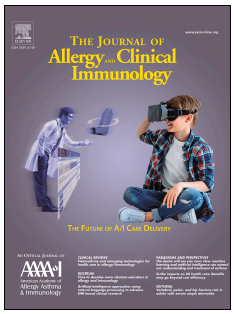
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Netherton syndrome subtypes share IL-17/IL-36 signature with distinct IFN-α and allergic responses

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PII: S0091-6749(21)01398-1

DOI: https://doi.org/10.1016/j.jaci.2021.08.024

Reference: YMAI 15271

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 3 March 2021

Revised Date: 12 August 2021

Accepted Date: 27 August 2021

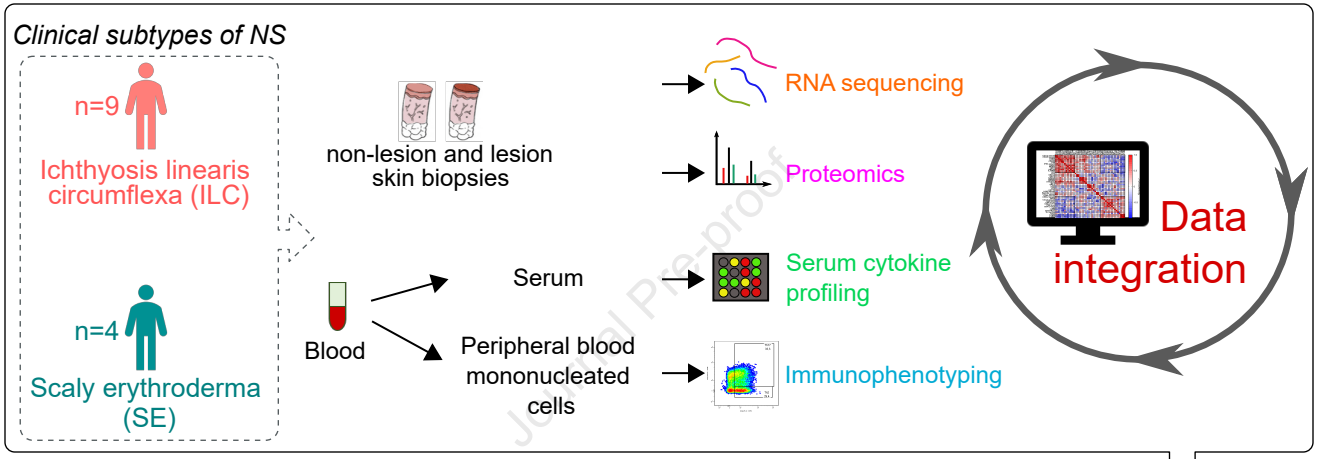
Please cite this article as: Barbieux C, des Claustres MB, Fahrner M, Petrova E, Tsoi LC, Gouin O, Leturcq F, Nicaise-Roland P, Bole C, Béziat V, Bourrat E, Schilling O, Gudjonsson JE, Hovnanian A, Netherton syndrome subtypes share IL-17/IL-36 signature with distinct IFN-α and allergic responses, *Journal of Allergy and Clinical Immunology* (2021), doi: https://doi.org/10.1016/j.jaci.2021.08.024.

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Netherton syndrome (NS) subtypes share IL-17/IL-36 signature with distinct type I IFN, Th2 and Th9 allergic responses



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Abbreviations
IL-17: Interleukin 17
IL36: Interleukin 36
IFN: Interferon
NS: Netherton syndrome
Th2: Type 2 helper lymphocytes
Th9: Type 9 helper lymphocytes

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Netherton syndrome subtypes share IL-17/IL-36 signature with distinct IFN-α and allergic responses

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Conflict of interest statement: The authors declare that they have no competing interests.

Funding: This project was funded by the French National Research Agency ANR-17-CE14-0025 Tfh Atopy (AH), ANR-19-CE17-0017 Target-NS (AH), the Imagine institute with Cross-Lab program and Association Ichtyose France (AH). AH and OS were supported by the European Union's Horizon 2020 research and innovation program under the ERA-NET Cofund action n° 643578 (KLKIN project), JEG and LCT are supported by NIH P30-AR075043 and AI130025.

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Abstract

Background: Netherton syndrome (NS) is a rare recessive skin disorder caused by loss-of-function mutations in *SPINK5* encoding the protease inhibitor LEKTI. NS patients suffer from a severe skin barrier defect, display inflammatory skin lesions and superficial scaling with atopic manifestations. They present with typical ichthyosis linearis circumflexa (NS-ILC) or scaly erythroderma (NS-SE). **Objective:** Here we employed a combination of several molecular profiling methods to comprehensively characterize the skin, immune cells and allergic phenotypes of NS-ILC and NS-SE patients.

Methods: We studied a cohort of 13 NS patients comprising 9 NS-ILC and 4 NS-SE.

Results: Integrated multi-omics revealed abnormal epidermal proliferation and differentiation and IL-17/IL-36 signatures in lesion skin and in blood in both NS endotypes. While the molecular profiles of NS-ILC and NS-SE lesion skin were very similar, non-lesion skin of each disease subtype displayed distinctive molecular features. Non-lesion and lesion NS-SE epidermis showed activation of the type I IFN signaling pathway, while lesion NS-ILC skin differed from non-lesion NS-ILC skin by increased complement activation and neutrophil infiltration. Serum cytokine profiling and immunophenotyping of circulating lymphocytes showed a Th2-driven allergic response in NS-ILC, whereas NS-SE patients displayed mainly a Th9 axis with increased CCL22/MDC and CCL17/TARC serum levels.

Conclusion: This study confirms IL-17/IL-36 as predominant signaling axes in both NS endotypes and unveils molecular features distinguishing NS-ILC and NS-SE. These results identify new therapeutic targets and could pave the way for precision medicine of NS.

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Key messages

- Multi-omic analysis revealed that IL-36 signature was a hallmark of Netherton syndrome.
- Cutaneous molecular signatures of NS-ILC involved neutrophil infiltration and complement activation, while NS-SE involved type I IFN.
- Allergic responses were predominantly associated with Th2 phenotype in NS-ILC patients, but a Th9 phenotype in NS-SE

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Capsule summary

Integrated multi-omics reveals IL-36 signature in Netherton syndrome, specific activation of type I IFN signaling in NS-SE and endotype-dependent Th2 and Th9 allergic responses. These results could pave the way for precision medicine of NS.

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Key words

Netherton syndrome; IL-36; ichthyosis linearis circumflexa; scaly erythroderma; type I IFN; Th2; Th9

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Abbreviations

AD	Atopic dermatitis
DEG	Differentially expressed gene
DEP	Differentially expressed protein
ECM	Extracellular matrix
FLG	Filaggrin
HC	Healthy control
IFN	Interferon
IgE/M/G1	Immunoglobulin E/M/G1
ILC	Ichthyosis linearis circumflexa
IVL	Involucrin
KLK	Kallikrein-related peptidase
KRT	Keratin
LEKTI	Lympho-epithelial Kazal-type-related inhibitor
LOR	Loricrin
MPO	Myeloperoxidase
NS	Netherton syndrome
PAR2	Protease activated receptor 2
PBMC	Peripheral blood mononucleated cells
SE	Scaly erythroderma
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin

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Introduction

Netherton Syndrome (NS) (OMIM 256500) is a rare and severe recessive genetic skin disease characterized by the diagnostic triad of ichthyosiform erythroderma, a specific hair shaft abnormality known as trichorrhexis invaginata and high serum IgE levels with atopic manifestations. NS is an orphan disease with currently no satisfactory treatment. NS is caused by recessive loss of function mutations in *SPINK5* [1], encoding the serine protease inhibitor LEKTI expressed at the granular layer-stratum corneum interface [2,3], leading to unopposed activity of cutaneous proteases, including kallikrein-related proteinases (KLK) 5, KLK7, and KLK14 and epidermal elastase 2 [4–8]. Unrestrained epidermal protease activity causes excessive desquamation resulting in a profound skin barrier defect and triggers the activation of inflammatory pathways [9–12]. NS patients display increased transepidermal water loss, superficial scaling, and redness of the skin. At birth or shortly thereafter, NS patients present with diffuse erythroderma and scaling. During childhood and adulthood, cutaneous lesions evolve in some patients towards ichthyosis linearis circumflexa (ILC), which refers to fluctuating polycyclic and serpiginous erythematous lesions bordered by a double collar of scales, while other patients retain a scaly erythrodermic phenotype (SE). Both NS-ILC and NS-SE patients usually develop increased allergen-specific serum IgE with associated multiple airborne and/or food allergies. The presence of immune system abnormalities remains debated [13,14].

Histological analyses of NS skin shows stratum corneum detachment, parakeratosis, reduced granular layer [15] with epidermal hyperplasia and hyper-papillomatosis with increased expression of proliferation markers such as keratin 16 (KRT16) [16]. Differentiation markers such as involucrin (IVL), loricrin (LOR), and filaggrin (FLG) are diffuse in NS upper epidermal layers with a reduction of desmosomal components [5], which contribute to the skin barrier defect. Several studies pointed to an altered lipid composition of NS stratum corneum [17,18], which is likely to aggravate the skin barrier defect and inflammation. Epidermal hyperplasia, abnormal differentiation, and altered lipid composition are features in common with other inflammatory skin diseases, including atopic dermatitis (AD), psoriasis and ichthyoses. A transcriptomic study comparing different forms of

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ichthyoses including NS, with psoriasis and AD, revealed that NS transcriptomic signature was closer to psoriasis, although psoriatic patients lack allergic manifestations [16].

In addition to skin barrier disruption, epidermal proteases such as KLK5 and KLK14 play a role in skin inflammation by activating Protease-activated receptor 2 (PAR2), which in turn triggers the expression of pro-inflammatory cytokines such as TSLP, CXCL8 and TNFα [9,19–21]. Bacterial- and allergen-derived proteases can also induce PAR2 signaling and activate downstream pro-inflammation pathways. In particular, NS patients are susceptible to infections by *Staphyloccocus aureus*, which also induces an immune response driven by the Th17 and IL-36 axis, thus enhancing skin inflammation [22,23]. Previous transcriptomic analyses of NS lesion skin showed an IL-17 signature with increased expression of target genes encoding anti-microbial peptides or IL-17-related cytokines [16,24]. NS patients also displayed an enrichment in circulating lymphocytes expressing IL-17 and IL-22 [25], suggesting that NS could be a Th17-driven disorder resulting from skin barrier impairment [14]. The involvement of the Th17 axis in NS pathogenesis is supported by the successful treatment of NS patients with anti-IL-17A antibodies [26–28]. Although anti-IL-17A therapy results in a significant clinical benefit, we recently reported that improvement could not be durably sustained, suggesting that other biological pathways are likely to contribute to NS pathogenesis.

NS is a disorder with a profound impact on skin homeostasis leading to skin inflammation and systemic allergic manifestations involving the immune system. The biological mechanisms underlying NS pathophysiology are not fully understood. In this study, we performed a comprehensive molecular characterization of a NS patient cohort to gain further insight into the consequences of loss of LEKTI expression in the skin and at the systemic level. We sought to define NS and endotype-specific molecular signatures which could pave the way for novel and precise therapeutic strategies.

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Methods

Patient cohort

A cohort of 13 NS patients from 10 kindreds with a median age of 32 years and 19 age- and sex-matched HC (median age of 30 years) was recruited. NS was confirmed by the identification of deleterious mutations in *SPINK5* and negative LEKTI immunostaining for the 13 NS patients including 9 NS-ILC and 4 NS-SE patients. Demographic information of patients are provided in Table 1. Treatments included emollient on a daily base and topical corticosteroids during acute flares. Skin biopsies and blood were collected prior to any biotherapy and at distance (at least 48 hours) from the application of a corticosteroid. The protocol was approved by the international review board of Necker Hospital (Clinical Trial NCT 020 813 13), and the study was conducted in accordance with the Declaration of Helsinki principles. All patients and HC gave written and informed consent.

RNA sequencing library of NS skin

Total RNA was isolated from 5mm skin biopsy. After a DNase treatment with HL-dsDNase (ArcticZymes), complemetary DNA (cDNA) was prepared using the NuGEN Ovation RNA-Seq System from 100 ng of total RNA. RNA-Seq libraries were sequenced on an Illumina HiSeq. Further details are provided in Supplementary Materials. The transcriptomic data were deposited at GEO data repository with the accession number GSE164285.

Proteomic analysis of NS skin

Proteins were extracted from frozen skin samples using RapiGest containing buffer followed by reduction and alkylation. Proteins were digested by first incubating with LysC followed by incubating with Trypsin. Data were acquired with a Q-Exactive Plus (Thermo Scientific, Bremen, Germany) mass spectrometer. Further details are provided in Supplementary Materials. Mass spectrometry data as well as data analysis results have been deposited to ProteomeXchange via MassIVE (ID: PXD023658).

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Statistical analyses

Statistical analyses were performed with GraphPad Prism v8.4.3 and R software (R Development Core Team, 3.5.0). Further details are provided in supplementary informations. Differences were considered significant for $p<0.05$.

Experimental procedures of immunostaining, immunophenotyping of PBMC, western-blotting and serum cytokine profiling by Luminex are described in supplementary information.

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Results

1. Patient characterization

To study the molecular mechanisms involved in NS, we analyzed a cohort of 13 patients, comprising 9 NS-ILC and 4 NS-SE (Table 1). NS diagnosis was confirmed by the identification of deleterious mutations in *SPINK5* and negative LEKTI immunostaining for each patient. NS-ILC patients presented with flares of erythematous, serpiginous lesions with a double scaly edge on their trunk and/or limbs. In contrast, NS-SE patients had extensive scaly and red skin and often displayed a lichenified appearance of flexion creases (Figure 1A).

In both endotypes, histological analyses revealed stratum corneum detachment, psoriasiform epidermal hyperplasia with elongated rete ridges and parakeratosis in non-lesion and lesion skin (Figure 1B). The stratum granulosum was thicker in non-lesion NS-SE skin and was almost completely absent in lesion skin from both NS endotypes. Subcorneal microabscesses were seen in lesion NS-ILC skin only. Histological observations were supported by immunostaining of skin sections (Figure E1). Marked inflammatory infiltrates were observed in NS lesion skin in both subtypes. Immunostaining with MPO and tryptase antibodies showed massive neutrophil and mast cell infiltrates in lesion NS-ILC and lesion NS-SE skin, respectively (Figure 1C). No eosinophils or infiltrating lymphocytes were observed (data not shown).

Total serum IgE levels were similarly increased by 3-to 40-fold in both NS-ILC and NS-SE patients (Table 1) and were associated with several atopic manifestations. Serum component allergen-specific IgE showed high levels of specific IgE against pollens, dust mites, pets and nuts (Figure 1D and Figure E2A). Likewise, all patients analyzed had elevated specific IgE against cross-species allergens such as profilin, tropomyosin or PR10 proteins (Figure 1D and S2B). These results showed that the two clinical subtypes of NS shared common histological and allergic sensitization features with distinct immune cell skin infiltrates.

2. NS patients display IL-17 and IL-36 driven immune response in skin and peripheral blood.

To investigate the transcriptomic signature of NS, RNAseq was performed of lesion and non-lesion skin biopsies obtained from NS patients or healthy donors. Principal component analysis (PCA)

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revealed a clear separation between NS lesion, non-lesion and healthy control samples (Figure 2A), with non-lesion skin of NS-ILC patients being close to HC and non-lesion NS-SE skin resembling lesion samples. A global analysis was performed to compare HC, NS non-lesion and NS lesion skin (Table E1) and the DEGs identified in each comparison were submitted to DAVID online tool. Epidermal differentiation, immune response, inflammation and proliferation were the most enriched biological processes in DEGs. Lesion and non-lesion skin showed similar features with DEGs mainly involved in epidermal differentiation and immune response when compared to healthy controls. Inflammation and proliferation were specifically enriched in lesion skin (Figure E3A).

In parallel, proteomic profiling of non-lesion and lesion skin biopsies from the same patients showed a clear separation between NS and healthy controls as revealed at the RNA level (Figure E3B). RNAseq and proteomic were positively correlated (Figure 2B) and showed enrichment for the same biological processes. Up-regulated proteins in NS non-lesion and lesion skin (Table E1) were mainly involved in epidermal differentiation, cell adhesion, anti-microbial response and the immune response, whereas multiple proteins crucial in extracellular matrix organization were downregulated compared to healthy skin biopsies (Figure E3C). Proteins involved in the immune response, especially in the IL-17/IL-36 pathways, were specifically upregulated in lesion skin.

Genes involved in IL-17/IL-36 pathways (*S100A7/8/9, PI3, IL36G*) were among the ones with the highest transcript and protein fold changes (Figure 2B). Immunostaining of skin sections revealed a significant increase in IL-36γ in lesion and non-lesion NS skin and S100 proteins in lesion NS skin compared to healthy controls (Figure 2C). Evidence for IL-17 and IL-36 signature was also sought in NS patients sera . IL-36γ and the IL-17-induced chemokine CCL20 serum levels were significantly increased in NS patient compared to HC (Figure 2D). Overall, the IL-17/IL-36 transcriptomic and proteomic signature was identified in lesion skin of both NS endotypes and in non-lesion NS-SE skin.

3. Transcriptomic and proteomic profiling distinguishes NS-ILC from NS-SE

Despite common features, omic analyses revealed that differences could be observed between the two NS endotypes. Therefore, transcriptomic data were further analyzed by calculating the Euclidian distance which clearly distinguished non-lesion NS-ILC samples from non-lesion NS-SE skin with

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2208 DEGs (Figure 3A, Table E2). In contrast, lesion skin from NS-ILC and NS-SE showed more similarities with only 168 DEGs identified (Figure E6A, Table E2). Extracellular matrix (ECM) organization, cell adhesion, and innate immune response were significantly enriched in NS-ILC, while the NS-SE signature involved the type I interferon signaling pathway and response to viruses (Figure 3B). NS-ILC and NS-SE skin transcriptomic profiles were compared to AD and psoriasis lesion skin [29,30] and showed that lesion NS-ILC and NS-SE and non-lesion NS-SE skin significantly correlated with psoriasis (Figure E6B).

In parallel, proteomic data were also further analyzed and revealed 66 significant DEPs between NS-ILC non-lesion and NS-SE non-lesion skin and 54 DEPs between NS-ILC lesion and NS-SE lesion skin (Table E2). Multiple proteins involved in the extracellular matrix organization as well as in neutrophil degranulation were present in higher abundance in NS-ILC compared to NS-SE biopsies (Figure 3C). Four SERPINs, some of which are known as protease inhibitors, were enhanced in NS-ILC skin compared to NS-SE. Of note, heavy chains of IgG1 and IgM (IGHG1 and IGHM, respectively) and C1S, which are involved in the classical pathway of complement activation, were also enriched in NS-ILC lesion skin (Figure 3C and E6B). In the NS-SE endotype, proteins involved in epidermal development, keratinocyte differentiation, cell proliferation and the inflammatory response were upregulated. Proteomic profiling of NS-SE skin revealed that the IFN pathway downstream effector STAT1 was more abundant in NS-SE non-lesion skin than in NS-ILC non-lesion skin (Figure E6B).

4. Complement activation is more pronounced in NS-ILC skin

Proteomic data showed increased expression of complement C1S, IgG and IgM heavy chains. Enhanced expression of classical complement components was confirmed at the transcript level by RNAseq data. In particular, C1QB and C1QC prensented significantly increased transcript levels in non-lesion and lesion skin compared to healthy controls (Figure 4A). C1R was also significantly increased in lesion NS-ILC skin compared to healthy controls. Since these components initiate the classical cascade, cleavage of complement C3 was assessed by western-blotting in NS skin (Figure 4B). Complement C3 could be detected in NS skin lysates but not in healthy controls. Complement

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C3α full length (115kDa) and fragments iC3b (68 kDa) and C3dg (40 kDa) were observed. The three bands were quantified and added to obtain total complement C3, which was significantly increased by 2-fold in lesion NS-ILC skin lysates compared to non-lesion NS-ILC and NS-SE (Figure 4B). In addition, the percentage of complement C3 cleavage was enhanced by 1.5-fold in non-lesion and lesion NS-ILC skin compared to NS-SE (Figure 4C). The increased cleavage of C3 in NS-ILC skin suggested that complement activation was involved in NS-ILC inflammatory response.

5. NS-SE patients show an IFN signature which predominates in the skin.

Transcriptomic profiling revealed a marked type I IFN signature in non-lesion and lesion skin from NS-SE patients, which was absent in non-lesion NS-ILC and minimal in lesion NS-ILC (Figure 4D and E7A). Strikingly, the intensity of increased expression of IFN-induced gene was comparable between non-lesion and lesion NS-SE skin. The IFN signaling pathway was further assessed in skin biopsies by immunostaining of STAT1 and its phosphorylated form. Increased expression and phosphorylation of STAT1 with nuclear localization were oberved in lesion epidermis of both NS subtypes compared to healthy control (Figure 4E). STAT1 expression and phosphorylation were also enhanced in non-lesion NS-SE skin, consistent with type I IFN pathway activation, in contrast to non lesion NS-ILC. Plasmacytoid dendritic cells (pDC) which are the main source of type I IFN, were not detected by immunostaining (data not shown). Evidence for a type I IFN signature was also shown at the systemic level with increased serum level of IFN-α in NS-SE patients compared to HC and NS-ILC patients (Figure 4F). However, patients' PBMC showed no evidence for IFN polarization (Figure E7B), except for patients 10.2 and 9. Immunophenotyping of peripheral DCs revealed no differences in pDC frequency and increased type 1 myeloid DC (mDC1) in NS-SE compared to HC (Figure E7C).

6. Distinct allergic responses are triggered in NS-ILC and NS-SE

Discrimination between the two endotypes was further assessed at the systemic level by analyzing their serum cytokine profiles. A supervised partial least square discriminant analysis revealed that these cytokine profiles differed between HC, NS-ILC and NS-SE (Figure 5A). Among the 49 cytokines, 15 were significantly discriminant and could explain the projection of the 3 groups along

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the 2 axes. The first axis was mainly driven by CCL22/MDC and CCL17/TARC, while the second axis was mostly explained by CCL27/CTACK, IL-4, IL-16 and CXCL2. CCL22/MDC and CCL17/TARC serum levels were significantly increased in NS-SE, but not in NS-ILC patients, compared to HC. CCL27/CTACK and IL-4 showed no statistical difference between NS-ILC and HC but were significantly decreased in NS-SE sera compared to NS-ILC and HC (Figure 5B).

The 4 most discriminant cytokines (CCL17/TARC, CCL22/MDC, CCL27/CTACK and IL-4) are known to be involved in allergic response. While IL-4 and CCL27 are Th2 cytokines, the major source of CCL17/TARC and CCL22/MDC is Th9 cells. Detailed immunophenotyping of PBMCs revealed a significant increased Th2 subset in NS-ILC compared to healthy controls, while NS-SE showed a significantly enhanced Th9 subset compared to both healthy controls and NS-ILC patients (Figure 5C). These results were supported by increased IL-4 and IL-13 positive memory CD4 T cells in NS-ILC patients compared to NS-SE. Of note, IL-9 producing memory CD4 T cells were not enhanced in neither NS-ILC nor NS-SE patients. Th2 and Th9 cells contribute to the maturation of B cells which are involved in the allergic response by producing IgE.

7. Skin and blood multi-omics show correlated signature

Each NS subtype could be distinguished based on their cutaneous molecular signature and their immune profile in peripheral blood. Spearman correlation and hierarchical clustering was built on omic, Luminex and phenotyping datasets to identify related cutaneous and systemic signatures and their correlation with NS clinical features (IgE serum levels and severity score). Four clusters, including two with positive correlations and two with negative correlations, could be observed (Figure 6). The first cluster consisted in a strong positive correlation between the expression level of genes involved in type I IFN, IL-36 pathways, Th9 subset frequency and CCL22, CCL20 and IFNα serum levels. This cluster also revealed a strong correlation with markers of epidermal proliferation (MKI67), wound healing (KRT16) and differentiation (IVL). The enhancement of these markers was more pronounced in NS-SE patients than in NS-ILC. The second correlation cluster gathered C1Q and MPO transcript levels, CXCL2, CCL27, IL-4 and IgE serum levels and the frequency of IL-17, IL-13 and IL-4 positive CD4 T cells, which were all increased in NS-ILC patients. Conversely, the last two

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clusters resulted from negative correlations between the markers of the first and the second cluster and vice versa. IgE serum levels were included in the NS-ILC-specific cluster but no significant correlation was observed, except for IL-4-positive memory T cells. The severity score showed a significant positive correlation with CCL17 serum levels and was negatively correlated with IL-9-positive memory CD4 T cells. The Spearman correlation matrix was consistent with the inflammatory signatures identified in each endotype.

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Discussion

Here, we describe the global changes in NS-ILC and NS-SE patients compared to HC, at the clinical, histological and molecular levels. In both clinical subtypes, transcriptomic and proteomic signatures of non-lesion skin revealed abnormalities in epidermal proliferation and differentiation. Epidermal hyperproliferation and impaired differentiation are major characteristics of NS lesion skin which have been previously reported [15,16,31]. Complete loss of differentiation markers seen in lesion NS-SE may result from increased serine protease activity in lesion NS-SE compared to NS-ILC [18], which is supported in our study by downregulation of protease inhibitors in NS-SE patients. Multi-omic profiling showed a prominent IL-17/IL-36 signature in lesion skin of both NS endotypes, which could contribute to epidermal hyperproliferation and impaired differentiation. IL-17 and IL-36 cytokines trigger epidermal proliferation [32,33] and inhibit epidermal differentiation in psoriasis [34,35]. We previously reported, and confirmed in this study, that lesion NS-ILC skin displayed marked neutrophil infiltrates, whereas lesion NS-SE skin was mainly infiltrated with mast cells [27]. Neutrophil gelatinase-associated lipocalin and histamine released by mast cells could participate in the dysregulation of epidermal differentiation in NS-ILC and NS-SE patients, respectively [36,37]. The expression of wound healing markers (KRT6 and KRT16) was enhanced in NS-SE non-lesion skin compared to NS-ILC non-lesion skin, suggesting that skin homeostasis is more disrupted in NS-SE patients. Non-lesion NS-SE skin also displayed increased expression of epidermal differentiation markers compared to NS-ILC, which could result from the enhanced IFN signature in non-lesion NS-SE skin. Indeed, IFN-β is required to induce differentiation of cultured keratinocytes [38].

Multi-omic analyses of NS skin revealed increased immune and inflammatory responses, which mainly relied on IL-17 and IL-36 cytokines. IL-17A and F are secreted by Th17 cells, and IL-17C is expressed by keratinocytes, while IL-36α, β and γ cytokines are mainly found in the epidermis [39]. *Staphyloccocus aureus* is the most represented strain in NS skin microbiome [22] and contributes to the IL-17/IL-36 signature by disrupting the skin barrier. In addition, a recent study showed that epicutaneous *Staphylococcus aureus* enhanced IgE production in an IL-36-dependent manner [40]. Therefore, the skin barrier defect is likely to trigger an immune response in NS patients with no evidence for an immunodeficiency as previously suggested [14]. Of note, the inflammatory properties of

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IL-17 and IL-36 pathways are enhanced by their capacity to mutually regulate each other [41–43]. Recent studies have shown that IL-36 cytokines are the most upstream mediator of skin inflammation in murine models of psoriasis [44–46]. In keratinocytes, IL-17 and IL-36 cytokines induce CCL20 and CXCL8 expression and secretion, which contribute to the inflammatory environment by recruiting neutrophils [47,48]. In psoriatic patients, IL-17A/F and IL-36α and γ cytokines have been shown to be crucial in the course of the disease and to correlate with disease severity [49,50]. Recently, a transcriptomic study showed increased expression of IL-36α and γ cytokines in NS skin, which also correlated with disease severity [24]. The present study is the first one describing non-lesion and lesion NS skin as well as systemic abnormalities found in both clinical manifestations of the disease. In addition to their increased expression in skin, IL-36α and IL-36γ serum levels were also enhanced in the sera from both NS endotypes. Although the IL-17/IL36 signature was found in lesion skin from both endotypes, only non-lesion NS-SE skin displayed increased expression of IL-17/IL-36-related genes, suggesting that each endotype could present a unique molecular signature.

Multi-omic analyses showed distinct signatures in NS-ILC and NS-SE, suggesting that the molecular mechanisms involved in skin inflammation are partially different between the two endotypes. Transcriptomic and proteomic analyses revealed an immune response involving the innate compartment in NS-ILC lesion skin. In particular, complement C1Q, heavy chains of immunoglobulins G1 and M and complement C3 cleavage were increased, suggesting classical complement activation, and are likely to participate in neutrophil recruitment in NS-ILC lesion skin. Indeed, subcorneal neutrophil collection requires C5a, one of the most potent neutrophil chemoattractant factors [51]. When psoriatic lesions were induced by imiquimod in a C3-deficient murine model, inflammation and neutrophil infiltrates were reduced [52], supporting the role of complement activation in neutrophil recruitment and skin inflammation.

In NS-SE lesion and non-lesion skin, transcriptomic analyses revealed a type I IFN-related signature, which was supported by increased nuclear pSTAT1 immunostaining in keratinocytes. Type I IFN cytokines are produced by pDCs, which massively infiltrate psoriatic skin [53] upon activation of the IL-36 pathway [54,55]. However, we could not observe pDC infiltrates in lesion or non-lesion NS-SE skin.

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In chronic plaque psoriasis lesions, almost no pDCs are detected but AMP self-RNA complexes and type I IFN trigger mDC activation [56,57], which is consistent with the increase in mDC1 subset observed in PBMC from NS-SE patients. The expression level of IL-36 cytokines was higher in non-lesion NS-SE skin and could contribute to the enhanced IFN signature. Indeed, IL-36 cytokines induce a strong and early expression of *STAT1, MX2* and oligoadenylate synthase (*OAS*) genes in human keratinocytes [47]. A recent transcriptomic analysis of blood from pustular and plaque psoriasis patients showed that IL-36 induces a type I IFN response in severe forms of psoriasis [54]. Overall, although no marked induction of the IFN pathway was observed in NS blood, it is likely that the IFN pathway contributes to chronic inflammation in NS-SE patient skin.

In addition to skin barrier defect and inflammation, all NS patients studied, except patient 8, had elevated serum IgE levels associated with atopic manifestations. Specific IgE profiling revealed that all NS patients were sensitized to at least one panallergen and presented sensitivity to food and airway allergens, as previously shown in a paediatric NS cohort [58]. Immunophenotyping of PBMC revealed increased Th2 subset in NS-ILC, suggesting that the Th2 axis contributes to allergic manifestations in NS-ILC patients. This hypothesis is supported by increased IL-4 and CCL27/CTACK serum levels, which distinguished NS-ILC from NS-SE patients. Th2 cells secrete cytokines, including but not limited to IL-4 and CCL27, which activate immunoglobulin secretion by B cells [59]. In contrast, the allergy-mediated immune response in NS-SE patients involved massive mast cell infiltrates, increased serum levels of CCL22/MDC and CCL17/TARC and an enhanced Th9 subset in circulating lymphocytes. Th9 subset has previously been involved in allergen sensitization and in the development of allergic asthma by producing CCL22/MDC and CCL17/TARC chemokines, leading to mast cell chemotaxis in mice and in human [60,61]. IL-36γ and type I IFN increase the differentiation of naïve T cells into Th9 [62,63] and could contribute to the allergic response in NS-SE.

The description of biological pathways led to considerable progress in the treatment of NS since classical NS treatments, including emollients, calcineurin inhibitors or topical corticosteroids, show a limited benefit [10]. The new current treatments of NS mainly rely on intravenous immunoglobulins in children with recurrent infections [13] and biotherapy targeting TNF-α, IL-4/IL-13, IL-12/IL-23 or IL-17A, which reduce skin inflammation [64,26,65,66]. Recently, the inhibition of the IL-17 axis [27] revealed a

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duality in the therapeutic response according to the NS endotype, suggesting that other pathways may contribute to NS pathogenesis. Here, we confirm the relevance of blocking IL-17A in both endotypes and the Th2 axis in NS-ILC patients. Our multi-omic approach also revealed a significantly increased IL-36 signature, which has become an attractive therapeutic target with promising results in pustular psoriasis [67,68]. We also reveal an IFN signature in NS-SE patients. Few therapies specifically inhibit the IFN signaling pathway but Janus kinase inhibitors are commonly used in AD and psoriasis to block the IFN pathway [69,70], and could be a new option in NS treatment. Although biotherapies are not curative treatment of NS, they substantially improve NS patients' quality of life.

Altogether, our study reveals a complex skin and systemic inflammatory phenotype in NS, which confirms the IL-17/IL-36 signature as a common characteristic of NS-ILC and NS-SE and distinguishes the two endotypes by the type of their immune response: a Th2/complement-driven (NS-ILC) *versus* a type I IFN/Th9-driven (NS-SE) inflammatory axis (Figure 6). The dual immune responses triggered in NS-ILC and NS-SE could pave the way for precision medicine, with a potential of improving NS patient care and quality of life.

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Acknowledgements

We are grateful to Dr Marie-Louis Frémond and Dr Hervé Bachelez for insightful discussions. The authors acknowledge Ranjitha Uppala, Fanny Louis, Snaigune Miskinyte and Nicolas Cagnard for technical support.

Authors' contribution: Conceptualization: CB, MBDC, EP, OG, VB, AH; Methodology: MF, OS (DIA proteomic analysis); Formal analysis: CB, MF, LCT; Investigation: CB, MBDC, MF, EP, LCT, OG, FL, CBo, VB; Resources: AH; Writing-Original Draft: CB, MBDC, MF; Writing-Review and Editing: CB, EP, LCT, OS, JEG, AH; Supervision: OS, JEG, AH

Data and materials availability: All data associated with this study are present in the paper or the supplementary materials. Deposition of RNAseq data on GEO (accession number: GSE164285) and of proteomic data on MassIVE (accession number: PXD023658)

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Patient	**Sex**	**Age (year)**	***SPINK5* mutations**	**Consequences**	**NS subtype**	**Trichorrexis invaginata**	**IgE[1] UI/mL**	**Atopy**	**Associated manifestations**	**Severity score**
1	F	27	c.880_882del; c.1820+2T>A	Altered splicing*; altered splicing	NS-ILC	-	6217	Allergic rhinitis, food allergies	Blepharitis, flat warts	5
2	F	17	HMZ: c.1048C>T	p.R350*	NS-SE	+	282	Allergic rhinitis, angiodema, multiple food allergies	Perinatal dehydratation, failure to thrive	8
3.1	M	16	c.238dup; c.891C>T	p.A217Gfs*19; p.C297C (altered splicing)	NS-ILC	+	783	Allergic rhinitis, asthma, allergic conjunctivitis		6
3.2	M	12	c.238dup; c.891C>T	p.A217Gfs*19; p.C297C (altered splicing)	NS-ILC	+	670	Multiple food allergies, angiodema		8
4	F	21	c.666+1G>A; c.2620_2621insT	Altered splicing; p.Y874Lfs*6	NS-ILC	-	519	Allergic rhinitis, food allergy (eggs)	Diarrhea in perinatal period	2
5	F	57	c.2468dup; c.1351del	p.K824Efs*4; p.C451Afs*53	NS-ILC	+	7827	Allergic conjunctivitis, asthma, angiodema, multiple food allergies	Failure to thrive in infancy	6
6	F	14	HMZ: c.2539-1501G>C	Retention of 79bp intronic fragment	NS-ILC	+	11657	Allergic rhinitis, allergic conjunctivitis, asthma, multiple food allergies	Perinatal dehydratation, blepharitis, visual impairement[#], obesity[#], developmental delay[#]	8
7	F	18	c.55+1G>A; c.2015+5G>A	Altered splicing; altered splicing	NS-ILC	-	14471	Allergic rhinitis, angiodema, multiple food allergies	Short stature (<2.5 percentile)	6
8	F	34	HMZ: 55kb deletion including exon 1 to 16	Partial *SPINK5* deletion	NS-SE	+	1259	-	Perinatal dehydratation, blepharitis, failure to thrive in infancy, short stature (<2.5 percentile)	5
9	M	28	c.238dup; 52kb deletion including exon 5 to 34	p.A217Gfs*19; partial *SPINK5* deletion	NS-SE	+	4676	Allergic rhinitis, allergic conjunctivitis, angiodema, multiple food allergies	Perinatal dehydratation, flat warts, short stature (<2.5 percentile)	8

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10.1	F	45	c.283-2A>T; c.2468dup	Altered splicing; p.K824Efs*4	NS-ILC	+	40918	Allergic rhinitis, allergic conjunctivitis, multiple food allergies		8
10.2	F	43	c.283-2A>T; c.2468dup	Altered splicing; p.K824Efs*4	NS-ILC	+	15538	Allergic rhinitis, allergic conjunctivitis, multiple food allergies	Failure to thrive in infancy, short stature (<2.5 percentile), flat warts	8
10.3	M	41	c.283-2A>T; c.2468dup	Altered splicing; p.K824Efs*4	NS-SE	+	8538	Allergic rhinitis, allergic conjunctivitis, angiodema, multiple food allergies	Perinatal dehydratation, failure to thrive in infancy, short stature (<2.5 percentile), papillomatous lesions, flat warts	8

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Table 1: Demographic, clinical and molecular characteristics of 13 NS patients included in the study. ILC, ichthyosis linearis circumflexa; SE, scaly erythroderma.

[1], The normal values of IgE serum level of the laboratory are below 114 UI/mL;

*, This mutation changes the last nucleotide of exon 10 and disrupts the donor splice site of intron 10.

#, These features are caused by the association of Bardet-Biedl syndrome confirmed by molecular diagnostic in this patient.

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Figure 1: Clinical and histological features, but not IgE profiles, distinguish 2 NS subtypes. A. Representative clinical pictures of NS-ILC and NS-SE patients. **B**. HES staining of skin sections of HC, lesion and non-lesion skin from NS-ILC and NS-SE patients (magnification x10), bar= 25µm. The enlargements (magnification x40) correspond to upper epidermal layers, bar= 50µm. The dotted black line delineates the dermal-epidermal junction. *sc*, stratum corneum; *sg*, stratum granulosum; *ma*, micro-abscess; *P*, parakeratosis. **C**. Neutrophil and mast cell infiltrates were detected with anti-MPO and tryptase antibodies, respectively. Nuclei were counterstained with DAPI (blue). The dotted white line delineates the dermal-epidermal junction. Bar= 50µM (magnification x40). **D.** Specific IgE were measured by ImmunoCap ISAC (NS-ILC, n=7; NS-SE, n=2) and the number of patients showing reactivity towards specific allergens or panallergens such as PR10 proteins, Lipid Transfert Proteins (LTP), profilins and tropomyosins, is represented.

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Figure 2: Transcriptomic and proteomic profiling of NS reveal an IL-36 signature. **A.** Principal component analysis of RNAseq data from healthy donor skin biopsies (n=19), non-lesion skin (n=13) and lesion skin (n=14) of 9 NS-ILC and 4 NS-SE patients. **B.** Pearson correlation analysis using RNA and protein expression values of cumulatively identified genes. Linear regression line is shown in blue with a 95% confidence interval (grey). **C.** IL-36γ (red) and anti-microbial peptides S100A7 (green) immunostaining of skin sections from HC, non-lesion and lesion skin from NS-ILC and NS-SE patients. Nuclei were counterstained with DAPI (blue). The dotted white line delineates the dermal-epidermal junction. Bar= 50 µm (magnification x40). **D.** Serum levels of proinflammatory IL-36γ and CCL20 in NS patients (n=13) and healthy controls (n=11). The lines correspond to the mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

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Figure 3: Transcriptomic and proteomic profiling of NS-ILC and NS-SE patients revealed molecular differences between each endotype. A. Correlation between non-lesion samples determined by calculating Euclidian distance, which is represented on the heatmap. **B.** Biological processes enriched in DEGs between NS-ILC and NS-SE skin. **C.** Volcano plot comparing proteome data from healthy donor skin, non-lesion and lesion skin from NS patients (n = 11). Proteins of interest and their respective GO ´biological process´ term are highlighted in different colors.

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Figure 4: NS-ILC and NS-SE skin display distinct inflammatory response involving complement and IFN pathway, respectively. A. Level of C1QA, C1QB, C1QC, C1R qnd C1S transcripts. **B.** Complement C3 expression level skin lysates assessed by western-blotting and its **C.** cleavage **p**ercentage in protein extracts from HC, non-lesion and lesion NS skin. **D.** The Log2 of the fold change for the genes involved in the IFN pathway is represented on the heatmap for each group. **E.** STAT1 and pSTAT1 immunostaining in skin sections from HC, non-lesion and lesion skin from NS-ILC and NS-SE patients. Nuclei were counterstained with DAPI (blue). The dotted white line delineates the dermal-epidermal junction. Bar= 50µM (magnification x40). **F.** Serum levels of IFN-α in NS patients (n=13) and healthy controls (n=11). The horizontal bars correspond to the mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

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691 **Figure 5: NS-ILC and NS-SE show allergic features involving either Th2 or Th9 axes A** Partial
692 least square discriminant analysis (PLS-DA) model followed by the determination of variable
693 importance projection (VIP, in bold) based on serum cytokine profiles. **B.** Serum concentrations of IL-
694 4, CCL27/CTACK, CCL22/MDC and CCL17/TARC. Horizontal bars correspond to the mean ± SEM.
695 **C.** Percentages of Th2 and Th9 in PBMC from HC, NS-ILC and NS-SE and production of their
696 cytokines after stimulation with PMA/ionomycin. Horizontal bars represent the mean ± SEM. *
697 $p<0.05$, ** $p<0.01$, *** $p<0.001$.

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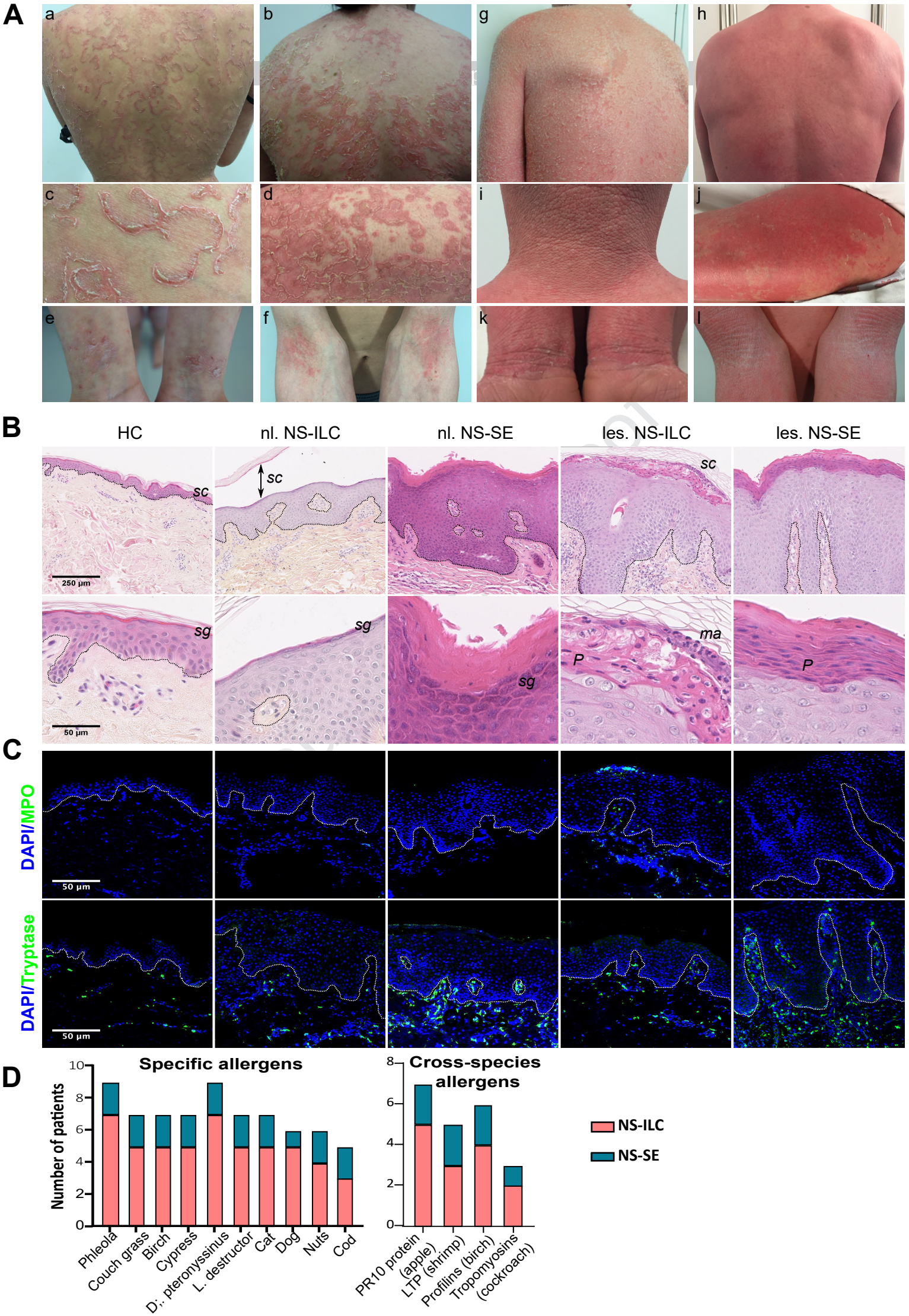
Figure 6: Cutaneous molecular features and peripheral blood phenotype are strongly correlated in each NS subtype. Spearman correlation heatmap of clinical (severity score, serum IgE level) markers, transcript levels, serum cytokine levels and PBMC immunophenotyping. The black boxes underline clustered markers. Statistitcal significance is indicated in the heatmap * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

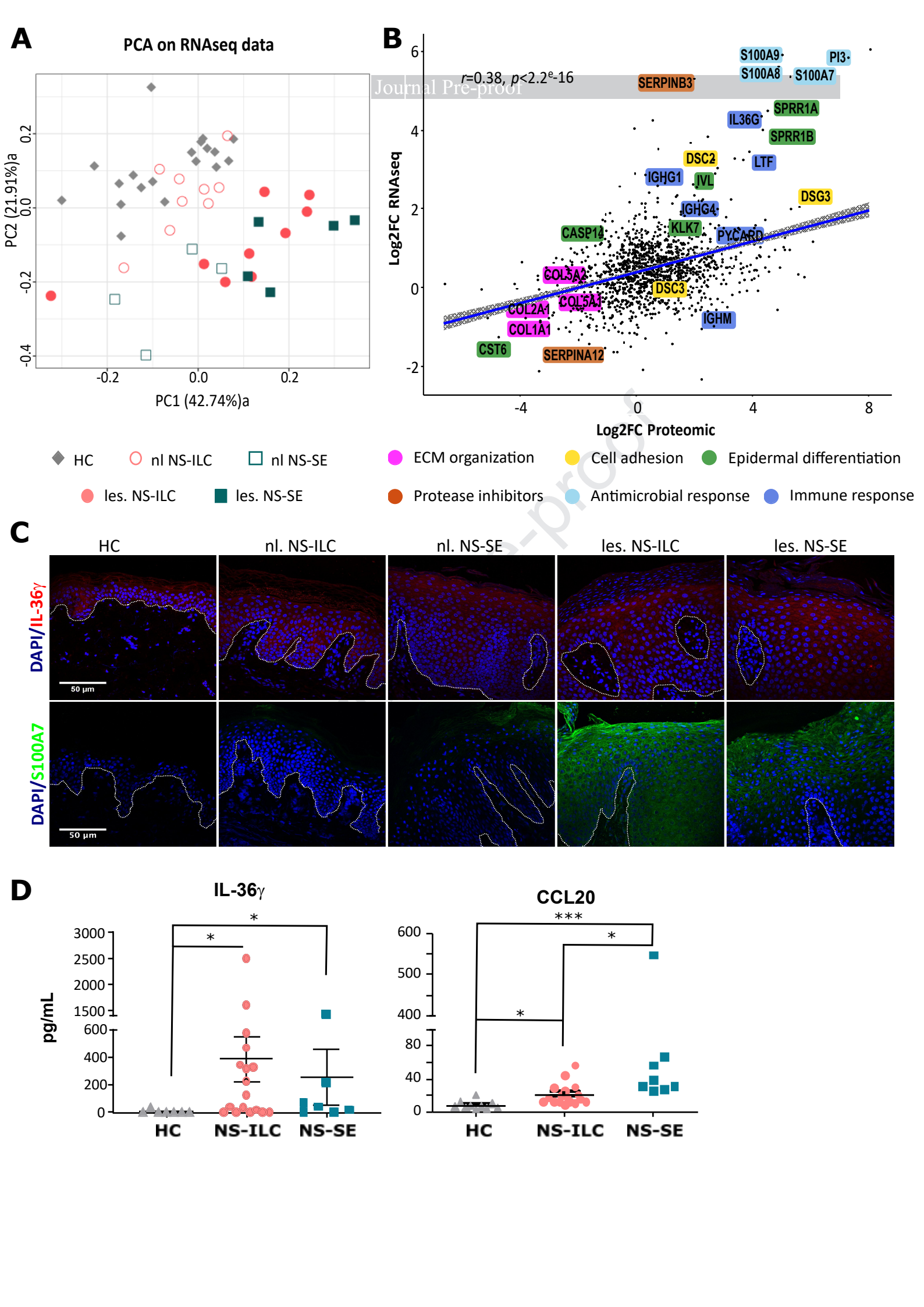
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Figure 7: Proposed model of NS physopathology in its two clinical forms. Loss of LEKTI function due to *SPINK5* mutations, leads to unopposed activity of cutaneous proteases and causes stratum corneum detachment, epidermal hyperplasia and parakeratosis. The profound skin barrier defect allows pathogen and allergen entry, triggering the production of pro-inflammatory mediators, the priming of naïve T lymphocytes. **A. In NS-ILC**, naïve T cells differentiate into Th2 cells, which release IL-4 triggering pruritus and allergic responses. Complement molecules and CXCL8 chemokine contribute to the formation of marked neutrophil infiltrates in the dermis and in the stratum corneum. **B. In NS-SE,** Th9 cells produce large amounts of CCL22/MDC and CCL17/TARC chemokines, which are mast cells chemoattractant. Th9 cells contribute to the allergic response by enhancing the secretion of IgE's, which induce mast cell degarnulation. Increased IFN signature could contribute to cutaneous inflammation.

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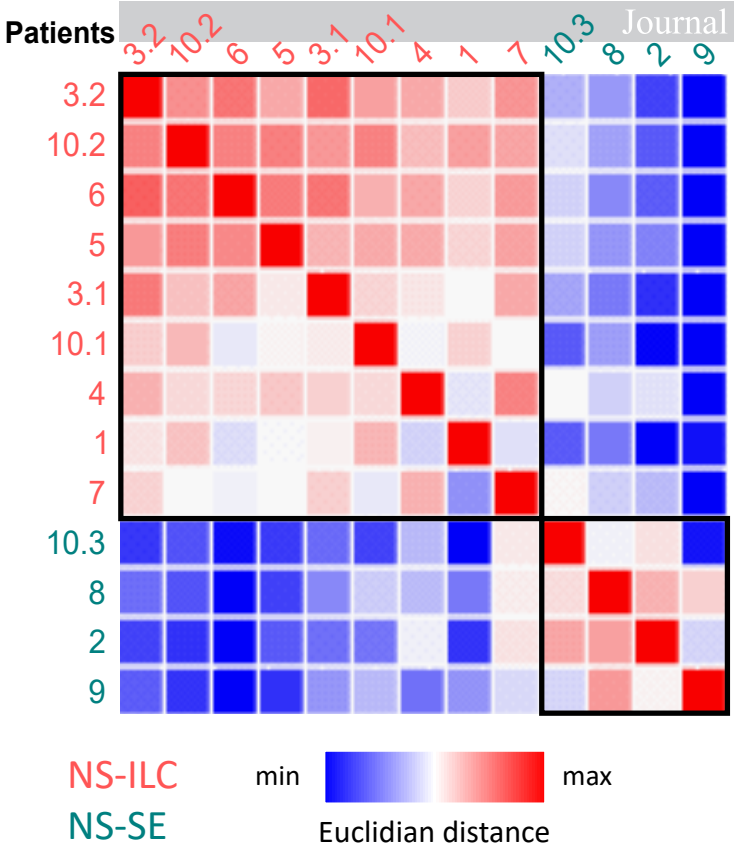

A
a
b
g
h
c
d
i
j
e
f
k
l
B
HC
nl. NS-ILC
nl. NS-SE
les. NS-ILC
les. NS-SE
sc
sg
ma
P
250 µm
50 µm
C
DAPI/MPO
DAPI/Tryptase
50 µm
D
Specific allergens
Number of patients
Phleolá
Couch grass
Birch
Cypress
D;. pteronyssinus
L. destructor
Cat
Dog
Nuts
Cod
Cross-species allergens
PR10 protein (apple)
LTP (shrimp)
Profilins (birch)
Tropomyosins (cockroach)
NS-ILC
NS-SE



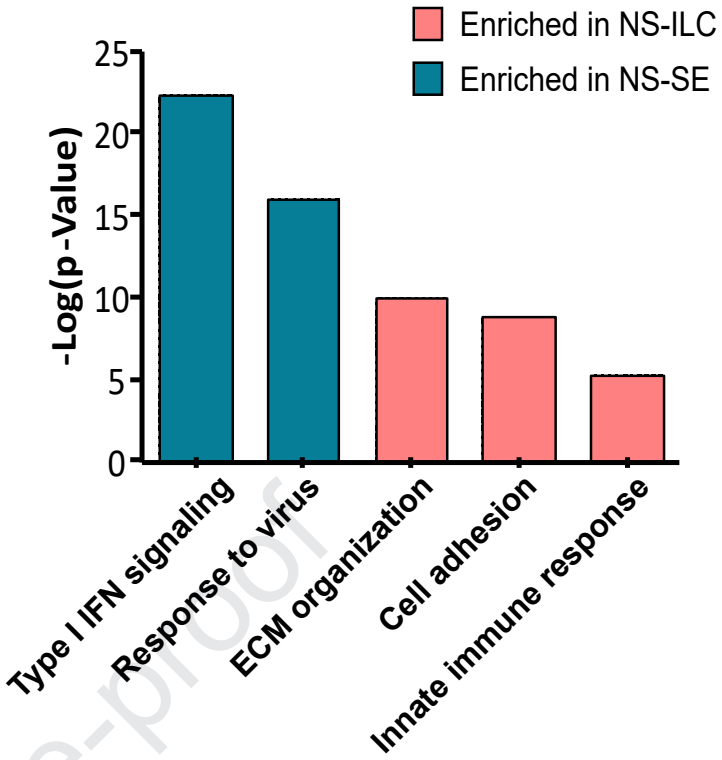
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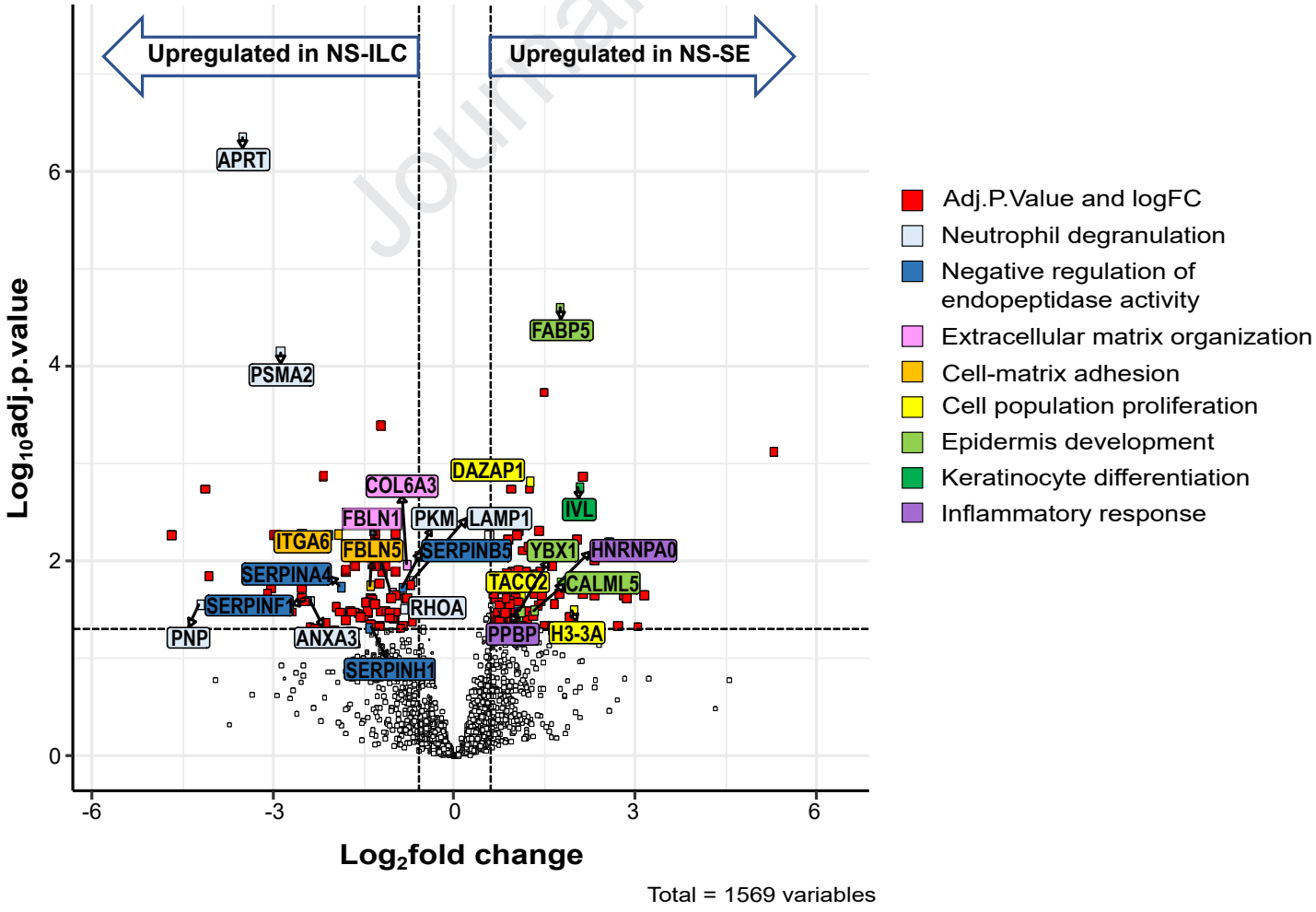
A Correlation between non-lesion skin transcriptomes

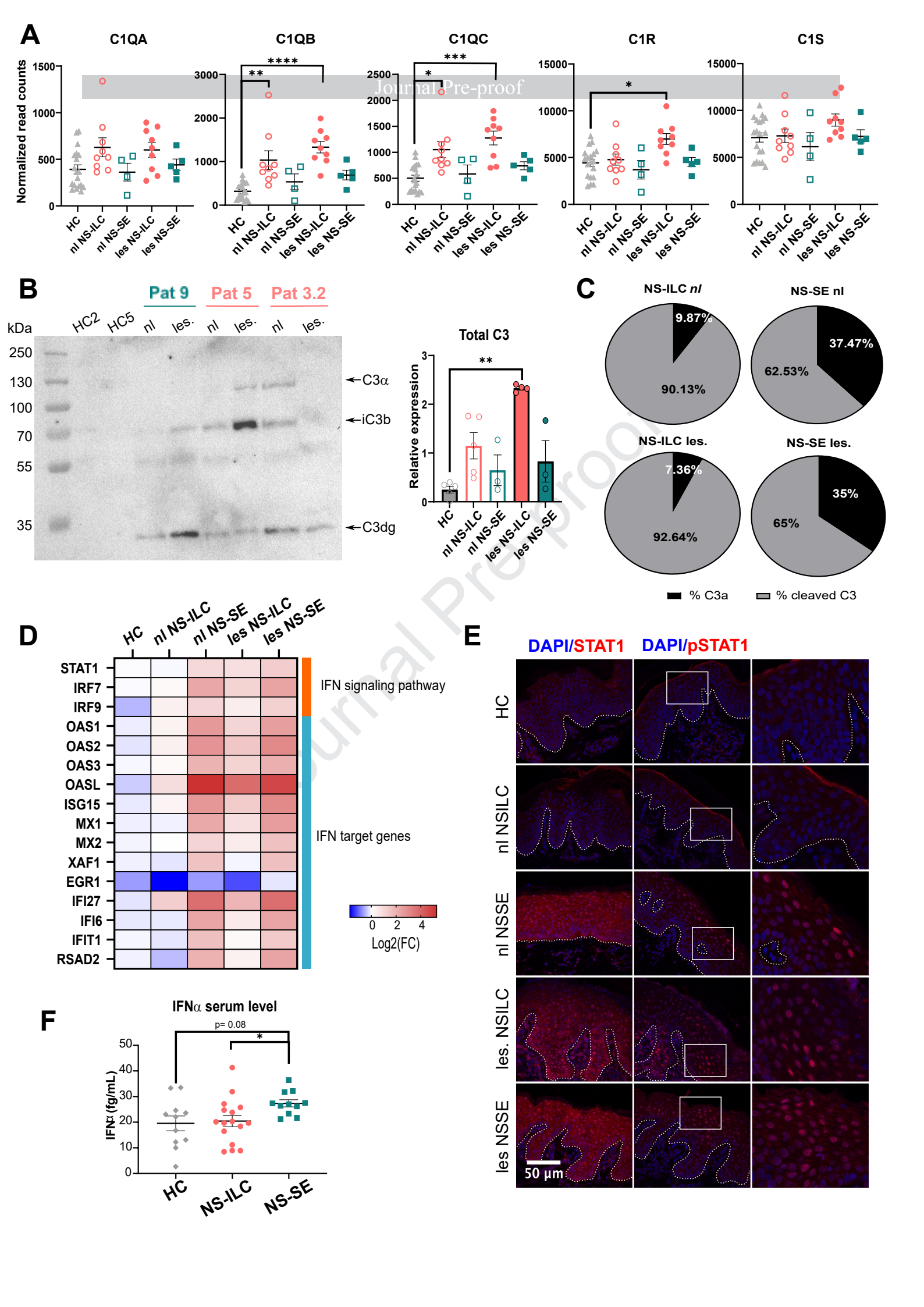


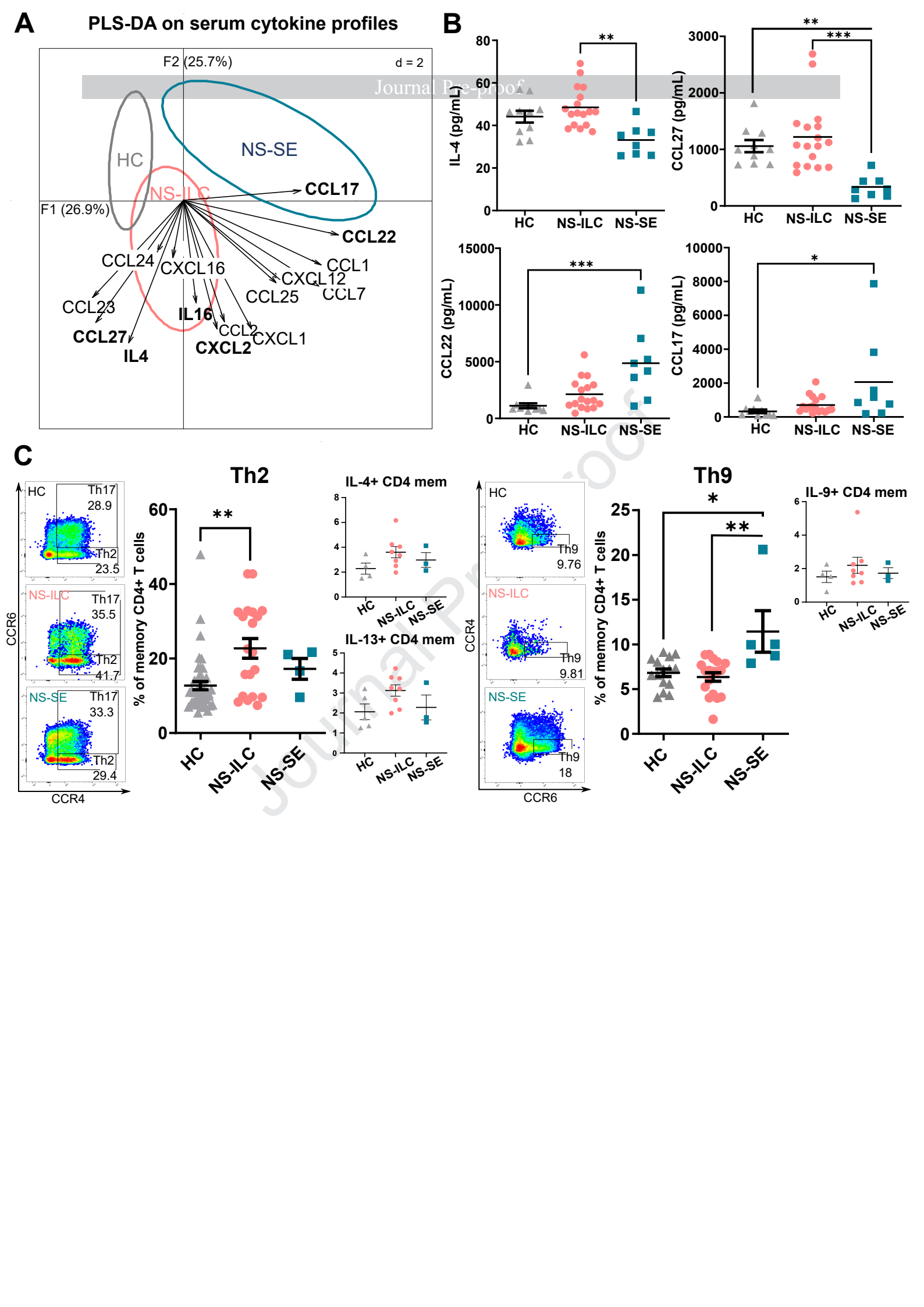
B Biological processes enriched in DEGs between NS-ILC and NS-SE



C DEPs global comparison NS-SE *vs* NS-ILC

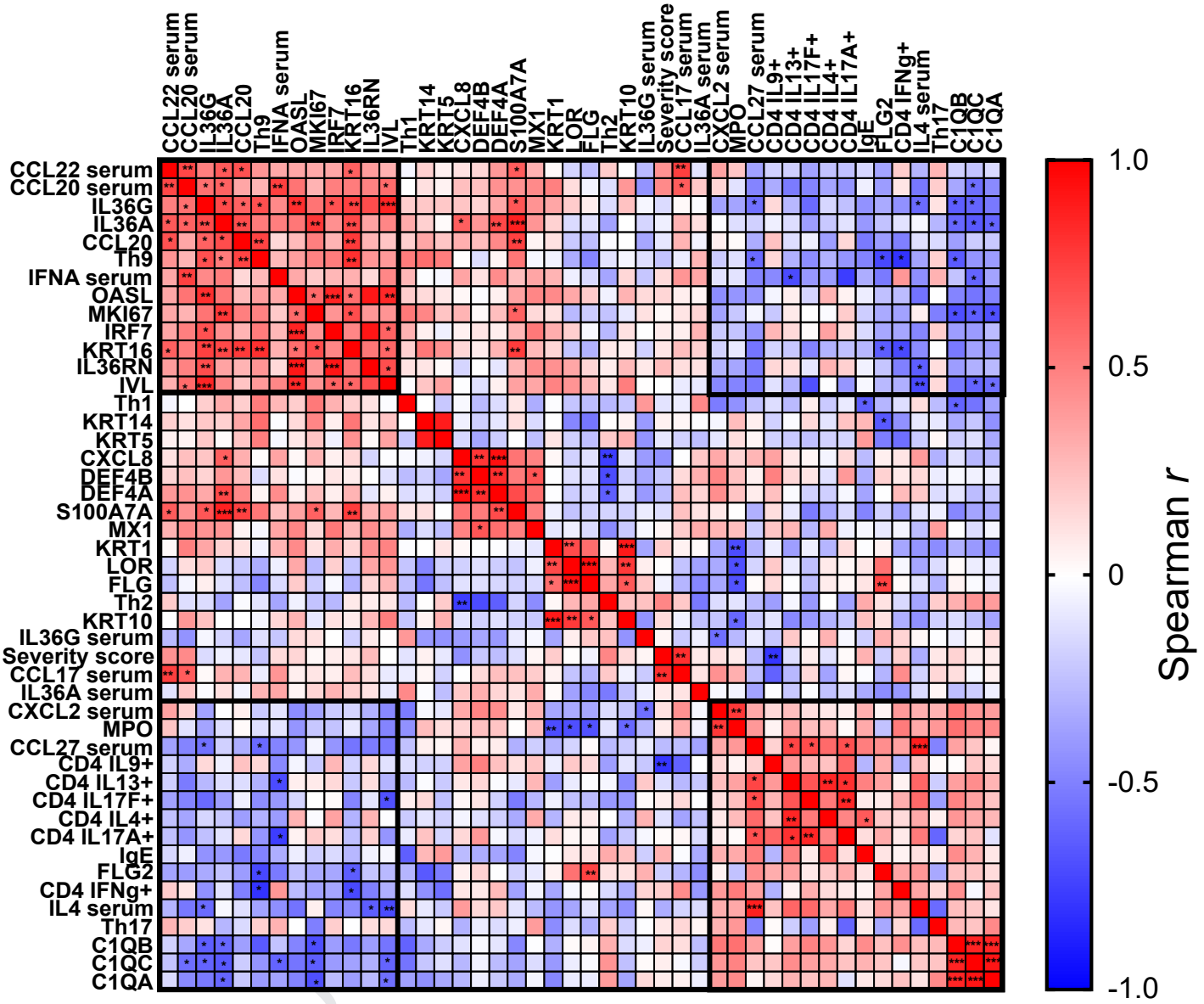


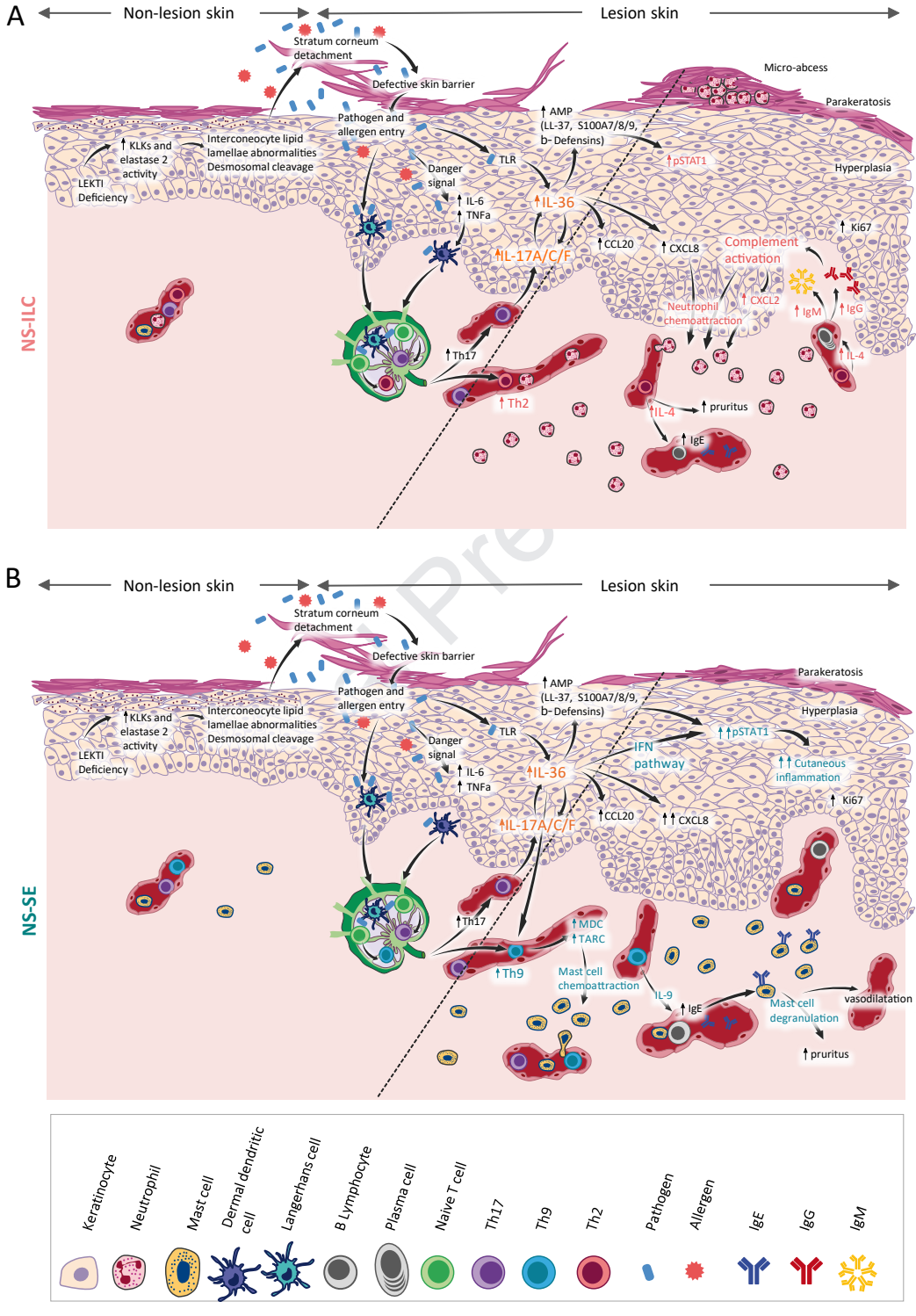

A
C1QA
C1QB
C1QC
C1R
C1S
Normalized read counts
HC
nl NS-ILC
nl NS-SE
les NS-ILC
les NS-SE
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B
Pat 9
Pat 5
Pat 3.2
HC2
HC5
nl
les.
kDa
250
130
100
70
55
35
C3α
iC3b
C3dg
Total C3
Relative expression
C
NS-ILC nl
NS-SE nl
NS-ILC les.
NS-SE les.
9.87%
90.13%
37.47%
62.53%
7.36%
92.64%
35%
65%
% C3a
% cleaved C3
D
STAT1
IRF7
IRF9
OAS1
OAS2
OAS3
OASL
ISG15
MX1
MX2
XAF1
EGR1
IFI27
IFI6
IFIT1
RSAD2
IFN signaling pathway
IFN target genes
Log2(FC)
E
DAPI/STAT1
DAPI/pSTAT1
HC
nl NSILC
nl NSSE
les. NSILC
les NSSE
50 µm
F
IFNα serum level
p= 0.08
IFNα (fg/mL)
NS-ILC
NS-SE

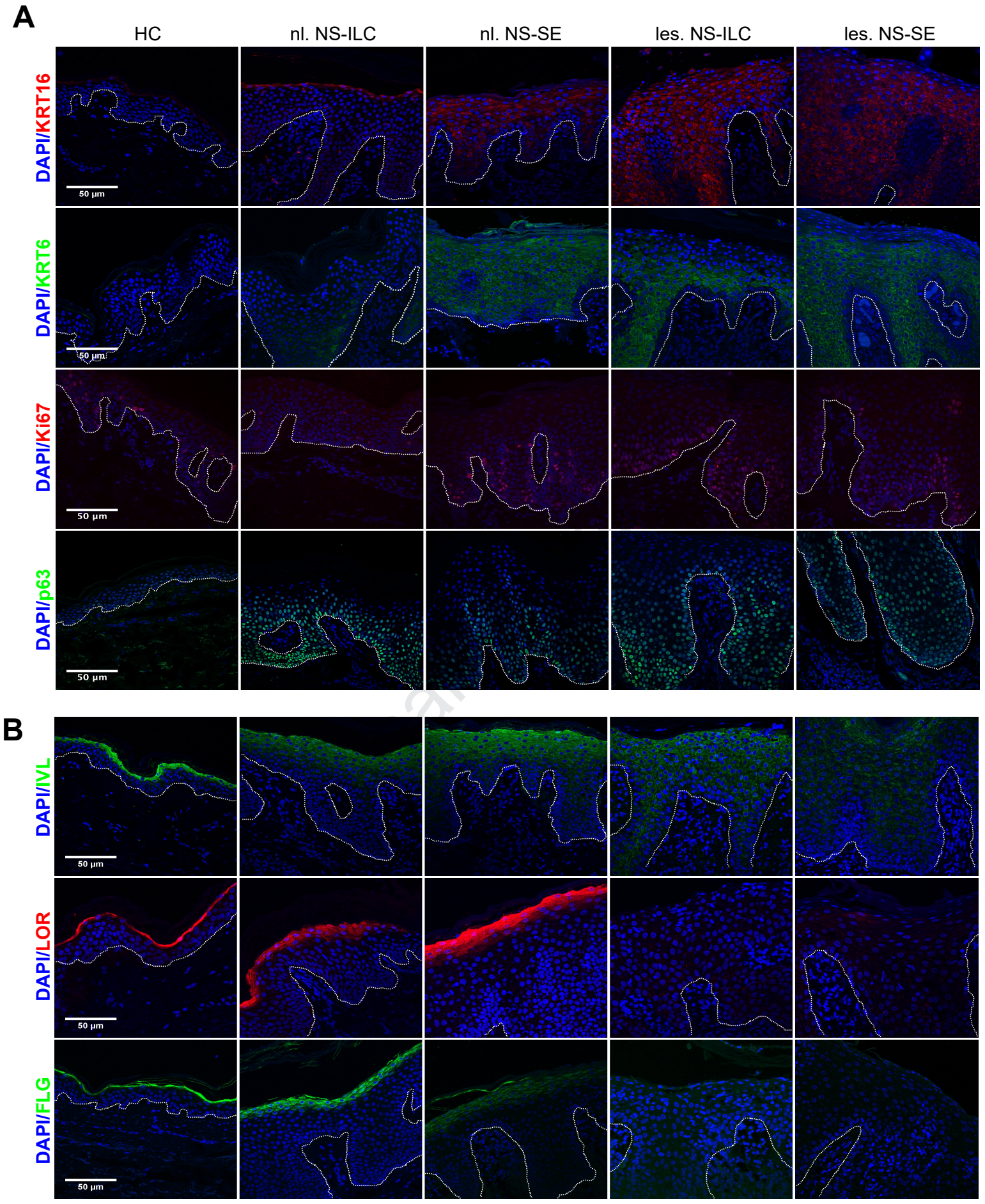


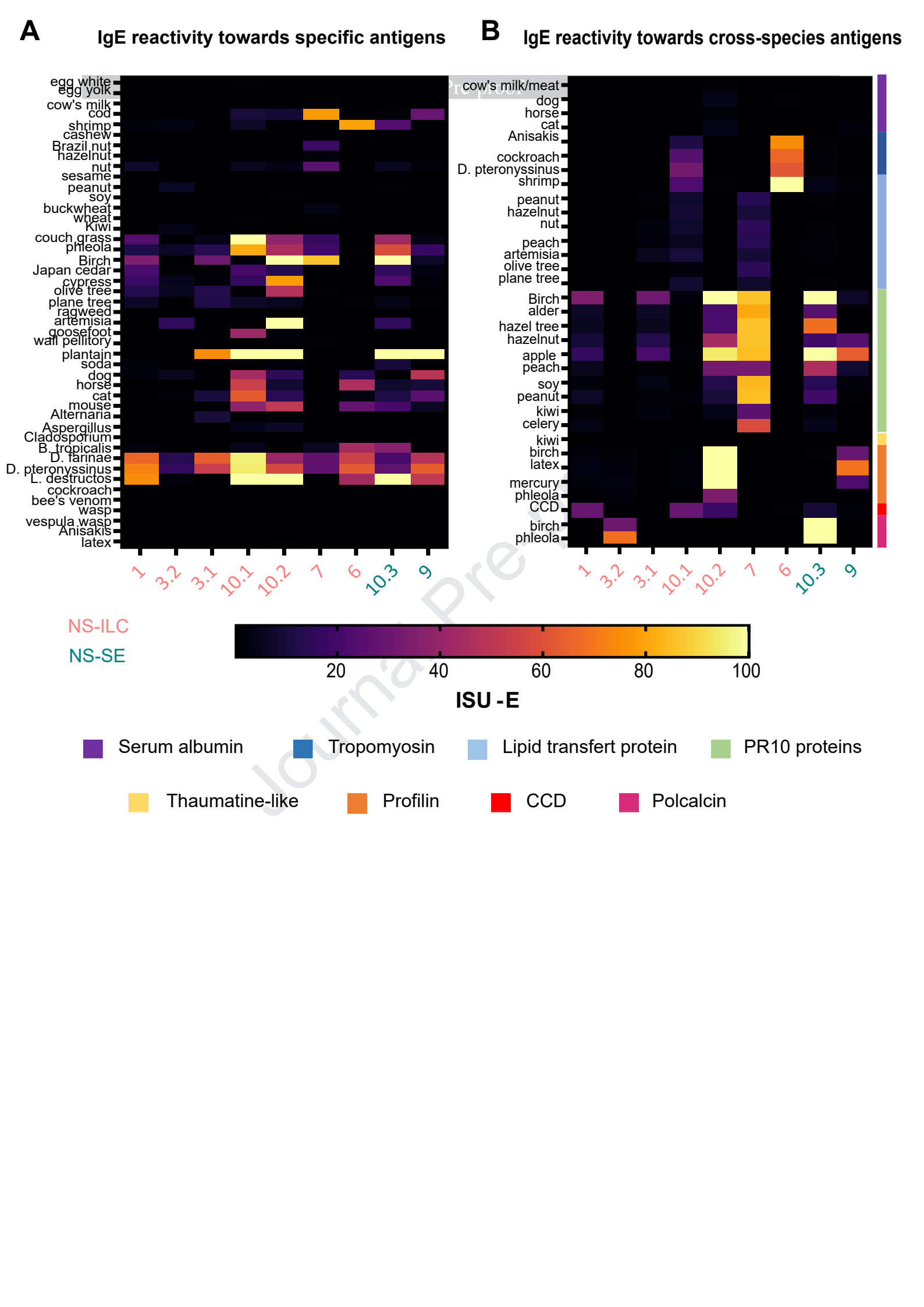
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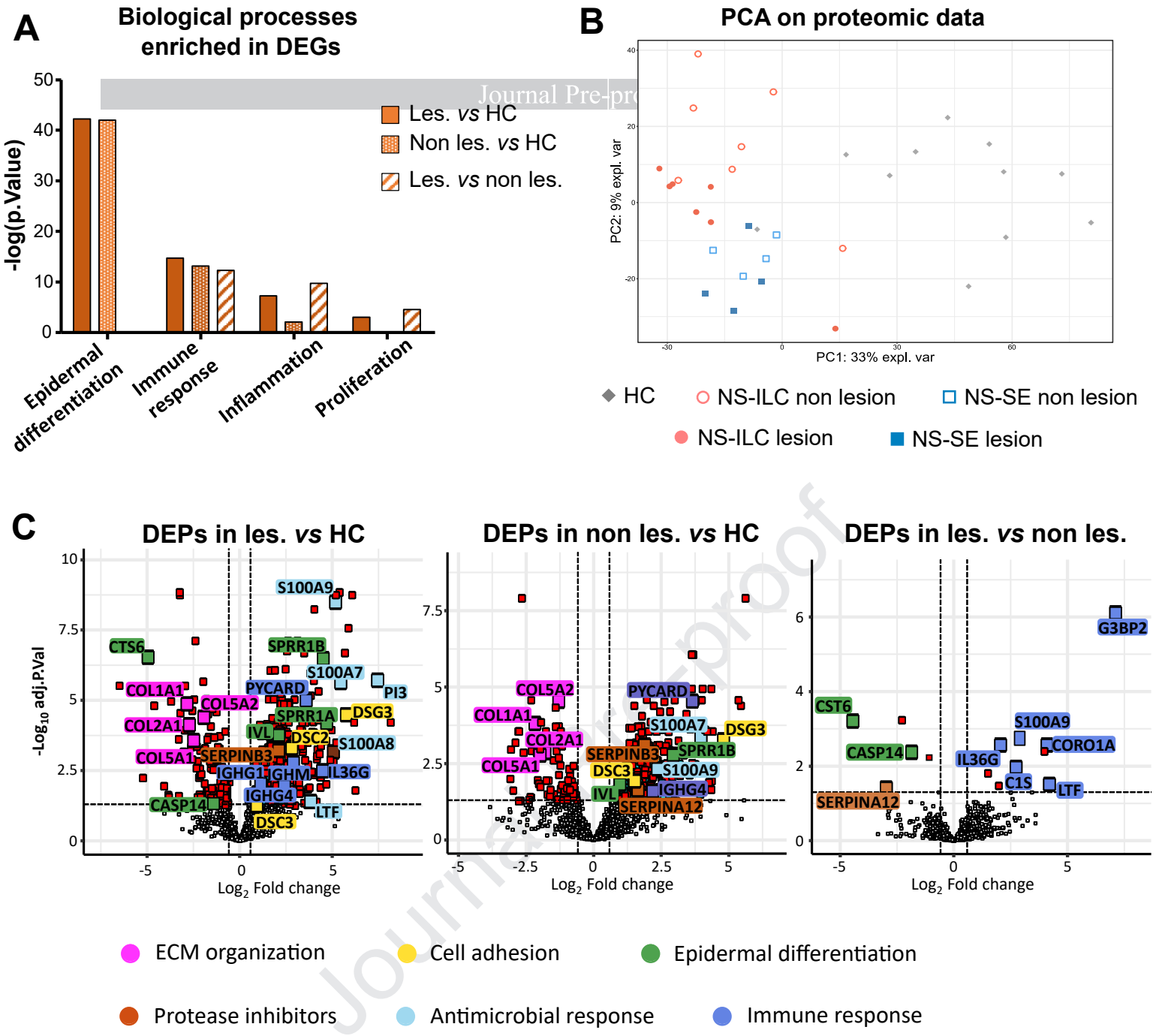
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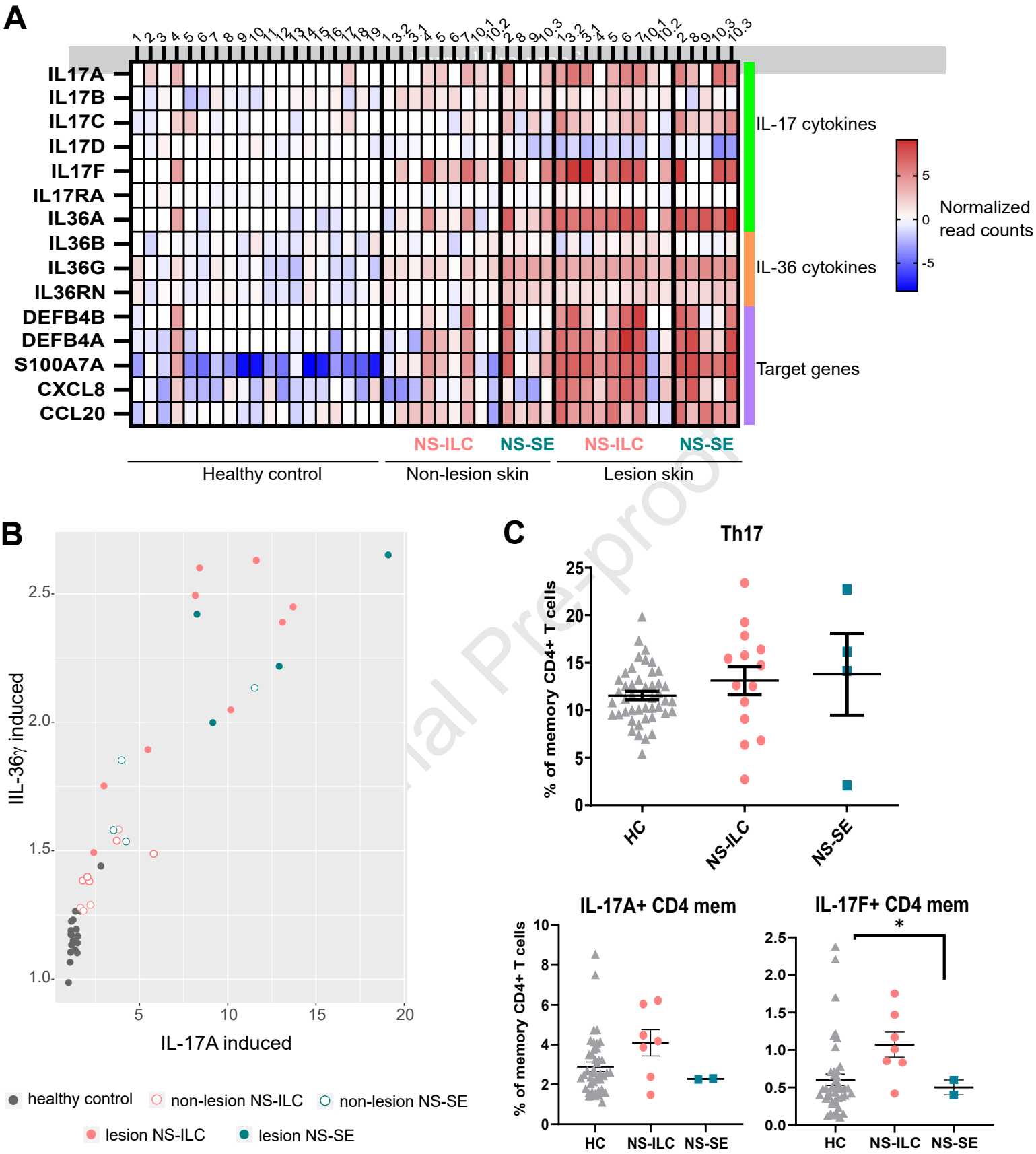

A
Non-lesion skin
Lesion skin
Stratum corneum detachment
Defective skin barrier
Pathogen and allergen entry
↑ AMP (LL-37, S100A7/8/9, b- Defensins)
Micro-abcess
Parakeratosis
Hyperplasia
↑ KLKs and elastase 2 activity
LEKTI Deficiency
Interconeocyte lipid lamellae abnormalities
Desmosomal cleavage
Danger signal
TLR
↑pSTAT1
↑ IL-6
↑ TNFa
↑IL-36
↑IL-17A/C/F
↑CCL20
↑CXCL8
Complement activation
↑ Ki67
↑CXCL2
Neutrophil chemoattraction
↑IgM
↑IgG
↑IL-4
NS-ILC
↑Th17
↑Th2
↑IL-4
↑ pruritus
↑IgE
B
Non-lesion skin
Lesion skin
Stratum corneum detachment
Defective skin barrier
Pathogen and allergen entry
↑ AMP (LL-37, S100A7/8/9, b- Defensins)
Parakeratosis
Hyperplasia
↑ KLKs and elastase 2 activity
LEKTI Deficiency
Interconeocyte lipid lamellae abnormalities
Desmosomal cleavage
Danger signal
TLR
↑↑pSTAT1
IFN pathway
↑↑ Cutaneous inflammation
↑ IL-6
↑ TNFa
↑IL-36
↑IL-17A/C/F
↑CCL20
↑↑CXCL8
↑ Ki67
NS-SE
↑Th17
↑MDC
↑TARC
↑Th9
Mast cell chemoattraction
IL-9
↑IgE
Mast cell degranulation
vasodilatation
↑ pruritus
Keratinocyte
Neutrophil
Mast cell
Dermal dendritic cell
Langerhans cell
B Lymphocyte
Plasma cell
Naive T cell
Th17
Th9
Th2
Pathogen
Allergen
IgE
IgG
IgM


A
HC
nl. NS-ILC
nl. NS-SE
les. NS-ILC
les. NS-SE
DAPI/KRT16
DAPI/KRT6
DAPI/Ki67
DAPI/p63
50 μm
B
DAPI/IVL
DAPI/LOR
DAPI/FLG
50 μm


A
IgE reactivity towards specific antigens
B
IgE reactivity towards cross-species antigens
egg white
egg yolk
cow's milk
cod
shrimp
cashew
Brazil nut
hazelnut
nut
sesame
peanut
soy
buckwheat
wheat
Kiwi
couch grass
phleola
Birch
Japan cedar
cypress
olive tree
plane tree
ragweed
artemisia
goosefoot
wall pellitory
plantain
soda
dog
horse
cat
mouse
Alternaria
Aspergillus
Cladosporium
B. tropicalis
D. farinae
D. pteronyssinus
L. destructos
cockroach
bee's venom
wasp
vespula wasp
Anisakis
latex
1
3.2
3.1
10.1
10.2
7
6
10.3
9
cow's milk/meat
dog
horse
cat
Anisakis
cockroach
D. pteronyssinus
shrimp
peanut
hazelnut
nut
peach
artemisia
olive tree
plane tree
Birch
alder
hazel tree
hazelnut
apple
peach
soy
peanut
kiwi
celery
kiwi
birch
latex
mercury
phleola
CCD
birch
phleola
1
3.2
3.1
10.1
10.2
7
6
10.3
9
NS-ILC
NS-SE
20
40
60
80
100
ISU -E
Serum albumin
Tropomyosin
Lipid transfert protein
PR10 proteins
Thaumatine-like
Profilin
CCD
Polcalcin

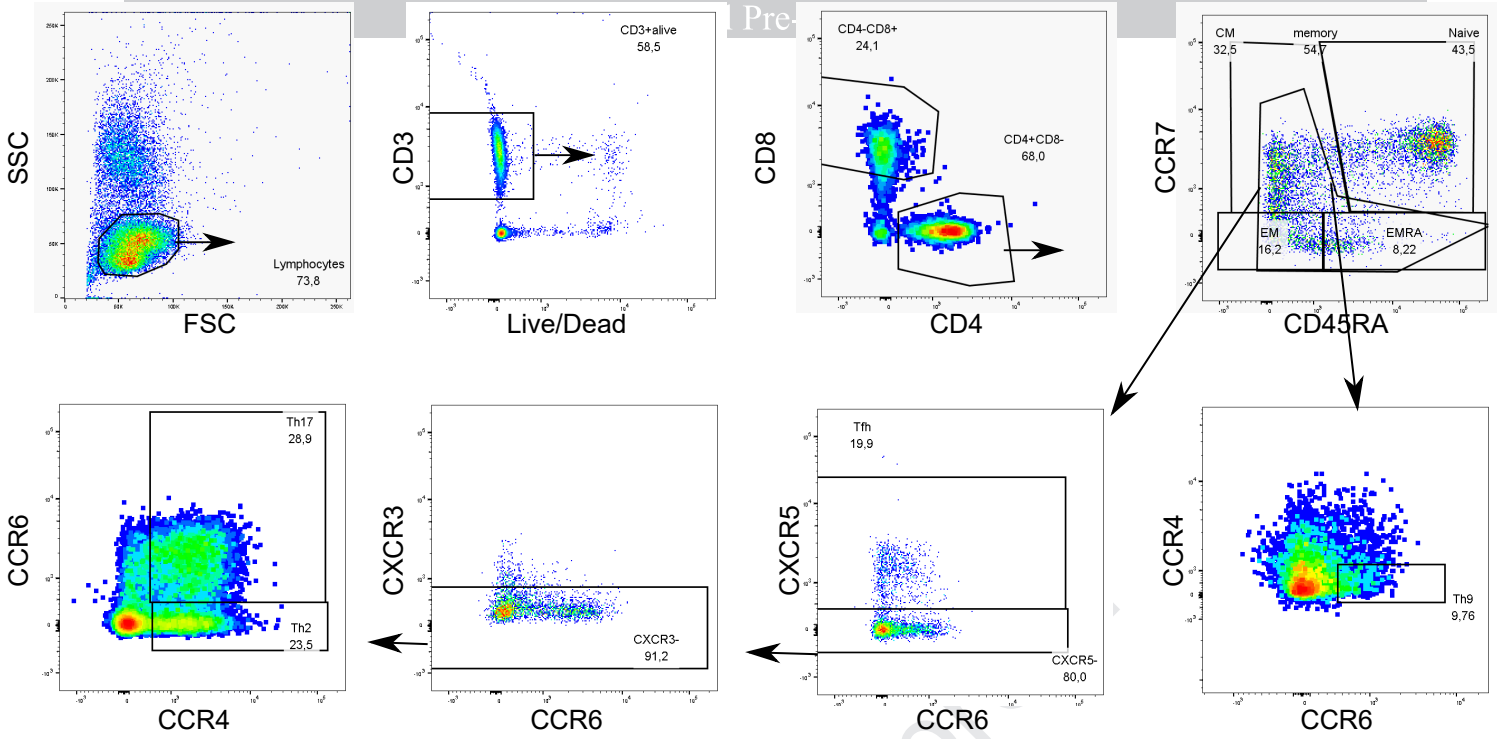


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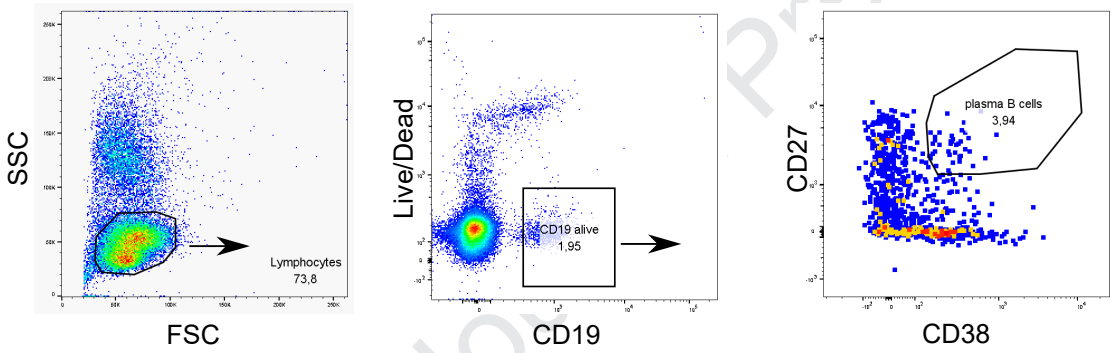


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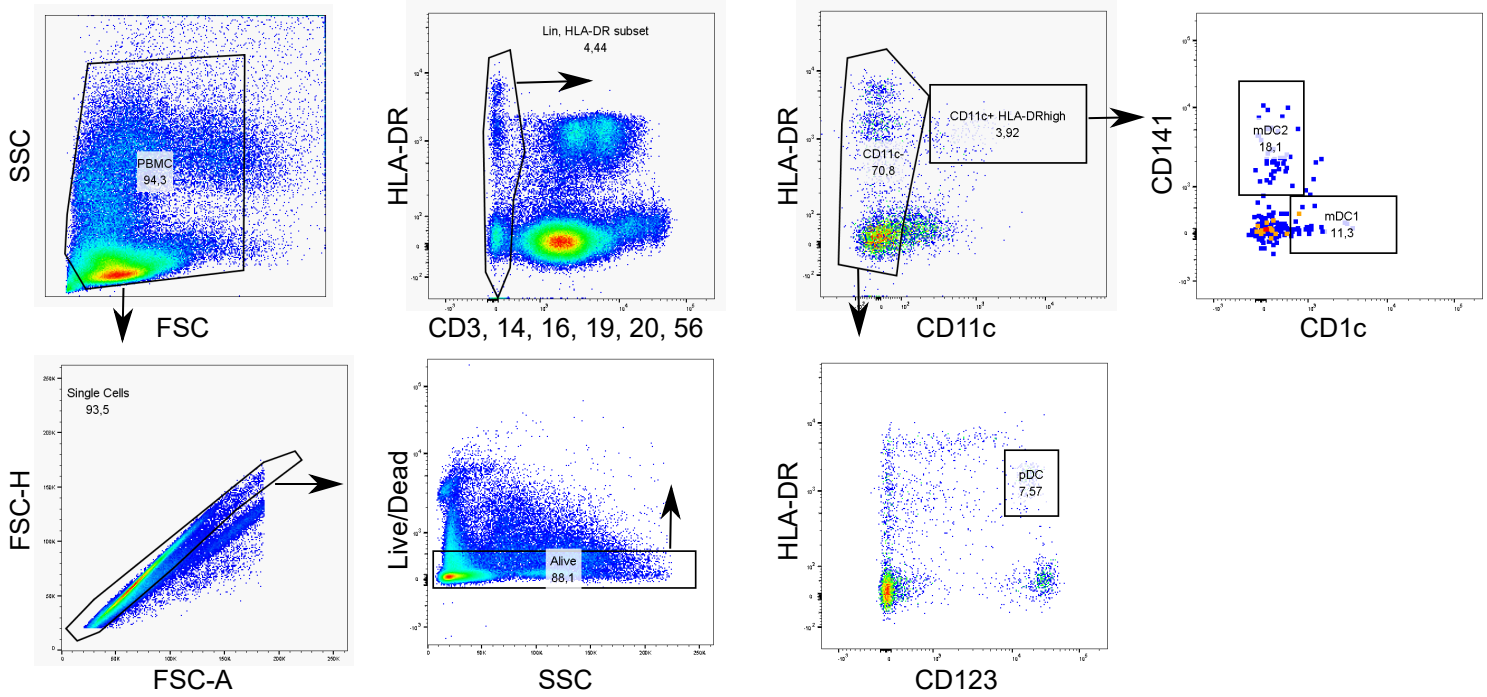
A Gating strategy for T cells

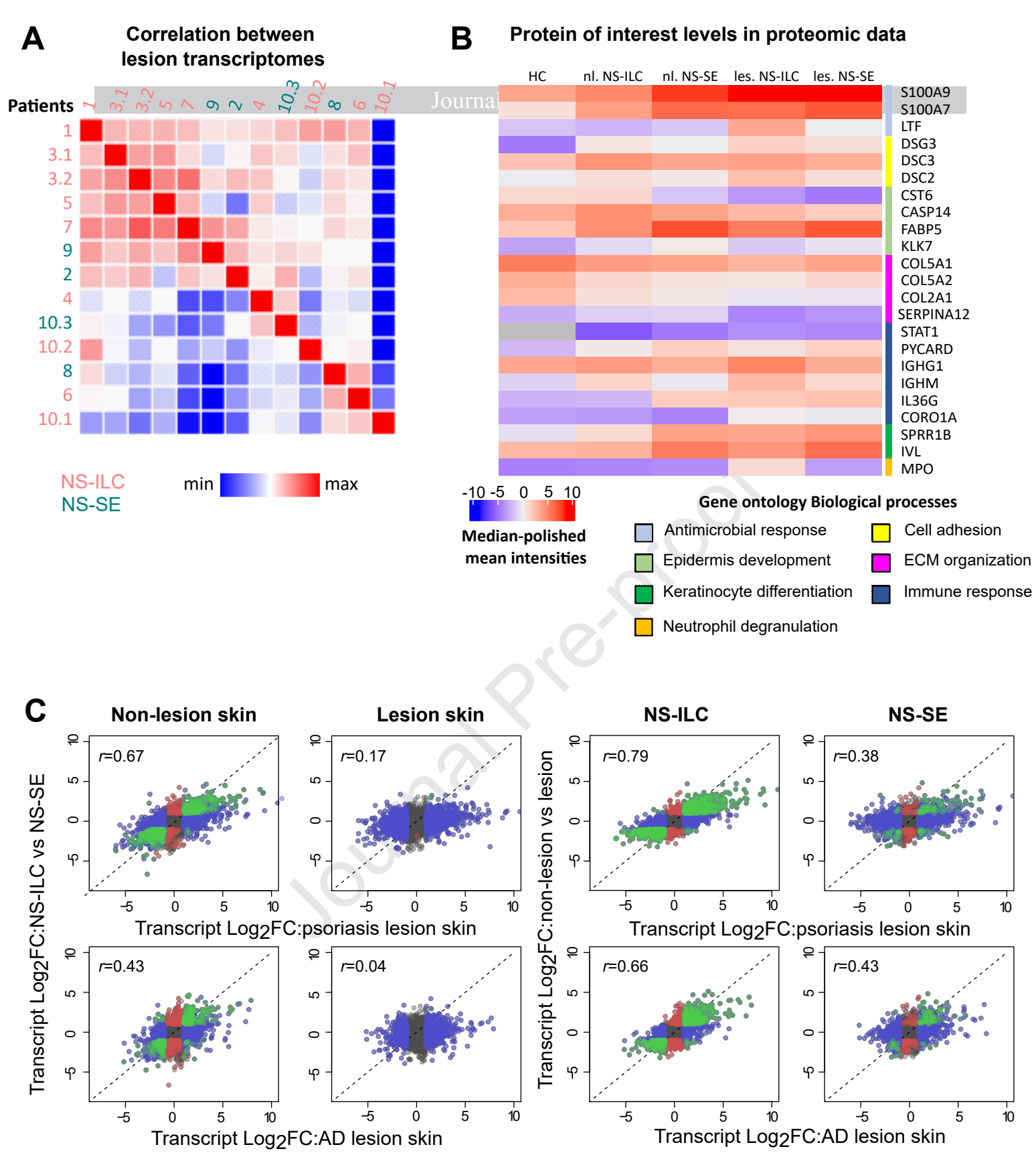


B Gating strategy for plasma B cells

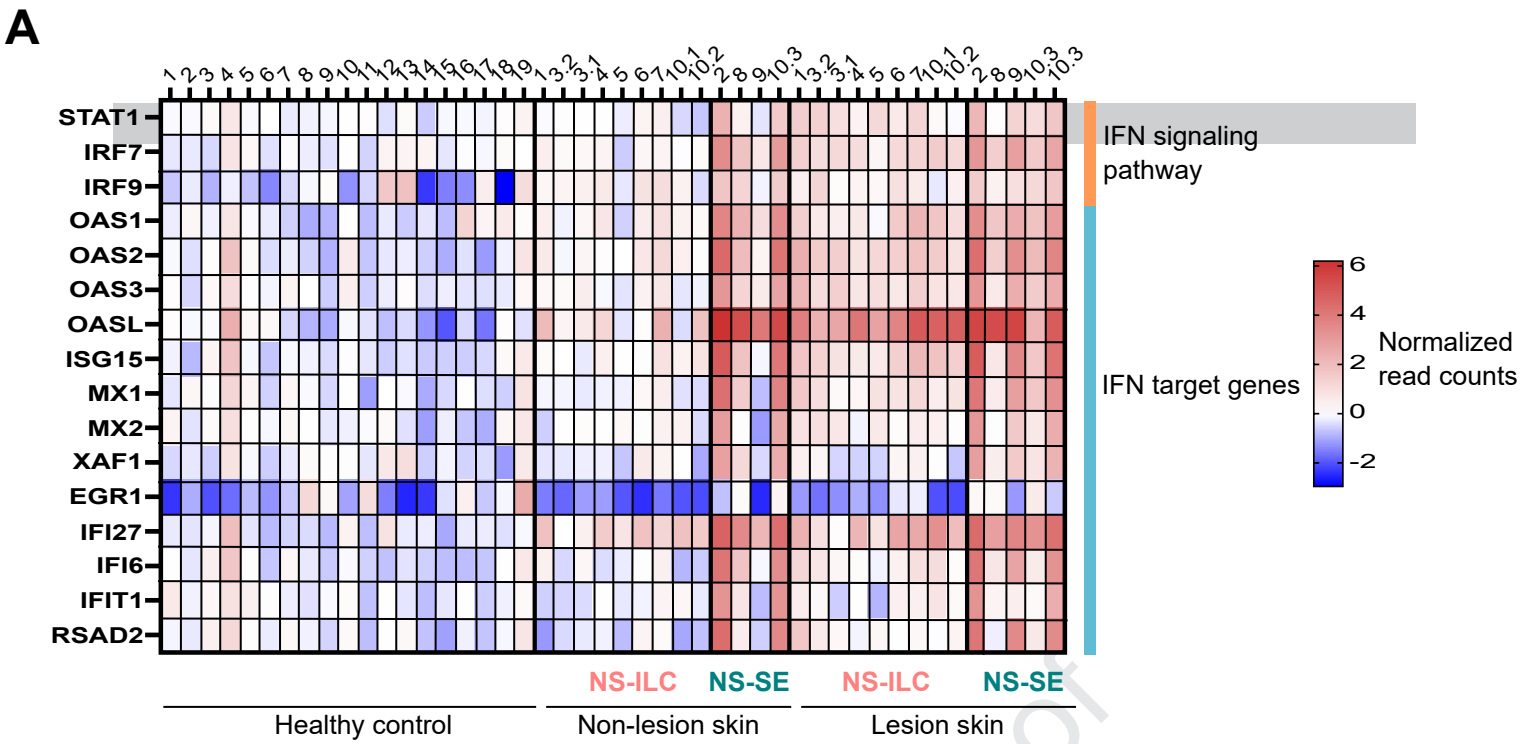


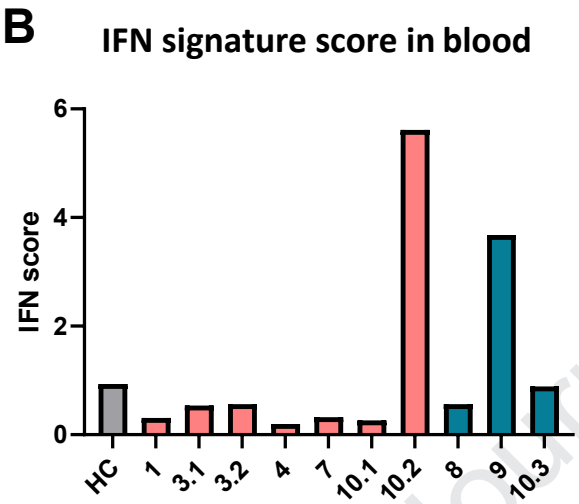
C Gating strategy for dendritic cells

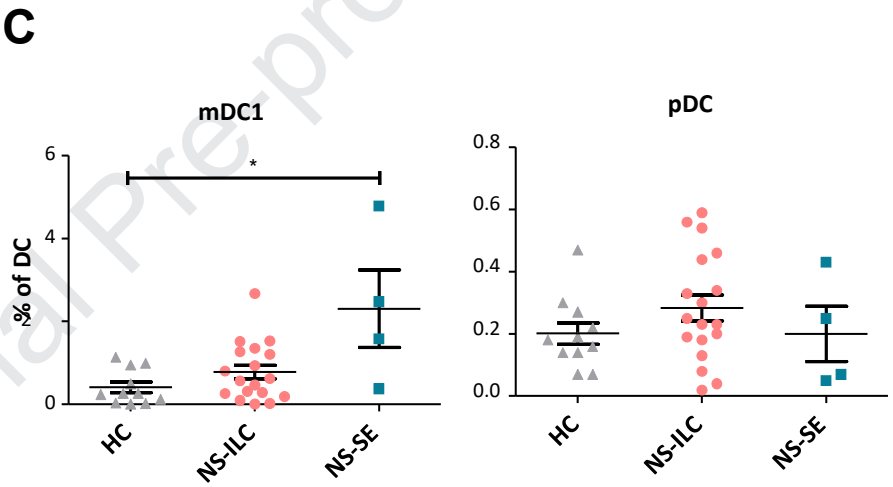




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