the paper, the CCA was washed with saline. The probe was carefully positioned in the upper intact part of the CCA, not the part that touched the paper, and the blood flow was monitored for 10 min. Blood flow data were analyzed using the iWorx Labscribe software (version 4.01; iWorx Systems, Inc., Dover, NH, USA). Time to occlusion was defined as the time from removing the filter paper to the time when blood flow decreased to zero. The CCA containing thrombus was excised 10 min after the occlusion, immediately fixed with 4% paraformaldehyde at 4°C overnight, and then embedded in paraffin. Blood was collected in a tube containing 10% EDTA by cardiac puncture immediately at the end of the blood flow recording lasting 10 min. Complete blood cell counts were obtained using a cell counter analyzer (MS9-5V; Melet Schloesing Laboratories, Cergy-Pontoise, France).

Thrombus size measurements

Paraffin blocks were sectioned into 4-μm slices and mounted on glass slides. A slice representing the largest part of the thrombus was stained with hematoxylin and eosin and used to measure thrombus size. Thrombus size (area) was determined using a light microscope (Axio Imager.D2; Carl Zeiss Imaging Solution, Oberkochen, Germany) and Zeiss Axio Vision software (AxioVs40 V 4.8.1.0; Carl Zeiss Imaging Solution) for each mouse.

Immunohistochemistry and histological analyses of thrombi

Immunohistochemistry was performed using antibodies against red blood cells (TER-119, 1:1600, BioLegend, San Diego, CA, USA), fibrinogen (1:1600, Abcam, Cambridge, UK), platelet (CD41, 1:800, Abcam), TF (1:800, Abcam), neutrophil (Ly-6G, 1:400, BioLegend), monocyte (CD 11b, 1:1600, Novus Biologicals LLC, Centennial, CO, USA), histone H3-cit (1:800, Abcam), and IL-17A (1:400, Abcam).

Paraffin sections were deparaffinized with xylene, passed through graded ethanol for rehydration, and then subjected to heat-mediated antigen retrieval using IHC-Tek epitope retrieval solution and a steamer (IHC World, Woodstock, MD, USA) for 40 min at 100°C. After cooling the sections for 20 min, they were washed with phosphate-buffered saline (PBS) and immersed in 10 mM glycine in PBS for 10 min at 25°C. The sections were blocked with 1% horse serum and 5% nonfat milk in tris-buffered saline for 20 min at 25°C and incubated with the primary antibodies for 2 h at 37°C (anti-TER-119 and anti-CD11b) or overnight at 4°C (anti-citrullinated histone H3, anti-TF, anti-fibrinogen, anti-CD41, anti-Ly-6G, and anti-IL-17A). The sections were incubated for 30 min at 37°C with goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) or goat anti-rat IgG (Vector Laboratories). Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. After reaction with avidin-biotin complexes (Vector Laboratories), the peroxidase signal was developed using a NovaRed substrate kit (Vector Laboratories). Following counterstaining with hematoxylin, the sections were dehydrated and mounted with Permount mounting medium (Fisher Scientific, Fair Lawn, NJ, USA).

Quantification of thrombus composition

The stained images were acquired using a ZEISS imager M2 microscope equipped with a camera (Axio Cam HRC, Carl Zeiss Imaging Solution) and Axio Vision software. The acquired images were analyzed using color deconvolution in ImageJ (NIH, Bethesda, MD, USA). Pixel density was adjusted at the auto threshold value throughout all measurements. The fraction (%) of each component (platelet, erythrocyte, fibrin, and TF) was calculated as the pixel-density percentage for the entire thrombus area. Ly-6G, Histone H3-cit, CD11b, and IL-17A-positive cells were counted using a Zeiss imager M2 microscope equipped with a motorized stage. All measurements were performed in a blinded manner.

Assessment of blood coagulation

The effect of IL-17A on blood coagulation was determined using rotational thromboelastometry (ROTEM; TEM innovation, Munich, Germany). Briefly, 20 mice received 25 μg/kg rIL-17A or normal saline intraperitoneally (five male and five female mice in each group). Thirty minutes after intraperitoneal injection, whole blood was obtained by cardiac puncture into a tube containing 1/10 (v/v) 3.2% sodium citrate and kept at 37°C for 10 min. ROTEM (NATEM) was performed according to the manufacturer's instructions using the NATEM reagent (CaCl2), which is very sensitive to any endogenous activator.

Assessment of plasma levels of coagulation factors and MCP-1

Plasma levels of factor XIIIa (FXIIIa), thrombin, monocyte chemoattractant protein-1 (MCP-1), and TF were determined using Western blotting. Briefly, 18 mice received rIL-17A or normal saline (nine mice in each group; four males and five females) 30 min before $FeCl_3$-induced arterial thrombosis. After 30 min, blood was collected by cardiac puncture and mixed with 3.2% sodium citrate. Plasma was separated by centrifugation at 3000 rpm at 4°C for 20 min and stored at -70°C until further analysis. Plasma was mixed with RIPA buffer (Biosesang, Seongnam, Korea) containing phosphatase and protease inhibitor cocktail (GenDEPOT, Katy, TX, USA), and protein concentrations were determined using a bicinchoninic acid protein assay. Proteins (10 μg for FXIIIa and thrombin, 35 μg for MCP-1, and 20 μg for

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TF) were separated using SDS-polyacrylamide gel electrophoresis (12% resolving gel for MCP-1 and 10% resolving gel for FXIIIa, thrombin, and TF), and the gels were transferred to polyvinylidene difluoride membranes (0.2 μm, Bio-Rad, Hercules, CA, USA) activated with 100% methanol. The membranes were blocked with 5% nonfat milk in tris-buffered saline with 1% Tween 20 at 25°C for 1 h and incubated with the primary antibodies against FXIIIa (Invitrogen, Waltham, CA, USA, 1:3000), thrombin (Novus Biologicals LLC, Centennial, CO, USA, 1:3000), MCP-1 (Cell Signaling Technology, Danvers, MA, USA, 1:500), TF (Abcam, 1:1000), or β-actin (Santa Cruz, Dallas, TX, USA, 1:1000) at 4°C overnight. The membranes were washed with tris-buffered saline with 1% Tween 20 and incubated with goat anti-rabbit IgG H&L secondary antibody (Abcam, 1:10000) at 25°C for 1 h. The corresponding protein level was detected using a Western blotting detection reagent kit (Ab Signal, Ab clone, Seoul, Korea). The protein bands were visualized using a ChemiDox imaging system (LAS4000, GE Healthcare, Chicago, IL, USA) and quantified using ImageJ. The densities of FXIIIa, thrombin, MCP-1, and TF were normalized to that of β-actin and are presented as relative fold changes. Western blotting was performed in triplicates.

Statistical analyses

Statistical analyses were performed using SPSS 23.0 software (IBM Corp., Armonk, NY, USA). The independent Student's t-test was used for comparison between the groups except for the determination of rIL-17A dose and for sex-dependent comparison of ROTEM parameters that were analyzed using the Mann-Whitney U test. Statistical significance was set at $p<0.05$. Data are presented as a means±SD or median with interquartile range.

RESULTS

Effects of IL-17A in early arterial thrombosis

Primary and secondary outcomes

In the study group, the time to occlusion did not differ significantly between the IL-17 group (94.6±86.9 sec) and the control group (121.0±84.4 sec) ($p=0.238$) (Fig. 1A). However, this difference was distinct between the male and female mice. The time to occlusion was significantly shorter in the IL-17A group than the control group of female mice (74.6±57.2 sec vs. 130.0±76.2 sec, $p=0.032$) (Fig. 1B), but not male mice (114.6±107.2 sec vs. 111.9±93.7 sec, $p=0.942$) (Fig. 1C). The sizes of thrombi were similar between the groups (0.2±0.1 mm^2 in the control group vs. 0.3±0.1 mm^2 in the IL-17A group, $p=0.401$).

Hematologic assessment

We compared blood cell counts between the two groups (Table 1). Eosinophil counts were significantly higher in the IL-17A group than in the control group (0.3±0.2 cells/μL vs. 0.2±0.1 cells/μL, $p=0.046$). Counts for other leukocytes and platelets did not differ between groups. There was no significant sex difference in blood cell counts between groups (Supplementary Table 1, only online).

Thrombus composition

We compared thrombus composition between the groups. The proportions of red blood cells, fibrin, and platelets did not differ between the groups (Table 2). No difference was found in the numbers of neutrophils (Ly-6G), monocytes (CD 11b), and IL-17A-positive cells between the IL-17A and control groups (Table 2). There was no significant sex difference in thrombus

Table 1. Effects of IL-17A on Hematologic Parameters in Mice with Ferric Chloride-Induced Thrombosis

	Control (n=28)	IL-17A (n=30)	*p* value
Erythrocytes, ×10^6 cells/μL	8.8±0.6	8.9±0.5	0.781
Hemoglobin, g/dL	14.5±0.7	14.6±0.7	0.700
Hematocrit, %	49.6±2.8	49.9±2.9	0.699
Red cell distribution width, %	12.8±1.0	12.7±0.8	0.692
Platelet count, ×10^3 cells/μL	943.7±275.3	869.6±183.8	0.237
White blood cells, ×10^3 cells/μL	4.8±1.3	5.1±1.5	0.434
Neutrophils	0.9±0.3	0.9±0.4	0.716
Lymphocytes	3.6±1.1	3.7±1.3	0.585
Monocytes	0.1±0.0	0.1±0.0	0.914
Eosinophils	0.2±0.1	0.3±0.2	0.046
Basophils	0.0±0.0	0.0±0.0	0.537

The values are presented as a mean±SD.

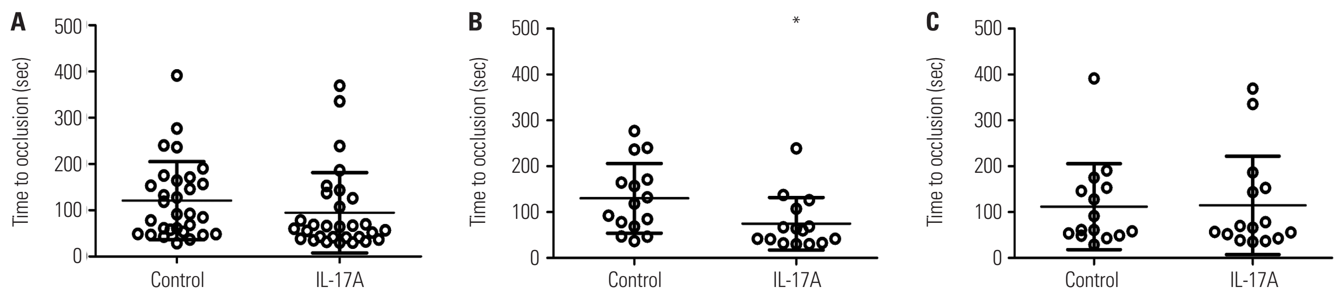

Fig. 1. Effect of rIL-17A treatment on time to occlusion. (A) Time to occlusion did not differ between the IL-17A (n=30) and the control (n=30) groups. (B) In female mice, time to occlusion was shortened in the IL-17A group (n=15), compared to the control group (n=15). (C) In male mice, time to occlusion did not differ between the groups (n=15/groups). The data were analyzed using Student's t-test. The bars represent means±SD. *$p<0.05$ vs. control.

composition between groups (Supplementary Table 2, only online).

Effects of IL-17A on blood coagulation, coagulation factors, and chemokine in thrombosis

While time to occlusion was shortened in the rIL-17A-treated female mice, there were no changes in thrombus composition and the numbers of IL-17A positive cells and leukocytes between the groups. Therefore, we next determined the effect of IL-17A on blood coagulation, coagulation factors, and chemokine in thrombosis. Blood coagulation in thrombosis reflects the viscoelastic properties of clot formation and dissolution (ROTEM), such as maximum clot firmness (MCF) and amplitude at 30 min after coagulation (A30), coagulation factors (FXIIIa, TF, thrombin), and chemokines (MCP-1). The results are shown in Figs. 2 and 3.

Table 2. Composition of Thrombi Isolated from Mice with Ferric Chloride-Induced Thrombosis

	Control (n=30)	IL-17A (n=30)	*p* value
Erythrocytes, %	6.5±6.8	6.8±6.8	0.870
Tissue factor, %	15.7±5.0	14.2±6.3	0.308
Fibrinogen, %	48.5±5.9	47.6±4.5	0.516
Platelets, %	28.1±12.5	25.7±14.3	0.495
Neutrophils, /mm^2	70.5±56.4	59.4±63.0	0.473
Monocytes, /mm^2	76.5±63.2	77.8±82.5	0.944
Neutrophil extracellular traps, /mm^2	228.0±123.1	199.6±117.1	0.364
IL-17A-positive cells, /mm^2	59.7±42.1	59.6±79.0	0.997

The values are presented as a mean±SD.

Coagulation in blood

The parameters for dynamic clot formation were compared between the IL-17A and control groups using ROTEM (Fig. 2A). The MCF, which indicates clot strength, was significantly higher in the IL-17A group than in the control group (71.3±4.5 mm vs. 66.7±4.7 mm, p=0.038) (Fig. 2B). The amplitude at 30 min after coagulation, which evaluates clot firmness, also increased in the IL-17A group (69.7±5.2 mm vs. 64.5±5.3 mm, p=0.040) (Fig. 2B). There were no significant differences in clotting time (242.4±108.4 sec vs. 311.7±123.1 sec, p=0.198), clot formation time (69.7±38.6 sec vs. 98.7±40.9 sec, p=0.120), and alpha angle (76.4±7.2° vs. 70.6±7.9°, p=0.104) between the IL-17A and control groups (Fig. 2B). There was no significant sex difference in ROTEM parameters between the IL-17 and control groups (Supplementary Fig. 2, only online).

Levels of FXIIIa, thrombin, MCP-1, and TF in blood

As clot strength and firmness were increased in the IL-17 group, we assessed the blood levels of FXIIIa. FXIIIa is associated with fibrin cross-linking and clot formation. The blood levels of FXIIIa were significantly higher in the IL-17A group than in the control group (2.2±1.5 vs. 1.0±0.9, p=0.008) (Fig. 3A). The levels of FXIIIa significantly increased between the groups in each sex, respectively (2.0±0.7 vs. 1.0±0.6, p=0.004 in female; 3.6±2.5 vs. 1.0±0.8, p=0.020 in male). The blood levels of thrombin were not significantly different between the groups (1.2±0.8 vs. 1.0±1.0, p=0.383) (Fig. 3B). There was also no significant dif-

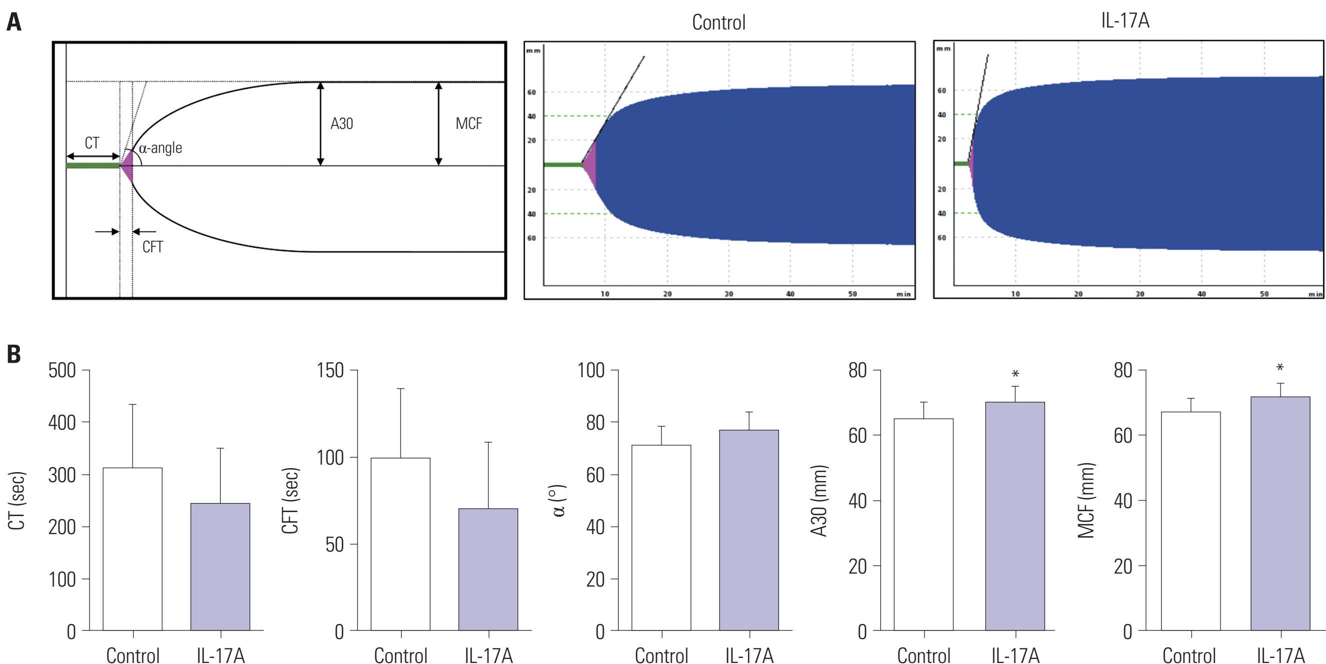

Fig. 2. Assessment of blood coagulation using rotational thromboelastometry (ROTEM). (A) Representative ROTEM traces in a control mouse and an IL-17A-treated mouse. CT, coagulation time (green); CFT, clot formation time (pink); α-angle, A30=amplitude at 30 min; MCF, maximum clot firmness (black line). (B) A30 and MCF increased in the IL-17A group (n=10) compared to the control group (n=10). The data was analyzed by using Student's t-test. The values are presented as means±SD. *p<0.05 vs. control.

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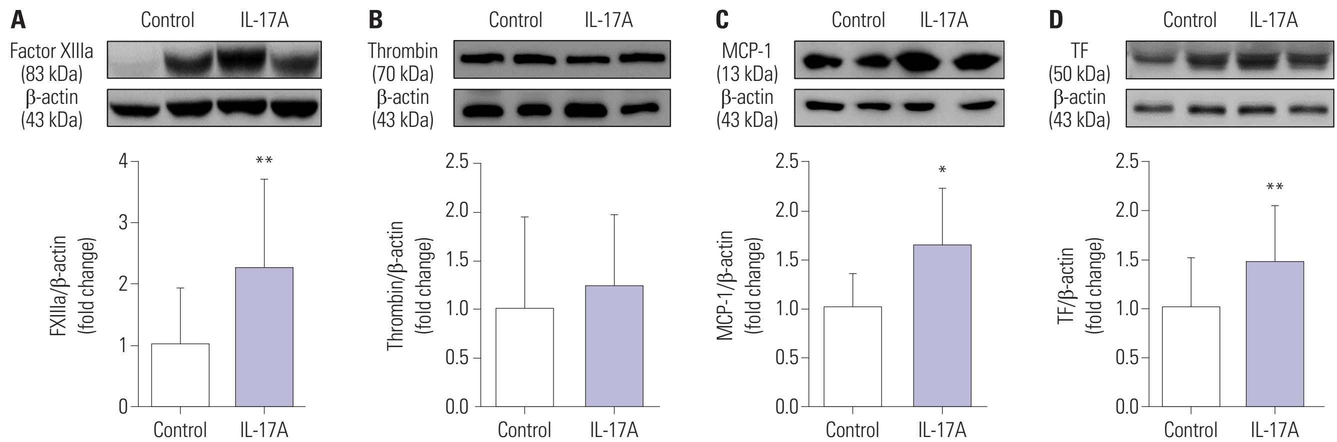

Fig. 3. Effects of IL-17A on plasma levels of factor XIIIa (FXIIIa), thrombin, monocyte chemoattractant protein-1 (MCP-1), and tissue factor (TF) in arterial thrombosis. Representative images of Western blot analysis showing the plasma levels of FXIIIa (A), thrombin (B), MCP-1 (C), and TF (D) in the control (n=8 for FXIIIa, thrombin, and MCP-1, n=9 for TF) and mice treated with IL-17A (n=8 for FXIIIa, thrombin, and MCP-1, n=9 for TF). (A) FXIIIa levels were significantly higher in the IL-17A group than in the control group. (B) Thrombin concentration did not differ between the groups. (C and D) MCP-1 and TF levels increased significantly in the IL-17A group, compared to those in the control group. Representative images of FXIIIa and MCP-1 were from female samples, and those of thrombin and TF were from male samples. The data were analyzed using Student's t-test. The values are presented as a means±SD. *p<0.05 and **p<0.01 vs. control.

ference in thrombin levels between the groups in female mice (1.5±0.9 vs. 1.0±0.7, p=0.114) and in male mice (1.2±0.7 vs. 1.0±0.6, p=0.671).

We also assessed blood levels of MCP-1 as it is an important factor of thrombus organization and resolution. MCP-1 levels were significantly higher in the IL-17A group than in the control group (1.6±0.6 vs. 1.0±0.4, p=0.023) (Fig. 3C). In comparison between the groups of male and female mice, respectively, the levels of MCP-1 in the IL-17A group were significantly higher among female mice (1.8±0.3 vs.1.0±0.3, p=0.010), but not male mice (1.4±0.5 vs. 1.0±0.4, p=0.246).

We also determined the blood levels of TF, which is a key initiating factor in the extrinsic coagulation pathway. The blood levels of TF were significantly higher in the IL-17A group than in the control group (1.5±0.6 vs. 1.0±0.5, p=0.003) (Fig. 3D). There was no significant difference in TF levels between female mice (1.2±0.5 vs. 1.0±0.5, p=0.283) and male mice (1.5±0.8 vs. 1.0±0.8, p=0.104).

DISCUSSION

In this randomized, blinded, placebo-controlled study performed in a mouse model of $FeCl_3$-induced thrombosis, we showed that the administration of rIL-17A in mice induces significantly faster thrombus formation in female mice. The findings of this study suggest that IL-17A plays a role in the initial stages of thrombus formation. However, no differences in the size and composition of the thrombi were observed between the IL-17A and control groups. Moreover, the number of leukocytes and IL-17A-positive cells in the thrombi did not differ between the groups.

Although IL-17A induced significantly faster thrombus formation in female mice, we were not able to find any notable changes in thrombi between the groups. Therefore, we examined the effect of IL-17A on blood coagulation. Results of ROTEM showed that the amplitude at 30 min and MCF were significantly higher in the IL-17A group than in the control group. Other parameters, such as clotting time and clot formation time, also revealed enhanced clotting in the IL-17A group, although the differences were not significant. This result suggests that IL-17A contributes to blood clotting, possibly by enhancing clot strength.

Further evaluation showed that the plasma levels of FXIII and MCP-1 were increased in the IL-17A group. Clot strength depends on fibrinolysis, fibrin polymerization, thrombin concentration, and coagulation factor XIII activity.[28,29] FXIII activation increases clot stability by cross-linking fibrin.[30,31] Cross-linked fibrin stabilizes clots and increases resistance to fibrinolysis.[29,31] MCP-1 was also associated with coagulation by recruiting peripheral blood monocytes and inducing TF accumulation.[32-34] Monocytes induced TF expression and directly affected thrombosis by enhancing the contraction of clots and thrombi.[10,35]

In this study, the blood levels of TF were also significantly higher in the IL-17A group than in the control group. TF is a membrane-bound protein that acts as the cellular receptor for factor VII/VIIa and is the primary initiator of the coagulation process.[36,37] IL-17A stimulates TF expression in human umbilical vein endothelial cells.[19] The expression of TF was synergistically induced by IL-17A and/or TNF-α in human endothelial cells.[38] Our findings corroborate previous findings on the role of IL-17A on the expression of TF. Collectively, our findings, along with previous findings, suggest that IL-17A might play a role in the initial stages of firm thrombus formation, which is mediated by the effect of IL-17A on TF, FXIII, and MCP-1.

In this study, a sex-related difference was observed regarding

the effect of IL-17A on arterial thrombosis: thrombosis was enhanced by IL-17A in female mice, but not in male mice. Outcomes were worse in females with acute coronary syndrome.[24] Sex-specific differences in platelet function or coagulation and the effect of sex hormones have been suggested as the potential explanations for the worse outcomes in females.[24] Young females have more mature and responsive blood neutrophils and an elevated capacity of NET formation.[25] Moreover, female patients show a more pronounced formation of leukocyte-platelet aggregates and increased protease-activated receptor-1 mediated platelet reactivity.[39] Increased plasma level of MCP-1 in the female mice of the IL-17A group in this study supports that inflammation-mediated thrombosis might be more prominent in female mice than in male mice. Platelets from female mice showed greater reactivity to adenosine diphosphate and collagen-related peptides than those from male mice.[26] Our findings along with those of previous studies suggest that females may be more susceptible to thrombosis that is mediated with IL-17A.

This study has several limitations. In this study, there were no differences in the size and composition of thrombi or the number of leukocytes and IL-17A-positive cells in thrombi between the IL-17A and control groups. However, the design of this study might not be sufficient to assess the long-term effect of IL-17A within thrombi because those evaluations were performed in fresh thrombi obtained 10 min after thrombotic occlusion. Therefore, the role of IL-17A during later stages of thrombosis is uncertain. Although sex-related differences were observed in time to occlusion after establishing $FeCl_3$-induced thrombosis and the plasma levels of MCP-1, no such differences were observed in ROTEM or plasma levels of FXIIIa and TF. Therefore, the exact mechanisms underlying the sex-related difference in time to occlusion remains not fully addressed from this study. While we used young and non-pregnant mice, altered sex hormones during pregnancy and the postmenopausal period may influence thrombosis,[40] which was not explored in this study. Also, the sex-related effect of IL-17A or inflammation on thrombosis may differ among species. Thus, interpretation of our findings on sex-related effects of IL-17A should warrant caution. In addition, the $FeCl_3$-induced arterial thrombosis model may not completely represent various complicated thrombosis mechanisms in patients with diverse thrombotic disorders.

In conclusion, our findings suggest that IL-17A plays a role in the initial stages of arterial thrombosis in female mice. MCP-1, TF, and FXIII may be associated with IL-17A-mediated thrombosis.

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AUTHOR CONTRIBUTIONS

Conceptualization: Il Kwon, Hyun-Jung Choi, and Ji Hoe Heo. Data curation: Youngseon Park and Yeseul Shim. Formal analysis: Youngseon Park, Il Kwon, and Ji Hoe Heo. Funding acquisition: Ji Hoe Heo. Investigation: Youngseon Park and Yeseul Shim. Methodology: Youngseon Park, Il Kwon, Heow Won Lee, and Ji Hoe Heo. Project administration: Hyo Suk Nam and Ji Hoe Heo. Resources: Youngseon Park, Yeseul Shim, Il Kwon, and Ji Hoe Heo. Software: Youngseon Park, Il Kwon, Heow Won Lee, and Ji Hoe Heo. Supervision: Ji Hoe Heo. Validation: Il Kwon, Heow Won Lee, and Ji Hoe Heo. Visualization: Youngseon Park, Heow Won Lee, and Ji Hoe Heo. Writing—original draft: Youngseon Park. Writing—review & editing: Heow Won Lee and Ji Hoe Heo. Approval of final manuscript: all authors.

ORCID iDs

Youngseon Park https://orcid.org/0000-0001-7723-7194
Yeseul Shim https://orcid.org/0000-0002-5618-8044
Il Kwon https://orcid.org/0000-0001-9449-5646
Heow Won Lee https://orcid.org/0000-0003-3733-4453
Hyo Suk Nam https://orcid.org/0000-0002-4415-3995
Hyun-Jung Choi https://orcid.org/0000-0003-3695-3420
Ji Hoe Heo https://orcid.org/0000-0001-9898-3321

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