Clin Exp Dermatol 2022; **00**: 1–12
https://doi.org/10.1093/ced/llac069
Advance access publication date: 11 November 2022

CED
Clinical and Experimental Dermatology
Original Article

Interleukin-18 as a severity marker and novel potential therapeutic target for epidermolytic ichthyosis

Osamu Ansai,[1] Toshinari Miyauchi,[2] Ryota Hayashi,[1] Tatsuya Katsumi,[1] Tomoki Nishiguchi,[1] Akito Hasegawa,[1] Satoru Shinkuma,[3] Ken Natsuga,[2] Toshifumi Nomura,[4] Yutaka Shimomura[5] and Riichiro Abe[1]

[1]Division of Dermatology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
[2]Department of Dermatology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Japan
[3]Department of Dermatology, Nara Medical University, Kashihara, Japan
[4]Department of Dermatology, Faculty of Medicine, University of Tsukuba, Tsukuba, Japan
[5]Department of Dermatology, Yamaguchi University Graduate School of Medicine, Ube, Japan

Correspondence: Riichiro Abe, Division of Dermatology, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Dori, Asahi machi, Chuo-ku, Niigata City, Niigata, 951-8510, Japan Email: aberi@med.niigata-u.ac.jp

OA and TM contributed equally to this work and should be considered joint first authors.

RH and RA contributed equally to this work and should be considered joint corresponding authors.

Abstract

Background Epidermolytic ichthyosis (EI) is a major form of nonsyndromic inherited ichthyosis, characterized by erythroderma, marked hyperkeratosis and scale, bulla and erosion at birth, associated with *KRT1/KRT10* mutations. The cytokine and chemokine profiles in EI are poorly understood, and specific treatment options have not been established.

Aim To explore novel biomarkers and therapeutic targets in patients with EI.

Methods We analysed cytokine levels in serum and skin samples from 10 patients with inherited ichthyosis, including seven patients with EI. Wild-type and mutant *KRT1* constructs were established and transfected into HaCaT cells, an immortalized keratinocyte cell line, for *in vitro* immunoblotting and immunocytochemistry analyses.

Results Multiplex cytokine/chemokine analysis revealed that 10 cytokines/chemokines [interleukin (IL)-1β, IL-4, IL-17A, IL-16, IL-18, IL-1 receptor-α, macrophage colony-stimulating factor, interferon-α2, basic fibroblast growth factor and monocyte chemotactic protein-3] were significantly increased in patients with EI. Furthermore, IL-18 levels were significantly higher in patients with EI [$n=7$; 2714.1 (1438.0) pg mL^{-1}] than in healthy controls [$n=11$; 218.4 (28.4) pg mL^{-1}, $P<0.01$]. Immunohistochemical analyses showed that IL-18 expression was elevated in skin samples from patients with EI. Serum IL-18 levels correlated with the severity of ichthyosis, as measured by the Ichthyosis Scoring System. Immunoblotting analysis revealed that mature IL-18 levels were increased in the supernatant of mutant *KRT1* expressing HaCaT cells. Additionally, these cells showed NLRP3 aggregation in the cytoplasm and ASC clustered around mutant keratin aggregations. These findings suggest that mutant keratin might promote the activation of the NLRP3 inflammasome and its downstream caspase-1-mediated IL-18 release in keratinocytes from patients with EI.

Conclusions Our results suggest that serum IL-18 is a severity marker released from the skin of patients with EI. Blockade of IL-18 may be a useful novel therapeutic option for patients with EI.

Introduction

Inherited ichthyosis is a heterogeneous group of genodermatoses characterized by generalized hyperkeratosis, dry skin and scales. Several ichthyosis subtypes are often associated with erythroderma.[1,2] Epidermolytic ichthyosis (EI; OMIM 113800), a major form of inherited ichthyosis, is characterized by erythroderma, marked scales, bullae and erosions at birth. EI is also classified as keratinopathic ichthyosis, and is mainly caused by heterozygous *KRT1* or *KRT10* genes encoding keratin (K)1 or K10, respectively.[1,2] Keratins are intermediate filament proteins expressed in epithelial cells and are divided into two groups: acidic Type I (K9–28, K31–40) and basic Type II (K1–8, K71–86). Keratins form heterodimers between these two groups in a tissue-dependent and differentiation-dependent manner.[3,4] Type II keratin K1 and Type I keratin K10 are the major keratins expressed in the suprabasal keratinocytes of the epidermis.[4] Furthermore, keratin is a crucial structural protein and its mutations are associated with keratin-related diseases, as they cause structural instability in keratinocytes. However, recent studies have also suggested that keratins regulate immune responses,[5–8] and an imbalance in the immune response may be an important pathomechanism in keratin-related diseases, such as EI.

Inherited ichthyosis, including EI, negatively affects patient quality of life.[9–11] There is no specific or effective treatment option for ichthyosis; treatment is limited to topical emollients, keratolytic agents and oral retinoids. Therefore, effective therapeutic options are needed to reduce the

Accepted: 9 November 2022

psychological and economic burden of the disease.[11,12] As biologics have recently been widely used as safe and effective treatments for several inflammatory skin diseases, such as atopic dermatitis (AD) and psoriasis,[13,14] elucidation of the systemic immunological profile of the disease may aid the expansion of therapeutic targets. Several recent studies have analysed immunophenotypes in the skin of patients with inherited ichthyosis and focused on T helper (Th)17-related cytokines as a common signature across the major orphan forms of ichthyosis.[15,16] Based on these findings, a clinical trial of secukinumab, an anti-interleukin (IL)-17 monoclonal antibody, was recently conducted against several subtypes of inherited ichthyosis, including EI, autosomal recessive congenital ichthyosis (ARCI) and Netherton syndrome (NS), but did not show significant efficacy.[17] These results suggest that the immunological features may not be consistent across ichthyosis subtypes with diverse backgrounds. Investigations focusing on individual subtypes are needed to identify specific therapeutic targets.

We analysed sera from patients with inherited ichthyosis (whose immunological profiles are not well understood), including EI, to investigate novel severity markers and therapeutic targets of EI. Several proinflammatory cytokines were found to be significantly elevated in patients with EI and we focused on the importance of IL-18, which is not well understood in the pathomechanism of EI.

Methods

Patients

Serum and skin samples of patients with ichthyosis were collected from Niigata University Medical and Dental Hospital and Hokkaido University Hospital. The diagnosis and classification of inherited ichthyosis were performed by board-certified dermatologists according to international criteria.[1] The severity of ichthyosis was assessed using the Ichthyosis Scoring System (ISS), a recently developed ichthyosis severity measurement tool.[18] In addition to the total ISS score, the combined scale or erythema scores for each site were evaluated and designated as ISS-Scale (ISS-S) and ISS-Erythema (ISS-E) scores, respectively. Serum samples from healthy volunteers without any dermatological disorders were collected as healthy controls (HCs) and patients with AD of various severities were also collected as controls.

Cytokine and chemokine measurement by multiplex bead assay kit

The serum levels of 48 cytokines and chemokines were measured (Bio-Plex Pro Human Cytokine Screening Panel, 48-Plex; Bio-Rad Laboratories, Hercules, CA, USA) as described previously,[19] and data acquisition of plates with fluorescent beads was performed (Bio-Plex 200 system; Bio-Rad Laboratories). Standards and serum samples were measured in duplicate. Values below the detection limit were rated as 0 and those above were rated as the value of the detection limit. Data analysis was performed using Bio-Plex Manager Software (V6.1; Bio-Rad Laboratories) to calculate concentrations in pg mL^{-1}.

Measurement of serum interleukin-18 levels by ELISA

Serum IL-18 levels were measured by sandwich ELISA (Human IL-18 ELISA kit; MBL, Tokyo, Japan) according to the manufacturer's instructions. Standards and serum samples were measured in duplicate. Absorbance was measured at 450 nm using a microplate reader (iMark; Bio-Rad Laboratories). The concentration of IL-18 was calculated from standard curves generated using Microplate Manager software (V6; Bio-Rad Laboratories).

Immunohistochemical staining

Immunohistochemistry of formalin-fixed paraffin-embedded sections was performed according to standard procedures. Antigen retrieval with citrate buffer (pH 6.0) was performed on the dewaxed sections. Mouse monoclonal anti-IL-18 antibody (clone25-2G, 1 : 200, MBL) was used as the primary antibody. A prediluted, horseradish peroxidase (HRP)-labelled antibody (Dako EnVision + System-HRP-labelled polymer antimouse; Agilent Technologies, Santa Clara, CA, USA) was used as the secondary antibody. Colour development was performed using HRP and 3,3′-diaminobenzidine.

Statistical analyses

Statistical analysis and visualization were performed using R statistical software (V4.1.3; https://www.r-project.org) with the R package ggplot2.[20] A significant difference in multiplex cytokine measurement between HCs and EI was detected using the Mann–Whitney *U* test. Comparison was performed of serum IL-18 concentrations between HCs, patients with AD, patients with non-EI ichthyosis and patients with EI, using Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn–Bonferroni test to detect significant differences between each group. Correlations with clinical severity were assessed using the Spearman rank correlation test. Differences were considered statistically significant at $P < 0.05$.

Results

Patients and healthy controls

We obtained sera from 10 patients with inherited ichthyosis (seven with EI, two with ARCI and one with NS), 7 patients with AD and 11 HCs. The clinical and genetic characteristics of patients with inherited ichthyosis are summarized in Table 1.

Multiplex cytokine/chemokine analysis revealed increased levels of proinflammatory cytokines and chemokines in the serum of patients with epidermolytic ichthyosis

To investigate the changes in immunological status in patients with EI, we performed a multiplex cytokine/chemokine analysis of 48 cytokines and chemokines in serum samples from 7 patients with EI (Table 1) and 7 HCs (Table 2). (IL)-1β, IL-4, IL-17A, IL-16, IL-18, IL-1 receptor-α,

Table 1 Clinical and genetic summary of the patients with epidermolytic ichthyosis

Patient	Age, years	Sex	Ichthyosis subtype	Causative gene	Mutation	Mutation type	ISS
1	20	Male	EI	KRT1	p.Glu490Gly	Heterozygous	6.42
2	35	Female	EI	KRT1	p.Asp188Lys	Heterozygous	5.98
3	59	Male	EI	KRT1	p.Leu484Arg	Heterozygous	4.35
4	56	Male	EI	KRT1	p.Glu490Lys	Heterozygous	4.89
5	28	Female	EI	KRT10	p.Arg156Cys	Heterozygous	5.16
6	15	Male	EI	KRT1	p.Leu208Pro	Heterozygous	0.57
7	32	Male	EI	KRT1	p.Glu478Gln	Heterozygous	0.56
8	19	Male	NS	SPINK5	p.Thr125fs/p.Glu948Ter	Compound heterozygous	4.79
9	35	Female	ARCI	TGM1	p.Asn125Thrfs	Homozygous	5.10
10	68	Male	ARCI	ABCA12	p.Arg1514His	Homozygous	2.60

ARCI, autosomal recessive congenital ichthyosis; EI, epidermolytic ichthyosis; ISS, ichthyosis severity score; NS, Netherton syndrome.

macrophage colony-stimulating factor, interferon (IFN)-α2, basic fibroblast growth factor and monocyte chemotactic protein-3 levels were all significantly increased in the serum of patients with EI (Figure 1).

Interleukin-18 is increased in both serum and skin samples of patients with epidermolytic ichthyosis

As K1 ablation reportedly caused a prenatal increase in IL-18 and impaired the inflammatory network in murine keratinocytes,[8] we focused on IL-18 among the cytokines our multiplex analysis had shown to be increased. To confirm increased serum IL-18, ELISA was used to measure IL-18 in seven patients with EI, five patients with other inherited ichthyoses (two with ARCI and one with NS), seven patients with AD and 11 HCs (Figure 2a). The EI group [2714.1 (1438.0)] showed significantly higher serum IL-18 levels than the HC group [218.4 (28.4), $P<0.01$]. No significant differences were observed in the non-EI ichthyosis group [237.4 (73.6)] compared with the HC group. The AD group [402.7 (93.0)] showed a trend towards higher IL-18 levels than the HC group; however, the difference was not statistically significant. Interestingly, serum IL-18 is reportedly a severity marker of AD.[21,22]

We further performed immunohistochemical staining of IL-18 in skin samples from EI, non-EI and HCs (Figure 2b–e, Supplementary Figure S1). HCs had only weak positivity in the basal layer and no expression in suprabasal keratinocytes, whereas patients with EI showed strong positivity in the entire layer of the epidermis. Patients with ARCI showed a similar pattern to HCs, while patients with NS only had weak positivity throughout the whole epidermis.

These findings suggest that IL-18 levels increase in both the epidermis and serum of patients with EI and might be a disease-specific marker of EI. Furthermore, immunohistochemical staining suggested that IL-18 was secreted by the epidermal keratinocytes of patients with EI.

Interleukin-18 levels correlate with clinical severity of epidermolytic ichthyosis

To examine whether serum IL-18 levels could be a marker of severity of EI, we compared serum IL-18 levels with ISS scores.[18] First, the EI group was classified by ISS into three subgroups [mild (ISS 0–2.0), moderate (ISS 2.0–5.0) and severe (ISS 5.0–8.0)],[18] and IL-18 levels were compared between subgroups (Figure 3a). The severe subgroup [5490.6 (2763.3)] had significantly higher levels of IL-18 than the mild subgroup [272.3 (66.9), $P<0.05$], suggesting that IL-18 levels might correlate with the severity of EI. Next, we examined the correlation between serum IL-18 levels and ISS scores, and found a strong correlation (Figure 3b). Furthermore, the combined scores of scale or erythema at each anatomical site of ISS were calculated as ISS-S or ISS-E, respectively, and the correlations between IL-18 levels and either ISS-S or ISS-E were examined (Figure 3c,d). Both ISS-S and ISS-E showed significant and strong correlations with IL-18 levels. However, ISS-E showed a stronger correlation, suggesting that IL-18 might be associated with the severity of erythema or erythroderma in EI. We further compared ISS with several proinflammatory cytokines, including IL-1β, IL-4 and IL-17A, which were significantly elevated in patients with EI. IL-1β showed no correlation with the severity of ichthyosis, while IL-4 and IL-17A showed relatively weak correlations (Supplementary Figure S2).

One patient (Patient 6) with mild EI showed marked hyperkeratosis localized to the palm, soles and flexural and extensoral areas of his limbs. The mutation in this patient (*KRT1* p.Leu208Pro) has also been reported in systemic EI, suggesting the presence of modifying factors (manuscript in submission). To investigate whether site-specific IL-18 expression correlated with phenotypic differences, immunohistochemical analysis was performed on skin samples from hyperkeratotic and nonhyperkeratotic areas of this patient. The skin sample from the hyperkeratotic area showed that IL-18 expression was upregulated in the whole skin layer, whereas the sample from the nonhyperkeratotic area showed that IL-18 expression was similar to that of HC skin samples (Figure 3e).

Based on these findings, we conclude that serum IL-18 levels are not only a disease-specific marker but also a severity marker for EI.

Mutant keratin 1 enhances mature interleukin-18 secretion from keratinocytes

EI is caused by mutations in *KRT1* or *KRT10*, which are expressed only in suprabasal keratinocytes. Furthermore, keratinocytes are one of the major sources of IL-18 production.[23–25] Therefore, we investigated whether EI-associated *KRT1* mutations could promote IL-18 production in *in vitro* experiments (see Supplementary Data S1 for detailed materials and methods). In addition to generating enhanced green fluorescent protein (EGFP)-tagged constructs harbouring wild-type *KRT1* and two *KRT1* mutations (Table 1) associated with a severe phenotype (p.Glu490Gly; Patient 1)

Table 2 Serum cytokine levels in patients with epidermolytic ichthyosis and healthy controls in Luminex bead-based cytokine/chemokine assays

	Group, median (interquartile range); pg mL^{-1}		
Cytokine	**HC ($n=7$)**	**EI ($n=7$)[a]**	***P*[b]**
IL-1β	1.40 (1.24–2.82)	8.51 (5.01–9.47) ↑	<0.01**
M-CSF	20.03 (16.73–22.42)	129.82 (76.02–193.63) ↑	<0.01**
IL-17A	4.13 (3.02–4.65)	10.91 (8.80–14.18) ↑	0.01*
IFN-α2	0 (0–1.68)	10.39 (8.03–11.97) ↑	0.01*
IL-18	16.39 (0–55.63)	4601.28 (1436.30–8477.69) ↑	0.02*
IL-16	32.33 (30.46–51.37)	86.53 (63.73–110.19) ↑	0.02*
Basic FGF	35.65 (32.38–51.83)	100.67 (65.25–120.85) ↑	0.03*
IL-4	3.25 (2.88–3.45)	4.97 (4.13–5.70) ↑	0.03*
MCP-3	0 (0–0.25)	1.23 (1.03–2.50) ↑	0.03*
IL-1Rα	262.44 (121.80–344.22)	607.19 (484.45–777.41) ↑	0.04*
IL-12 (p40)	16.00 (11.15–24.51)	60.81 (36.90–76.81)	0.06
IL-8	8.53 (6.11–11.74)	12.88 (11.13–14.99)	0.10
IFN-γ	3.99 (2.84–10.97)	12.56 (7.46–22.38)	0.13
MIF	628.9 (411.86–735.74)	936.75 (588.48–1186.40)	0.13
TRAIL	51.35 (0–54.48)	0 (0–18.21)	0.14
TNF-α	36.92 (34.55–44.63)	50.23 (47.70–55.46)	0.14
CTACK	1064.63 (794.27–1131.54)	822.11 (431.07–1004.08)	0.17
IL-9	281.47 (263.68–1606.87)	1863.76 (979.60–2102.53)	0.17
SCF	108.6 (81.32–115.86)	156.60 (90.91–236.69)	0.17
TNF-β	436.70 (395.55–5224.42)	6247.06 (3284.44–7412.78)	0.17
IL-3	0 (0–0)	0 (0–0.025)	0.17
VEGF	0 (0–0.83)	0 (0–0)	0.17
HGF	351.51 (316.13–453.08)	559.75 (387.75–601.69)	0.21
IL-7	12.85 (3.33–16.81)	19.62 (12.10–19.62)	0.24
MIG	155.25 (119.15–202.65)	111.91 (96.17–129.36)	0.26
MIP-1β	236.26 (208.89–1619.89)	2129.38 (1076.20–2227.74)	0.26
SCGF-β	199 646.74 (169 666.18–573 583.05)	570 829.22 (347 780.11–891 201.62)	0.26
GM-CSF	0.37 (0–1.86)	1.8 (1.31–2.52)	0.27
IL-10	2.39 (0.48–5.27)	6.03 (2.83–8.38)	0.34
GRO-α	762.18 (502.13–1233.44)	1404.6 (719.83–1540.60)	0.34
IL-1α	0 (0–4.24)	6.52 (0–18.51)	0.34
β-NGF	0 (0–0)	0 (0–0)	0.39
IL-12 (p70)	0 (0–0)	0 (0–0)	0.39
IL-15	0 (0–0)	0 (0–0)	0.39
RANTES	14 175.91 (10 116.69–15 516.19)	15 516.19 (12 739.61–15 516.19)	0.44
PDGF-BB	9909.81 (2477.07–16366.73)	18 712.6 (3234.71–22 055.26)	0.46
IL-6	0.09 (0–0.55)	0.57 (0.08–1.415)	0.47
SDF-1α	1527.58 (976.03–6766.38)	6766.38 (3823.47–6766.38)	0.52
LIF	4.59 (0–9.02)	5.76 (0–80.09)	0.55
IL-2	0 (0–0)	0 (0–0.15)	0.59
Eotaxin	103.09 (78.36–123.35)	138.67 (85.97–231.05)	0.62
MCP-1	38.41 (28.97–49.64)	46.51 (38.28–72.40)	0.70
IP-10	347.67 (267.51–370.90)	351.48 (219.90–373.58)	0.71
IL-5	0 (0–12.62)	0.19 (0–5.92)	0.79
IL-2α	54.03 (52.61–58.62)	92.22 (47.19–95.52)	0.81
G-CSF	48.72 (14.40–67.33)	21.72 (19.79–27.19)	0.85
IL-13	1.60 (1.09–6.54)	1.92 (1.29–6.68)	0.90
MIP-1α	3.39 (1.69–3.70)	2.72 (2.19–3.64)	1.00

CTACK, cutaneous T cell-attracting chemokine; EI, epidermolytic ichthyosis; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; GRO, growth regulated oncogene; HC, healthy control; IL, interleukin; IFN, interferon; IP, IFN gamma-induced protein; LIF, leukaemia inhibitor factor; MCP, monocyte chemoattractant protein; M-CSF, macrophage colony-stimulating factor; MIF, macrophage migration inhibitory factor; MIG, monokine induced by interferon-gamma; MIP, macrophage inflammatory protein; NGF, nerve growth factor; PDGF-BB, platelet-derived growth factor BB; RANTES, regulated upon activation, normal T cell expressed and presumably secreted; SCF, stem cell factor; SCGF, stem cell growth factor; SDF, stromal cell-derived factor; sIL-2R, soluble interleukin-2 receptor; TNF, tumour necrosis factor; TRAIL, TNF related apoptosis-inducing ligand; VEGF, vascular endothelial growth factor. [a]Cytokines that were significantly elevated compared with healthy controls are indicated by ↑; [b]significant differences between two groups are detected by Mann–Whitney *U* test; $P<0.05$ was considered statistically significant. *$P<0.05$, **$P<0.01$.

and a mild phenotype (p.Leu208Pro; Patient 6), we generated EGFP-tagged *KRT1* constructs associated with the other two mutations (p.Asn188Ser and p.Ile479Thr) located on the helix initiation motif and helix termination motif, which are hotspots for keratin mutations. The wild-type and mutant K1 constructs were transiently transfected into HaCaT cells and analysed. Confocal microscopic observation showed that mutant K1 caused small ball-like aggregations in HaCaT cells, whereas wild-type K1 formed filament structures (Figure 4a).

Next, we performed immunoblotting analysis of cell lysates and supernatants from transfected cells to assess

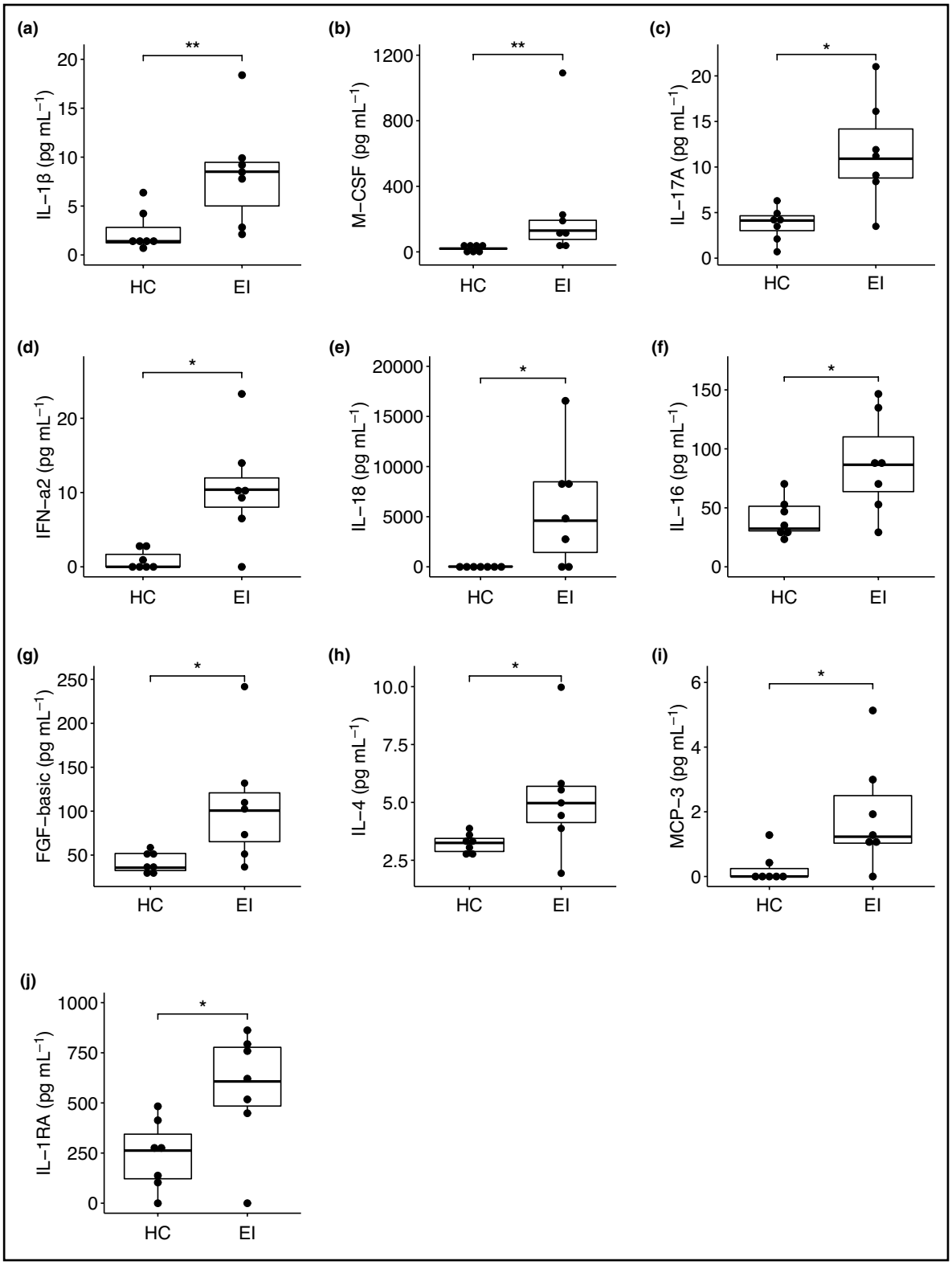

Figure 1 (a–j) Multiplex cytokine/chemokine analysis in patients with epidermolytic ichthyosis (EI) and healthy control (HC) individuals. Serum levels of (a) interleukin (IL)-1β; (b) macrophage colony-stimulating factor (M-CSF); (c) IL-17A; (d) interferon-α2; (e) IL-18; (f) IL-16; (g) basic fibroblast growth factor (FGF-basic); (h) IL-4; (i) monocyte chemotactic protein (MCP)-3; and (j) IL-1RA in the HC and EI groups. $*P<0.05$, $**P<0.01$.

IL-18 secretion by mutant K1-expressing HaCaT cells. IL-18 is cleaved from pro-IL-18 (approximately 22 kDa) by caspase-1 to form mature IL-18 (18 kDa) during activation;[26] caspase-1 is also found as a precursor form (45 kDa) and an active cleaved form (cleaved caspase-1; 20 kDa). Immunoblotting analysis showed that EGFP-tagged K1 was expressed in the lysates

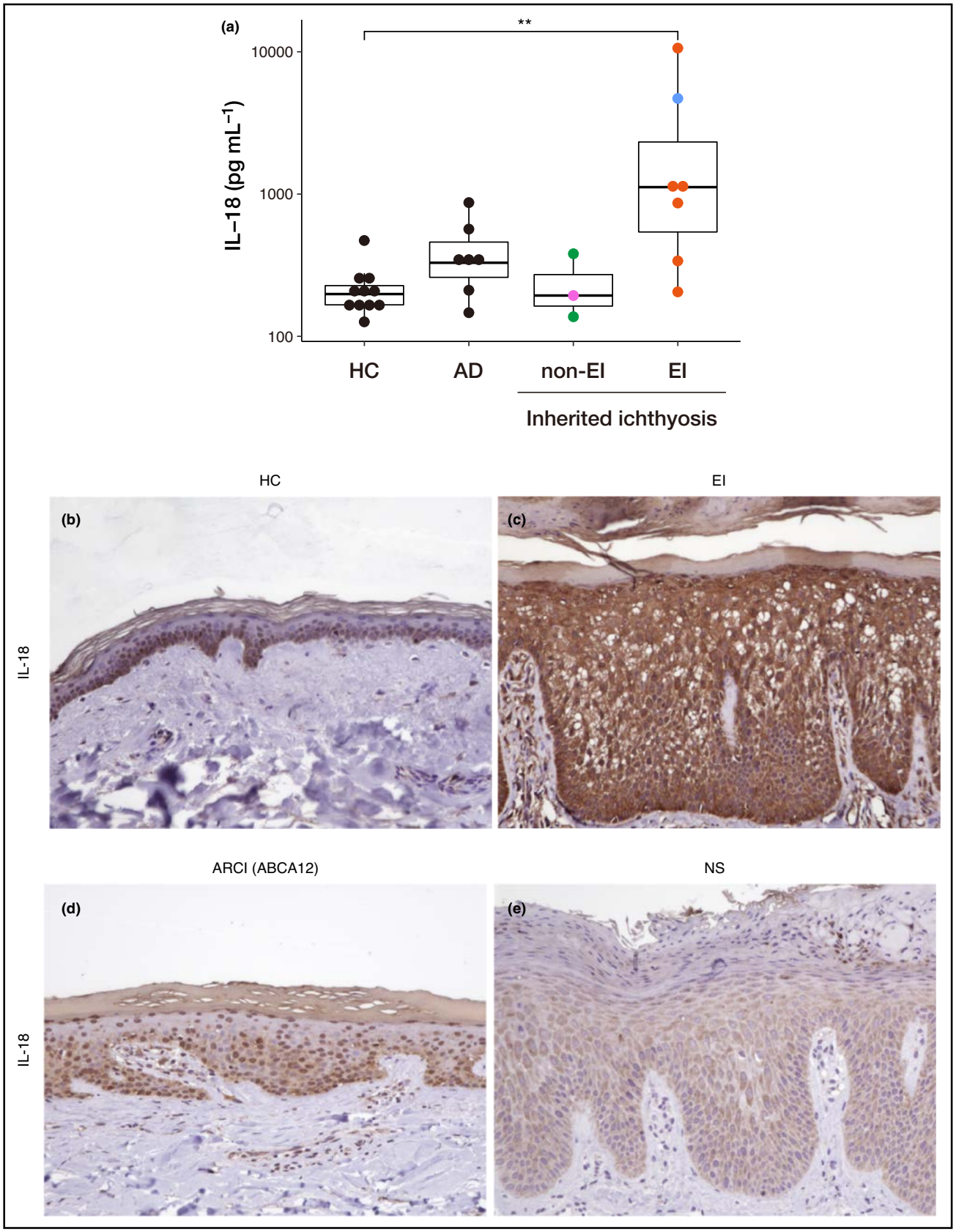

Figure 2 (a–e) Interleukin (IL)-18 levels in the serum and skin samples of patients with epidermolytic ichthyosis (EI). (a) Serum IL-18 levels in healthy control (HC) individuals, patients with atopic dermatitis, patients with non-EI and patients with EI were analysed by ELISA. Mutations are coloured: patients with EI associated with *KRT1* (orange) and *KRT10* (blue) mutations; patients with non-EI: Netherton syndrome (NS, magenta) and autosomal recessive congenital ichthyosis (ARCI, green). Statistical analysis was performed using Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn–Bonferroni test $^{**}P < 0.01$. (b–e) Immunohistochemical staining for IL-18 in (b) healthy controls; (c) EI (Patient 1); (d) NS (Patient 8); and (e) ARC (Patient 10). Original magnification (b–e) × 200.

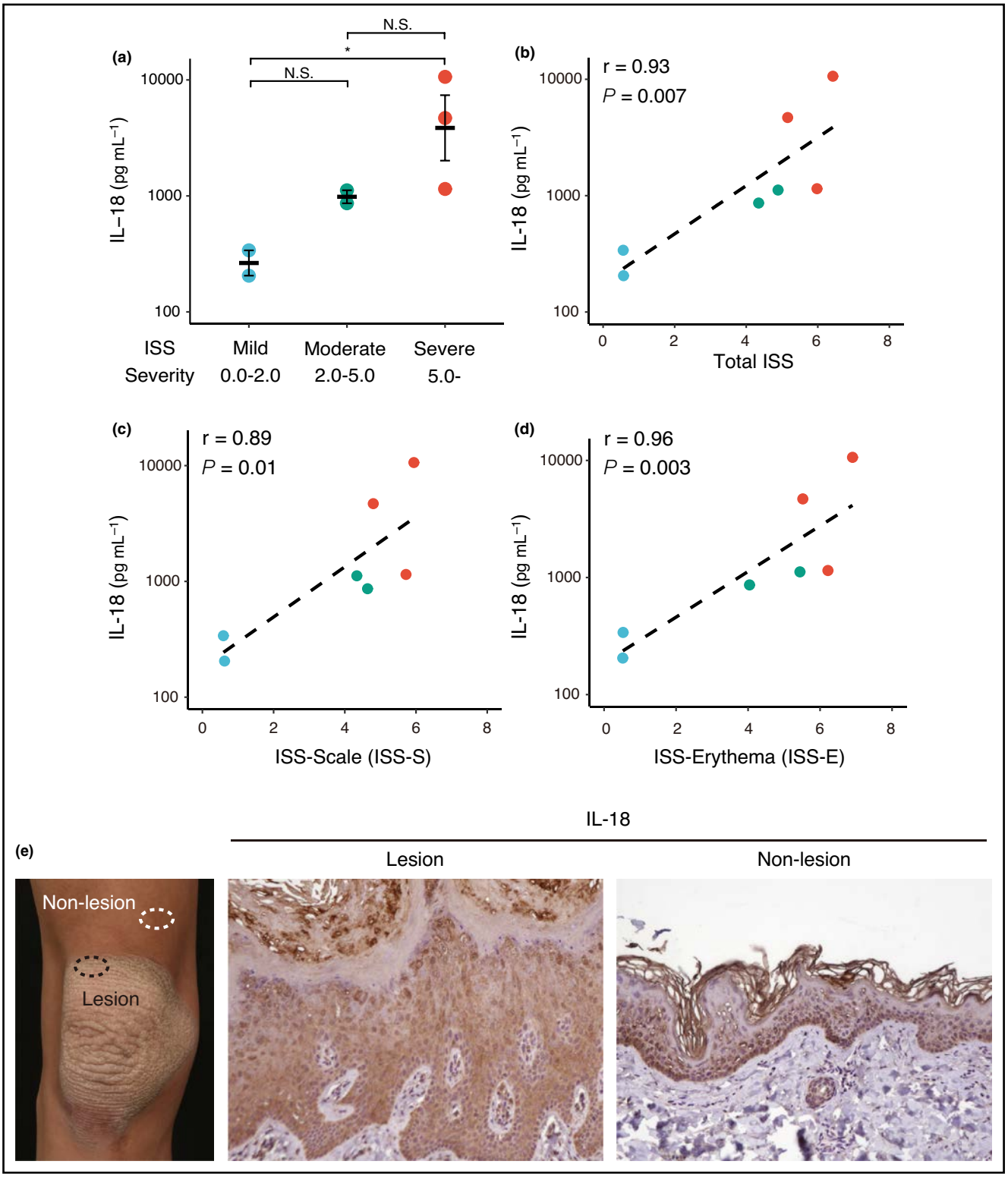

Figure 3 (a–e) Interleukin (IL)-18 correlates with the severity of epidermolytic ichthyosis (EI). (a) Comparison of serum IL-18 levels across subgroups classified by ichthyosis severity as measured by the Ichthyosis Scoring System: mild (blue) moderate (green) and severe (red) subgroups. Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn–Bonferroni test, $*P<0.05$. (b–e) Serum IL-18 levels correlated with (b) total ISS scores; (c) ISS-Scale (ISS-S) scores; and (d) ISS-Erythema (ISS-E) scores. Correlations were assessed using Spearman rank correlation test. (e) Clinical manifestation and immunohistochemical staining for IL-18 in the skin samples from the hyperkeratotic and nonhyperkeratotic areas of a patient with mild EI (Patient 6) (original magnification × 200). The locations of the biopsy are indicated by dashed circles in the clinical picture.

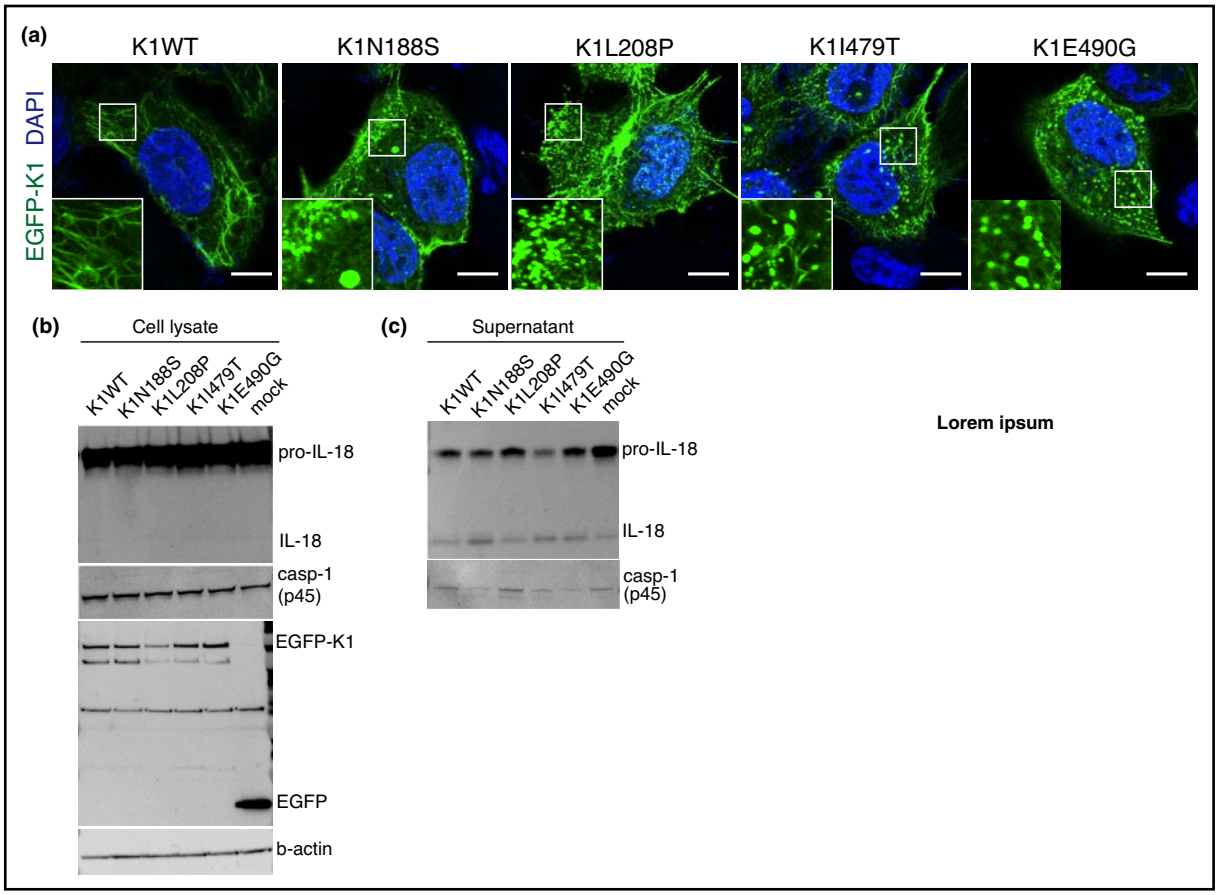

Figure 4 (a–e) Mutant keratin (K)1 expression promotes interleukin (IL)-18 release from keratinocytes. (a) Confocal microscopy of enhanced green fluorescent protein (EGFP)-tagged wild-type and mutant K1-transfected HaCaT cells (scale bars = 10 μm). (b,c) Immunoblotting analysis of IL-18 and caspase (casp)-1 in (b) cell lysate and (c) supernatant of wild-type and mutant K1-transfected HaCaT cells. DAPI, 4′,6-diamidino-2-phenylindole.

of transfected cells (Figure 4b). The amount of mature IL-18 in the supernatant was higher in mutant K1-expressing cells than in either the wild-type or mock-transfected cells (Figure 4c). However, as in previous research on human primary keratinocytes,[8] cleaved caspase-1 (p20; 20 kDa form) could not be detected in either lysate or supernatant and only the premature form (45 kDa) could be detected.

The NLRP3 inflammasome is activated in mutant keratin 1-expressing keratinocytes

As the activation and release of IL-18 are mediated by the activation of the NLRP3 inflammasome and its downstream caspase-1,[8,25–27] we examined the NLRP3 inflammasome in EGFP-K1-transfected HaCaT cells. NLRP3 aggregated in the cytoplasm of mutant K1-expressing HaCaT cells (Figure 5a). In addition, after inflammasome activation, ASC, an adapter protein associated with the NLRP3 inflammasome, assembles into a large protein complex, which is termed 'speck'.[28,29] Interestingly, immunofluorescent staining of ASC showed 'speck'-like assemblies around mutant K1 aggregations (Figure 5b).

Discussion

In this study, we performed multiplex cytokine/chemokine analysis and found that several proinflammatory cytokines, including IL-1β, IL-4, IL-17, IL-18, MCP-3 and M-CSF, were elevated in patients with EI. Recent studies have clarified the immune profiles of patients with inherited ichthyosis. In particular, gene expression profiles of skin samples analysed using quantitative reverse transcription-PCR, and the immunophenotyping profiles of peripheral blood cells showed that a Th17 dominant profile is shared across various types of ichthyosis.[15,16,30] Although increased levels of several proinflammatory cytokines (tumour necrosis factor-α, IL-1β, IL-2 and IL-18) have been reported in patients with NS, which often present with immunodeficiency symptoms,[31,32] similar proinflammatory cytokine profiles are poorly understood in EI.

We found that IL-18 is specifically elevated in both the serum and skin of patients with EI, and that serum IL-18 levels correlate with the severity of EI. IL-18 is a proinflammatory cytokine that facilitates IFN-γ production by Th1 cells, and was initially described as 'IFN-γ inducing factor'.[33,34] Human keratinocytes constitutively express IL-18, and dysregulation of the IL-18 pathway has been reported in several dermatological diseases.[35,36] Serum IL-18 levels are elevated in patients with adult-onset Still disease (AOSD), and IL-18 is considered to be an established biomarker for the diagnosis of AOSD.[37–43] In addition, IL-18 has been proposed as a disease activity marker in various cutaneous diseases, such as cutaneous lupus erythematosus,[44] dermatomyositis,[45] psoriasis,[46] AD[22] and autoinflammatory diseases.[47,48] However, there is no significant evidence regarding serum or skin levels of IL-18 in EI or keratin-related genodermatoses, and our

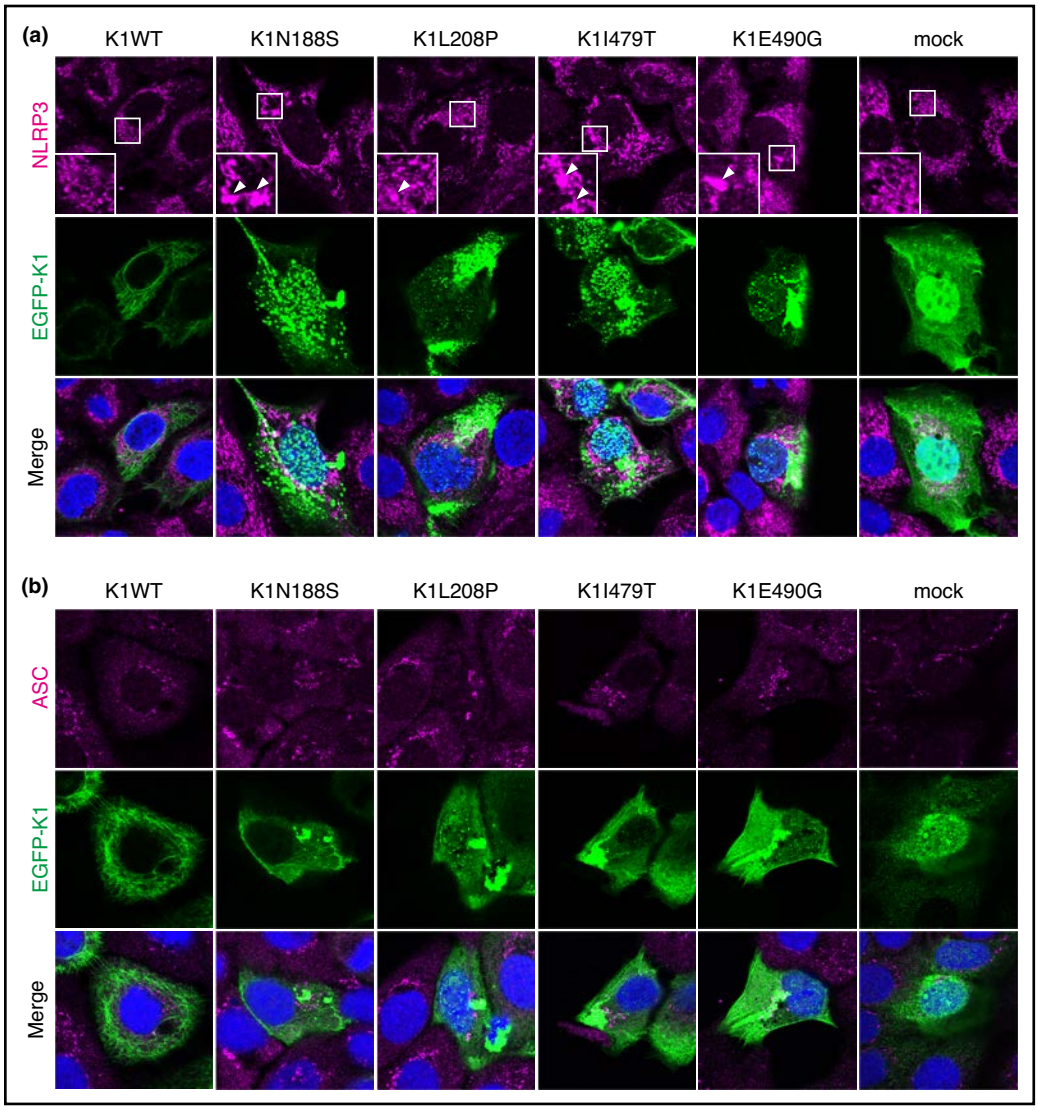

Figure 5 (a–e) Mutant keratin aggregations activate the NLRP3 inflammasome in keratinocytes. (a,b) Immunofluorescence staining for (a) NLRP3 and (b) ASC in wild-type and mutant K1-transfected HaCaT cells. Arrowheads indicate cytoplasmic NLRP3 aggregation Scale bars = 10 μm.

research provides new insights into the important role of IL-18 in these diseases.

Recently, as the detailed molecular pathology of ichthyosis has been clarified, clinical trials of novel therapies, such as biologics, small molecules and gene therapies, have been conducted.[49] Biologics, represented by specific monoclonal antibodies targeting cytokines and their receptors, have recently become available in a wide range of areas to regulate various pathways. In inherited ichthyosis, several case reports or series have shown that biologics, including

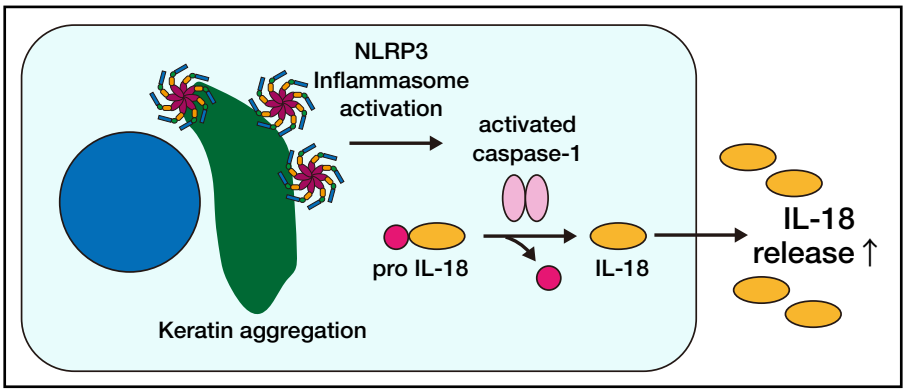

Figure 6 Graphical summary of this study. IL, interleukin.

infliximab,[50] dupilumab,[51–53] ustekinumab,[54] secukinumab[55] and ixekizumab[56] are effective for NS. Clinical trials of secukinumab in patients with inherited ichthyosis, such as EI, ARCI and NS, were recently reported to have failed to show significant effects.[17] However, other clinical trials of biologics for ichthyosis are still ongoing. Moreover, IL-18 is elevated in *krt1* knockout mice, which form aggregations of K10 and exhibit morphological features similar to those of patients with EI. The phenotype of *krt1* knockout mice was rescued by pharmacological and genetic ablation of IL-18.[8] These findings indicate that the phenotypes of EI caused by mutant keratin aggregation can also be rescued by IL-18 blockade. In fact, a human recombinant IL-18-binding protein (tadekinig alfa) has been developed as an IL-18 antagonist, and has been used in clinical trials on patients with AOSD.[57] Tadekinig alfa had demonstrated favourable safety and efficacy in AOSD, and might be one of the therapeutic options expected to be expanded for patients with EI.

Our data also suggest that mutant keratins associated with EI might promote IL-18 release from keratinocytes via activation of the NLRP3 inflammasome (Figure 6). Keratin-related diseases, such as epidermolysis bullosa simplex (EBS) and EI, are believed to be caused by the disruption of keratin filaments, one of the crucial cytoskeletal proteins in keratinocytes.[58] Meanwhile, keratins reportedly regulate the immune system, particularly innate immunity.[8,5,7] Aberrant distribution of NLRP3 has been reported in K1 knockout keratinocytes,[8] and K8 in colonic epithelia and vimentin in alveolar macrophages have been reported to regulate the NLRP3 inflammasome.[59,60] Our study suggests that mutant keratin aggregations also affect NLRP3 distribution and function. Furthermore, we found that ASC clustered around keratin aggregates, suggesting that mutant keratin may directly activate the inflammasome and its downstream proinflammatory cytokines.

Interestingly, Patient 6, who had mild EI, showed localized hyperkeratosis had markedly increased IL-18 in the skin sample from hyperkeratotic areas, whereas samples from non-hyperkeratotic areas had a similar pattern to HCs and mildly elevated serum IL-18 levels. Genotype–phenotype correlation has been observed in patients with EBS,[58,61] which is caused by *KRT5/14* mutations, but it is less clear in diseases associated with *KRT1*/*KRT10* mutations, including EI. The same *KRT1* mutation could result in clinical phenotypic variations, indicating that some modifying factors might exist in KRT1/10-associated keratinizing diseases.[62] The correlation between systemic or cutaneous IL-18 expression and clinical phenotypes of EI led us to hypothesize that the profiles of proinflammatory cytokines, including IL-18, might be a crucial factor in determining the phenotypes of EI and other KRT1/KRT10-associated keratinizing diseases.

Conclusion

In this study, we showed that increased IL-18 levels are a severity marker of EI and that mutant keratin aggregations could promote IL-18 release from keratinocytes via NLRP3 inflammasome activation. We therefore propose that IL-18 blockade may become a crucial therapeutic option for EI in the near future.

What is already known about this topic?

- EI is a major form of nonsyndromic inherited ichthyosis and associated with *KRT1* or *KRT10* mutations.
- As immunophenotypes in the skin of patients with inherited ichthyosis have been gradually revealed through research, clinical trials with biologics have been conducted.
- Specific treatment options for EI have not been established.

What does this study add?

- Serum IL-18 may be a severity marker released from the skin of patients with EI.
- Mutant K1 associated with EI might promote activation of the NLRP3 inflammasome and downstream caspase-1-mediated IL-18 release.
- This study provides the detailed cytokine profiles of patients with EI, and indicates that IL-18 blockade may be a crucial future therapeutic option for EI.

Acknowledgement

We thank Editage (www.editage.com) for English language editing.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This work was supported in part by a research grant (no. J20G0146) from Niigata Medical Association to RH.

Ethics statement

Ethics approval: the study was approved by the Human Research Ethics Committees of Niigata University Medical and Dental Hospital (approval number: 2022-0018) and the Institutional Review Board of the Hokkaido University Graduate School of Medicine (approval number: 14-063) and was conducted in accordance with the principles of the Declaration of Helsinki. Informed consent: written informed consent was obtained from all subjects for participation and for publication of their case details and images.

Data availability

Data are available on request from the corresponding author.

References

1 Oji V, Tadini G, Akiyama M *et al.* Revised nomenclature and classification of inherited ichthyoses: results of the First Ichthyosis Consensus Conference in Sorèze 2009. *J Am Acad Dermatol* 2010; **63:**607–41.
2 Takeichi T, Akiyama M. Inherited ichthyosis: non-syndromic forms. *J Dermatol* 2016; **43:**242–51.

3 Coulombe PA, Omary MB. 'Hard' and 'soft' principles defining the structure, function and regulation of keratin intermediate filaments. *Curr Opin Cell Biol* 2002; **14:**110–22.
4 Moll R, Divo M, Langbein L. The human keratins: biology and pathology. *Histochem Cell Biol* 2008; **129:**705–33.
5 Lessard JC, Piña-Paz S, Rotty JD *et al.* Keratin 16 regulates innate immunity in response to epidermal barrier breach. *Proc Natl Acad Sci U S A* 2013; **110:**19537–42.
6 Chung BM, Arutyunov A, Ilagan E *et al.* Regulation of C-X-C chemokine gene expression by keratin 17 and hnRNP K in skin tumor keratinocytes. *J Cell Biol* 2015; **208:**613–27.
7 Kumar V, Behr M, Kiritsi D *et al.* Keratin-dependent thymic stromal lymphopoietin expression suggests a link between skin blistering and atopic disease. *J Allergy Clin Immunol* 2016; **138:**1461–4.e6.
8 Roth W, Kumar V, Beer H-D *et al.* Keratin 1 maintains skin integrity and participates in an inflammatory network in skin via interleukin-18. *J Cell Sci* 2012; **125:**5269–79.
9 Gånemo A, Lindholm C, Lindberg M *et al.* Quality of life in adults with congenital ichthyosis. *J Adv Nurs* 2003; **44:**412–19.
10 Gånemo A, Sjöden P-O, Johansson E *et al.* Health-related quality of life among patients with ichthyosis. *Eur J Dermatol* 2004; **14:**61–6.
11 Mazereeuw-Hautier J, Dreyfus I, Barbarot S *et al.* Factors influencing quality of life in patients with inherited ichthyosis: a qualitative study in adults using focus groups. *Br J Dermatol* 2012; **166:**646–8.
12 Styperek AR, Rice ZP, Kamalpour L *et al.* Annual direct and indirect health costs of the congenital ichthyoses. *Pediatr Dermatol* 2010; **27:**325–36.
13 Gooderham MJ, Hong HC, Eshtiaghi P, Papp KA. Dupilumab: a review of its use in the treatment of atopic dermatitis. *J Am Acad Dermatol* 2018; **78:**S28–36.
14 Smith CH, Yiu ZZN, Bale T *et al.* British Association of Dermatologists guidelines for biologic therapy for psoriasis 2020: a rapid update. *Br J Dermatol* 2020; **183:**628–37.
15 Paller AS, Renert-Yuval Y, Suprun M *et al.* An IL-17–dominant immune profile is shared across the major orphan forms of ichthyosis. *J Allergy Clin Immunol* 2017; **139:**152–65.
16 Malik K, He H, Huynh TN *et al.* Ichthyosis molecular fingerprinting shows profound TH17 skewing and a unique barrier genomic signature. *J Allergy Clin Immunol* 2019; **143:**604–18.
17 Lefferdink R, Rangel SM, Chima M *et al.* Secukinumab responses vary across the spectrum of congenital ichthyosis in adults. *Arch Dermatol Res* 2022; doi: 10.1007/s00403-022-02325-3.
18 Sun Q, Asch S, Bayart C *et al.* Development and initial validation of a novel system to assess ichthyosis severity. *JAMA Dermatol* 2022; **158:**359.
19 Nguyen HH, Shinkuma S, Hayashi R *et al.* New insight of itch mediators and proinflammatory cytokines in epidermolysis bullosa. *J Cutan Immunol Allergy* 2022; **5:**78–87.
20 Wickham H. *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag, 2016.
21 Konishi H, Tsutsui H, Murakami T *et al.* IL-18 contributes to the spontaneous development of atopic dermatitis-like inflammatory skin lesion independently of IgE/stat6 under specific pathogen-free conditions. *Proc Natl Acad Sci U S A* 2002; **99:**11340–5.
22 Trzeciak M, Gleń J, Bandurski T *et al.* Relationship between serum levels of interleukin-18, IgE and disease severity in patients with atopic dermatitis. *Clin Exp Dermatol* 2011; **36:**728–32.
23 Wittmann M, Purwar R, Hartmann C *et al.* Human keratinocytes respond to interleukin-18: implication for the course of chronic inflammatory skin diseases. *J Invest Dermatol* 2005; **124:**1225–33.
24 Companjen AR, Prens E, Mee JB, Groves RW. Expression of IL-18 in human keratinocytes. *J Invest Dermatol* 2000; **114:**598–9.
25 Dai X, Sayama K, Tohyama M *et al.* Mite allergen is a danger signal for the skin via activation of inflammasome in keratinocytes. *J Allergy Clin Immunol* 2011; **127:**806–14.e1–4.
26 Dai X, Tohyama M, Murakami M, Sayama K. Epidermal keratinocytes sense dsRNA via the NLRP3 inflammasome, mediating interleukin (IL)-1β and IL-18 release. *Exp Dermatol* 2017; **26:**904–11.
27 Zheng J, Yao L, Zhou Y *et al.* A novel function of NLRP3 independent of inflammasome as a key transcription factor of IL-33 in epithelial cells of atopic dermatitis. *Cell Death Dis* 2021; **12:**871.
28 Stutz A, Horvath GL, Monks BG, Latz E. ASC speck formation as a readout for inflammasome activation. *Methods Mol Biol Clifton NJ* 2013; **1040:**91–101.
29 Venegas C, Kumar S, Franklin BS *et al.* Microglia-derived ASC specks cross-seed amyloid-β in Alzheimer's disease. *Nature* 2017; **552:**355–61.
30 Czarnowicki T, He H, Leonard A *et al.* The major orphan forms of ichthyosis are characterized by systemic T-cell activation and Th-17/Tc-17/Th-22/Tc-22 polarization in blood. *J Invest Dermatol* 2018; **138:**2157–67.
31 Renner ED, Hartl D, Rylaarsdam S *et al.* Comèl-Netherton syndrome defined as primary immunodeficiency. *J Allergy Clin Immunol* 2009; **124:**536–43.
32 Hosomi N, Fukai K, Nakanishi T *et al.* Caspase-1 activity of stratum corneum and serum interleukin-18 level are increased in patients with Netherton syndrome. *Br J Dermatol* 2008; **159:**744–6.
33 Yasuda K, Nakanishi K, Tsutsui H. Interleukin-18 in health and disease. *Int J Mol Sci* 2019; **20:**E649.
34 Okamura H, Tsutsi H, Komatsu T *et al.* Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature* 1995; **378:**88–91.
35 Macleod T, Berekmeri A, Bridgewood C *et al.* The immunological impact of IL-1 family cytokines on the epidermal barrier. *Front Immunol* 2021; **12:**808012.
36 Calabrese L, Fiocco Z, Satoh TK *et al.* Therapeutic potential of targeting interleukin-1 family cytokines in chronic inflammatory skin diseases. *Br J Dermatol* 2022; **186:**925–41.
37 Kawashima M, Yamamura M, Taniai M *et al.* Levels of interleukin-18 and its binding inhibitors in the blood circulation of patients with adult-onset Still's disease. *Arthritis Rheum* 2001; **44:**550–60.
38 Kawaguchi Y, Terajima H, Harigai M *et al.* Interleukin-18 as a novel diagnostic marker and indicator of disease severity in adult-onset Still's disease. *Arthritis Rheum* 2001; **44:**1716–17.
39 Chen D-Y, Lan J-L, Lin F-J, Hsieh T-Y. Proinflammatory cytokine profiles in sera and pathological tissues of patients with active untreated adult onset Still's disease. *J Rheumatol* 2004; **31:**2189–98.
40 Girard C, Rech J, Brown M *et al.* Elevated serum levels of free interleukin-18 in adult-onset Still's disease. *Rheumatol Oxf Engl* 2016; **55:**2237–47.
41 Kudela H, Drynda S, Lux A *et al.* Comparative study of interleukin-18 (IL-18) serum levels in adult onset Still's disease (AOSD) and systemic onset juvenile idiopathic arthritis (sJIA) and its use as a biomarker for diagnosis and evaluation of disease activity. *BMC Rheumatol* 2019; **3:**4.
42 Nam SW, Kang S, Lee JH, Yoo DH. Different features of interleukin-37 and interleukin-18 as disase activity markers of adult-onset Still's disease. *J Clin Med* 2021; **10:**910.
43 Ogita A, Ansai S-I, Saeki H. Serum interleukin-18 provides a clue to the diagnosis of adult-onset Still's disease: findings from 6 Japanese patients with adult-onset Still's disease. *J Nippon Med Sch Nippon Ika Daigaku Zasshi* 2022; **89:**114–18.
44 Wang D, Drenker M, Eiz-Vesper B *et al.* Evidence for a pathogenetic role of interleukin-18 in cutaneous lupus erythematosus. *Arthritis Rheum* 2008; **58:**3205–15.
45 Tsoi LC, Gharaee-Kermani M, Berthier CC *et al.* IL18-containing 5-gene signature distinguishes histologically identical dermatomyositis and lupus erythematosus skin lesions. *JCI Insight* 2021; **5:**e139558.
46 Gangemi S, Merendino R, Guarneri F *et al.* Serum levels of interleukin-18 and s-ICAM-1 in patients affected by psoriasis: preliminary considerations. *J Eur Acad Dermatol Venereol* 2003; **17:**42–6.

47 Kanameishi S, Nakamizo S, Endo Y *et al*. High level of serum human interleukin-18 in a patient with pyogenic arthritis, pyoderma gangrenosum and acne syndrome. *J Eur Acad Dermatol Venereol* 2017; **31:**e115-16.
48 Wada T, Toma T, Miyazawa H *et al*. Longitudinal analysis of serum interleukin-18 in patients with familial Mediterranean fever carrying MEFV mutations in exon 10. *Cytokine* 2018; **104:**143–6.
49 Joosten MDW, Clabbers JMK, Jonca N *et al*. New developments in the molecular treatment of ichthyosis: review of the literature. *Orphanet J Rare Dis* 2022; **17:**269 .
50 Fontao L, Laffitte E, Briot A *et al*. Infliximab infusions for Netherton syndrome: sustained clinical improvement correlates with a reduction of thymic stromal lymphopoietin levels in the skin. *J Invest Dermatol* 2011; **131:**1947–50.
51 Steuer AB, Cohen DE. Treatment of Netherton syndrome with dupilumab. *JAMA Dermatol* 2020; **156:**350–1.
52 Süßmuth K, Traupe H, Loser K *et al*. Response to dupilumab in two children with Netherton syndrome: Improvement of pruritus and scaling. *J Eur Acad Dermatol Venereol* 2021; **35:**e152-5.
53 Murase C, Takeichi T, Taki T *et al*. Successful dupilumab treatment for ichthyotic and atopic features of Netherton syndrome. *J Dermatol Sci* 2021; **102:**126–9.
54 Volc S, Maier L, Gritsch A *et al*. Successful treatment of Netherton syndrome with ustekinumab in a 15-year-old girl. *Br J Dermatol* 2020; **183:**165–7.
55 Luchsinger I, Knöpfel N, Theiler M *et al*. Secukinumab therapy for Netherton syndrome. *JAMA Dermatol* 2020; **156:**907–11.
56 Barbieux C, Bonnet des Claustres M, de la Brassinne M *et al*. Duality of Netherton syndrome manifestations and response to ixekizumab. *J Am Acad Dermatol* 2021; **84:**1476–80.
57 Gabay C, Fautrel B, Rech J *et al*. Open-label, multicentre, dose-escalating phase II clinical trial on the safety and efficacy of tadekinig alfa (IL-18BP) in adult-onset Still's disease. *Ann Rheum Dis* 2018; **77:**840–7.
58 Coulombe PA, Kerns ML, Fuchs E. Epidermolysis bullosa simplex: a paradigm for disorders of tissue fragility. *J Clin Invest* 2009; **119:**1784–93.
59 Misiorek JO, Lähdeniemi IAK, Nyström JH *et al*. Keratin 8-deletion induced colitis predisposes to murine colorectal cancer enforced by the inflammasome and IL-22 pathway. *Carcinogenesis* 2016; **37:**777–86.
60 dos Santos G, Rogel MR, Baker MA *et al*. Vimentin regulates activation of the NLRP3 inflammasome. *Nat Commun* 2015; **6:**6574.
61 Ansai O, Shinkuma S, Kabata Y *et al*. Amino acid charge and epidermolysis bullosa simplex severity: genotype-phenotype correlations. *J Eur Acad Dermatol Venereol* 2020; **34:**e87–90.
62 Murase Y, Tanahashi K, Takeichi T *et al*. Mild epidermolytic ichthyosis with palmoplantar keratoderma due to the KRT1 mutation p.Ile479Thr. *J Dermatol* 2020; **47:**e336–9.

Supporting Information

Additional Supporting Information can be found in the online version of this article at the publisher's website.