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

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The ichthyoses are rare skin disorders with immune and barrier aberrations. Identifying blood phenotypes may advance targeted therapeutics. We aimed to compare frequencies of skin homing/cutaneous lymphocyte antigen (+) versus systemic/cutaneous lymphocyte antigen (−) “polar” CD4+/CD8+ and activated T-cell subsets in ichthyosis versus atopic dermatitis, psoriasis, and control blood, with appropriate clinical correlations. Flow cytometry was used to measure IFN-γ, IL-13, IL-9, IL-17, and IL-22 cytokines in CD4+CD8+ T cells, with inducible co-stimulator molecule and HLA-DR defining mid- and long-term T-cell activation, respectively. We compared peripheral blood from 47 patients with ichthyosis (congenital ichthyosiform erythroderma, lamellar ichthyosis, epidermolytic ichthyosis, and Netherton syndrome) with 43 patients with atopic dermatitis and 24 patients with psoriasis and 59 age-matched controls. Clinical measures included the ichthyosis severity score, with subsets for erythema and scaling, trans-epidermal water loss, and pruritus. All ichthyoses had excessive inducible co-stimulator molecule activation (P < 0.001), particularly epidermolytic ichthyosis. Significantly elevated IL-17- (P < 0.05) and IL-22-producing (P < 0.01) T cells characterized ichthyoses, mainly Netherton syndrome and congenital ichthyosiform erythroderma (P < 0.05). Increased T helper 2/cytotoxic T cell 2/T helper 9 (P < 0.05) and similar IFN-γ frequencies (P > 0.1) versus controls were also noted. IL-17/IL-22-producing cells clustered with clinical measures, whereas IFN-γ clustered with age. Our data show peripheral blood IL-17/IL-22 activation across the ichthyoses, correlating with clinical measures. Targeted therapies should dissect the relative contribution of polar cytokines to disease pathogenesis.


INTRODUCTION

The ichthyoses encompass a spectrum of syndromic and nonsyndromic dermatoses with varying underlying genetic bases (Oji et al., 2010). Their shared features include a defective epidermal barrier (Kawashima et al., 2005) with scaling and variable cutaneous erythema. Patients suffer from reduced quality of life and recurrent infections, posing an economic burden on health care (Dreyfus et al., 2015). The best-characterized and most common phenotype is ichthyosis vulgaris, owing to FLG (encoding filaggrin) gene mutations (Smith et al., 2006). The more prevalent rare forms of ichthyosis are lamellar ichthyosis (LI; usually TGM1, encoding transglutaminase 1) and congenital ichthyosiform erythroderma (CIE; multiple causative genes) (Takeichi and Akiyama, 2016), collectively known as autosomal recessive congenital ichthyosis (ARCI; Oji et al., 2010), keratinopathic ichthyosis, epidermolytic ichthyosis (EI), involving mutations in KRT1 and KRT10 (Vahlquist et al., 2017), and Netherton syndrome (NS), with its atopic manifestations and underlying deficiency of a serine peptidase inhibitor, Kazal type 5 (SPINK5) mutations (Sarri et al., 2017). Recently, the cutaneous ichthyosis phenotype of these orphan ichthyoses was linked to robust IL-17/tumor necrosis factor-α cytokine activation, which is highly associated with disease severity and transepidermal water loss (TEWL), the functional barrier measure (Paller et al., 2017b). These data were supported by studies on a limited number of patients with restricted panels, mostly in NS (Briot et al., 2009; Fontao et al., 2011), showing a pro-Th2/TARC/CCL17/TSLP signature...
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(Akagi et al., 2013; Briot et al., 2009) with innate (IL-1β, tumor necrosis factor-α) and T-cell activation (IL-2) cytokine increases (Renner et al., 2009). A previous study in nine children with NS using flow cytometry found normal numbers of T, B, and T-regulatory cells (Tregs), with small increases in natural killer cells in affected individuals (Renner et al., 2009). Another study on 10 children and one adult with NS showed altered B-cell expression and decreased natural killer cytotoxic activity (Hannula-Jouppi et al., 2016). Comprehensive blood phenotyping of a larger number of these pediatric and adult patients with ichthyosis is not available.

There remains a huge unmet need for safer, more effective treatments for ichthyoses. Current treatments are empirical, often ineffective, and largely directed at reducing scale. These include topical moisturizers, keratolytics, retinoids, vitamin D analogs, corticosteroids, and calcineurin inhibitors, all off-label. Systemic retinoids have variable efficacy and multiple potential side effects (DiGiovanna and Robinson-Bostom, 2003). Experimental gene therapies are early in preclinical development (Gorell et al., 2014). Although cutaneous and systemic immune inflammation has been successfully targeted in atopic dermatitis (AD) and psoriasis (Czarnowicki et al., 2015c; Guttmann-Yassky et al., 2017), little attention has been paid to targeting inflammation in ichthyosis. Indeed, specific immune-targeting therapeutics have shown efficacy for NS (anti-tumor necrosis factor-α) (Fontao et al., 2011; Paller et al., 2017a) and ichthyotic disease resulting from mutations in desmoglein 1 encoding desmolplakin (ustekinumab) (Paller et al., 2017a).

Cutaneous lymphocyte antigen (CLA$^+$) memory T cells are peripheral biomarkers in inflammatory skin disorders (Czarnowicki et al., 2017b). We evaluated activated CD4$^+$/CD8$^+$ T cells and their differentiation to IFN-γ, IL-13, IL-22, IL-17A, and IL-9-producing T cells within skin homing/CLA$^+$ and systemic/CLA$^-$ compartments in blood of patients with ichthyosis, compared to patients with AD and psoriasis and healthy controls. Blood of patients with ichthyosis had increases in T-cell activation, and in IL-22, IL-17A, IL-13, and IL-9-producing T cells. IL-17/IL-22 axes were linked with disease severity, whereas the IFN-γ pathway was associated with disease chronicity.

RESULTS

Flow cytometry was used to measure frequencies of IFN-γ$, IL-9, IL-13, IL-17, and IL-22-producing T cells, defining T helper (Th) 1/cytotoxic T cell (Tc)1, Th9/Tc9, Th2/Tc2, Th17/Tc17, and Th22/Tc22 subsets in CD4$^+$/CD8$^+$ T cells, respectively. Cell surface staining was used to measure expression of mid (inducible co-stimulator molecule [ICOS]) and late (HLA-DR) activation markers in central (Tcm/CCR7$^+$CD45RO$^+$) and effector (Tem/CCR7$^+$CD45RO$^+$) memory T cells in skin homing/CLA$^+$ and systemic/CLA$^-$ subsets. To assess immune polarity, samples were analyzed in parallel with blood from healthy controls and previously published cohorts of moderate-to-severe AD and psoriasis (Czarnowicki et al., 2015b, 2015c), representing different immune polarizations (Th2/Th22 and Th1/Th17, respectively). To understand the common immune skewing in ichthyosis, we first compared frequencies of T-cell populations between ichthyosis overall and healthy controls, patients with psoriasis and AD, and subsequently evaluated differences among the various ichthyosis subtypes (ARCI-LL, ARCI-CIE, EI, and NS).

To enhance representation of the distribution of values on the significance of results, comparison plots incorporate both means (black) and medians (red) ± standard error, with respective P-values. Although results discuss mean values, both are detailed in Supplementary Tables S1 and S2 online.

Ichthyosis is characterized by increased mid/ICOS and late/HLA-DR T-cell activation

Tcm and Tem cells, key components of adaptive immunity, are characterized by diverse homing capacities (Sallusto et al., 2004). Both express the skin homing marker CLA, but Tcm are also CCR7$^+$, allowing them to migrate into lymph nodes, creating an immunologic reserve (Sallusto et al., 1999). Ichthyosis showed the highest skin-homing CD4$^+$Tem frequencies (normal: 20%, psoriasis: 21%, AD: 23%, ichthyosis: 28%; $P < 0.01$; Figure 1a and b) and higher CLA$^+$CD8$^+$ Tem (12% vs. 9%, $P = 0.05$; Figure 1c) and Tcm (13.5% vs. 9.8%, $P = 0.03$; Figure 1d) cells than controls (Supplementary Table S1).

T-cell activation via the T-cell receptor leads to sequential expression of T-cell activation markers. ICOS, a mid-activation marker, is upregulated within 24 hours (Tafuri et al., 2001), whereas HLA-DR indicates chronic T-cell activation (Ferenzci et al., 2000). Ichthyosis showed increased pan activation of ICOS and HLA-DR in both the skin-homing/CLA$^+$ and systemic/CLA$^-$ compartments, particularly among CD4$^+$ populations (Figure 1e–l). Most striking was ICOS activation, in both CD4$^+$ and CD8$^+$ subsets (CD4$^+$ Tem: CLA$^+$: ichthyosis 43.5% vs. normal 20.5%, $P < 0.0001$; CLA$^-$: ichthyosis 18.5% vs. normal 11.7%, $P < 0.001$ [Figure 1c] and TCM: CLA$^+$: ichthyosis 29.8% vs. normal 15.6%, $P < 0.0001$; CLA$^-$: ichthyosis 6.3% vs. normal 4.2%, $P = 0.001$ [Supplementary Figure S1a, b, e, and f online]). These frequencies were even higher than in AD, a highly inflammatory skin disease with excessive T-cell activation (Czarnowicki et al., 2015c). Conversely, HLA-DR demonstrated differential expression, with increases restricted to CD4$^+$ subsets (Tem: CLA$^+$: ichthyosis 10% vs. normal 5.5%, $P = 0.004$; CLA$^-$: ichthyosis 4.2% vs. normal 2.2%, $P = 0.05$; TCM: CLA$^+$: ichthyosis 8.8% vs. normal 4.6%, $P = 0.004$; CLA$^-$: ichthyosis 4% vs. normal 4%, $P = 0.03$ [Figure 1g, h, k, and l]), but significantly lower frequencies in CD8$^+$ Tem/Tcm/CLA$^+$/CLA$^-$ cells ($P < 0.02$; Supplementary Figure S1c, d, g and h, Supplementary Table S1).

Prominent Th17/Th22 signatures characterize ichthyoses

T-cell activation is followed by T-cell subset differentiation. Thus, we next evaluated the frequencies of polar T-cell subsets, including IFN-γ$, IL-9, IL-13, IL-17, and IL-22-producing CD4$^+$/CD8$^+$ T cells, in both the cutaneous/CLA$^+$ and systemic/CLA$^-$ compartments.

Congruent with our recent skin findings (Paller et al., 2017b), IL-17 was increased in ichthyosis versus control blood, particularly among skin-homing CD4$^+$ T cells (CLA$^+$: 4.5% vs. 2.4%, $P = 0.004$; Figure 2a), demonstrating levels either similar to or higher than in psoriasis (CD8$^+$/CLA$^+$/CLA$^-$:...
much higher than in psoriasis across all subsets ([Cla] or higher (CD8+ systemic compartments. IL-22 levels in ichthyosis were similar versus controls, seen consistently across the cutaneous and [P]4%; [Cla]P 0.004; CLA+) activation in CLA+CD4+ Tcm/Tem cells in control (red), psoriasis (green), AD (purple), and ichthyosis (blue). Bar plots represent means (black)/medians (red) ± SEMs. P-values are designated as ***<0.001, **<0.01, *<0.05, <0.1. AD, atopic dermatitis; CLA, cutaneous lymphocyte antigen; ICOS, inducible co-stimulator molecule; SEM, standard error of the mean/median; Tcm, central memory T cell; Tcm, effector memory T cell.

Figure 1. T-cell memory subset activation. (a–d) CLA+CD4+/CD8+ Tcm (CD45RO+CCR7+), Tem (CD45RO+CCR7-) subset frequencies, and (e–h) ICOS+ and HLA-DR+ activation in CLA+CD4+ Tcm/Tem cells in control (red), psoriasis (green), AD (purple), and ichthyosis (blue). Bar plots represent means (black)/medians (red) ± SEMs. P-values are designated as ***<0.001, **<0.01, *<0.05, <0.1. AD, atopic dermatitis; CLA, cutaneous lymphocyte antigen; ICOS, inducible co-stimulator molecule; SEM, standard error of the mean/median; Tcm, central memory T cell; Tcm, effector memory T cell.

To detect whether inflammatory profiles of ichthyosis subtypes vary, we next compared disease subcategories and controls. Although all subtypes had increased Tem ICOS activation, El showed highest expressions in both CLA+ (55.3% vs. 20.5%, P = 0.0001; Figure 4a) and CLA- (29%...
NS and CIE demonstrate the highest IL-17/IL-22 frequencies
NS showed elevated IL-17 and IL-22 frequencies in CLA⁺ or CLA⁻, CD4⁺ or CD8⁺ cells (P < 0.05 for all), not just versus controls but also compared with other ichthyosis subtypes except CIE (P > 0.1). Contrary to the Th2 fingerprint reported in past NS serum studies (Akagi et al., 2013; Briot et al., 2009), our results showed comparable IL-13 frequencies in NS and controls (Supplementary Figure S2a–d online). In contrast, CIE and LI had increased IL-13 frequencies, particularly among the CLA⁻ CD4⁺ (CIE 1.8% vs. control 0.7%, P = 0.007; Supplementary Figure S3b) and CD8⁺ (CIE 1.3% vs. control 0.3%, P = 0.01; LI 1.4% vs. control 0.3%, P = 0.005; Supplementary Figure S3d) subsets. Th9/Tc9 occurrence was upregulated in NS and LI (Supplementary Figure S3e–h). Only EI showed significantly lower IFN-γ levels than controls (P < 0.05; Supplementary Figure S3i–l).

Th17/Tc17 and Th22/Tc22 cells cluster with ichthyosis clinical measures
We also assessed how clinical characteristics, including ichthyosis severity (total ichthyosis area severity index [IASI]), with subsets for erythema [IASI-E] and scaling [IASI-S]), pruritus, and TEWL, a functional barrier measure, relate to polar T-cell subsets. Unsupervised hierarchical clustering of all parameters was performed using Pearson correlations, as shown in the correlation heatmap and dendrogram in Figure 6 (red: positive; blue: negative correlations, stars and plus signs: significant correlations). Selected individual correlation scatter plots are presented in Supplementary Figures S4 and S5 online. As shown in Figure 6, a tight cluster gathering of TEWL, IASI-S, IASI, IASI-E, and IL-17-producing cells (purple square) was adjacent to a Th22/Tc22 cluster (green square). Clinical severity and TEWL measures (red ellipses) branched around IL-17/IL-22 axes, whereas pruritus clustered with IL-13-producing cells (yellow square). IFN-γ clustered with age (blue square). Correlations are listed in Supplementary Table S3 online.

IASI-E/inflammation and IASI-S/scaling components highly correlated with total IASI (P < 0.0001; Supplementary Figure S4a and b). Th17 (r = 0.4, P = 0.004; Supplementary Figure S4c/Tc17 (r = 0.34, P = 0.016; Supplementary Figure S4d) and Th22 (r = 0.43, P = 0.001; Supplementary Figure S4e/Tc22 (r = 0.32, P = 0.02; Supplementary Figure S4f) correlated significantly with total IASI. Analogous correlations were observed between Th17/
Tc17/Th22/Tc22 and IASI-E (Supplementary Figure S5a–d). IASI-S correlated with both CD8+/CLA+ cells (r = 0.31, P = 0.02; Supplementary Figure S5e) and Th9/Tc9 cells (P < 0.05; Supplementary Figure S5f and g). Although Th22 cells correlated positively with TEWL (r = 0.28, P = 0.06; Supplementary Figure S4g), negative correlations were observed between Th9/Tc9 (r = −0.35, P = 0.02 for both; Supplementary Figure S4h and i) and TEWL. Tc2 (r = 0.44, P = 0.003; Supplementary Figure S4j) significantly correlated with the 5D-pruritus scale, with lesser correlations found with Th17 (r = 0.32, P = 0.04) and Th22 (r = 0.28, P = 0.07; Supplementary Figure S4k and l). To evaluate for the effect of disease chronicity, we also correlated parameters with age. IFN-γ, previously associated with disease chronicity (Czarnowicki et al., 2015a, 2017a; Gittler et al., 2012), was significantly correlated with age (P < 0.04; Supplementary Figure S4m and n). Ichthyosis severity showed a trend of amelioration with age (Supplementary Figure S4o). Lastly, IL-22-producing cells highly correlated with both IL-17 and IL-13 CD4+/CD8+ T-cell subsets (P < 0.01 for all; Supplementary Figure S5e–k).

To assess how skin-homing T cells in blood correlate with cutaneous T cells and disease severity in ichthyosis, we performed immunostaining for CD3+ T cells in skin from available ichthyosis samples (n = 27). CD3+ cell counts correlated significantly with ICOS-activated systemic Tcm cells (r = 0.45, P = 0.02; Supplementary Figure S5l), with a trend toward significance for IASI-E (r = 0.34, P = 0.08; Supplementary Figure S5m), but not IASI-S (P = 0.9; Supplementary Figure S5n, Supplementary Table S3).
Total Tregs are increased in ichthyosis and correlate with T-cell activation.

Ichthyosis showed significantly increased total (defined as CD25<sup>+</sup>CD127<sup>−</sup>CCR4<sup>+</sup>) (Gorski et al., 2010) (P < 0.01), but not skin-homing (CCR4<sup>+</sup>CLA<sup>+</sup>) Treg frequencies versus control, psoriasis, and AD, with largely similar distribution among ichthyosis subtypes (Supplementary Figure S6a–d online). Similar to AD report (Czarnowicki et al., 2015c), Tregs significantly correlated with skin-homing Tcm/Tem, HLA-DR activated T-cell subsets (P < 0.013 for all; Supplementary Figure S6e–h, Supplementary Table S3).

**DISCUSSION**

This study details all recognized effector T-cell subsets within CLA<sup>+</sup> and CLA<sup>−</sup> compartments in the circulation of the four more common subtypes among orphan ichthyoses (ARCI-LI, ARCI-CIE, EI, and NS) compared with healthy controls. Because of the suggested phenotypical similarities to psoriasis and AD in skin, ichthyosis blood samples were analyzed in parallel with previously published AD (Th2/Th22-skewed) and psoriasis (Th1/Th17-polarized) samples (Czarnowicki et al., 2015c). We not only found a common blood phenotype in the largest group of patients with ichthyosis, but also characterized the unique blood profiles of different subtypes. Expanding the ichthyosis phenotyping to blood, including identification of activation markers and polarized cytokines, carries the potential for the development of noninvasive biomarkers to monitor treatment responses and advance targeted therapeutic development.

Similar to our recent findings of Th17-dominant inflammation in skin (Paller et al., 2017b), the current data establish shared elevation of IL-17 frequencies in ichthyoses blood across skin homing/CLA<sup>+</sup> and systemic/CLA<sup>−</sup> CD4<sup>+</sup> and CD8<sup>+</sup> populations, supporting their systemic immune dysregulation and hinting at possible associated comorbidities. IL-22 was profoundly upregulated in ichthyosis to levels even higher than in AD, a disease with a prominent IL-22 signature (Czarnowicki et al., 2015b; Gittler et al., 2012). Furthermore, Th17/Tc17/Th22/Tc22 subsets correlate with total IASI and IASI-E, and cluster close to TEWL, 5D pruritus score and IASI-S, suggesting their possible role in ichthyosis skin manifestations. The close link of barrier measures to dysregulated cytokines indicates ongoing crosstalk between these components in ichthyosis, similar to AD and psoriasis (Czarnowicki et al., 2014; Guttmann-Yassky et al., 2011). NS and CIE show the highest IL-17/IL-22 expression, reflecting their more erythrodermic phenotype (Richard, 1993) and higher IASI-E and TEWL values (Supplementary Table S4 online), further highlighting the association between inflammation and barrier impairment. IL-22 and IL-17 are extensively involved in protective antibacterial immune responses (Cho et al., 2010; Jin and Dong, 2013), antimicrobial peptide secretion, regulation of chronic inflammation, and maintenance of epithelial integrity (Sonnenberg et al., 2011; Wolk et al., 2004). The
increased IL-17/IL-22 blood subsets are consistent with the elevated IL-17/IL-22-related markers in skin, including antimicrobial peptides (Paller et al., 2017b). Although positive correlations between IL-13 and IL-22 observed here are similar to AD findings (Czarnowicki et al., 2015b), IL-17 and IL-22 associations might partially derive from the ability of Th17 cells to produce both cytokines (Liang et al., 2017). The ichthyosis blood profile blends characteristics of both AD and psoriasis, with increased ICOS/HLA-DR and IL-22 activation, features of both AD and psoriasis, but also elevated IL-17 level characteristic of psoriasis and increased IL-13 frequencies typifying AD. Clustering (and positive correlations) of Th2/Tc2 cells and the 5D pruritus characteristics of both AD and psoriasis, with increased ICOS/HLA-DR activation marker HLA-DR (Reddy et al., 2004) is increased in ichthyosis blood, perhaps perpetuating the Th17/Th22 deviation. Although positively correlated with IASI-S, IL-9 correlates negatively with TEWL, suggesting a complex interaction between Th9/Tc9 subsets and barrier homeostasis. Similar to alopecia areata (Czarnowicki et al., 2017a) and AD (Czarnowicki et al., 2015a; Gittler et al., 2012), in which IFN-γ marks disease chronicity, Th1/Tc1 subsets correlate with age. Ichthyosis has similar or even higher Tem/Tcm cell frequencies than AD, suggesting excessive circulatory activation with concurrent immune reserve accumulation in lymph nodes, as indicated by increased Tcm cells, potentially explaining disease chronicity, recurrent exacerbations, and treatment failures.

ICOS, the mid-activation marker, is necessary for intact immune circuits (Tafuri et al., 2005) and shapes adaptive immunity (Coyle et al., 2000). Although ICOS augments Th2 responses (Tesciuba et al., 2008), it is also critical for Th17 development (Nurieva, 2005; Paulos et al., 2010). ICOS activation is profoundly increased across all Tcm/Tem/CD4+/CD8+/CLA+/systemic subsets in ichthyosis to levels even higher than the exceedingly activated AD (Czarnowicki et al., 2015c), probably augmenting IL-17 responses in ichthyosis blood and skin (Paller et al., 2017b). The fact that the chronic activation marker HLA-DR (Reddy et al., 2004) is increased in CD4+/Tem/Tcm cells, but significantly low among CD8+ subsets, suggests a specific T-cell activation sequence in ichthyosis with persistent ICOS/HLA-DR CD4+ activation, and a minor contribution of CD8+ cells in chronic disease.
Figure 6. Unsupervised hierarchical clustering of polarized cytokine subsets with ichthyosis clinical measures, using Pearson correlation as a similarity metric. CLA⁻/⁻ Th17/Tc17 clustered with or adjacent to total IASI, IASI-E, IASI-S and TEWL (blue box), and close to CLA⁺/⁺ Th22/Tc22 (orange box). Th2/Th2 clustered with the 5-D Pruritus Scale (teal box). All clinical measures clustered close to each other (red ellipses). CLA⁻/⁻ Th1/Tc1 cells clustered with age (red box). The heatmap shows the positive (red) or negative (blue) correlations of all parameters with color intensity reflecting the strength of the correlation (−1 to +1). Dendrograms are shown as a tree, representing the distance between variables. P-values are designated as **p < 0.001, *p < 0.01, *p < 0.05, +p < 0.1.

CLA, cutaneous lymphocyte antigen; IASI, ichthyosis area severity index; IASI-E, ichthyosis area severity index-erythema; IASI-S, ichthyosis area severity index-scaling; Tc, cytotoxic T cell; TEWL, transepidermal water loss; Th, T helper.
Tregs are crucial in maintaining immune tolerance (Long and Buckner, 2011), and their levels may influence activation of effector cells (McHugh and Shevach, 2002). Our data show significantly increased systemic Treg frequencies in ichthyosis, even higher than in AD. Conversely, CLA+/CLA− Tregs were lower than AD and psoriasis, possibly resulting from the complex counterequilibrium between Th17 cells and Tregs (Diller et al., 2016; Eisenstein and Williams, 2009). The positive correlations between activated skin-homing memory T cells and Treg may be secondary to the possible perpetuation of inflammation via Tregs, in addition to their suppressive effects.

Whether expanded Th17/Th22 cytokines are primary pathogenic factors in ichthyosis or secondary to chronic antigenic stimulation and barrier impairment still needs further investigation. Our preliminary skin and blood data showing Th1/Th17/IL-23 elevation in a patient with an ichthyotic syndrome resulting from DSP mutation led to initiation of targeted Th1/Th17/IL-23 blockade with ustekinumab in two patients. Ustekinumab treatment reduced skin erythema, scaling, and TEWL, supporting a possible pathogenic role for IL-17 (Paller et al., 2017a). Furthermore, the significant Th17 skewing in our recent ichthyosis skin phenotyping in 21 patients with the four orphan forms studied herein (Paller et al., 2017b) engendered an ongoing clinical trial of secukinumab (anti-IL17-antibody) in patients with ichthyosis (NCT03041038).

Ichthyoses are characterized by overt systemic inflammation, as reflected by increased T-cell activation and immune dysregulation of multiple polar cytokines among CLA− subsets. These data further highlight ichthyosis as a systemic, rather than skin-limited disease, similar to psoriasis and AD, both currently conceptualized as systemic disorders (Guttmann-Yassky and Krueger, 2017; Guttmann-Yassky et al., 2017). Both were linked to systemic comorbidities, including the increased risk of cardiovascular disease (Brunner et al., 2017; Puig, 2017; Silverberg and Greenland, 2015). In psoriasis, IL-17 was suggested to drive cutaneous and systemic inflammation, as well as the comorbidities (Krueger and Brunner, 2017). Although there is no consensus on comorbid associations in ichthyoses, our results suggest the need for early monitoring for systemic extracutaneous involvement, particularly for metabolic and cardiovascular disease. It is possible that excessive T-cell activation and IL-17 polarization in ichthyosis promote systemic manifestations that, similar to psoriasis (Conrad and Gilliet, 2018), might be addressed or even prevented through targeted therapeutics.

Our study had limitations, including the fact that a small subset of ichthyosis patients was younger than 18 years, limiting our ability to compare absolute numbers between age groups. However, their immune polarity was comparable to that of adults (data not shown). Also, although our study presents the largest dataset of ichthyoses blood profiles, there were patient number variations across different subtypes due to their rarity.

In sum, ichthyosis blood is accompanied by increased systemic and skin-homing T-cell activation and multicytokine polarization, with IL-17/IL-22 predominance, similar to the skin compartment. Although the pathogenic contribution of each cytokine pathway will only be determined through targeted treatment investigation, positive correlations between CLA+/CLA− IL-17/IL-22-producing cells and clinical measures support their possible roles in ichthyosis. Furthermore, our findings suggest that “nonsyndromic/skin-limited” ichthyoses may have systemic manifestations and support systemic therapeutic approaches for severely affected patients.

**MATERIALS AND METHODS**

**Patient characteristics and samples**

Blood was obtained from 47 patients with ichthyosis (30 females, 17 males; ages 1–57 years, mean 22 years; 13 ARCI-LI, 18 ARCI-CIE, 8 NS, 8 EI) (Paller et al., 2017b). 43 patients with moderate-to-severe AD (19 females, 24 males; ages 18–81 years; mean 43 years), 24 patients with moderate-to-severe psoriasis (19 females, 24 males; ages 18–81 years; mean 43 years), and 59 controls (30 females, 29 males; ages 6–66 years; mean 25 years). No age (P = 0.32, one-way analysis of variance), ethnicity (P = 0.57, chi-square test), or sex (P = 0.18, chi-square test) disparities were observed between ichthyosis patients and controls. Eighteen patients with ichthyosis were younger than 18 years. All patients were classified clinically, but genotyping was performed on 96% of patients and was consistent with the clinical diagnosis. All NS resulted from SPINK5 mutations; seven of the eight EI had a KRT10 and one a KRT1 mutation; clinically patients with LI had TGM1 mutations, but one had a NIPAL4 mutation; and the patients with CIE included three with harlequin ichthyosis (all ABCA12 mutations), but others had one of various genes mutated as have been described with CIE (ABCA12, ALOXE3, ALOX12B, CorS3, NIPAL4, and PNPLA1). Of the 47 patients, 2 (4%) took a retinoid orally (one acetretin, one isoretinoin), 3 (6%) used topical tazarotene to localized areas, 1 had used budesonide ointment, and 1 tacrolimus ointment for pruritus in the previous few days. No patient was treated with an oral immunosuppressant medication.

Although cytokine polarization was similar between children and adults by sensitivity analysis, adults showed higher T-cell activation than children, probably secondary to disease chronicity and ongoing immune stimulation. Written institutional review board-approved informed consents were obtained from subjects (≥12 years) and parents (<18 years). Ichthyosis severity was calculated using the IASI score, as previously described (Paller et al., 2017b), a nonvalidated scale that combines the IASI-E/inflammation and IASI-S into a total IASI score. Additional clinical measures, including TEWL on the upper arm and 5D pruritus score, were captured as well. Demographic data are summarized in Supplementary Tables S4 and S5 online.

**Isolation of peripheral blood mononuclear cells**

Peripheral blood mononuclear cells were isolated from whole blood by Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden), as previously described (Czarnowicki et al., 2017a) (see Supplementary Materials and Methods online).

**Stimulation of blood cell populations for cytokine responses**

Ex vivo cell activation is required to detect cytokine production, as less than 1% of nonstimulated cells produce cytokines. Whole blood was incubated with phorbol 12-myristate 13-acetate (25 ng/ml) plus ionomycin (2 μg/ml) in the presence of brefeldin A (10 μg/ml) for 4 hours at 37°C to induce cytokine responses. After stimulation, red blood cells were lysed with FACS lysing solution to obtain leukocytes (see Supplementary Materials and Methods).
Cell surface staining and intracellular staining on peripheral blood mononuclear cells and stimulated and nonstimulated CD4/CD8 T cells

Peripheral blood mononuclear cells were stained with fluorochrome-labeled antibodies to cell surface markers (CD3, CD8, CD4, CD45RO, CCR7, ICOS, HLA-DR, and CLA). Stimulated and nonstimulated blood cells were stained for cell surface markers (CD3, CD4, CD69, and CLA) and permeabilized with FACS/perm to stain for cytokines, including IL-13, IL-22, IL-9, IFN-γ, and IL-17A (see Supplementary Materials and Methods).

Immunohistochemistry

Immunohistochemistry staining for CD3 was performed on frozen OCT-embedded cryostat sections using purified mouse anti-human mAb (Clone SK7, BD Biosciences), as described (Esaki et al., 2015; Suarez-Farinas et al., 2015). Immunohistochemistry staining was performed on a subset of 27 available patient samples. Cell counts were quantified with ImageJ V1.42 (National Institute of Health, Bethesda, MD). See Supplementary Materials and Methods for details.

Statistical analysis

Welch’s t-test and the Wilcoxon Mann-Whitney test were used to compare means and medians of variables, respectively. Unsupervised hierarchical clustering of variables was performed using the R package hclust with Pearson correlation as a similarity metric and a dendrogram (using the R package ape). Individual scatter plots display coefficients, 95% confidence intervals, and P-values. P-values were designated as ***<0.001, **<0.01, *<0.05, +<0.1.

CONFLICT OF INTEREST

JGK: Institutional research grants and funding from Novartis, Pfizer, Boehringer, Janssen, Abbvie, Amgen, Innovaderm, BMS, EMD Serono, Paraxial, Leo Pharma, Vitae, Kinta, Regeneron, Novan, AkrosNovartis, Sienou, UCB; consulting fees from Novartis, Pfizer, Boehringer, Amgen, Lilly, Biogeniclec, Janssen, Abbvie,Leo Pharma, Escalor, Acros, Roche, Valiant, Allergen, UC..B Aurigene. EGY: Institutional grants from Celgene, Dermira, Janssen, Leo, Merck Novartis, Regeneron BMS; consulting fees from Abbvie, Allergen, Amgen Anacor, Asanu,BMS, Celgene, Celsus,Curadim, Dermira, Drais, Escalier, Galdemenventech, GlaxoSmithKline, Glenmark, Kymab Limited, Kyowa Kirin, LEAD, Leo, Novartis, Pfizer, Regeneron, Sarost, Sienna, Therave. The remaining authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org. and at https://doi.org/10.1016/j.jid.2018.03.1523.

REFERENCES