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Neutrophil Phagocytosis of Platelets in the Early Phase of 2,4,6-trinitro-1-chlorobenzene (TNCB)-induced Dermatitis in Mice

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Activated platelets form platelet–leukocyte aggregates in the circulation in inflammatory diseases. We investigated whether activated platelets in inflamed skin tissues are phagocytized and removed by neutrophils. To investigate the kinetics of platelets and neutrophils, we immunohistochemically examined the spatiotemporal distribution of them in a murine model of 2,4,6-trinitro-1-chlorobenzene (TNCB)-induced dermatitis by using confocal and structured illumination microscopy. Four hours after elicitation, aggregates of CD41-positive platelets were adhered to CD31-positive endothelial cells within the vessels, and CD62P and PF4, markers of activated platelets, were expressed on platelet aggregates. At 8 hour post-elicitation, fragmented CD41-positive platelets were located both inside and outside vessels. Twenty-four hours after elicitation, the number of Ly-6G-positive neutrophils ingesting fragmented CD41-positive platelets outside vessels was increased, and CD62P and PF4 expression on the phagocytosed platelets was no longer observed. Disc-shaped CD41-positive platelets were not found outside vessels at any time during the experiment. Our data revealed that aggregates of activated platelets inside vessels were ingested and removed by neutrophils in the early stage of TNCB-induced dermatitis, suggesting that the process of removal of activated platelets by neutrophils may play an important role not only in the early phase of skin inflammation but also in other types of acute inflammation.

Key words: platelet, neutrophil, spatiotemporal distribution, phagocytosis, dermatitis

I. Introduction

Platelets play an eminent role not only in production of hemostasis and thrombosis but in the inflammatory process [1, 2, 13, 15, 17]. Following activation, platelets release chemokines, such as platelet factor 4 (PF4), stored in α-granules, and induce neutrophil recruitment at inflamed sites [4]. Activated platelets also rapidly express various kinds of ligands, such as P-selectin (CD62P), on their membrane, resulting in increased intensity of inflammatory reactions [13, 22]. Platelets form platelet–leukocyte aggregates via P-selectin in the circulation to recruit leukocytes [3].

Neutrophils are the most numerous of the leukocytes, and their activities are important for the control of inflammation. Neutrophils are recruited to sites of infection, where they phagocytose and remove pathogens. In addition, neutrophils have recently been shown to phagocytose platelets in peripheral blood in acute myocardial infarction, polycythemia vera and essential thrombocythemia [8, 9].

In the field of dermatology, we have shown that platelets play various important roles in the process of skin inflammation [4, 19–21]. Platelets are shown to stimulate leukocytes, endothelial cells and other platelets; traffic leukocytes to dermal tissue; induce fibrosis; and control inflammation in the skin [4, 16]. However, spatiotemporal localization of platelets in inflammatory tissues is not well

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understood. Especially, it has not been elucidated whether platelet–leukocyte aggregates migrate intact from vessels into tissues and subsequently stimulate inflammation in inflamed tissues. In this study, we hypothesized that activated platelets in inflamed dermis are phagocytized and removed by neutrophils inside vessels and that platelet–leukocyte aggregates do not transmigrate intact from vessels into tissues. To investigate this issue, we immunohistochemically examined the spatiotemporal distribution of platelets and neutrophils in murine inflamed dermis with 2,4,6-trinitro-1-chlorobenzene (TNCB)-induced contact dermatitis.

II. Materials and Methods

Sensitizing agent

TNCB was purchased from Tokyo Kasei (Tokyo, Japan). TNCB was dissolved in acetone/olive oil (4:1) as a 1% solution and used for sensitization and elicitation.

Experimental mouse model

Seven- to ten-week-old male BALB/c mice were obtained from Shimizu Laboratory Supplies (Kyoto, Japan). They were sensitized with 20 µl (10 µl on the dorsal side and 10 µl on the ventral side) of 1% TNCB solution applied to the right external ear as described previously [5, 19]. Seven days after sensitization, 20 µl of 1% TNCB solution was applied once again to the original, sensitized, right ear. An identical amount of acetone/olive (4:1) was administered topically on the left ear as a control. Ear thickness was measured with a caliper in the base of each ear at 0, 2, 4, 8, 12, and 24 hr post-elicitation. The excised ear tissues were fixed in 2% paraformaldehyde with phosphate-buffered saline (PBS, pH 7.4) for 6 hr. The samples were stored at –80°C until cryostat sectioning. Right and left ears from five mice per each time point of sacrifice were used for the subsequent histological experiments. All animal experiments were approved by the Committee for Animal Research (M21-186, M22-143, M23-124, M24-249, and M25-160), Kyoto Prefectural University of Medicine.

Histological examination

H&E staining of ear sections was performed according to standard methods. The number of infiltrating cells in the dermis was counted within five separate fields per tissue section from five mice each.

The applied primary antibody species for immunohistochemical analysis were anti platelet antibody (CD41, AbD Serotec, Oxford, UK, 1:100 dilution), anti neutrophil antibody (Ly-6G-FITC, Abcam, Cambridge, England, 1:500 dilution), anti T-cell antibody (CD3, Abcam, 1:100 dilution), anti endothelial cell antibody (CD31, Santa Cruz Biotechnology, Dallas, TX, 1:200 dilution), anti P-selectin antibody (CD62P, Santa Cruz, 1:100 dilution), anti PF4 antibody (CXCL4, R&D Systems, Minneapolis, MN, 1:500 dilution) and myeloperoxidase (MPO, Abcam, 1:100 dilution).

Ten-micrometer-thick frozen sections of ears were cut using a cryostat, mounted on glass slides, and air dried. The sections were immersed in PBS, and then nonspecific binding was blocked with PBS containing 5% donkey serum and 0.1% Triton-X for 30 min at room temperature. The sections were incubated for 40 min at 37°C with the primary antibodies. After washes in PBS containing 0.1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO), incubation with Alexa 488 anti-goat IgG (Molecular Probes, Eugene, OR, 1:500 dilution) or with Cy3 anti-rat IgG (Jackson Immunoresearch Laboratories, West Grove, PA, 1:500 dilution) was performed for 30 min at room temperature. After final rinses in PBS containing 0.1% BSA, the sections were mounted in Vectorshield (Vector Laboratories, Burlingame, CA). All antibody dilutions were made using PBS containing 1% BSA. As negative controls, specimens were incubated in the absence of the primary antibodies. Nuclei were counterstained with TO-PRO-3 iodide (Invitrogen, Carlsbad, CA, 1:1000 dilution).

Fluorescence microscopy

The immunohistochemically stained sections were examined by using a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan) equipped with an oil immersion objective lens (Plan Apo ×60, NA=1.4; Olympus) [11, 12]. A multi Ar laser and HeNe laser produced excitation bands at 488 nm for Alexa 488, at 543 nm for Cy3, and at 633 nm for TO-PRO-3. Fluorescence images were collected with emission wavelengths at 500–530 nm for Alexa 488, at 555–585 nm for Cy3, and at >650 nm for TO-PRO-3. To analyze immunofluorescence images in three dimensions, optical z-sections of the samples were collected at 1-µm steps using the confocal microscope (60× magnification, NA=1.4), and the multiple focal-plane confocal images were reconstituted with the FV10-ASW software (Olympus) (Supplementary Movie 1).

A structured illumination microscope (N-SIM; Nikon, Tokyo, Japan) equipped with laser excitation lines at 488, 561 and 640 nm and an oil-immersion objective lens (CFI Apo TIRF 100×, NA=1.49; Nikon) were also used to examine samples immunohistochemically stained with CD41, Ly-6G, and TO-PRO-3 iodide with high spatial resolution. N-SIM analysis software (Nikon) was used to construct fluorescence images. Optical z-sections of the samples were acquired at 0.1-µm steps using the structured illumination microscope, and the multiple focal-plane images were reconstructed with the software (Supplementary Movie 2).

In-vitro time-lapse imaging

Seven- to nine-week-old male C57BL/6 mice and seven- to nine-week-old male C57BL/6-green fluorescent protein (GFP) mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan) and RIKEN (Saitama, Japan), respectively. The two groups of five mice were sensitized

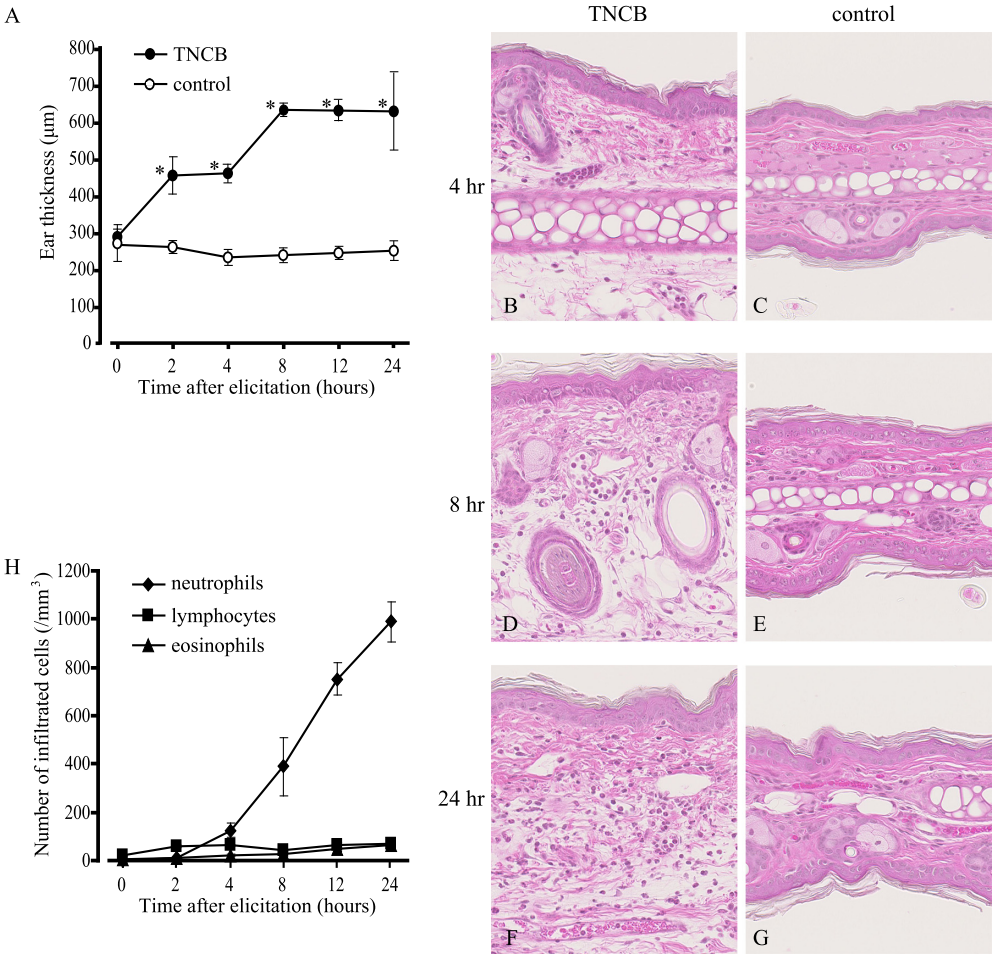

Fig. 1. Murine model of 2,4,6-trinitro-1-chlorobenzene (TNCB)-induced dermatitis. (**A**) Changes in external ear thickness after elicitation with TNCB. Values represent the mean±standard deviation (SD) (vertical bars). *P<0.01 versus mice injected with vehicle (control). (**B–G**) Histologic examination of ear skin sections. H&E-stained TNCB-treated right ear skin sections (left panels) and the control left ear skins (right panels) at indicated time points after elicitation are shown. (**H**) The number of infiltrating inflammatory cells in the dermis per mm^3. Values represent the mean±SD (vertical bars).

with 1% TNCB. Seven days after sensitization, they were elicited with 1% TNCB. At 24 hr post-elicitation, whole blood samples were collected from C57BL/6-GFP mice and C57BL/6 mice to separate GFP-labeled platelets and phycoerythrin (PE)-labeled neutrophils by using anti-Gr-1-PE antibody in MACS separation system (Miltenyi Biotec, Bergisch Gladbach, Germany). After the separation, GFP-labeled platelets and PE-labeled neutrophils were co-incubated in Dulbecco's modified Eagle's medium supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 30 mM HEPES, 0.05 mM β-mercaptoethanol and 10% bovine growth serum in a humidified incubator containing 5% CO_2 at 37°C. The incubated cells were chronologically examined with the confocal scanning microscope. Fluorescence images were collected with emission wavelengths at 500–530 nm for GFP and at 555–655 nm for PE.

Statistical analysis

Statistical analysis was performed with commercial software (Microsoft Office Excel; Microsoft Corp., Redmond, WA). Unpaired Student's t test was used to assess the difference.

III. Results

TNCB-induced dermatitis model

In our murine model of dermatitis, a marked increase in ear thickness was observed in the 1% TNCB-treated right ears, while no increase in ear thickness was found in the left control ears (Fig. 1A). Histologic examination revealed dermal edema but not hemorrhage at 4–24 hr post-elicitation in the TNCB-treated right ears (Fig. 1B, 1D, and 1F). Many neutrophils aggregated in vessels at 4 hr post-elicitation in the right ears (Fig. 1B). As time passed, the number of infiltrating neutrophils outside vessels was markedly increased, while that of infiltrating lymphocytes and eosinophils was not apparently changed (Fig. 1D, 1F,

and 1H). In the control left ears, no significant histological change was observed (Fig. 1C, 1E, and 1G).

Changes of platelet distribution in dermatitis

To reveal the distribution of platelets in inflamed dermis, we immunohistochemically stained sections of TNCB-treated ears with platelet marker CD41 and endothelial cell marker CD31. At 2 hr post-elicitation (Fig. 2A1–2A3), CD41-positive (red) platelets started to adhere to CD31-positive (green) endothelial cells within the vessels. At 4 hr post-elicitation (Fig. 2B1–2B3), CD31-positive vessels filled with aggregates of CD41-positive platelets and TO-PRO-3-positive (blue) nucleated cells were observed. At 8 hr post-elicitation (Fig. 2C1–2C3), fragmented CD41-positive platelets surrounding TO-PRO-3-positive cellular nuclei were observed within and without vessels (arrows and arrowheads in Fig. 2C3). At 24 hr post-elicitation (Fig. 2D1–2D3 and Supplementary Movie 1), the number of infiltrating TO-PRO-3-positive nucleated cells outside vessels surrounded by fragmented CD41-positive platelets (arrowheads in Fig. 2D3) was increased. On the other hand, there was no apparent change of platelet distribution over the entire monitoring period in the left control ears (data not shown). As shown in Figure 2E, TO-PRO-3-positive cellular nuclei surrounded by shattered CD41-positive platelets outside vessels appeared from 8 hr after elicitation, and the number of such cells increased as time passed. In contrast, no disc-shaped platelets were found outside vessels during the experiment (Fig. 2A3, 2B3, 2C3, and 2D3).

To clarify the nuclei of which types of cells were surrounded by the fragmented platelets, immunohistochemistry for CD41, Ly-6G, and TO-PRO-3 iodide was performed. N-SIM super-resolution microscopic analysis revealed that Ly-6G-positive (green) multinuclear neutrophils internalized fragmented CD41-positive (red) platelets at 24 hr post-elicitation (Fig. 2F and Supplementary Movie 2). Confocal microscopic analysis showed that CD3-positive T cells did not internalize CD41-positive platelets at 24 hr post-elicitation (Supplementary Fig. 1).

Activation markers of platelets and neutrophils

To elucidate whether platelets and neutrophils are in an activated state or not in inflamed ears, immunohistochemistry for CD62P, PF4, and MPO was performed (Fig. 3). At 4 hr post-elicitation, CD62P and PF4, activation markers of platelets, were expressed on platelet aggregates (Fig. 3A and 3C), whereas they were not expressed on internalized fragmented CD41-positive platelets 24 hr after the elicitation (arrows in Fig. 3B and 3D). MPO expression was markedly observed at 4 hr post-elicitation (Fig. 3E). However, MPO expression in cells surrounded by shattered CD41-positive platelets at 24 hr post-elicitation was reduced or absent (arrows in Fig. 3F).

In-vitro time-lapse imaging analysis

In-vitro imaging analysis was performed to confirm whether aggregates of platelets and neutrophils were formed in this murine model of dermatitis. Several minutes after incubation, platelets adhered to neutrophils and aggregations of platelets and neutrophils were formed (Fig. 4). However, platelets phagocytized by neutrophils were not observed in these *in-vitro* experiments.

IV. Discussion

In this study, we examined the spatiotemporal localization of platelets and neutrophils in the dermis of mouse dermatitis. As far as we know, this is the first report on neutrophil phagocytosis of platelets in dermatitis. Clots of activated platelets inside vessels adhered to endothelial cells in the early phase of TNCB-induced dermatitis, and platelet aggregates were subsequently phagocytosed and cleared by neutrophils. Disc-shaped CD41-positive platelets having the form of platelet–neutrophil aggregates were not found outside vessels at any time during the experiment (Fig. 5).

Recent studies demonstrated that circulating platelets and leukocytes interact productively, and the formation of heterotypic aggregates of platelets and leukocytes is a feature of acute coronary syndromes and systemic inflammatory diseases [7]. The formation of the heterotypic aggregates was also observed in our model of dermatitis (Figs. 2B3 and 4). Neutrophils are speculated to possess a phagocytosing ability [7]. In our mouse model of dermatitis, neutrophilic ingestion of platelets was revealed after the formation of aggregates of platelets and neutrophils by super resolution microscopy (Fig. 2F and Supplementary Movie 2). Phagocytosis of platelets by neutrophils in circulating blood has been observed in acute myocardial infarction, polycythemia vera, and essential thrombocythemia [8, 9]. These previous reports by Maugeri *et al.* were based solely on the results of circulating blood analysis. Our results based on tissue analysis seemed to be consistent with the previous studies and also provide additional data.

In this model of dermatitis, platelets began to be phagocytosed by neutrophils 4–8 hr after the elicitation, and the number of extravasated neutrophils internalizing fractured platelets was increased 8 hours after the elicitation. Activation markers of platelets, CD62P and PF4, were not expressed on internalized platelets, and MPO expression was markedly decreased 24 hr after the elicitation in phagocytosing cells (Fig. 3). MPO is reported to be rapidly released by activated polymorphonuclear neutrophils, and it is decreased or absent when neutrophils phagocytize activated platelets [6, 8], although some additional factors, such as transforming growth factor β signaling, may partially influence the MPO expression in neutrophils [10, 14, 18]. Platelet phagocytosis by activated neutrophils could be regarded as clearance of aggregated platelets in the vessels and may act in an inflammation-suppressing manner in inflamed dermis.

We should remark that in our study, macroscopic and microscopic observation showed no apparent hemorrhage

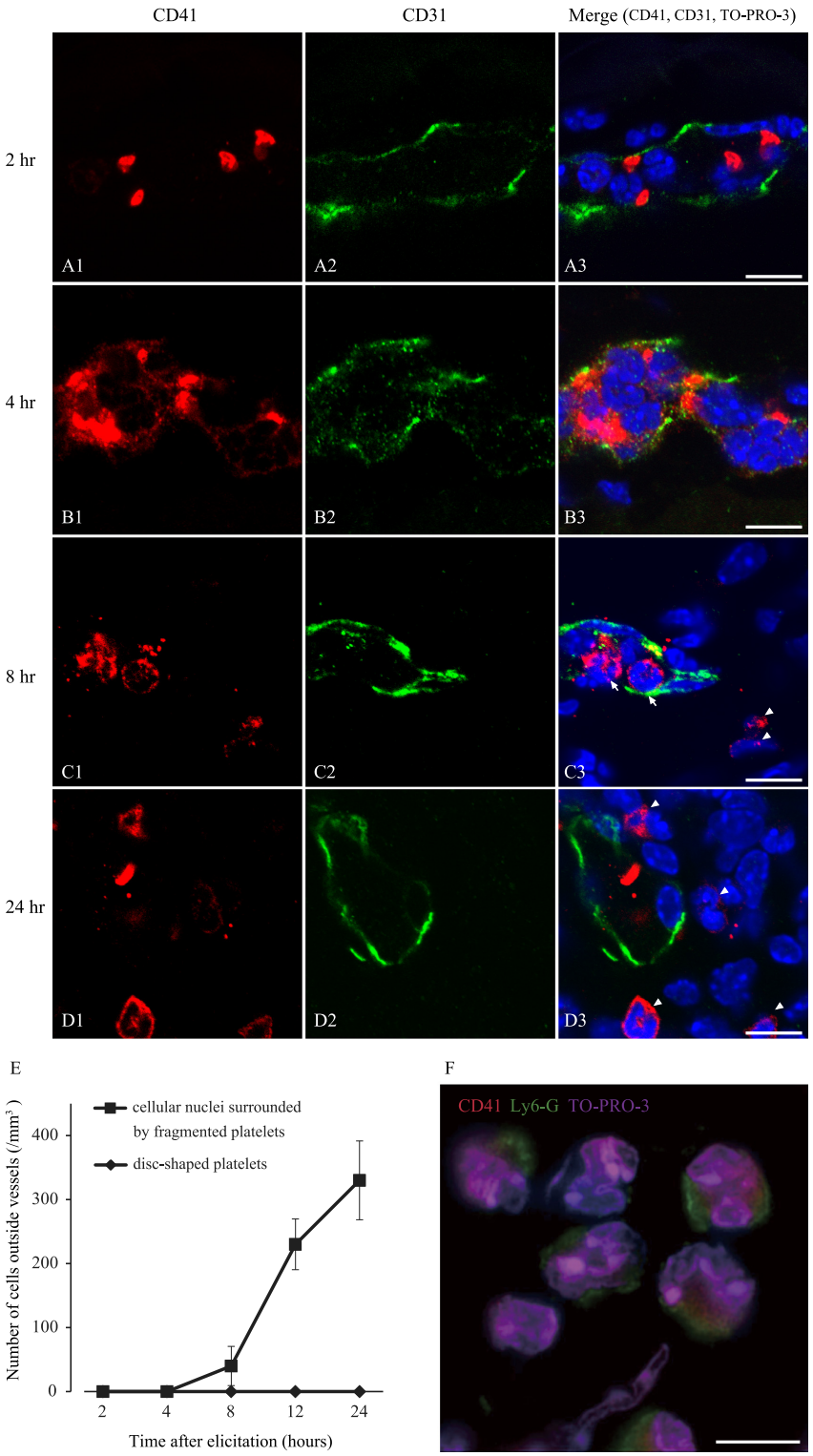

Fig. 2. Imaging analysis of TNCB-treated ear skin sections stained with CD41, CD31, Ly-6G, and TO-PRO-3. (**A, B, C, D**) Confocal microscopic images were acquired at indicated time points after the elicitation. Platelet aggregates adhered to CD31-positive cells within the vessels 4 hr after elicitation. At 8 hr post-elicitation, cellular nuclei surrounded by fragmented CD41-positive platelets were observed inside vessels (arrows in **C3**) and outside vessels (arrowheads in **C3**). At 24 hr post-elicitation, the number of nuclei of infiltrating cells outside vessels surrounded by shattered CD41-positive platelets (arrowheads in **D3**) is increased. Red: CD41 for platelet marker (excitation: 543 nm, emission: 555–585 nm); Green: CD31 for endothelial marker (excitation: 488 nm, emission: 500–530 nm); Blue: TO-PRO-3 for nuclear marker (excitation: 633 nm, emission: >650 nm). Bar=10 μm. (**E**) The number of disc-shaped platelets outside vessels and that of cellular nuclei surrounded by fragmented CD41-positive platelets outside vessels. Values represent the mean±SD (n=5). (**F**) Structured illumination microscopic analysis of TNCB-treated ear skin sections stained with Ly-6G, CD41, and TO-PRO-3 at 24 hr after elicitation. Green: Ly-6G for neutrophil marker (excitation: 488 nm); Red: CD41 (excitation: 561 nm); Violet: TO-PRO-3 (excitation: 640 nm). Bar=5 μm.

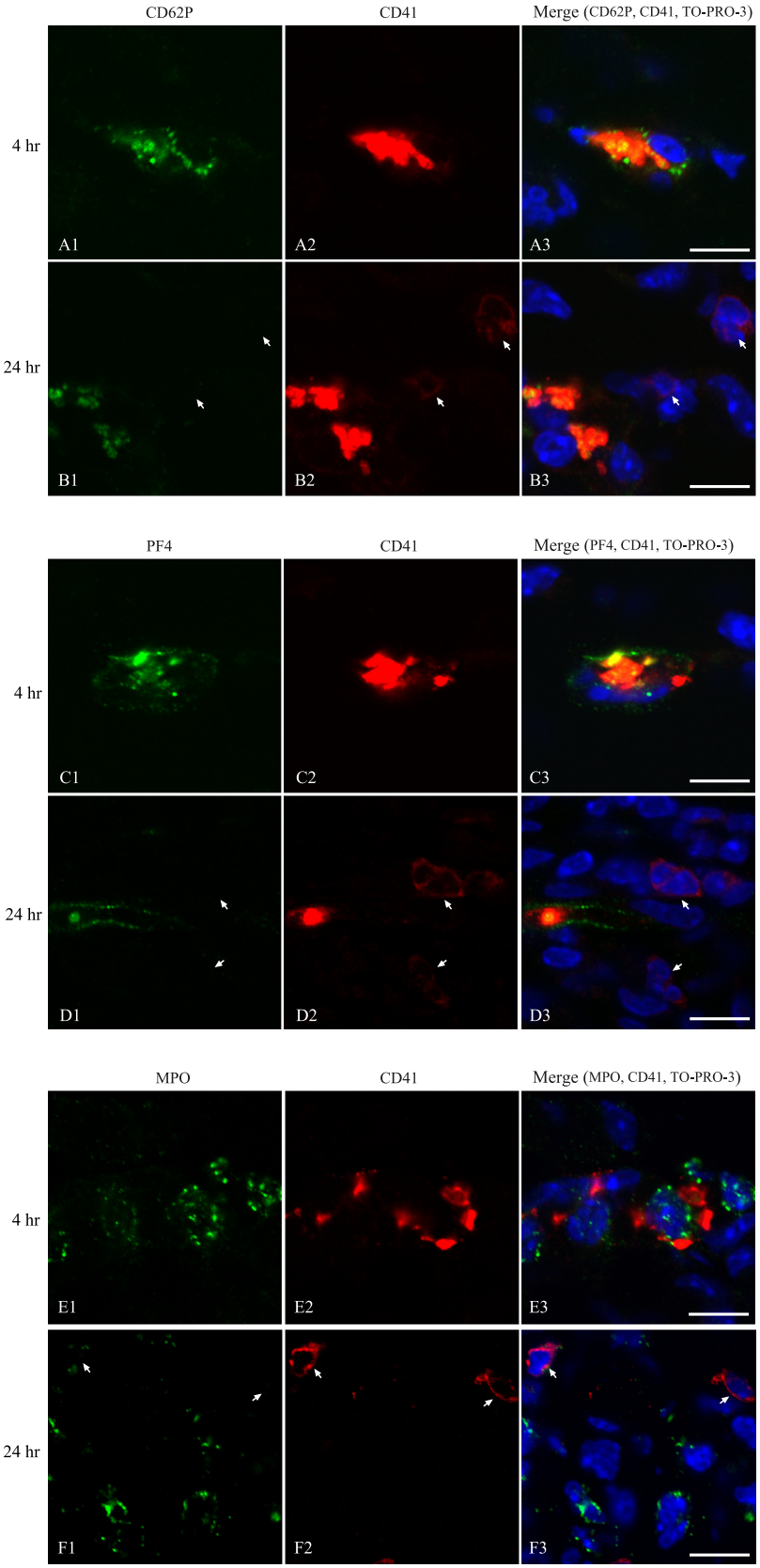

Fig. 3. Confocal images of TNCB-treated ear skin sections stained with CD62P, CD41, and TO-PRO-3, platelet factor 4 (PF4), and myeloperoxidase (MPO). The specimens analyzed were acquired at indicated time points after the elicitation. Note the phagocytosed and fragmented platelets express neither CD62P (arrows in **B**) nor PF4 (arrows in **D**) at 24 hr post-elicitation, whereas MPO expression is reduced or absent in neutrophils phagocytosing platelets. Green: CD62P (**A1** and **B1**), PF4 (**C1** and **D1**), and MPO (**E1** and **F1**) (excitation: 488 nm, emission: 500–530 nm); Red: CD41 (excitation: 543 nm, emission: 555–585 nm); Blue: TO-PRO-3 (excitation: 633 nm, emission: >650 nm). Bar=10 μm.

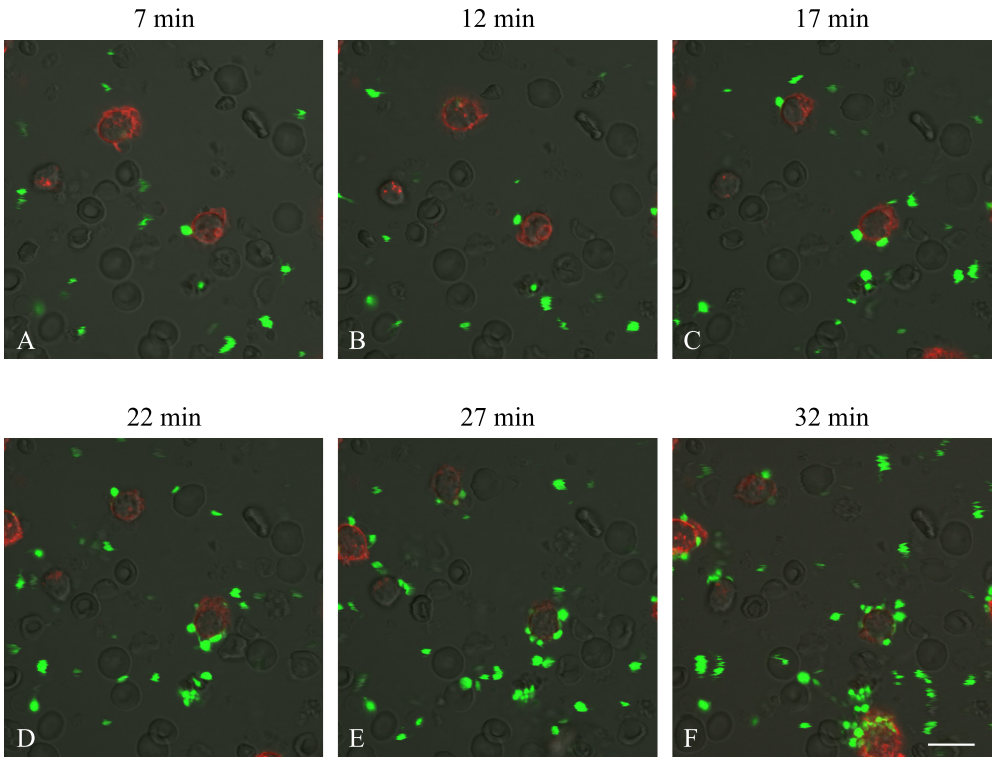

Fig. 4. *In-vitro* time-lapse imaging of Green fluorescent protein (GFP)-labeled platelets co-cultured with phycoerythrin (PE)-labeled neutrophils. Confocal images were acquired at indicated time points after the co-culture. Green: GFP (excitation: 488 nm, emission: 500–530 nm); Red: PE (excitation: 543 nm, emission: 555–655 nm). Bar=10 μm.

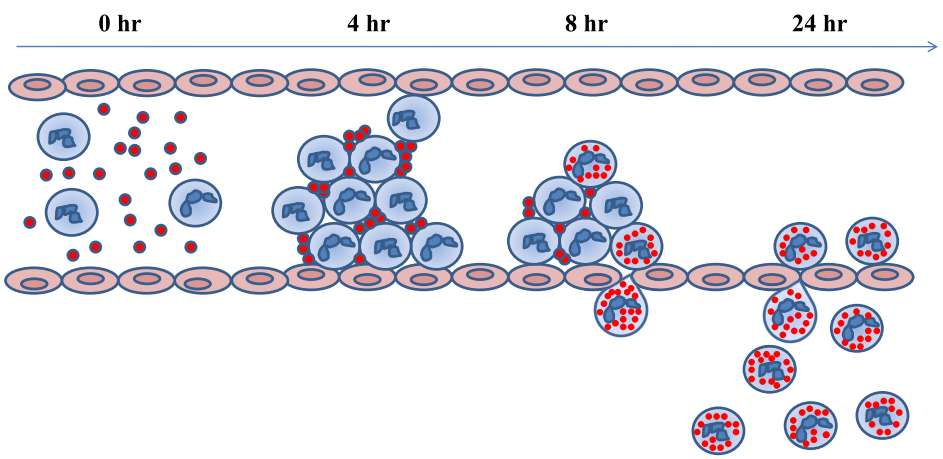

Fig. 5. Schematic representation of the spatiotemporal distribution of platelets and neutrophils inside and outside vessels of TNCB-treated ear dermis.

on TNCB-treated ears. Hemorrhage due to scratching behavior often results in exacerbating dermatitis. We have previously shown that increased frequency of scratching behavior seen in the mouse chronic dermatitis model significantly exacerbated dermatitis [4, 19]. In the case of dermatitis accompanied by hemorrhage, spatiotemporal distribution of platelets and neutrophils would change, although further studies are needed to examine this question.

In our *in-vitro* imaging experiment, heterotypic aggregates of neutrophils and platelets were observed, whereas phagocytosis of activated platelets by neutrophils was not evident (Fig. 4). Some additional factors are apparently required for the reproduction of phagocytosis in *in-vitro* experiments. Further examinations are required for the purpose of determining what those factors are.

In conclusion, we immunohistochemically analyzed the kinetics of platelets and neutrophils in the murine model of TNCB-induced dermatitis. Although further studies including *in-vivo* imaging studies are needed for revealing underlying mechanisms of the platelet-neutrophil-interactions, our results showed that platelet clots adhered to endothelial cells in the vessels and they were phagocytized by neutrophils during extravasation,

suggesting the possible pathophysiology involved in the removal process of activated platelet aggregates in inflamed sites of dermatitis.

V. Conflict of Interest

The authors have no conflict of interest to declare.

VI. Acknowledgments

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