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# CXCL1-Triggered PAD4 Cytoplasmic Translocation Enhances Neutrophil Adhesion through Citrullination of PDIA1

Jiro Aoyama[1, 2], Mizuko Osaka[1, 3], Michiyo Deushi[1], Shoichi Hosoya[4], Akihito Ishigami[5], Taketoshi Maehara[2] and Masayuki Yoshida[1]

[1]Department of Life Science and Bioethics, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan
[2]Department of Neurosurgery, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan
[3]Department of Nutrition and Metabolism in Cardiovascular Disease, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan
[4]Research Core, Research Facility Cluster, Institute of Research, Tokyo Medical and Dental University, Tokyo, Japan
[5]Research Team for Functional Biogerontology, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan

**Aims:** Vascular inflammation is critical for the development and progression of atherosclerosis. Previously, we reported that neutrophils adhere to the vascular endothelium in low-density lipoprotein receptor null mice fed a high-fat diet through hypercitrullination of histone H3 by peptidylarginine deiminase 4 (PAD4) in neutrophils. However, the involvement of PAD4 and citrullination of proteins other than histone H3 in neutrophil adhesion is not well known. In this study, we investigated the function of PAD4 and identified citrullinated proteins during vascular inflammation.

**Methods:** We pefformed flow assay under physiological flow conditions using differentiated HL-60 (dHL-60) cells stimulated with CXCL1 and human umbilical vein endothelial cells (HUVECs). Furthermore, phalloidin stain for dHL-60 stimulated with CXCL1 to observe F-actin polymerization and immunohistochemistry for the activated $\beta$2-integrin was conducted. To identify a target of citrullination in the cytoplasm of dHL-60 cells, liquid chromatography-mass spectrometry (LC-MS/MS) for dHL-60 stimulated with CXCL1 was performed.

**Results:** Inhibition or knockdown of PAD4 significantly decreased adhesion of under physiological flow conditions. Thr-Asp-F-amidine trifluoroacetate salt (TDFA), a PAD4 inhibitor, inhibited cytoplasmic translocation of PAD4 by CXCL1. TDFA or knockdown of PAD4 significantly decreased expression of $\beta$ 2-integrin and F-actin polymerization activated by CXCL1. Moreover, LC-MS/MS identified protein disulfide isomerase A1 (PDIA1) as a target of citrullination in the cytoplasm of dHL-60 cells. Knockdown of PDIA1 significantly decreased adhesion of dHL-60 cells to HUVECs, expression of $\beta$2-integrin, and F-actin polymerization.

**Conclusions:** Cytoplasmic translocation of PAD4 by CXCL1 induces neutrophil adhesion to vascular endothelial cells and citrullination of PDIA1.

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**Key words:** Vascular inflammation, PAD4, Neutrophil, Citrullination

**Abbreviations:** HFD: high-fat diet, LDL: low-density lipoprotein, PAD4: peptidylarginine deiminase 4, NES: nuclear transport signal, PDIA1: protein disulfide isomerase A1, dHL-60 cells: differentiated HL-60 cells, HUVECs: Human umbilical vein endothelial cells, TDFA: Thr-Asp-F-amidine trifluoroacetate salt, LC-MS/MS: liquid chromatography-mass spectrometry

## Introduction

Vascular inflammation is crucial in the early stages of atherosclerosis, which develops upon leukocyte adhesion to vascular endothelial cells. In our earlier studies, we found that a high-fat diet (HFD)

Address for correspondence: Masayuki Yoshida, Department of Life Sciences and Bioethics, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail: masa.vasc@tmd.ac.jp

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that triggers atherosclerosis also induces neutrophil adhesion[1]. Moreover, we reported that neutrophil adhesion in low-density lipoprotein receptor null (LDLR–/–) mice fed a HFD was enhanced by the upregulation of CXCL1 in the blood[2]. Neutrophil adhesion progresses through the activation of integrins, which undergo a conformational change from closed to open structure[3]. This change is dependent on chemokines followed by actin polymerization[4]. Therefore, CXCL1 may activate integrins such as $\beta$2-integrin on neutrophils.

Neutrophils are the most abundant immune cells and are crucial to the defense against pathogenic microorganisms. These cells release neutrophil extracellular traps and perform phagocytosis[5]. Peptidylarginine deiminase 4 (PAD4) activity leads to the formation of these extracellular traps and decondensation of chromatin structure through histone citrullination[4]. Our recent report also demonstrated that hypercitrullination of histone H3 in neutrophils via CXCL1 enhanced neutrophil adhesion in the femoral artery of LDLR–/– mice fed HFD. This enhancement involved PAD4, suggesting that this enzyme acts in neutrophil adhesion[2].

Citrullination is induced by the conversion of an arginine residue to citrulline by PAD[6, 7]. PAD4 is specifically expressed in neutrophils[8, 9]. These enzymes translocate from the nucleus to the cytoplasm via a nuclear export signal (NES) and citrullinate proteins other than histones[10]. Non-histone targets of PAD include glycogen synthase 3b in breast cancer cells, NF-$\kappa$B essential modulator in renal tubular cells, inhibitor of growth 4, and a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 [11-14]. However, citrullinated proteins other than histones involved in neutrophil adhesion are unknown. Hence, we hypothesized that citrullination of cytoplasmic proteins related to integrin activation by translocation of PAD4 are involved in neutrophil adhesion to vascular endothelial cells.

In this study, we found that CXCL1 induces cytoplasmic translocation of PAD4 and that protein disulfide isomerase A1 (PDIA1) is subsequently citrullinated by this enzyme. This process is integral to neutrophil adhesion. Thus, the translocation of PAD4 to cytoplasm is important for vascular inflammation.

## Materials and Methods

### Cell Culture

Human umbilical vein endothelial cells (HUVECs; LONZA, Walkersville, MD, USA) were purchased and cultured as previously described[15, 16]. Briefly, HUVECs were cultured in RPMI-1640 medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) with 20% fetal bovine serum (FBS), 10 ng/mL human fibroblast growth factor (R&D Systems, Minneapolis, MN, USA), 5 units/mL novoheparin (Mochida, Tokyo, Japan), and 50 units/mL penicillin and streptomycin (Gibco, Life Technologies Japan, Tokyo, Japan). Cells were incubated at 37℃ in an atmosphere containing 5% $CO_2$. HUVECs at passages 3 or 4 were used in all experiments. Human promyelocytic leukemia cells (HL-60; ATCC, USA) were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL penicillin, 0.17 mmol/L streptomycin, and 2 mmol/L L-glutamine. HL-60 cells were cultured in complete medium with 1.3% dimethyl sulfoxide (FUJIFILM Wako Pure Chemical Corporation) for 5 days to induce differentiation into neutrophil-like cells[17]. Differentiated HL-60 cells (dHL-60 cells) were stimulated with 10 nM of recombinant human CXCL1 (BioLegend, San Diego, CA, USA) in the presence or absence of 10 nM Thr-Asp-F-amidine trifluoroacetate salt (TDFA; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for flow assays, liquid chromatography-mass spectrometry (LC-MS/MS), and immunohistochemistry.

### Neutrophil Isolation

The research was approved by the ethics committee of the Tokyo Medical and Dental University (M2015-568). Ten milliliters of blood was collected from healthy donors in EDTA tubes, and 2% Dextran T500 (PHARMACOSMOS, Holbeak, Denmark) solution was added for 20 min. The buffy coat was then collected after centrifugation at 1200 rpm for 10 min. The buffy coat suspension containing leukocytes was gently layered onto 4 mL of Histopaque-1077 (Sigma-Aldrich, Merck KGaA) and centrifuged at 1500 rpm for 25 min. The lower layer was collected and 3 mL of water was added for hemolysis. Human neutrophils stimulated with 10 nM of human recombinant CXCL1 were used in the flow assay in the presence or absence of 10 nM TDFA or 3.5 μM KSC-34.

### Flow Assay

HUVEC monolayers on coverslips were stimulated with 48 units/mL of recombinant human IL-1$\beta$ (BioLegend, San Diego, CA, USA) for 4 h, and set into a flow chamber mounted on an inverted microscope (TX-10; Nikon, Tokyo, Japan). Isolated human neutrophils or dHL-60 cells transfected with siRNA ($1 \times 10^6$/mL) were stimulated with 10 nM of recombinant human CXCL1 (GRO-$\alpha$; BioLegend,

San Diego, USA) for 2 h. These cells were perfused onto HUVEC monolayers with a syringe pump (PHD2000, Harvard Apparatus Inc., Holliston, MA) for 5 min under a shear stress of 1.0 dyne/cm$^2$. The interaction of cells with HUVECs in the monolayers was recorded on PC. These images were analyzed to determine the number of adherent and rolling cells in about eight randomly selected ×20 microscope fields[18].

### Immunohistochemistry for PAD4 or Activated-β2-Integrin

dHL-60 cells ($4\times10^5$ cells) were seeded onto L-lysin-coated coverslips and reacted with anti-PAD4 antibody (kindly gifted from Dr. Ishigami)[19] or anti-activated-β2-integrin antibody, KIM 127 (Manassas, VA, USA). After staining with goat anti-mouse IgG (H&L) cross-absorbed, Alexa Fluor 488 (Thermo Scientific, PA, USA) and 4',6-diamidino-2-phenylindole (DAPI; FujiFilm Wako Junyaku, Japan), images of stained cells were captured by confocal laser scanning microscopy (THUNDER; Leica, Wetzlar, Germany.)

### LC-MS/MS

Trypsin-digested peptide samples were analyzed using a combination of mass spectrometry (LTQ Orbitrap Velos; Thermo Scientific) and nano-LC (EASY-nLC II; Thermo Scientific). Peptides were desalted and concentrated on a trap column (Capillary Ex-Nano Mono cap C18 Trap Column, 0.075 mm i.d.×50 mm; GL Sciences) and then transferred to an analytical column (Capillary Ex-Nano Mono cap C18 Nano-flow, 0.075 mm i.d.×150 mm; GL Sciences). For LC conditions, the following gradient was used : A, 0.1% formic acid/$H_2O$; B, 70% MeCN, 0.1% formic acid in $H_2O$; 0 min, 0% B; 0–2 min, 0%–29% B; 2–42 min, 29%–85% B; 42–47 min, 85%–100% B; 47–52 min, 100% B; flow rate 300 nL/min.

### siRNA Transfection

dHL-60 cells ($4\times10^5$/mL) in RPMI with 20% FBS in 6-well plates were transfected with duplexes of small interfering RNA (siRNA) using GenomONE-Si (Ishihara Sangyo, Japan) following the manufacturer's instructions for transient silencing of PAD4 or PDIA1. Mixtures containing each siRNA were added and cells were incubated for 24 h. The target sequences for transient silencing were 5'-GGACGGUCAUUGAUUACAAtt-3' for siPDIA1#1, 5'-UUGUAAUCAAUGACCGUCCtg-3' for siPDIA1#2, 5'-AGCUCAAAGAGUUCUCUAAtt-3' for siPADI4#1 (Nippon Gene, Toyama, Japan), and 5'-CAGAGACAAUCUCGAAUCUtt-3' for siPADI4#2 (Thermo Scientific). The universal negative control siRNA (Nippon Gene) was used as non-targeting siRNA.

### Western Blotting

dHL-60 cells were lysed in RIPA buffer with protease inhibitors (Roche, Penzberg, Germany), and the protein concentration was determined using DC$^{TM}$ Protein Assays (Bio-Rad, Hercules, CA, USA). Ten micrograms of each lysate was applied to 10% polyacrylamide gels. After electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes (GE Healthcare UK Ltd., Buckinghamshire, UK). The membranes were incubated with anti-human PDIA1 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-GAPDH (Santa Cruz Biotechnology, Inc.), or anti-human PAD4 antibodies followed by anti-mouse or anti-rabbit secondary antibody (GE Healthcare UK Ltd.). The proteins were visualized using EzWestLumi plus reagent (ATTO Corporation, Tokyo, Japan). Signals were detected using LAS-1000 (FUJIFILM Wako Pure Chemical Corporation).

### Phalloidin Staining

dHL-60 cells ($1\times10^6$ cells) were seeded onto 0.001% L-lysin-coated coverslips (Sigma-Aldrich, Merck KGaA) and stimulated with 10 nM CXCL1 in the presence or absence of 10 nM TDFA for 2 h. The cells were stained with phalloidin-Alexa Fluor 488 (Abcam, Cambridge, UK) for 50 min followed by with DAPI for 20 min. Cells were then fixed in 2% paraformaldehyde for 15 min. Images were captured using a confocal laser scanning microscope (THUNDER; Leica, Wetzlar, Germany).

### Statistical Analysis

Data are expressed as means ± standard deviation (SD). One-way analysis of variance with Tukey's post hoc test or two-tailed unpaired *t*-test was used to estimate statistical significance. A *p*-value of $<0.05$ was considered statistically significant. Prism software version 5 (GraphPad Prism Software Inc, La Jolla, CA, USA) was used for all statistical analyses.

## Results

### CXCL1 Stimulation Induced Adhesion of dHL-60 and Neutrophils to HUVECs

We performed *in vitro* flow assays under laminal flow using neutrophil-like dHL-60 cells. CXCL1 significantly increased the adhesion of dHL-60 cells to HUVECs ($p<0.05$, **Fig. 1A**), though it did not change the number of rolling cells. Likewise, CXCL1 also significantly increased the adhesion of human

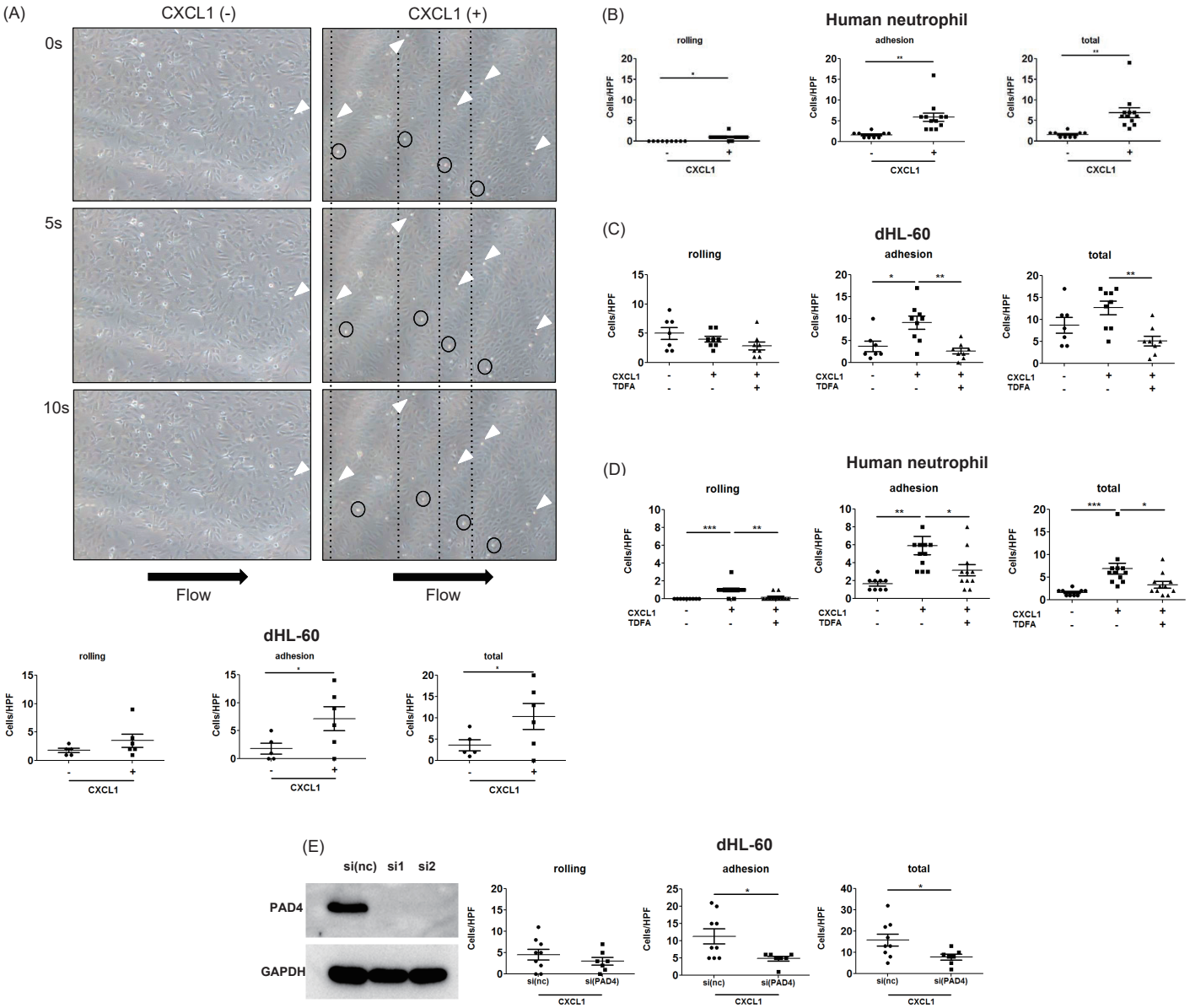


**Fig. 1.** Knockdown of PAD4 decreased neutrophil adhesion to HUVECs

HUVECs seeded on coverslips coated with fibronectin were stimulated by 48 units/mL of IL-1$\beta$, and adhesion to HUVECs was observed under physiological flow. (A) dHL-60 cells were stimulated by 10 nM CXCL1. White arrowheads indicate adhesion cells and opened circles indicate rolling cells (upper panel). The number of total recruitment dHL-60 cells and adhered cells were significantly increased by CXCL1 stimulation. The randomly selected ×20 microscope fields for analysis were five or six. (B) Human neutrophils isolated from peripheral blood were stimulated with 10 nM CXCL1. The number of total recruitment cells, rolling cells, and adhered cells were significantly increased by CXCL1 stimulation. The randomly selected ×20 microscope fields for analysis were nine or twelve. (C) dHL-60 cells were stimulated by 10 nM CXCL1 with or without 10 nM TDFA. The adhesion induced by CXCL1 significantly decreased in the presence of TDFA. The randomly selected ×20 microscope fields for analysis were seven or eight. (D) Isolated peripheral human neutrophils were stimulated by 10 nM CXCL1 with or without 10 nM TDFA. The number of total recruitment cells, rolling cells, and adhered cells were significantly decreased by TDFA. The randomly selected ×20 microscope fields for analysis were nine or eleven. (E) siRNA for PAD4 were diminished the expression of PAD4 in dHL-60 (left images). The number of total recruitment cells, rolling cells, and adhered cells were significantly decreased by knockdown of PAD4. The randomly selected ×20 microscope fields for analysis were seven or nine. Data are representative of three similar experiments and are presented as mean ± SD. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ by one-way ANOVA with Tukey's test or unpaired two-tailed Student's *t*-test. HPF indicates high-power field (×20 microscope field).

peripheral neutrophils ($p<0.01$) and rolling cells ($p<0.05$) **(Fig. 1B)**. These results suggest that stimulation by CXCL1 induces neutrophil adhesion to vascular endothelial cells.

## Inhibition of PAD4 Activity or Expression Decreased Neutrophil Adhesion to HUVECs

We investigated the involvement of PAD4 in neutrophil adhesion by *in vitro* flow assays using TDFA or knockdown of PAD4 by siRNA. TDFA, which specifically inhibits PAD4 activity, significantly decreased the adhesion of dHL-60 cells stimulated with CXCL1 to HUVECs ($p<0.01$) **(Fig. 1C)**. Similarly, TDFA also significantly reduced adhesion and rolling of human peripheral neutrophils to HUVECs (adhesion: $p<0.05$; rolling: $p<0.05$) **(Fig. 1D)**. Moreover, knockdown of *PAD4* in dHL-60 cells by siRNA significantly decreased adhesion ($p<0.05$) **(Fig. 1E, Supplemetary Fig. 1A)**. Thus, these results showed that inhibition of either PAD4 activity or expression decreases neutrophil adhesion. Moreover, translocation of PAD4 to the cytoplasm is likely involved in neutrophil adhesion.

## PAD4 Translocation from the Nucleus to the Cytoplasm is Induced by CXCL1

We previously demonstrated that CXCL1 induced citrullination of protein in a PAD4-dependent manner[2]. Furthermore, inflammation caused by renal ischemia and reperfusion also induced PAD4 translocation from nuclei to cytoplasm[10]. Therefore, we hypothesized that citrullination of other cytoplasmic proteins induced by translocation of PAD4 from the nucleus to the cytoplasm by CXCL1 is involved in neutrophil adhesion. CXCL1 induced translocation of PAD4 to the cytoplasm **(Fig. 2)**. Furthermore, TDFA inhibited PAD4 translocation **(Fig. 2)**. These results suggested that PAD4 functions as a catalyst for the citrullination of some proteins not only in the nucleus but also in the cytoplasm.

## Inhibition of PAD4 Activity and Expression Decreased β2-Integrin and F-Actin Polymerization

We assessed the expression of activated β 2-integrin in dHL-60 cells stimulated with CXCL1 in the presence or absence of TDFA using an antibody against activated β2-integrin. The number of activated-β2-integrin-positive cells was significantly increased upon CXCL1 stimulation ($p<0.001$) **(Fig. 3A)**. Moreover, TDFA significantly decreased the number of activated-β2-integrin-positive cells by CXCL1 stimulation ($p<0.001$) **(Fig. 3A)**. Similarly, knockdown of *PAD4* by siRNA significantly decreased the cells compared with control siRNA ($p<0.05$) **(Fig. 3B)**.

We also tested F-actin polymerization in dHL-60 cells stimulated with CXCL1 in the presence of TDFA or siRNA for *PAD4* using phalloidin staining to further confirm these findings. CXCL1 significantly increased the number of F-actin-positive dHL-60 cells ($p<0.001$) **(Fig. 3C)**. Consistently, TDFA significantly decreased the number of F-actin-positive cells ($p<0.001$) **(Fig. 3C)**, as did siRNA for *PAD4* ($p<0.05$) **(Fig. 3D)**. These results suggested that PAD4 is involved in activation of β2-integrin.

## PDIA1 is Citrullinated by CXCL1 Stimulation

We used LC-MS/MS for dHL-60 cells with or without CXCL1 stimulation. LC-MS/MS identified two citrullinated proteins without CXCL1 stimulation and three citrullinated proteins with CXCL1 stimulation **(Table 1)**. Comparing these two conditions, we focused on PDIA1 as a specific citrullinated protein targeted by CXCL1 stimulation. TDFA led to a reduction in PDIA1 citrullination upon CXCL1 exposure **(Table 1)**. These results suggest that PDIA1 was citrullinated by translocation of PAD4 to the cytoplasm.

## Inhibition of PDIA1 Activity and Expression Decreased Neutrophil Adhesion to HUVECs

We used *in vitro* flow assays with the PDIA1 activity inhibitor KSC-34 or using siRNA to knockdown *PDIA1*. KSC-34 significantly decreased adhesion of dHL-60 cells and human neutrophils to HUVECs (dHL-60 cells, $p<0.001$; human neutrophils, $p<0.001$) **(Fig. 4A, B)**. Knockdown of *PDIA1* in dHL-60 cells by siRNA also significantly decreased this adhesion ($p<0.05$) **(Fig. 4C, Suppremental Fig 1B)**. Thus, these results show that inhibition of PDIA1 activity and expression decreased neutrophil adhesion.

## Inhibition of PDIA1 Activity and Expression Decreased β2-Integrin and F-Actin Polymerization

We monitored the expression of activated β 2-integrin in dHL-60 cells stimulated with CXCL1 in the presence or absence of siRNA for *PDIA1*. Knockdown of PDIA1 significantly decreased the number of activated-β2-integrin-positive dHL-60 cells compared with those treated with the control siRNA ($p<0.01$) **(Fig. 4D)**. siRNA for PDIA1 also significantly decreased F-actin-positive cells under CXCL1 stimulation ($p<0.01$) **(Fig. 4E)**. These results suggest that inhibition of PDIA1 reduced the activation of β2-integrin.

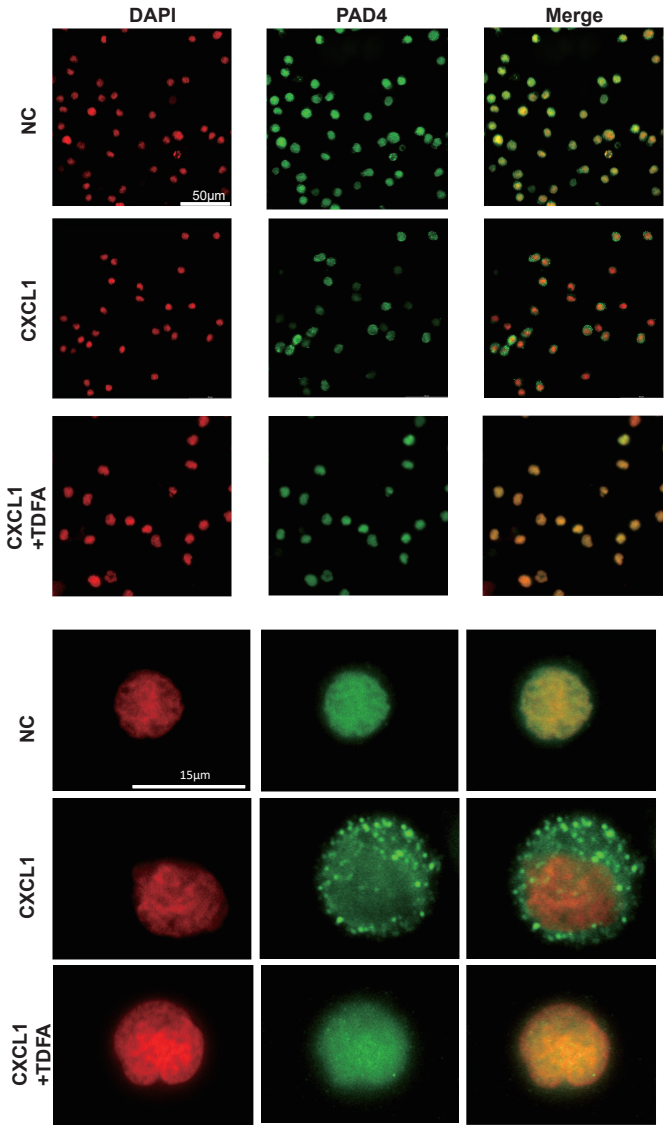


**Fig. 2.** CXCL1 stimulation induced cytoplasmic translocation of PAD4

PAD4 translocated from the nucleus to the cytoplasm by CXCL1 stimulation for 2 hours. Green; PAD4, red; nucleus. Bar, 50 or 15 μm. Magnification, ×63.

## Discussion

In this study, we showed that the cytoplasmic translocation of PAD4 by CXCL1 induced β2 integrin activation and F-actin polymerization, and that the PAD4 translocated to the cytoplasm citrullinated PDIA1 involved in integrin activation. Interactions of integrins on leukocytes and adhesion molecules on endothelial cells are important in vascular inflammation[4, 20]. Previously, we reported that histone citrullination in neutrophils induced by CXCL1 enhances neutrophil adhesion in the femoral artery of LDLR−/− mice fed a HFD[2]. However, the mechanism through which citrullination of proteins in the nucleus affects the interaction between neutrophils and endothelial cells, the activation of integrins and signal pathway related to integri activation, remains unknown. Thus, we hypothesized that citrullination of cytoplasmic or cell-surface proteins involved in adhesion or integrin activation enhances neutrophil adhesion. Some proteins such as IKKγ, p65, fibrinogen, and extracellular matrix have been reported as citrullinated proteins[12, 21-23]. However, the involvement of such proteins in vascular inflammation is not well understood. Here, we report the involvement of PAD4 in neutrophil adhesion and a novel citrullinated protein in neutrophils. Our findings contribute on the clarification of a new

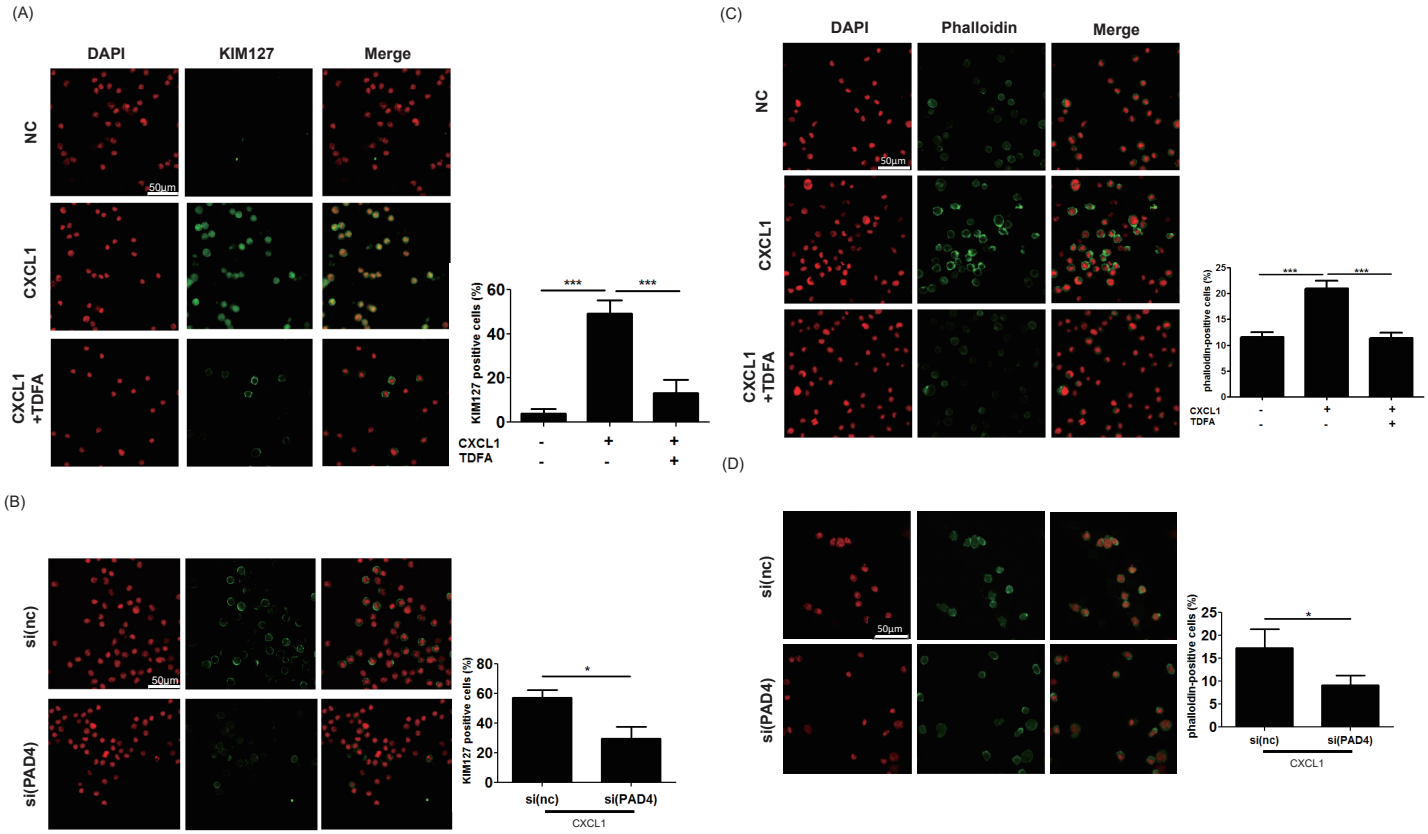


**Fig. 3.** Inhibition of PAD4 decreased the expression of activated-β2-integrin and F-actin polymerization upregulated by CXCL1 in dHL-60 cells

(A) dHL-60 cells stimulated with 10 nM CXCL1 in the presence or absence of 10 nM TDFA were stained with KIM 127, an anti-activated human β2-integrin. Green; activated β2-integrin, red; nuclei. Bar, 50 μm. The right graph shows the proportion of activated-β2-integrin-positive cells for each condition. Data are representative of three similar experiments and are presented as means ± SD. ***$p < 0.001$ by one-way ANOVA with Tukey's test. (B) dHL-60 cells stimulated with 10 nM CXCL1 in the presence of siRNA were stained with KIM 127. Green; activated-β2-integrin, red; nuclei. Bar, 50 μm. The right graph shows the proportion of activated-β2-integrin-positive cells in each condition. Data are representative of three similar experiments and are presented as means ± SD. *$p < 0.05$ by unpaired two-tailed Student's *t*-test. (C) dHL-60 cells stimulated with 10 nM CXCL1 in the presence or absence of 10 nM of TDFA were stained with phalloidin. Green; F-actin polymerization, red; nuclei. Bar, 50 μm. The right graph shows the proportion of F-actin polymerization-positive cells in each condition. Data are representative of three similar experiments and are presented as means ± SD. ***$p < 0.001$ by one-way ANOVA with Tukey's test. (D) dHL-60 cells stimulated with 10 nM CXCL1 in the presence of siRNA for *PAD4* or control siRNA were stained with phalloidin. Green; F-actin polymerization, red; nuclei. Bar, 50 μm. Magnification, ×63. The lower graph shows the proportion of actin polymerization-positive cells in each condition. Data shown are representative of three similar experiments and are presented as means ± SD. *$p < 0.05$ by unpaired two-tailed Student's *t*-test.

mechanism of vascular inflammation.

The transport of proteins from the nucleus to the cytoplasm is mediated by transport factors such as NES. NES is bound by the export karyopherin chromosomal region maintenance protein (CRM)-1/exportin 1, which escorts cargo proteins through the nuclear pore complex[24-26]. Most NES contain short sequences consisting of hydrophobic amino acids. PAD4 contains two NES sequences **(Supplementary Fig. 2)**. Renal ischemia and reperfusion induce translocation of PAD4 from the nucleus to the cytoplasm[10]. Our results showed that CXCL1 induces the translocation of PAD4 from nuclei to cytoplasm, which is inhibited by PAD4 inhibitor **(Fig. 2)**. Furthermore, PDIA1 in cytoplasm but not nuclei was citrullinated by CXCL1 **(Table 1)**. Therefore, these results suggested that the PAD4 has also potential to citrullinate cytoplasmic proteins.

PAD4 inhibition and knockdown of *PAD4* decreased F-actin polymerization and the number of activated-β2-integrin-positive cells **(Fig. 4)**, indicating that PAD4 induces actin polymerization and

**Table 1.** An LC-MS/MS analysis of citrullinated proteins in dHL-60 cells with or without CXCL1 stimulation

| CXCL1 | TDFA | Protein ID | Name | MW (kDa) | Citrullinated peptide |
|---|---|---|---|---|---|
| (-) | (-) | P06733 | Alpha+enolase | 47.1 | LAQANGWGVMVSHr |
| | | P04075 | Fructose bisphosphate aldolase A | 39.4 | GVVPLAGTNGETTTQ |
| | | | | | GLDGLSEr |
| (+) | (-) | P06073 | Alpha-enolase | 47.1 | LAQANGWGVMVSHr |
| | | P04066 | Glyceraldehyde-3-phosphate dehydrogenase | 36 | VGVNGFGr |
| | | P07237 | Protein disulfide isomerase A1 | 57.1 | TVIDYNGEr |
| (+) | (+) | P06733 | Alpha-enolase | 47.1 | LAQANGWGVMVSHr |
| | | | | | YASIcQQNGIVPIVEPEI |
| | | P04075 | Fructose bisphosphate aldolase A | 39.4 | LPDGDHDLKr |
| | | | | | GVVPLAGTNGETTTQ |
| | | | | | GLDGLSEr |
| | | P04066 | Glyceraldehyde-3-phosphate dehydrogenase | 36 | VGVNGFGr |
| | | P16402 | Histone H1 | 22.3 | ALAAAGYDVEKNNSr |
| | | P22626 | Heterogeneous nuclear ribonucleoproteins A2/B1 | 37.4 | YHTINGHNAEVr |

activation of β2-integrin. Furthermore, PAD4 inhibition and knockdown also decreased neutrophil adhesion to HUVECs under the physiological blood flow velocity **(Fig. 1C, D, E)**. PAD4 thus regulates the activation of β2-integrin directly or indirectly.

We identified PDIA1 as a citrullinated protein targeted by CXCL1 stimulation using LC-MS/MS analysis **(Table 1)**. PDI catalyzes cleavage and modification of disulfide bonds between cysteine residues of proteins in the endoplasmic reticulum during protein folding[11]. Knockdown or inhibition of PDIA1 decreased adhesion of dHL-60 cells and human neutrophils to HUVECs in flow assays **(Fig. 4A, B, C)**. PDI A1in lipid rafts on th cell surface regulates clustering of αMβ2-integrin through binding directly to the integrin by fMLF or TNFα, followed by induction of neutrophil adhesion onto HUVECs[27]. We further show that knockdown or inhibition of PDIA1 decreased F-actin polymerization and the number of activated-β2-integrin-positive cells **(Fig. 4D, E)**. These results indicate that PDIA1 induces not only clustering of αMβ2-integrin but also activation of β2-integrin. Future studies are required to elucidate the involvement of citrullinated PDIA1 in neutrophil adhesion.

Our data point to a novel inflammatory mechanism during atherosclerosis. Inhibition of PAD4 and/or PDIA1 may become new therapeutic targets for atherosclerosis.

In conclusion, we demonstrate that cytoplasmic translocation of PAD4 induces neutrophil adhesion through the activation of β2-integrin, suggesting that PAD4 may be involved in the development of atherosclerosis **(Fig. 5)**.

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## Author Contribution

JA, MO and MY wrote manuscript, JA performed flow assay, western blotting, phalloidin stain, immunohistochemistry and statistical analysis, MD managed cells and reagents. SH performed LC-MS/MS analysis. AI provided anti-human PAD4 antibody. MO decided conception, design, methodology, TM, AI and MY were supervisors.

## Disclosure of Potential Conflicts Interest

All authors declear that they have no conflict of interest.

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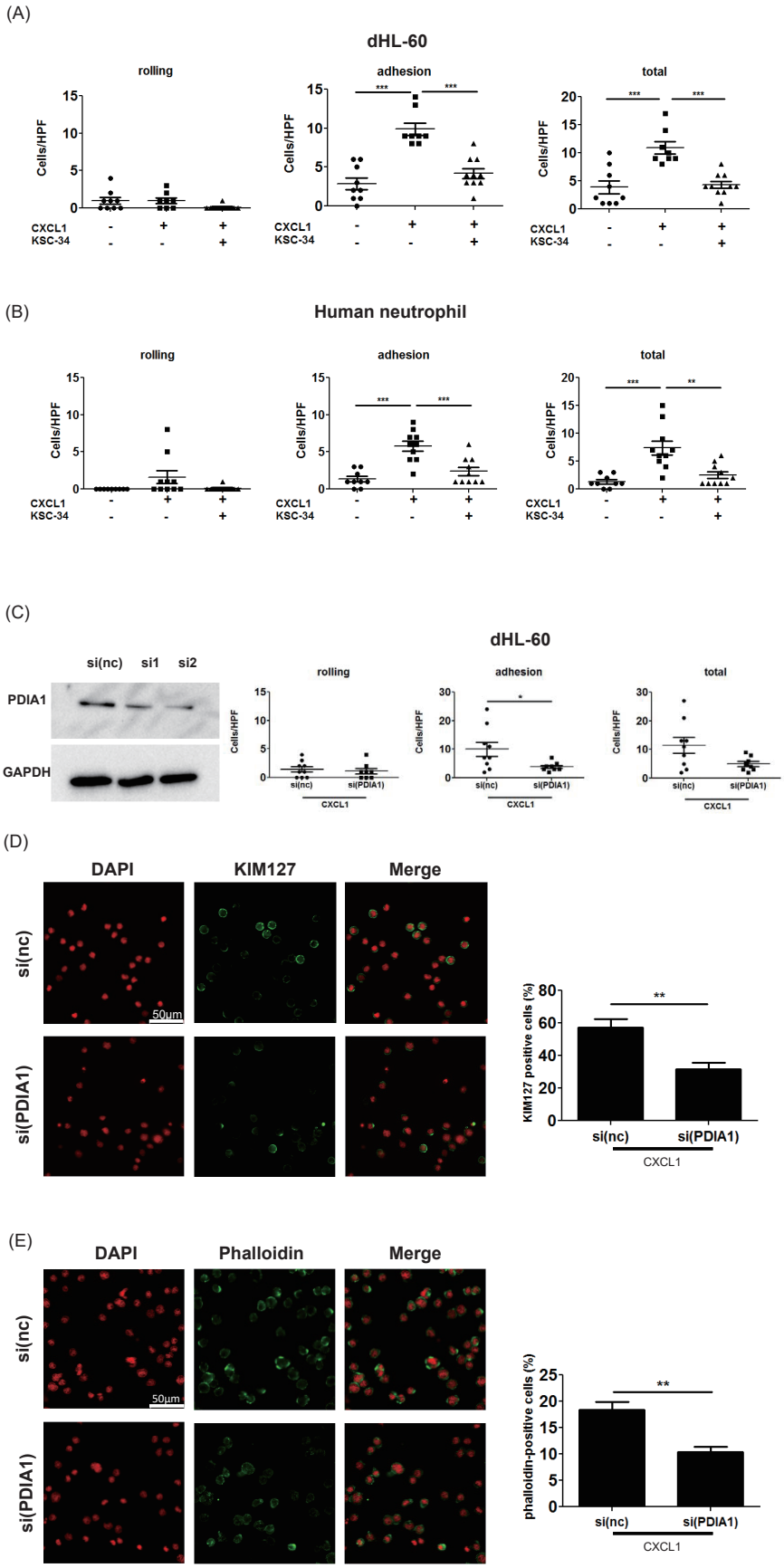


**Fig. 4.** Inhibition or knockdown of PDIA1 decreased neutrophil adhesion to HUVECs and decreased the activated-$\beta$2-integrin and the F-actin polymerization upregulated by CXCL1 in dHL-60

(A) dHL-60 cells were stimulated by 10 nM CXCL1 with or without 3.5 μM of KSC-34. Adhesion increased by 10 nM CXCL1 stimulation was significantly decreased by KSC-34. Data are representative of three similar experiments. (B) Isolated peripheral human neutrophils were stimulated by 10 nM CXCL1 with or without 3.5 μM of KSC-34. The number of total recruitment cells, rolling cells, and adhered cells were significantly decreased by KSC-34. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ by one-way ANOVA with Tukey's test. (C) siRNA for *PDIA1* decreased the expression of PDIA1 in dHL-60 cells (left images). The numbers of adhered cells were significantly decreased by siRNA of *PDIA1* (right graph). HPF indicates high-power field (×20 microscope field). $^{*}p<0.05$ by unpaired two-tailed Student's *t*-test. (D) dHL-60 cells stimulated with 10 nM CXCL1 in the presence of siRNA for *PDIA1* or control were stained with KIM 127. Green; activated-$\beta$2-integrin, red; nuclei. Bar, 50 μm. The right graph shows the proportion of activated-$\beta$2-integrin-positive cells in each condition. (E) dHL-60 cells were stained with phalloidin staining following stimulation with 10 nM of CXCL1 in the presence of siRNA for *PDIA1* or control. Green; F-actin polymerization, red; nuclei. Bar, 50 μm. Magnification, ×63. The lower graph shows the proportion of F-actin polymerization-positive cells in each condition. $^{**}p<0.01$ by unpaired two-tailed Student's *t*-test. Data are representative of three similar experiments and are presented as the mean ± SD.

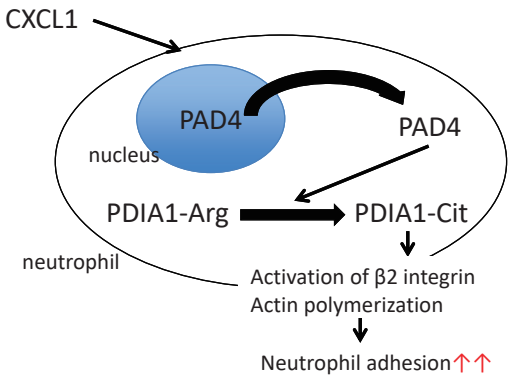


**Fig. 5.** Scheme of the mechanism through which cytoplasmic translocation of PAD4 induces neutrophil adhesion
The translocation of PAD4 from the nucleus to the cytoplasm by CXCL1 activated $\beta$2-integrin.


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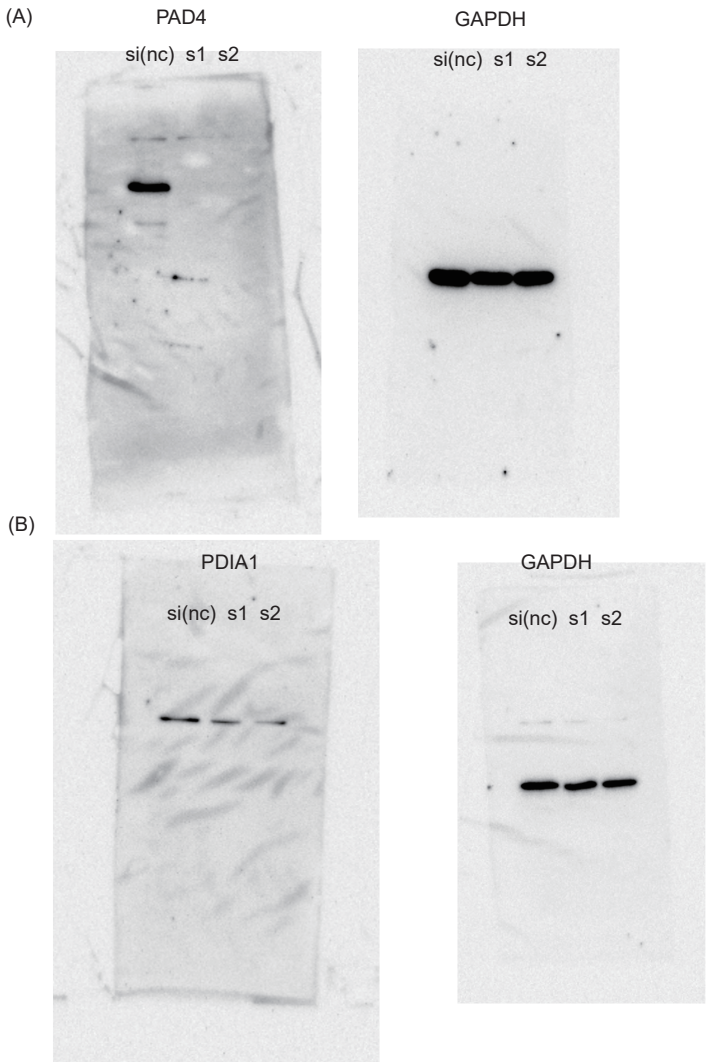


**Supplementary Fig. 1.** Western blotting for PAD4 and PDIA1

(A) Western blotting for PAD4 and GAPDH in dHL-60 cells treated with siRNA(nc) or siRNA(PAD4)
(B) Western blotting for PDIA1 and GAPDH in dHL-60 cells treated with siRNA(nc) or siRNA(PDIA1)

Amino acid sequence of PAD4

MAQGTLIRVTPEQPTHA**VCVLGTLTQLDI**CSSAPEDCTSFSINASPGVVVDIAHGPPAKK
KSTGSSTWPLDPGVEVTLTMKVASGSTGDQKVQISYYGPKTPPVKALLYLTGVEISLCA
DITRTGKVKPTRAVKDQRTWTWGPCGQGAILLVNCDRDNLESSAMDCEDDEVLDSEDL
QDMSLMTLSTKTPKDFFTNHTLVLHVARSEMDKVRVFQATRGKLSSKCSVVLGPKWPS
HYLMVPGGKHNMDFYVEALAFPDTDFPGLITLTISLLDTSNLELPEAVVFQDSVVFRVAP
WIMTPNTQPPQEVYACSIFENEDFLKSVTTLAMKAKCKLTICPEEENMDDQWMQDEMEI
GYIQAPHKTLPVVFDSPRNRGLKEFPIKRVMGPDFGYVTRGPQTGG**ISGLDSFGNLEV**
SPPVTVRGKEYPLGRILFGDSCYPSNDSRQMHQALQDFLSAQQVQAPVKLYSDWLSV
GHVDEFLSFVPAPDRKGFRLLLASPRSCYKLFQEQQNEGHGEALLFEGIKKKKQQKIKN
ILSNKTLREHNSFVERCIDWNRELLKRELGLAESDIIDIPQLFKLKEFSKAEAFFPNMVNM
LVLGKHLGIPKPFGPVINGRCCLEEKVCSLLEPLGLQCTFINDFFTYHIRHGEVHCGTNV
RRKPFSFKWWNMVP

**Supplementary Fig. 2.** Amino acid sequence of PAD4 and nuclear export signal

Underlining indicates NES sequences.