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RESEARCH ARTICLE

Decreased epidermal AXL expression and increased infiltration of AXL-expressing dendritic cells in psoriasis

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Abstract

Background: Psoriasis is a chronic skin inflammatory disease characterized by epidermal proliferation and inflammatory cell infiltration. AXL is a tyrosine kinase receptor that promotes cell proliferation and invasion in cancer cells, and its expression is elevated in multiple tumors. However, less is known about its expression and function in inflammatory diseases.

Objectives: The aim of this study is to examine AXL expression in psoriasis and investigate the biological function of AXL under psoriatic conditions.

Results: AXL mRNA expression was decreased in psoriatic skin compared to healthy skin, and an inverse correlation with neutrophil-to-lymphocyte ratio and platelet-to-lymphocyte ratio was observed. Immunohistochemical staining of psoriatic skin revealed decreased AXL expression of the epidermis and an increased number of AXL-positive dendritic cells in the dermis. Stimulation of epidermal keratinocytes with antimicrobial peptide LL37, but not with IL-17A, resulted in decreased AXL expression. Knockdown of AXL in epidermal keratinocytes did not affect cell proliferation or expression of psoriasis-associated cytokines and chemokines.

Conclusion: Decreased epidermal AXL expression and increased infiltration of AXL-expressing dendritic cells were revealed in psoriasis.

KEYWORDS
AXL, psoriasis

1 | INTRODUCTION

Psoriasis is a common chronic inflammatory skin disease that is characterized by well-demarcated scaly, thick erythematous plaques, leading to a reduced quality of life.[1,2] In the epidermis, the outermost part of the skin, LL37 is induced upon environmental stimuli and is highly expressed in psoriasis compared to normal skin.[3–5] While LL37 produced by the psoriatic epidermis has a direct effect on the surrounding keratinocytes, LL37 also activates plasmacytoid dendritic cells together with autologous DNA in the dermis, triggering immunological activation of the IL-23/IL-17 axis in the dermis.[6–8] Subsequently, epidermal keratinocytes enter a proliferative cycle in response to IL-17A/F and growth factors that are excessively produced in the psoriatic microenvironment.[9–11] Epidermal keratinocytes activated by IL-17A/F and growth factors further enhance the production of pro-inflammatory factors of cytokines.[12,13] This

TABLE 1 Clinical features of psoriasis patients and healthy controls.

	Psoriasis ($N=7$)	Healthy controls ($N=9$)
Gender	M6, F1	M7, F2
Age: Mean ± SD (range), year	56.1 ± 18.4 (25–83)	66.8 ± 13.5 (46–82)
Body mass index: Mean ± SD (range)	24.1 ± 9.5 (17.5–27.7)	–
Psoriasis area and severity index (PASI) score: Mean ± SD (range)	15.9 ± 11.4 (1.8–34)	–
Disease duration: Mean ± SD (range), year	14.5 ± 9.5 (3–30)	–

results in an inflammatory cascade of psoriatic inflammation amplification that leads to manifestations of psoriasis.[2,14] As such, epidermal keratinocytes are activated both by innate stimuli from the outside and by immunological signals from the dermis, leading to the complete pathogenesis of the chronic phase of psoriasis.

AXL is the receptor tyrosine kinase involved in various cellular processes, including proliferation, migration, and inflammation.[15,16] Since its discovery, overexpression of AXL has been observed in a variety of solid tumors and hematologic malignancies.[17–19] Evidence has been accumulating that AXL promotes tumor growth and immune escape of tumor cells, which may result in the aggressive phenotype of tumors.[15,16,19] Inhibitors targeting AXL are in clinical development for several cancers these days. AXL inhibitors exert their anti-tumor effects in drug-resistant cancers by suppressing tumor cell proliferation through inhibition of JAK/STAT and MEK/ERK and NF-kB signaling.[15,16,20] With the recent advances in immune checkpoint blockade, the function of AXL in immune surveillance has also been focused. AXL blockage induces de-repression of TLR inflammatory signaling and cytokine release in antigen-presenting cells, thus strengthening anti-tumor immunity.[21]

While the function of AXL in tumor cells and its significance as a therapeutic target has been well established, little is known about AXL expression or its function in non-malignant conditions. AXL expression of airway epithelium in patients with severe asthma is rather decreased and negatively correlates with eosinophilic inflammation, which is contradictory to the results observed under tumor conditions.[22] AXL has been reported to promote inflammation in the setting of myocardial infarction and peritonitis.[23] These conflicting findings between malignant- and non-malignant- settings indicate that AXL has cell-specific functions and that its effects are likely to be condition dependent.

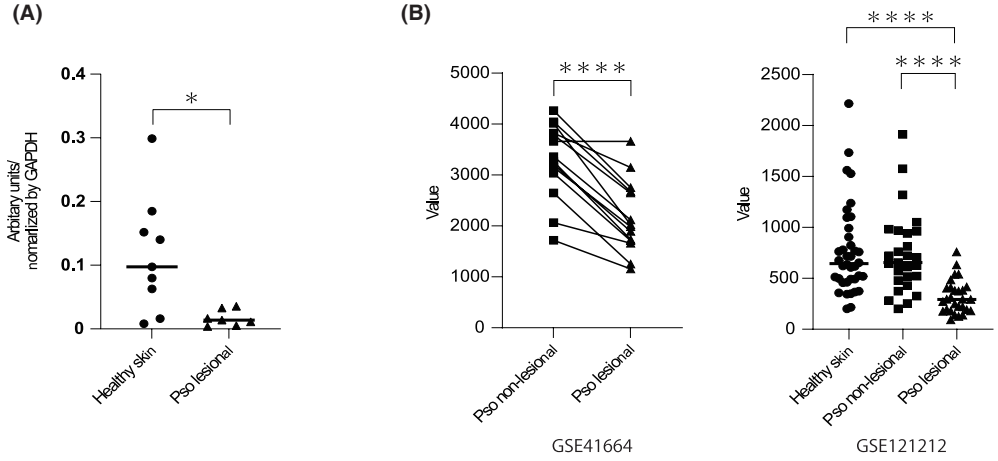
The current study examined the expression levels and distribution pattern of AXL in psoriatic skin, one of the representative inflammatory skin diseases. The study aimed to investigate factors that regulate AXL expression in epidermal keratinocytes under psoriatic conditions and to assess the function of AXL in the regulation of cell proliferation and expression of psoriasis-associated cytokines and chemokines. The findings of this study will provide new information on AXL in inflammatory diseases.

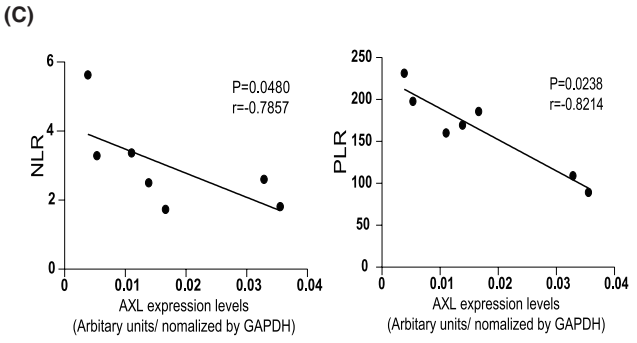
2 | RESULTS

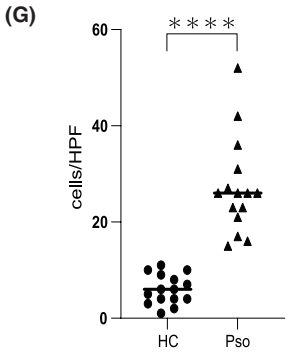
2.1 | Decreased epidermal expression of AXL in the psoriatic skin

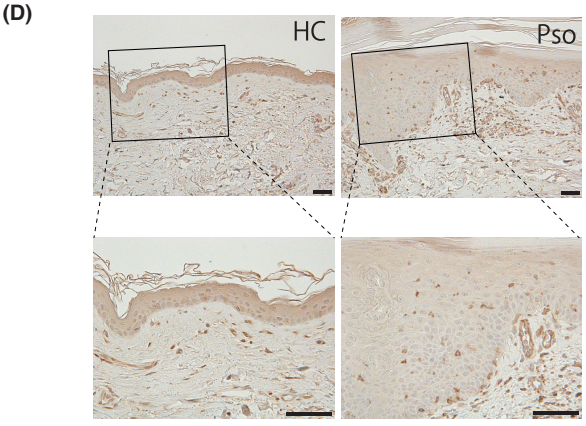
The gene expression levels of AXL in the skin of seven patients with psoriasis and nine healthy controls were evaluated by quantitative real-time polymerase chain reaction (Table 1). AXL mRNA levels were significantly decreased in the skin of psoriasis patients compared to healthy controls (Figure 1A). Consistent with our data, microarray datasets (GS4E4166 and GSE121212) from other groups[24–26] observed decreased AXL expression in the lesional skin of psoriasis compared to the non-lesional skin lesions of the same patients (Figure 1B). The AXL expression levels of the lesional skin of psoriasis patients negatively correlated with systemic inflammatory scores of neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) from blood tests (Figure 1C).

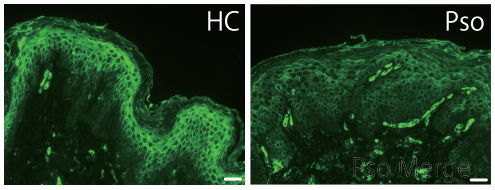
FIGURE 1 Decreased AXL expression levels in the psoriatic skin and its distribution in the skin. (A) Gene expression levels of AXL in the lesional skin of psoriasis patients ($N=7$) and the skin of healthy controls ($N=9$). The horizontal lines indicate the median values. Mann–Whitney *U*-test was conducted. (B) Gene expression levels in the lesional and non-lesional skin of psoriasis patients as well as the skin of healthy controls from a public data set (GSE41664 and GSE121212). The horizontal lines indicate the median values. Wilcoxon signed-rank test GSE41664 and Kruskal Wallis test for GSE121212 were conducted. (C) Correlations between skin mRNA levels of AXL and NLR or PLR scores from blood tests in psoriasis patients ($N=7$). The solid lines indicate a linear regression line. Spearman's rank correlation coefficient for two continuous variables was used. (D) Immunohistochemical images for AXL in the skin sections from healthy controls and psoriasis patients. Results are representative of four Healthy controls and four psoriasis patients. Scale bar, 50 μm. (E) Immunofluorescence images for AXL in the skin sections from healthy controls and psoriasis patients. Results are representative of three healthy controls and four psoriasis patients. Scale bar, 50 μm. (F) Immunofluorescence images for AXL and DC-LAMP/CD208 in the skin sections from healthy controls and psoriasis patients. AXL (green) was co-stained with dendritic cells-lysosomal associated membrane protein (DC-LAMP)/CD208 (red). Results are representative of three psoriasis patients. Scale bar, 50 μm. (G) The number of AXL and DC-LAMP-positive cells in the skin sections from psoriasis patients and healthy controls. Mann–Whitney *U*-test was conducted. $^{*}p<0.05$, $^{****}p<0.0001$.

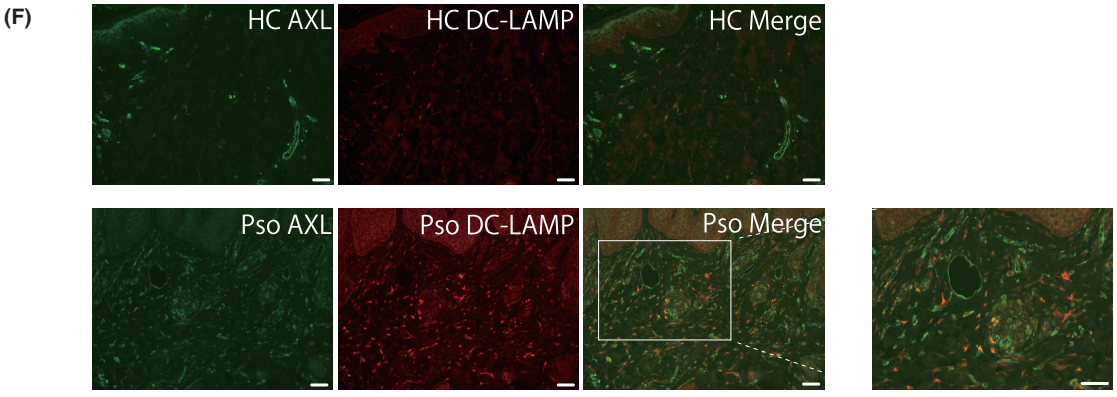












Immunohistochemical staining was then performed to examine the expression and distribution of AXL protein in the psoriatic skin and the skin from healthy controls (Figure 1D). The overall epidermal AXL expression was weaker in the psoriatic skin than in the healthy control skin, which was also confirmed by immunofluorescence staining (Figure 1D,E). Within the epidermis of psoriatic skin,

AXL-positive cells, which were smaller than epidermal keratinocytes and presumably Langerhans cells or inflammatory dendritic epidermal cells, were scattered. AXL-positive cells were also observed in the dermis of both psoriasis and healthy control skin, with a greater abundance in the psoriatic skin. Vascular endothelial cells in the dermis were moderately positive for AXL (Figure 1D). We next performed fluorescent immunodouble staining with AXL and dendritic cell-lysosomal-associated membrane protein (DC-LAMP)/CD208 to identify cells that are positive for AXL in the dermis (Figure 1F). AXL was expressed on infiltrates and vessels with luminal structures, which was compatible with immunohistochemical staining results (Figure 1F). DC-LAMP, a dendritic cell maturation marker, was positive for infiltrates in the dermis of psoriasis and psoriatic epidermis, as previously reported[27,28] (Figure 1F). The AXL-positive cells in the dermis were partially colocalized with DC-LAMP (Figure 1F) and the number of AXL and DC-LAMP- double-positive cells in the dermis was significantly higher in the psoriatic skin (Figure 1G). Thus, the decreased gene expression of AXL detected in the whole psoriatic skin was likely due to the downregulation of AXL expression in the epidermal keratinocytes of the psoriatic skin.

2.2 | Decreased AXL expression induced by LL37 in epidermal keratinocytes

We next examined whether psoriatic conditions may induce decreased expression levels of AXL in epidermal keratinocytes. Injured keratinocytes and psoriatic epidermis excessively produce the antimicrobial peptide LL37, and keratinocytes are autoreactive to the LL37 to exhibit a biological effect.[7] IL-17A was used as a stimulus that reflects the immunological microenvironment from the dermis in psoriasis. Normal human epidermal keratinocytes (NHEKs) were stimulated with LL37 or IL-17A to mimic in vivo conditions of the psoriatic epidermis, and the protein expression levels of AXL were examined by immunoblotting. Results showed that NHEKs expressed substantial amounts of AXL in homeostasis, and stimulation with LL37 induced a clear decrease in AXL expression (Figure 2A). AXL expression was comparable between IL-17A-stimulated and unstimulated NHEKs. Since the ligand for AXL, Gas6 has been reported to be expressed in the mouse epidermis,[29] we also examined the expression of Gas6 in NHEKs under LL37 or IL-17A treatment. Gas6 expression was reduced by LL37 treatment but not by IL-17A (Figure 2A). Given that AXL can be cleaved at the cell membrane and be yielded extracellularly as a soluble form of AXL,[30] we next examined the amount of soluble AXL in the supernatant of LL37- or IL-17A- stimulated NHEKs. Results showed that the amount of soluble AXL in the supernatant was significantly decreased in LL37- stimulated NHEKs but not in IL-17A- stimulated NHEKs (Figure 2B). Thus, the cellular protein expression of AXL detected by immunoblotting corresponded to the amount of soluble AXL in the supernatant of the respective cell conditions. Collectively, together with the result of Figure 1, AXL expression was downregulated in the psoriatic epidermis and in keratinocytes of LL37-stimulated psoriatic conditions.

2.3 | AXL silencing does not affect the proliferative potential of keratinocytes induced by growth factors and IL-17A

Epidermal hyperplasia is a characteristic feature of psoriasis. Epidermal keratinocytes proliferate in response to IL-17A and growth factors.[31] In the present study, NHEKs did not show stable proliferative effects upon stimulation with growth factors or IL-17A (data not shown). Therefore, we used HaCaT cells to examine the proliferative effects of growth factors and IL-17A and to explore the function of AXL on cell proliferation. Cell proliferation was assessed by newly synthesized DNA using a bromodeoxyuridine (BrdU) incorporation assay. HaCaT cells showed a concentration-dependent proliferation when stimulated with epidermal growth factor (EGF) or transforming growth factor (TGF)-α, with a significant difference at 10 ng/mL of EGF and 5 ng/mL of TGF-α compared to no stimulation (Figure 3A,B). Co-stimulation of 200 ng/mL of IL-17A with EGF or TGF-α enhanced the proliferative effects of HaCaT cells compared to the stimulation by EGF or TGF-α alone. We next examined whether AXL was involved in the proliferative effects of these factors. HaCaT cells were transfected with small interfering (si)-RNA for AXL (si-AXL1 and si-AXL2) or negative control siRNA (si-NC), and cell proliferation was compared between si-NC and si-AXL transfected HaCaT cells in response to EGF or TGF-α with or without IL-17A stimulation. The efficiency of siRNA-mediated gene knockdown was confirmed by quantitative polymerase chain reaction and immunoblotting (Figure 3C,D). Results showed that no significant difference in the proliferative effects was observed between si-NC and si-AXL transfected HaCaT cells in response to these factors (Figure 3E,F). Thus, proliferation induced by growth factors in combination with IL-17A was not dependent on AXL signaling in HaCaT keratinocytes.

2.4 | AXL silencing does not affect the expression of psoriasis-associated cytokines and chemokines in epidermal keratinocytes

We next investigated the function of AXL in the production of inflammatory factors in epidermal keratinocytes under IL-17A and TLR3 ligand poly (I:C). Since AXL is reported to function as a negative regulator of TLR signaling,[32] we have hypothesized that AXL reduction may increase the expression of target genes regulated by TLR signaling in keratinocytes. Koebner phenomenon, where a new psoriatic lesion develops at the site of skin injury, is associated with the activation of TLR3 signaling in response to dsRNA released by injured keratinocytes.[33,34] To investigate the involvement of AXL in the induction of TLR3-regulated genes, NHEKs transfected with si-RNA for AXL or negative control were stimulated with TLR3 ligand poly (I:C). Genes evaluated here were *IFNB1*, *CXCL8*, *TNFA*, *IL36G*, and *GMCSF* after 24 h after poly (I:C) stimulation. As shown in Figure 4A, treatment of si-NC transfected NHEKs with poly (I:C) led to a significant increase in mRNA expression of genes evaluated. However, AXL silencing did not further increase or decrease the expression of

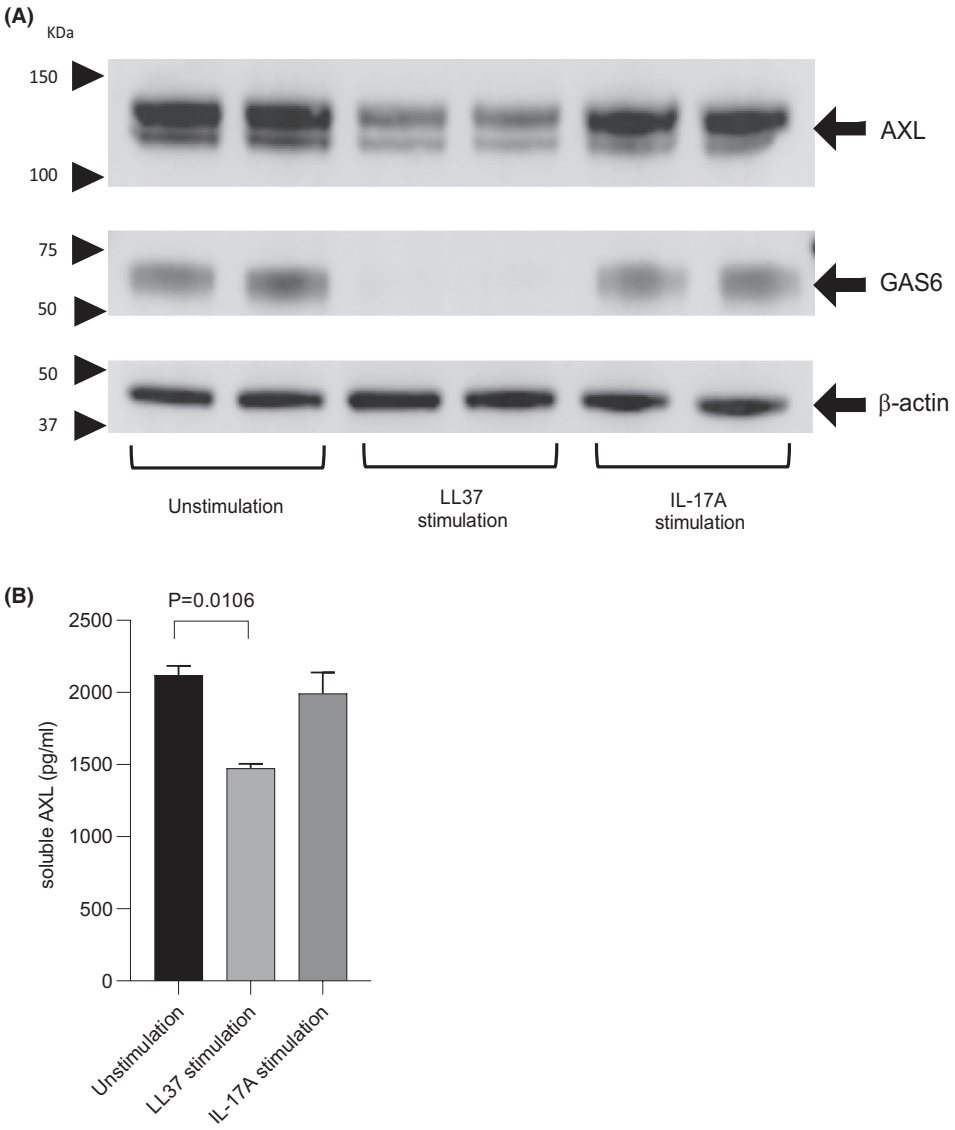


FIGURE 2 Decreased AXL expression levels induced by LL37 in epidermal keratinocytes. (A) Representative immunoblotting images of AXL, GAS6, and β-actin protein expression in NHEKs stimulated by LL37 or IL-17A. The results shown are representative of four independent experiments with similar results. Black arrows on the right sides indicate the bands for AXL, GAS6, and β-actin. Molecular weight is indicated as black triangles on the left sides. (B) Supernatant AXL levels of NHEKs stimulated by LL37 or IL-17A. Data are presented as mean ± SD. One-way ANOVA analysis of variance with Dunn's post hoc test was conducted.

these genes (Figure 4A). We further investigated whether AXL was involved in the IL-17 signaling pathway in keratinocytes. Transfected NHEKs, si-NC, si-AXL1, and si-AXL2, were stimulated with IL-17A for 24 h, and IL-17A-associated genes were evaluated. Genes evaluated here included S100 protein-coding genes, *DEFB4A*, *IL36G*, *CXCL8*, and *CCL20*. The expressions of these genes were significantly induced by IL-17A stimulation, however, no further induction or reduction was observed in AXL-silenced keratinocytes (Figure 4B).

3 | DISCUSSION

The aim of this study was to investigate the expression and function of AXL in non-malignant epidermal keratinocytes and to explore its link to the pathogenesis of psoriasis. Psoriasis is a chronic inflammatory skin disease characterized by epidermal proliferation and sustained inflammation. Whereas AXL expression is upregulated in many malignant tissue or cells, its expression was decreased in the psoriatic skin tissue and epidermis in the present study. The protein expression of AXL and its ligand Gas6 were downregulated by stimulation by LL37 in keratinocytes. The function of AXL with respect to both regulation of psoriasis-associated immune factors and cell proliferation was not confirmed in the present study.

Several studies have investigated the expression of AXL in non-malignant inflammatory conditions. AXL expression is downregulated in dendritic cells of patients with rheumatoid arthritis, and microRNA-34a contributes to the regulation of AXL expression.[32] Decreased expression of AXL has also been reported in airway macrophages of

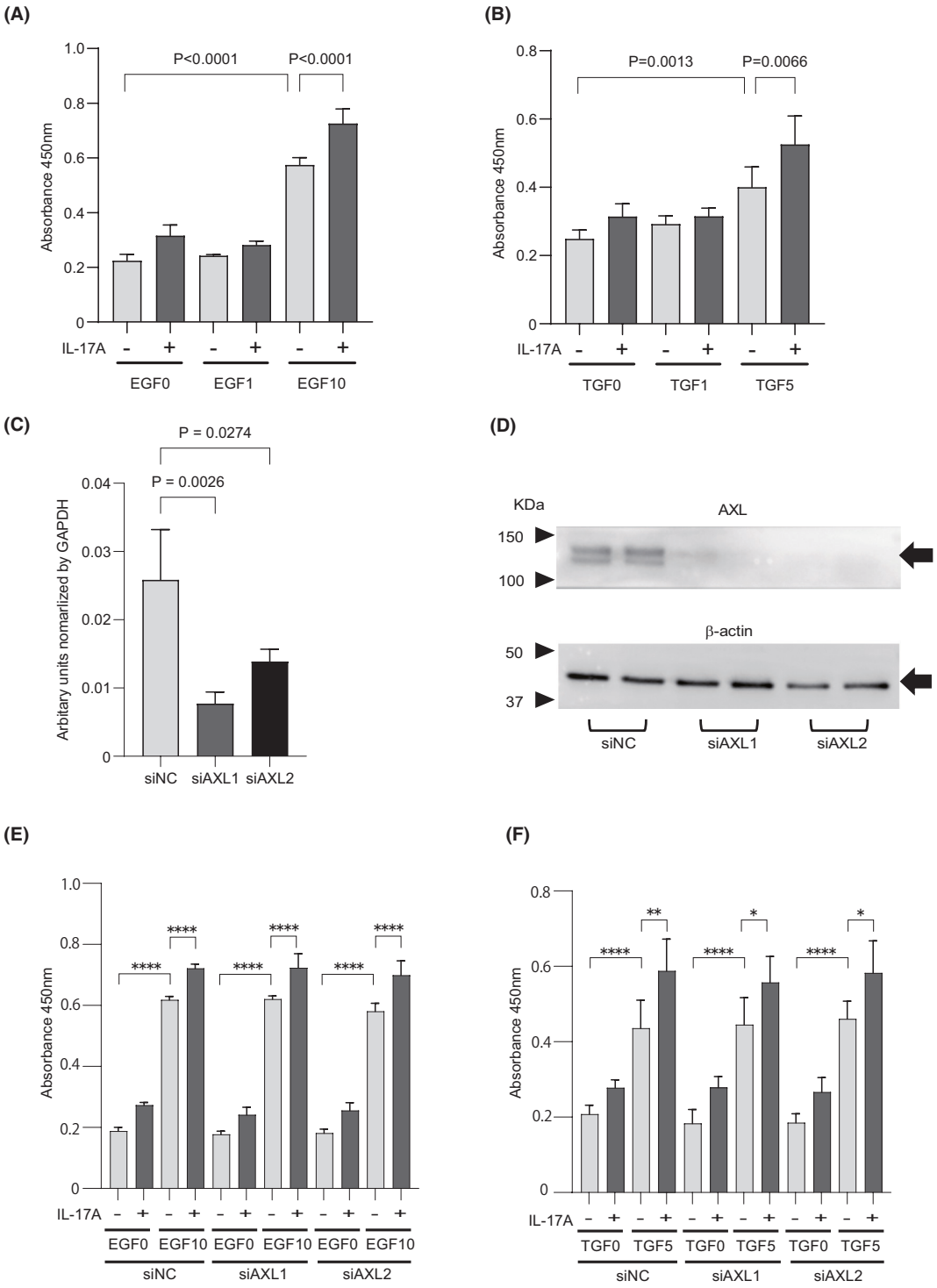


FIGURE 3 Cell proliferation of HaCaT cells induced by growth factors and IL-17A and evaluation of AXL involvement in cell proliferation. Cell proliferation of HaCaT cells was evaluated by bromodeoxyuridine (BrdU) assay. HaCaT cells were stimulated with (A) epidermal growth factor (EGF) (0, 1, 10 ng/mL) or (B) transforming growth factor (TGF)-α (0, 1, 5 ng/mL) with or without IL-17A (200 ng/mL), and BrdU incorporation was measured. HaCaT cells were transfected with si-RNA for AXL (si-AXL1 and si-AXL2) or negative control siRNA (si-NC). The efficiency of siRNA-mediated gene knockdown was confirmed by quantitative polymerase chain reaction (C) and immunoblotting (D). HaCaT cells transfected with si-AXL1, si-AXL2, or si-NC were evaluated for cell proliferation by BrdU assay. Transfected HaCaT cells were stimulated with (E) EGF (0, 10 ng/mL) or (F) TGF-α (0, 5 ng/mL) with or without IL-17A (200 ng/mL), and BrdU incorporation was measured. Data are presented as mean ± SD. One-way ANOVA analysis of variance with Dunn's post hoc test was conducted. $^{*}p < 0.0001$ versus EGF0/IL-17A- or TGF0/IL-17A- in each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus EGF10/IL-17A+ or TGF5/IL-17A+ in each group.

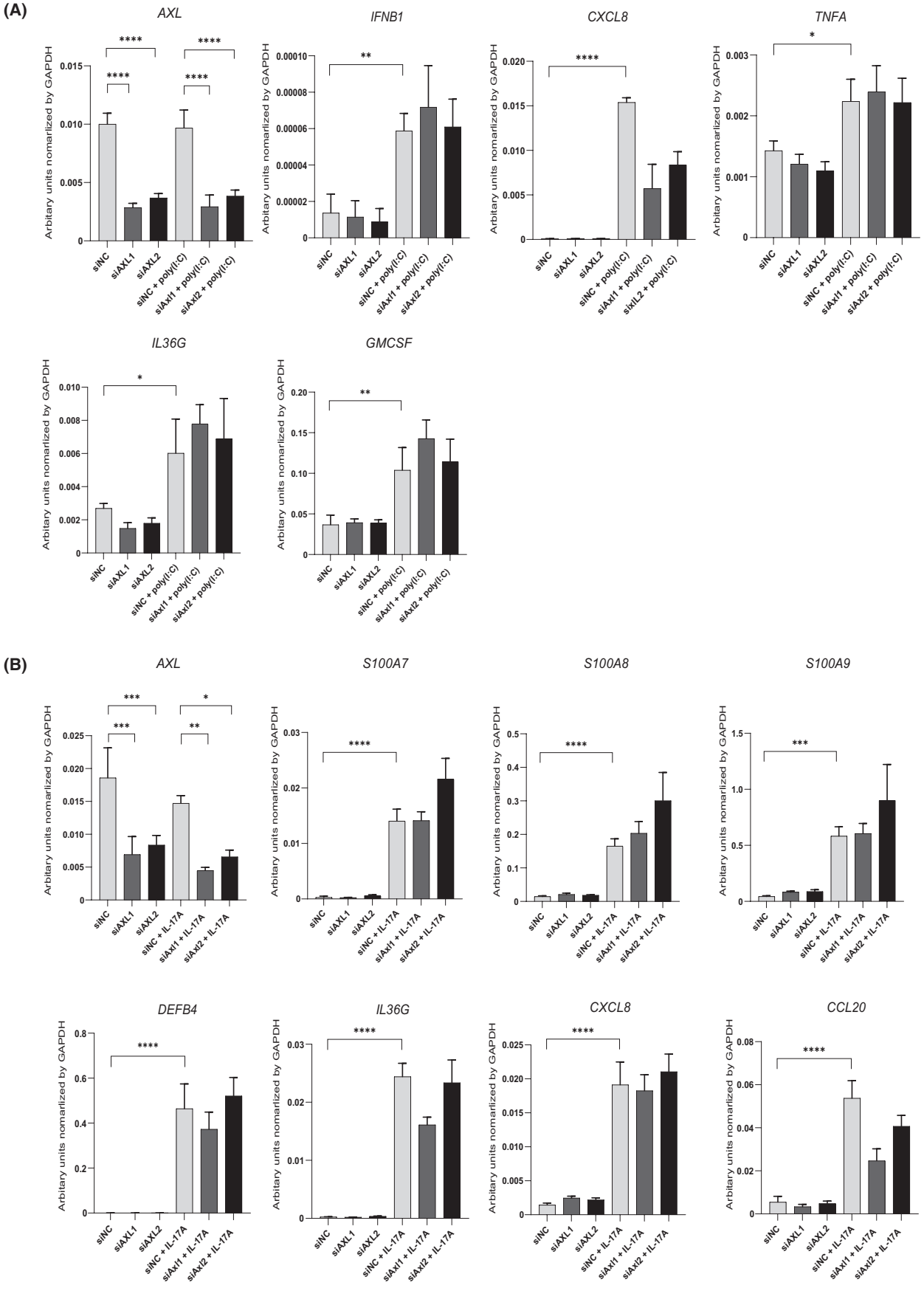


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FIGURE 4 Evaluation of AXL involvement in the expression of psoriasis-associated genes in normal human epidermal keratinocytes (NHEKs). NHEKs were transfected with si-RNA for AXL (si-AXL1 and si-AXL2) or negative control siRNA (si-NC). Transfected cells were stimulated with (A) poly (I:C) for 16 h or (B) IL-17A for 24 h, and gene expression levels were evaluated. The results shown are representative of six independent experiments with similar results. Data are presented as mean ± SD. One-way ANOVA analysis of variance with Dunn's post hoc test was conducted for gene expression analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

asthmatic patients, which has been linked to impaired efferocytosis of macrophages.[35] Conversely, AXL expression is rather elevated in macrophages during the reperfusion phase of myocardial infarction.[23] Thus, AXL expression and its function may vary depending on the type of disease and the stage of pathogenesis. Regarding AXL expression in the epithelial system, recent research has indicated a reduction in AXL expression levels in the airway epithelium of asthma patients, with a negative correlation between AXL expression levels and the number of infiltrating eosinophils.[22] The epidermal AXL expression in the present study was also reduced, and a negative correlation was observed between skin AXL expression levels and systemic inflammatory scores. Although the direction of T cell activation differs between asthma and psoriasis, the epithelium, located at the interface between the external environment and the internal body, contributes to disease development by facilitating communication with immune cells in both innate and adaptive immune responses.[36,37] The location and function of the epithelium, which is common in both diseases, may potentially regulate AXL expression.

In tumor cells, AXL is activated by the Gas6 ligand and forms dimers to phosphorylate its receptor. The intracellular downstream pathways include JAK/STAT, MEK/ERK, p38, PI3K/Akt, and NF-kB signaling, which promote tumor cell growth, invasion, and epithelial-mesenchymal transition.[38] Additionally, AXL contributes to immunoregulation by suppressing TLR signaling in antigen-presenting cells.[32] Inhibiting AXL in antigen-presenting cells within the tumor microenvironment enhances the expression of proinflammatory cytokines triggered by innate TLR signaling, which is advantageous for enhancing tumor immunity.[38] In this study, epidermal keratinocytes were stimulated with factors such as poly (I:C), IL-17A, EGF, and TGF-α to activate these intracellular and TLR signaling pathways.[7,31,34] Although these factors induced cell proliferation and cytokine production, the present study did not confirm the involvement of AXL in this process. Given the limited factors for activation and time points for evaluation in this study, further studies may be required to elucidate the role of AXL in epidermal keratinocytes in inflammatory conditions.

There are several limitations to this study. The sample size was small and accurate results may be lost. In addition, in vivo evaluation using mice with a genetic deletion of AXL would be desirable to assess the potential contribution of AXL to the development and maintenance of psoriasis.

In summary, the current study demonstrated a decreased expression of AXL specifically in the epidermis in patients with psoriasis. The present study may provide new data on AXL expression in inflammatory diseases, and further studies are expected to fully understand the functions of AXL in the pathogenesis of psoriasis.

4 | METHODS

4.1 | Samples and clinical information collected from patients with psoriasis and healthy controls

Skin samples were collected from seven psoriasis patients and nine healthy controls after obtaining written informed consent. Samples of psoriasis patients were taken from the lesional skin that had not received topical treatment for at least 2 weeks and prior to systemic treatment. Skin samples were snap-frozen and stored at −80°C until use. Clinical and hematological data were collected from the patient's medical records upon diagnosis. The medical ethics committee of the University of Tokyo approved all described studies (No. 0695-22 and No. 3360) and the study was conducted according to the principles of the Declaration of Helsinki.

4.2 | Immunohistochemistry and immunofluorescence staining

Formalin-fixed paraffin-embedded skin sections were deparaffinized and permeabilized with 0.2% Triton-X 100 in PBS. After heat-induced antigen retrieval with a citrate-based antigen unmasking solution (Vector Laboratories), sections were blocked with 5% BSA and 5% normal goat serum in PBS for 1 h at room temperature. The sections were then incubated with primary antibodies against AXL (R&D Systems) and DC-LAMP/CD208 (Abclonal Technology) at 4°C overnight, followed by 2 h of incubation at room temperature with matched secondary antibodies (Thermo Fisher Scientific). For immunofluorescence, DNA staining and section mounting were performed using DAPI Fluoromount-G (Southern Biotechnology Associates). Immunofluorescent images were obtained using an all-in-one fluorescence microscope BZ-X810 (Keyence). For immunohistochemistry, after incubation with secondary antibodies, sections were incubated with the avidin-biotin-peroxidase complex and then stained with 3,3′-Diaminobenzidine (Vector Laboratories).

4.3 | Cell culture of NHEKs and HaCaT cells

Primary NHEKs (Kurabo) were cultured in EpiLife medium (Thermo Fisher Scientific) with supplements (penicillin G sodium, streptomycin sulfate, and amphotericin B) in 5% CO2 at 37°C. HaCaT keratinocytes were provided by Dr. Kuroki (Showa University) with the permission of Dr. Fusenig (Institut für Zell- und Tumorbiologie, Deutsches Krebsforschungszentrum). HaCaT cells were cultured in Eagle's minimum essential medium (Sigma-Aldrich) containing 10%

fetal bovine serum in 5% CO2 at 37°C. NHEKs and HaCaT cells were cultured to approximately 70% confluence just before the experiments.

4.4 | RNA interference with AXL

Regents for siRNA against AXL or non-target control siRNA were obtained from Thermo Fisher Scientific. All transfections were performed using INTERFERin (Polyplus-transfection) or Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, cultured NHEKs or HaCaT cells were incubated with siRNA reagents with delivery medium for 4 h and then replaced with fresh medium. After 24 h, transfected cells were used for further studies.

4.5 | RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from NHEKs and skin samples from psoriasis patients with healthy controls using Direct-zol RNA Microprep (Zymo Research) and cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). Gene expression was quantified using THUNDERBIRD SYBR qPCR Mix (TOYOBO). GAPDH gene expression was used as an internal control and the relative expression levels of each gene were determined by the $2^{-\Delta\Delta CT}$ method. The primer sequences used were listed in Table 2.

TABLE 2 Sequences of the primers used for quantitative real-time PCR.

Gene	Forward	Reverse
AXL	5′- GTGGGCAACCC GGGAATATC- 3′	5′- GTACTGTC CCGTCG GAAG- 3′
IFNB1	5′- ATGACCAACAA GTGTCTCCTCC- 3′	5′- GGAATCCA AGCAAGTTG TAGCTC- 3′
CXCL8	5′- ACTGAGAG TGATTGAGA GTGGAC- 3′	5′- AACCCTCT GCACCCAGT TTTC- 3′
TNFA	5′- GAGGCCAAGCC CTGGTATG- 3′	5′- CGGGCCGA TTGATC TCAGC- 3′
IL36G	5′- AGGAAGGGCCG TCTATCAATC- 3′	5′- CACTGTCA CTTCGTGGA ACTG- 3′
GMCSF	5′- TCCTGAACCTG AGTAGAGACAC- 3′	5′- TGCTGCTT GTAGTG GCTGG- 3′
S100A7	5′- ACGTGATG ACAAGATTG ACAAGC- 3′	5′- GCGAGGTA ATTTGTGCC CTTT- 3′
S100A8	5′- ATGCCGTCTAC AGGGATGAC- 3′	5′- ACTGAGGA CACTCGGTC TCTA- 3′
S100A9	5′- GGTCATAG AACACATCA TGGAGG- 3′	5′- GGCCTGGC TTATGG TGGTG- 3′
DEFB4	5′- CTCCTCTTCTCGTT CCTCTTCA- 3′	5′- GCAGGTAA CAGGATCGC CTAT- 3′
CCL20	5′- TGCTGTACCAA GAGTTTGCTC- 3′	5′- CGCACACA GACAACTTT TTCTTT- 3′

4.6 | SDS-polyacrylamide gel electrophoresis and immunoblotting

Proteins from NHEKs were extracted using RIPA lysis buffer (Santa Cruz Biotechnology). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to the Immobilon-P transfer membrane (Millipore). The membrane was blocked in 5% non-fat milk buffer for 1 h at room temperature and then incubated with primary antibodies against β-actin (Santa Cruz Biotechnology), AXL (Cell Signaling Technology), and GAS6 (R&D Systems) at 4°C overnight, followed by incubation with the appropriate secondary antibodies (Proteintech) for 1 h at room temperature. Visualization was performed with enhanced chemiluminescence technology (Thermo Fisher Scientific).

4.7 | Enzyme-linked immunosorbent assay

The protein concentrations of cell culture supernatants from NHEKs were measured using the enzyme-linked immunosorbent assay (ELISA) kit for AXL (R&D Systems). The experiment was performed according to the manufacturer's instructions.

4.8 | Bromodeoxyuridine cell proliferation assay

The proliferation of cultured HaCaT cells was evaluated by a BrdU cell proliferation ELISA kit (Abcam). Briefly, cells were plated in a 96-well plate (Iwaki) at a density of 1×10^5 cells per well. After overnight growth, cells were transfected with the indicated siRNA (Thermo Fisher Scientific) and followed by stimulation with EGF (R&D Systems) or TGF-α (PeproTech) for 48 h. At the end of treatment, BrdU incorporation was measured according to the manufacturer's instructions.

4.9 | Statistical analysis

The data are presented as the mean ± standard deviation (SD). Statistical calculations were performed using GraphPad Prism version 8 (GraphPad Software Inc). For human sample analyses, Mann–Whitney *U*-test or Wilcoxon signed-rank test for two-group

comparison and Kruskal Wallis test for multiple-group comparison were conducted. Spearman's rank correlation coefficient was used for the evaluation of correlations between two continuous variables. Data were presumed to be normally distributed for in vitro experiments. One-way analysis of variance (ANOVA) was performed and multiple comparisons were corrected using Dunn's post hoc test for multiple-group comparison. Significant differences are illustrated as $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, and $^{****}p<0.0001$.

AUTHOR CONTRIBUTIONS

Yukiko Ito, Sayaka Shibata and Shinichi Sato designed the experiments. Yukiko Ito and Sayaka Shibata wrote the manuscript. Yukiko Ito performed most of the experiments and analyzed the data, with contributions from Asumi Koyama, Lixin Li, Eiki Sugimoto, Haruka Taira, Yuka Mizuno, and Kentaro Awaji. Sayaka Shibata and Shinichi Sato supervised the project. All authors discussed the results and commented on the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare. Dr. Shinichi Sato is a member of the Journal of Cutaneous Immunology and Allergy Editorial Board. Management of the peer review process, and all editorial decision-making, for this article was undertaken by Editor in Chief.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

Approval of the research protocol: The medical ethics committee of the University of Tokyo approved all described studies (No. 0695–22) the study was conducted according to the principles of the Declaration of Helsinki.

Informed Consent: All patients were provided written informed consent.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: N/A.

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