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Neutrophil extracellular traps, B cells, and type 1 Interferons contribute to immune dysregulation in Hidradenitis Suppurativa

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Abstract

Hidradenitis suppurativa (HS), also known as acne inversa, is an incapacitating skin disorder of unknown etiology manifested as abscess-like nodules and boils resulting in fistulas and tissue scarring as it progresses. Given that neutrophils are the predominant leukocyte infiltrate

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List of Supplementary Materials

Fig. S1. Analysis of neutrophil stimulation

Table S1. Correlation analysis of anti-TNF treatment with B cells and autoantibodies

Fig. S2. Analysis of PAD expression and BMI in HS

Fig. S3. Type I IFN serum activity is not increased in HS serum.

Data file S1. Primary data

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in HS lesions, the role of neutrophil extracellular traps (NETs) in the induction of local and systemic immune dysregulation in this disease was examined. Immunofluorescence microscopy was performed in HS lesions and detected the prominent presence of NETs. NET complexes correlated with disease severity, as measured by Hurley staging. Neutrophils from the peripheral blood of patients with HS peripheral also displayed enhanced spontaneous NET formation when compared to healthy control neutrophils. Sera from patients recognized antigens present in NETs and harbored increased antibodies reactive to citrullinated peptides. B cell dysregulation, as evidenced by elevated plasma cells and IgG, was observed in the circulation and skin from patients with HS. Peptidylarginine deiminases (PADs) 1–4, enzymes involved in citrullination, were differentially expressed in HS skin, when compared to controls, in association with enhanced tissue citrullination. NETs in HS skin co-existed with plasmacytoid dendritic cells, in association with a type I interferon (IFN) gene signature. Enhanced NET formation and immune responses to neutrophil and NET-related antigens may promote immune dysregulation and contribute to inflammation. This, along with evidence of upregulation of the type I IFN pathway in HS skin, suggests that the innate immune system may play important pathogenic roles in this disease.

One Sentence summary:

Innate and adaptive immune systems are dysregulated in hidradenitis suppurativa.

Introduction

Hidradenitis suppurativa (HS), also known as acne inversa, is a debilitating inflammatory disorder of the hair follicles. HS is characterized by recurrent painful abscess-like nodules with malodorous purulent drainage, sinus tracts, and scarring in the axillae, groin, anogenital region, and other intertriginous areas. Conflicting reports estimate the prevalence of HS as 0.053–4% (1), with a 3:1 female:male ratio, and women of childbearing age bearing the burden of this disease (2). Although it is also more prevalent among African Americans (3, 4), very few studies have focused on this population.

HS is associated with stigmatization and significant morbidity, with quality of life scores worse than psoriasis and most other dermatologic conditions (5). Patients with HS are more likely to suffer from depression, anxiety, and sexual dysfunction, and the impact of the disease on quality of life is worse in women (5, 6). Common current treatment options include intralesional corticosteroids, antibiotics, biologics (tumor necrosis factor (TNF) inhibitors), and surgical resection.

The etiology of HS is unknown, with obesity and smoking among risk factors (7). Neutrophils are prominent in affected lesional skin (8). Indeed, this is associated with recent associations between dermal IL-17 and Th-17 enhanced responses in HS that may drive neutrophil migration into affected areas and promote tissue damage (8, 9). However, the role of neutrophils in the pathogenesis of HS remains unclear. Among the several functions of neutrophils, these cells have the ability to form neutrophil extracellular traps (NETs) following exposure to certain microbes or sterile stimuli. NETs are web-like structures composed of nucleic acids and granule or cytosolic proteins that are extruded to the extracellular space following exposure to various danger signals (10, 11) During

NET formation, peptidylarginine deiminase 4 (PAD4) is activated, promoting histone citrullination. Indeed, histone H3 and H4 citrullination has been detected in tissues where NETs are present (10). Dysregulation of NET formation has been implicated in various autoinflammatory and autoimmune conditions, as NETs can activate several aberrant innate and adaptive immune responses, including the induction of type I interferons (IFNs) and the NLRP3 inflammasome, leading to amplification of inflammation (12, 13, 14).

NETs are considered a putative source of modified autoantigens targeted by the immune system in individuals with systemic lupus erythematosus (SLE) (15, 16) rheumatoid arthritis (RA) (10, 11, 17) and anti-neutrophil cytoplasmic antibody vasculitis (AAV) (18). Both enhanced NET formation and impaired NET clearance have been associated to the development of autoantibodies in various autoimmune conditions (12, 19, 20). It has been a matter of debate in the recent literature if HS displays features of autoimmunity, although this condition has been associated with several chronic inflammatory diseases (21, 22, 23, 24).

Infiltration of neutrophils in affected lesions during active disease has been reported in HS (8), but their role in the initiation and or perpetuation of this condition has not been systematically explored. Here, we analyze mostly biospecimens from African American subjects with HS and report substantial systemic and lesional innate and adaptive immune dysregulation in this condition, in association with disease progression.

Results

Netting neutrophils are present in HS lesions and peripheral blood

Histopathologic analysis of affected skin lesions from HS patients by Hematoxylin & Eosin (H&E) staining demonstrated infiltration of neutrophils when compared to skin from healthy donors. Multilobulated neutrophils were found primarily around the hair follicles (Fig. 1A) and this was corroborated by the presence of myeloperoxidase (MPO)-positive cells in the same areas in HS dermis but not in healthy control dermis (Fig. 1A, **lower panel**). To assess whether NETs are present in HS skin, we stained the dermis with antibodies recognizing MPO and citrullinated Histone 4 (cit-H4). HS lesional skin was characterized by the presence of NET structures that were not observed in controls (Fig. 1B). To corroborate the presence of NETs in HS skin, we analyzed homogenized tissue lysates to detect cit-H3/DNA complexes by ELISA. Indeed, complexes of citrullinated histone H3/DNA were significantly ($p=0.029$) increased in HS skin when compared to healthy controls (Fig. 1C); this was validated by Western blot to detect cit-H3 (Fig. 1D). There was a significant positive correlation between amount of NETs disgorged within lesions and HS severity index (Hurley stage; $r^2=0.7537$, $p<0.0001$ with 95% confidence) (Fig. 1E). To assess whether enhanced NET formation is confined to the skin, HS peripheral blood neutrophils were analyzed *ex vivo*. These neutrophils displayed enhanced NET formation in the absence of exogenous *in vitro* stimulation when compared to control neutrophils (Fig. 1F). This difference is also in the absence of clinically assessed infectious etiologies. Serum NET complexes were not found increased in HS (figs. S1A and S1B); however, HS serum failed to properly degrade healthy control NETs *in vitro* (fig. S1C), suggesting that NET clearance mechanisms may be impaired in the circulation of these patients. Taken

together, these results indicate that NET formation is enhanced in HS, both systemically and in lesional skin, in association with disease severity and that NET degradation mechanisms may be impaired in this disease.

Autoantibodies to NET components and to citrullinated proteins develop in HS

Given that HS neutrophils display enhanced NET formation, we hypothesized that individuals affected by this condition would display autoantibodies to NET components. Purified, ionophore-generated NETs from healthy donors were probed with control or HS sera. Western blot analysis showed differential recognition of peptides by HS serum IgG when compared to control IgG (Fig. 2A), suggesting HS serum recognizes autoantigens present in NETs, and the putative presence of autoantibodies in HS. Total IgG was significantly increased in HS sera ($p=0.0011$) when compared to sera from healthy volunteers (Fig. 2B). HS skin displayed intercellular IgG deposition in the epidermis, as well as infiltration by IgG-positive plasma cells (Fig. 2C), suggesting dysregulation in antibody responses. Flow cytometry analysis of HS peripheral blood mononuclear cells demonstrated significantly increased frequency of plasma cells ($p=0.0424$) and increased B cells when compared to controls. Also, activated $CD86^{+}$ plasma B cells ($p=0.0095$) were significantly elevated in HS, supporting dysregulation of the B cell compartment in this disease (Fig. 2D–I). TNFα is increased in HS serum and anti-TNFα therapy has been reported effective in some HS patients (25). Anti-TNFα treatment can promote autoimmunity and autoantibody development (26). However, the cohort of HS patients we studied were not prescribed or had discontinued use of any anti-TNFα agents at least 2–4 weeks prior to sample collection. Furthermore, there was no correlation between use of anti-TNFα treatment and B cells, activated B cells, plasma cells, IgG or autoantibody quantities (Table S1).

Since NETs contain citrullinated proteins that can serve as autoantigens in RA (10, 17), we next tested whether HS patients develop autoantibodies to citrullinated proteins. Clinical testing to detect antibodies to citrullinated peptides (anti-CCP-1 test) was performed in HS sera. Although anti-CCP positivity, particularly at high titers, is considered highly specific for RA (27, 28, 29), HS sera also showed significantly higher titers of anti-CCP antibodies ($p=0.0127$) when compared to healthy control sera, albeit lower than the titers found in RA (Fig. 3A). To further corroborate this finding, we employed a bead-based assay against citrullinated antigens that has been previously described (30). An antigen bead assay assessed the presence of autoantibodies to native and citrullinated proteins in HS serum. HS sera displayed significantly elevated antibodies recognizing citrullinated vimentin 1–16 ($p=0.0078$), citrullinated fibrinogen 27–43($p=0.0329$), citrullinated filaggrin 48–65 ($p=0.0326$), and citrullinated enolase-1A 5–21($p=0.0006$) (Fig. 3B–E), when compared to control sera. HS sera also showed more antibodies against citrullinated H2A, H2B, tenascin, ApoA1 231–248, and ApoE 277–296 (Fig. 3F). Unsupervised clustering of autoantibodies to citrullinated proteins demonstrated distinct enrichment in association with specific Hurley stages. Autoantibodies against citrullinated fibronectin and citrullinated filaggrin were more prevalent in Hurley stage 1 and decreased in Hurley stages 2 and 3. In contrast, autoantibodies against citrullinated ApoE, citrullinated vimentin, and citrullinated fibrinogenA 211–230 were more prevalent in Hurley stage 2, compared to stages 1 and 3. Hurley stage 3 was characterized primarily by autoantibodies against H2B and tenascin C1

(Fig. 3G). Taken together, HS is characterized by increased plasma cells and IgG and by the development of autoantibodies against citrullinated cellular and matrix proteins, some of them previously reported to be present in neutrophils and NETs.

Enzymes involved in citrullination are elevated in HS skin

PADs are enzymes involved in the conversion of arginine to citrulline. There are five PAD isoforms and two of them have been well-studied in myeloid cells, PAD2 and PAD4 (10, 31). As histone citrullination was increased in HS skin and both of these PADs can citrullinate histones, we hypothesized that PADs can increase protein citrullination in HS skin. In addition to myeloid-relevant PADs, PAD1 and PAD3 have been reported to be expressed in normal skin (32) and no difference in *PADI1* or *PADI3* gene expression detected in HS skin when compared to controls (fig. S2A). In contrast, *PADI2* gene ($p=0.0158$) was significantly higher in comparison to control skin (Fig. 4A). Likewise, protein expression was higher in HS skin when compared to control skin (Fig. 4B). Although we were not able to detect *PADI4* gene expression in HS skin (fig. S2A), PAD4 protein was higher in HS when compared to healthy skin (Fig. 4B). We then assessed the activity of the PAD enzymes in HS skin by using a probe that specifically recognizes citrullinated proteins. Confirming the observations of enhanced histone citrullination, this citrulline probe assay demonstrated that HS skin contains a number of increased citrullinated proteins when compared to control skin (Fig. 4C). The pattern of citrullination found in HS skin was comparable to the pattern generated with recombinant PADs 1–4, suggesting that PADs are involved in the generation of citrullinated proteins in HS skin. Of notice, immunofluorescence of HS skin showed differential expression of PADs compared to control skin (Fig. 4D–F). PAD1 and PAD3 were localized throughout the epidermis in control skin, whereas this expression was more basal in HS lesions. Furthermore, compared to control skin, PAD2 appeared to be more localized to the stratum corneum in HS. Taken together, these results suggest that PADs are active in HS lesional skin and may contribute to enhanced citrullination of autoantigens detected in HS lesions, potentially promoting the formation of autoantibodies targeting citrullinated peptides.

PADs are increased in the lungs of smokers (33) and smoking is a major risk factor for HS. The majority of the HS patients analyzed in our cohort were current or former smokers; therefore, we cannot exclude that tobacco use could have contributed to increased PAD expression in these patients. However, we did not detect differential PAD expression based on smoking status of patients with HS (fig. S2B, $\beta = -3.415$; $p=0.6628$, $SD= 7.7$). Likewise, since obesity is a known risk factor for HS, we assessed whether BMI correlated with enhanced NET formation and disease severity. Although the majority of the patients are overweight or obese, there was no direct correlation between elevated BMI and disease severity ($\beta = -0.068$; $p=0.353$, $SD= 0.073$)(fig. S2C).

Type I interferon pathway is activated in HS lesional skin

IFNs can amplify innate and adaptive immune responses and previous studies have demonstrated that NETs can promote induction of type I IFN signatures in skin, kidney and peripheral blood in various autoimmune and chronic inflammatory conditions (34, 35, 36, 37, 38). We tested whether HS patients display a type I IFN gene signature in skin by

homogenizing HS lesional skin and isolating RNA and proteins for analysis. Quantitative PCR analysis showed significantly increased expression of type I IFN regulated genes *IFI44L* (p=0.0026), *MX1* (p=0.0044), *CXCL10* (p=0.0219), *RSAD2* (p= 0.0428), and *IFI27* (p=0.0086) (Fig. 5A). To corroborate these findings, we performed Western blot analysis of homogenized skin lysates to determine the presence of activated key transcription factors involved in the type I IFN pathway, phosphorylated (p) IRF3 and IRF7 (39, 40). As shown in Fig. 5B, enhanced expression of p-IRF3 and p-IRF7 was detected in HS skin, further supporting activation of the type I IFN pathway in HS lesions.

Western blot analysis also showed enhanced IFN-alpha protein expression in HS when compared to control tissues. As it is known that NETs can activate pDCs to produce IFN-alpha (13, 38, 41), we tested whether pDCs and NETs may interact in HS skin. Confocal analysis demonstrated that NETs co-exist with pDCs in HS skin (Fig. 5C), suggesting that NETs priming pDCs may be involved in the upregulation of type I IFN responses detected in HS skin. To further address whether the type I IFN signature is a systemic or localized feature of HS, we performed a reporter assay to quantify IFN activity in the serum by incubating HeLa cells with HS and control sera for 6 h. Quantitative PCR showed no differences between control and HS induction of IFN-inducible genes in the cell line, suggesting no enhanced type I IFNs in the circulation of HS patients (fig. S3A). To further corroborate this finding, PBMCs and neutrophils from HS patients and controls were analyzed for a type I IFN gene signature. Although no differences were observed between HS and control PBMCs (Fig. 5D), *CXCL10* (p=0.0317) and *IFI27* (p=0.0190) genes were significantly elevated in the peripheral blood HS neutrophils when compared to controls (Fig. 5E). These results indicate that the type I IFN signature present in HS patients is primarily restricted to lesional skin and may also involve peripheral blood neutrophils.

Discussion

Severe dermal inflammation with substantial neutrophil infiltration is characteristic of HS lesions (8). However, how neutrophils contribute to HS pathogenesis and their role in activating other immune cell types and amplifying inflammation in this disease had not been systematically investigated. We now report that HS neutrophils are primed to form NETs and that these structures associate with disease severity and progression and with the activation of a type I IFN response in the skin. Furthermore, we describe that HS sera contains autoantibodies targeting NET components and citrullinated proteins and increased circulating plasma cells.

A major histologic finding of HS lesions is the infiltration of various inflammatory cells (42). Infiltrating macrophages in HS lesions release TNFα, IL-12, and IL-23, which can lead to the production of IL-17 by T helper cells (9). Besides Th17 cells, innate cells may contribute to IL-17 synthesis in HS (8, 43). In turn, increases in IL-17 in HS lesional skin may promote neutrophil migration to the skin. It is possible that some of the cytokines increased in HS lesions promote the formation of NETs. Previous studies in other inflammatory conditions have proposed a role for NETs as autoantigens and amplifiers of inflammation and innate and adaptive immune responses (10). We now provide evidence that enhanced NET formation in HS externalizes autoantigens that are recognized

by HS serum antibodies. Some of the antibodies recognizing citrullinated peptides that were detected in the serum of HS patients are considered important in the pathogenesis of RA (10, 17). In addition, the enhanced NET response exhibited by HS circulating and skin neutrophils may contribute to enhanced inflammation and activation of other immune cells, including skin pDCs, thereby promoting an enhanced type I IFN response in HS lesions.

Inadequate NET clearance has been proposed to play a role in enhancing the half-life of NET autoantigens in autoimmune diseases such as SLE (12, 19, 20) and appears to be a mechanism present in HS, at least *in vitro*. NET complexes in circulation were not enhanced in HS and no enhanced systemic type I IFN response was detected, except for increased *CXCL10* and *IFI27* in the HS neutrophils. In contrast, enhanced NET formation in HS skin may promote localized immune dysregulation and enhanced skin type I IFN responses. The discrepancy between the lack of enhanced NET complexes in circulation with evidence of enhanced *in vitro* NET formation by HS neutrophils, along with impaired *in vitro* NET degradation by HS serum, remains to be determined. One possibility is that the degree of NET formation in the circulation of HS patients remains low enough that, even with impairments in the degradation of these complexes, no significant elevations are observed in serum. This is in contrast to HS skin tissue where, the degree of NET formation along with the enhanced proinflammatory milieu in the skin, may enhance the half-life of these structures and promote immune dysregulation. It is also possible that some of the neutrophils isolated from the peripheral blood of HS patients are more prone to form NETs *in vitro* and show enhanced type I IFN responses because they were previously exposed to the HS lesional skin milieu, then undergone reverse transmigration (44) back to the circulation and on their way into the bone marrow.

B cells and plasma cells are present in chronic HS lesions (42, 45). IL-10 is upregulated in HS (46) and this cytokine can influence B cell differentiation to plasma cells and antibody production (47). It is possible that enhanced generation of autoantigens in HS lesional skin and peripherally, through increased NET generation, could play a role in activating B cells, generating plasma cells, and promoting an aberrant adaptive immune response. This is also supported by the identification of elevated IgG in HS patients, deposition of IgG in HS lesions, as well as increased plasma cells that we identified in HS. The role of the autoantibodies detected in HS in disease pathogenesis, as well as dependence on T cells, remains to be determined. Autoantibodies to ApoA1 and ApoE were detected in the serum of HS patients. Emerging data demonstrates the potential role of anti-apolipoprotein A1 in atherothrombosis (52), which could have implications for the described increased risk for vascular disease in HS (53).

One of the known hallmarks of RA pathogenesis is the preferential presentation of citrullinated peptides to the adaptive immune system and the development of autoantibody responses to citrullinated peptides, including autoantibodies that recognize antigens present in NETs (17, 48, 49). Of interest, autoantibodies recognizing citrullinated H2A, H2Bcit, α-enolase, vimentin, and clusterin, considered specific to RA (17), were also detected in HS subjects. Furthermore, PADs 1–4 were present in HS skin, with PAD2 and PAD4 proteins being more abundant in HS skin. Of interest, there was differential skin distribution of PADs 1–3 protein when compared to controls. As PAD1 and PAD3 can citrullinate extracellular

antigens, we cannot exclude their contributions to the enhanced citrullination of proteins in HS skin, in addition to the putative role of myeloid PAD2 and PAD4. Considering the complexity of chronic inflammation and the infiltration of multiple cellular types in HS lesions, it is possible that other cells besides neutrophils also contribute to the heightened citrullination found in HS skin through the activation of PAD2 and perhaps other PADs. PAD2 is expressed by different inflammatory cells, including macrophages (50), which are increased in HS lesions (9, 42, 51).

An increase in type I IFN responses was detected in HS lesional skin but not in PBMCs, although isolated neutrophils showed an increase in *CXCL10* and *IFI27*, suggesting a possible role for IFNγ. We currently cannot exclude the contributions of other IFNs to the HS signature. We hypothesize that enhanced NET formation may contribute to pDC activation in HS skin and this is partially supported by observing that netting neutrophils and pDCs are present in same areas in HS skin. NETs have been shown to activate pDCs to produce abundant type I IFN in SLE in a DNA- and TLR7/9-dependent manner (37). Whether other pathways driven by the presence of skin microbes or by the production of type I IFNs by other skin cell types play important roles in immune dysregulation in HS is unknown.

Importantly, these data were generated from the analysis of mostly African American patients, a cohort largely understudied in HS. It is not known if there are pathogenic differences across ethnicities in HS to confirm whether these observations are recapitulated in these other groups. Other limitations in this study include the relatively small sample size; the need to further understand in future experiments the role that neutrophil/type I IFN dysregulation plays in the clinical manifestations of the disease, as well as how these abnormalities in immune responses regulate other innate and adaptive immune cell types in HS.

Overall, we have characterized a putative axis of immune dysregulation characterized by enhanced NET formation and type I IFN responses associated to autoantibody generation in HS. This could potentially dictate the development of new therapeutics for this devastating condition.

Materials and Methods

Study design

The study investigated the role of neutrophils and NETs in promoting immune dysregulation in hidradenitis suppurativa. HS lesions from patients who underwent surgery were collected or tissue from healthy control patients following abdominoplasties and mammoplasties as explained in the *Collection of tissue samples*. Pilot studies were done to test our hypotheses as reported herein; subsequently, the number of patients were increased to corroborate the findings and for statistical analysis. Other biospecimens (blood, serum, etc.) were obtained from consented patients as needed. Patients who were diagnosed clinically and correlated with histopathologic characteristics of HS (42) were included. Patients younger than 16 years of age were excluded. None of the patients were on immunosuppressants and immunosuppressive therapy had been stopped at least 2–4 weeks prior to tissue collection.

No data was excluded. There were at least three biological replicates for all experiments. The number of patients included are indicated in the figure legends and was determined in part by the number of samples available at specific time points. Symbols denoted in the figures represent one patient or biological replicate. Investigators were blinded for the Hurley staging of the patients during experiments. Once the data were obtained, the Hurley Stage (disease severity) was known for interpretation of the data. Primary data are reported in data file S1.

Collection of tissue samples

The study was approved by the Johns Hopkins (JH) University Institutional Review Board (IRB) and informed consent was obtained. Normal (non-HS) and lesional HS skin were obtained from surgical resections during cosmetic surgery and from JH's tissue bank obtained from patients undergoing surgical removal of HS skin. Patients with Hurley stage 2 and 3 were considered to have chronic HS, i.e., sinus tracts and fibrotic tissue. Visual assessment of the patient at the time of surgery as well as clinical and histopathological diagnosis were verified. Each sample was collected and stored in liquid nitrogen for transport. Samples were then embedded in optimal cutting temperature (OCT) media and placed at 20°C. Once solidified, 0.5–0.8 μm cryostat sections were placed on Superfrost Plus slides (Thermo) and placed at 20°C until immunofluorescent staining was done as described below. Some skin sections were fixed in 10% formalin and embedded in paraffin. H&E staining was performed for histological characterization.

Antibodies

Rabbit polyclonal antibodies used were anti-IFNa (1:500, Abcam), anti-phospho-IRF7 Ser471/472 (1:500, Cell Signaling), anti-beta actin (1:1000, Abcam), anti-PAD1 (1:100, Proteintech), anti-PADI2 (1:500 dilution (WB); 1:200 (IF), Proteintech), anti-PAD3 (1:200, arigo Biolaboratories), anti-MPO (1:1000, Dako), and anti-H3 (1:500, Abcam). Monoclonal rabbit anti-phospho-IRF3 Ser386 (1:500, Abcam) and mouse anti-PADI4 clone 6A2 (1:500 dilution, Lifespan Biosciences) were also used. CD303 (BDCA-2) FITC-coupled (1:100) was from Miltenyi. The secondary antibodies purchased from LI-COR and used at a 1:10,000 dilution, were donkey anti-mouse 800, donkey anti-mouse 680, donkey anti-rabbit 800, and goat anti-rabbit 680. Anti-CCP analysis was done by the Clinical Immunology Laboratory in Johns Hopkins Hospital, following established chemiluminescent immunoassay protocols (QUANTA Flash® CCP3; Inova Diagnostics).

NET isolation

NETs were isolated, as previously described (9, 16). Briefly, HS neutrophils were plated in 24-well tissue culture plates in RPMI without phenol red. Cells were incubated for 3 hours at 37°C. Supernatants were harvested, and NETs were digested with micrococcal nuclease (10 U/ml; Thermo Fisher) for 15 min at 37°C. Supernatants were collected and centrifuged at 5,000 rpm for 5 min at 4°C. NET supernatants were transferred to a fresh tube and stored at −80°C until used.

Detection of NETs, PADs, and IgG in skin

To detect NETs in skin, frozen sections were blocked with 10% BSA for one hour at room temperature. Where indicated, anti-PAD1 (1:100, Proteintech), anti-PAD2 (1:200, Proteintech), anti-PAD3 (1:200, arigo Biolaboratories), anti-MPO (1:1000, Dako), anti-human histone H4 (citrulline 3) rabbit polyclonal (1:1000) (EMD Millipore) diluted in 5% BSA was added to sections and placed at 4°C overnight. Each slide was washed three times with PBS and Alexa Fluor 555 donkey anti-rabbit IgG (Invitrogen) secondary antibody (1:400) was added and incubated for 1 hour at RT. Nuclei were counterstained with (1:1000) Hoechst for 10 min at RT followed by 5 more washed with PBS. Tissue sections were sealed with a coverslip containing ProLong Gold solution (Thermo). Images were acquired on a Zeiss LSM780 confocal laser-scanner microscope. IgG immunohistochemistry was done by the JHMI Immunopathology Core.

Quantitative PCR analysis in skin and PBMCs

3–5 mm tissue sections were homogenized using liquid nitrogen, mortar and pestle. One-half of the homogenized tissue was resuspended in 500 μL of TRI Reagent (Sigma-Aldrich). After 30 mins, solution was centrifuged for 10 min at 14,000 rpm, and supernatant was transferred to a fresh Eppendorf tube. RNA was isolated using Direct-zol RNA Miniprep Kit (Zymo Research) according to the manufacturer's instructions. RNA from HS and control PBMCs was isolated through the same method. Total RNA (500 ng) was reverse transcribed using iScript RT single strand cDNA (Bio-Rad). qPCR was performed using Taqman Gene Expression Master Mix (ThermoFisher Scientific), human *GAPDH* primers (Hs99999905_m1), and sequence-specific primers for *IFI44* (Hs00197427_m1), *IFI44L* (Hs00915292_m1), *IFI27* (Hs01086373_g1), *RSAD2* (Hs00369813_m1), *CXCL10* (Hs00171042_m1), *HERC5* (Hs00180943_m1), *MX1* (Hs00895608_m1), *IFNA1* (Hs04189288_g1), and *IFNB1* (Hs01077958_s1), *PADI1* (Hs00203458_m1), *PADI2* (Hs01042505_m1), *PADI3* (Hs01042496_m1), and *PADI4* (Hs01057483_m1). Fold difference was calculated using the delta Ct equation.

Serum type I IFN activity assay

HeLa cells were cultured in a humidified incubator with 5% CO2 at 37°C. in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were plated in a 24 well plate and treated with 50% HS or control serum for 24 hours. Cells were washed twice in PBS and were suspended in TRI Reagent (Sigma-Aldrich). RNA was extracted, cDNA was prepared, and qPCR was performed as described above.

Citrullinated histone H3-DNA complex ELISA

Citrullinated Histone 3 – DNA NET complexes were quantified by ELISA. A 96-well plate was coated with rabbit polyclonal anti-citrullinated Histone 3 (Abcam) at 1:400 in PBS overnight at 4°C. Wells were washed three times in washing buffer (0.5% tween in PBS) and blocked in blocking buffer (1% BSA in PBS) at room temperature for 1 hour. After washing twice, 5 μg of total protein isolated from HS or control skin was added to the wells in blocking buffer and incubated overnight at 4°C. The wells were washed

three times and incubated with mouse monoclonal anti-double stranded DNA antibody (EMD Millipore) at 1:100 in blocking buffer. After washing three times, goat anti-mouse conjugated HRP antibody (Bio-Rad) was added to the wells in blocking buffer at 1: 10,000. Wells were washed five times followed by the addition of TMB substrate (Sigma Aldrich) and stop solution (Sigma Aldrich). The absorbance was measured at 450 nm and values were calculated as an OD index. Assay was performed in duplicate.

Citrullinated autoantibodies bead-assay

As previously described (54), serum ACPA antibodies targeting 40 RA-associated autoantigens were measured using a bead-based immunoassay on a Bio-Plex platform. Serum was diluted to a 1:30 ratio in a proprietary sample dilution buffer (Bio-Rad), mixed with the antigens conjugated to spectrally distinct fluorescent microspheres (Bio-Rad), and then incubated with an anti-human phycoerythrin–labeled antibody (Jackson ImmunoResearch). The resulting fluorescence intensities were analyzed on a Luminex 200 platform (Luminex Corporation).

Serum-induced degradation of NETs

This was performed as previously described (55). Briefly, control neutrophils were resuspended in unsupplemented RPMI (1×10^6 cells/mL) and were stimulated with PMA (500ng/mL) for 4 h at 37°C to induce NETs. Following stimulation, PMA-treated neutrophils without serum were fixed with 4% PFA and stored at 4°C. Neutrophils were treated with 1% serum from either healthy controls, HS and RA subjects for 16 h at 37°C. Neutrophils were fixed with 4% PFA and stored at 4°C before immunofluorescence was done to visualize NETs.

Western blot

3–5mm tissue sections were flash-frozen with liquid nitrogen. Frozen skin was ground with pestle and mortar. One-half of the homogenized tissue was then resuspended in 500 μL of lysis buffer (50mM Tris-HCl pH 7.4, 300 mM NaCl, 0.5% w/v Triton X-100, 5mM EDTA) enriched with a cocktail of protease inhibitors (Roche) (16) and placed at 4°C on a rotator for approximately 1 hour. The supernatants were transferred to a fresh Eppendorf tube. Total protein was quantified using BCA assay (ThermoFisher). Equal amounts of total protein were resolved in a 4–12% gradient Bis-Tris gel (Invitrogen), transferred onto a nitrocellulose membrane and blocked with 10% BSA for 30 min at room temperature. After overnight incubation with primary antibodies, membranes were washed three times with PBS-Tween (PBS-T) and incubated with secondary antibody coupled to IRDye 800CW. Membranes were developed using Li-COR Odyssey Clx scanner (Li-COR).

Immunophenotyping of B cells by Flow Cytometry

Following Ficoll centrifugation, HS and matched healthy control PBMCs were obtained and counted and 2×10^6 cells were resuspended in FACS buffer (phosphate-buffered saline containing 0.5% BSA) and spun at 1,500 rpm for 5 minutes. Cells were resuspended in Fc Receptor blocking solution (Human Trustain FcX, 1:100 in FACS buffer) for 10 minutes on ice. Cells were then spun at 1,500 rpm for 5 minutes and resuspended

in a cocktail of fluorescently labelled antibodies and stained for 30 minutes on ice. Single stains of Ultracomp eBeads (Invitrogen) were used as compensation controls. Cells were washed twice with FACS buffer, spun at 1,500 rpm for 5 minutes, then fixed with 2% Paraformaldehyde (Chem Cruz) for 15 minutes on ice. Fluorescently labelled antibodies were obtained from Biolegend unless otherwise specified. The following antibodies were obtained for this study: CD19-Pacific Blue, CD27-APC-Cy7, CD138-FITC, CD80-PE, CD69-AF700, CD86-APC, CD44-PE, CD25-AF700 and CD30-APC. Antibody concentrations were chosen based on titration and guided by developer recommendations. Analysis of cells was performed using a FACSCanto-II flow cytometer and FlowJo Software (v10.3).

Peptidylarginine deiminase activity using Rh-PG probe

Citrulline probe was used as previously described (16). Briefly, ten micrograms of total protein from control and HS skin were incubated in the presence of 10 mM CaCl2 (Quality Biological), 1mM DTT (Sigma) overnight at 37°C. Samples were incubated with 100 μM of citrulline-Rh-PG probe (Cayman) in 20% trichloroacetic acid (TCA) for 30 min at 37°C. Human recombinant PAD1 (Cayman Chemical Company), PAD2 (Sigma), PAD3 (Cayman Chemical Company) and PAD4 (Sigma) were used as positive controls. Samples were washed with cold acetone twice and separated in a 4–12% gradient Bis-Tris gel (Invitrogen). Citrullinated proteins were visualized in an Azure c600 Imaging system (Azure Biosystems).

Statistical analysis

Data were analyzed using GraphPad Prism software. For samples with non-Gaussian distribution, we used Mann Whitney U-test. Where indicated, one-way analysis of variance (ANOVA) Brown-Forsythe test analysis was used. Results are presented as the mean +/− SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data and Materials availability:

All data associated with this study are in the paper or Supplementary Materials. Hidradenitis suppurativa biospecimens are available from Johns Hopkins University School of Medicine under a material transfer agreement with the University.

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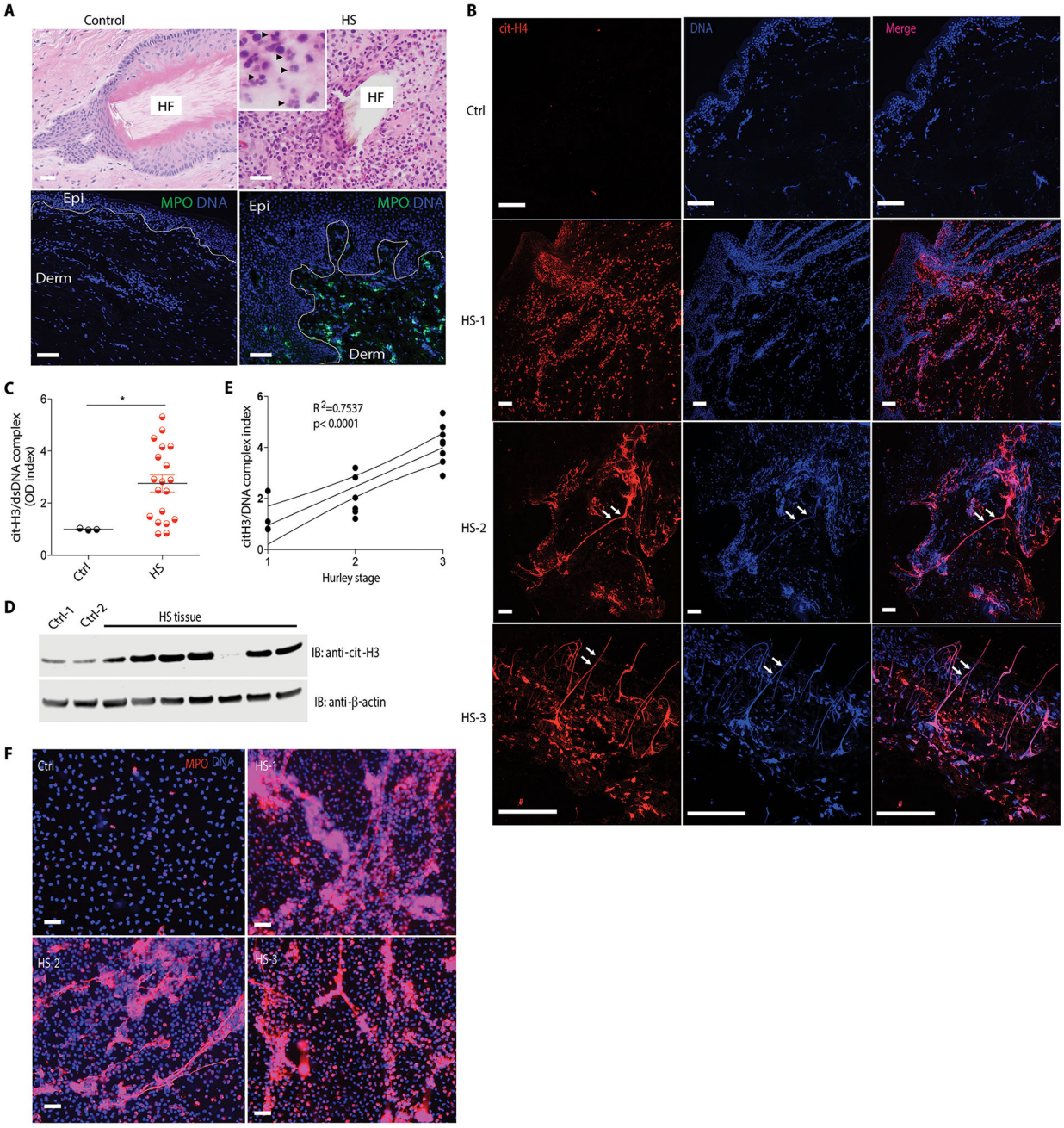

Figure 1. NETs are present in HS lesions and correlate with disease severity.

(**A**) Biopsies from HS patients stained with H&E (*upper panel, neutrophils indicated with arrows*) and immunofluorescence staining against myeloperoxidase (MPO, green) and Hoechst (DNA, blue) (*lower panel*). Epi= epidermis, Derm= dermis, HF=hair follicle. Scale bars, 50 μm. (**B**) Representative confocal images of lesions from HS patients stained for citrullinated histone H4 (cit-H4, red) and Hoechst (DNA, blue), *arrows show co-localization of cit-H4 and DNA*; Scale bars, 100 μm. (**C**) Homogenized lysates from resected tissues from various Hurley stages were tested for citrullinated histone H3 and DNA complexes. Results are the mean +/− SEM, *p<0.05, Mann-Whitney *U* test analysis was used. (**D**)

Cit-H3 protein abundance was evaluated in tissue from HS patients and assessed by Western blot. (**E**) Correlation of citrullinated histone H3 and DNA complexes with HS severity as determined by the Hurley stage. One-way ANOVA Brown-Forsythe test analysis was used. (**F**) NETosis of peripheral blood neutrophils from HS patients and control neutrophils. MPO is red and DNA is blue. Scale bars, 50 μm.

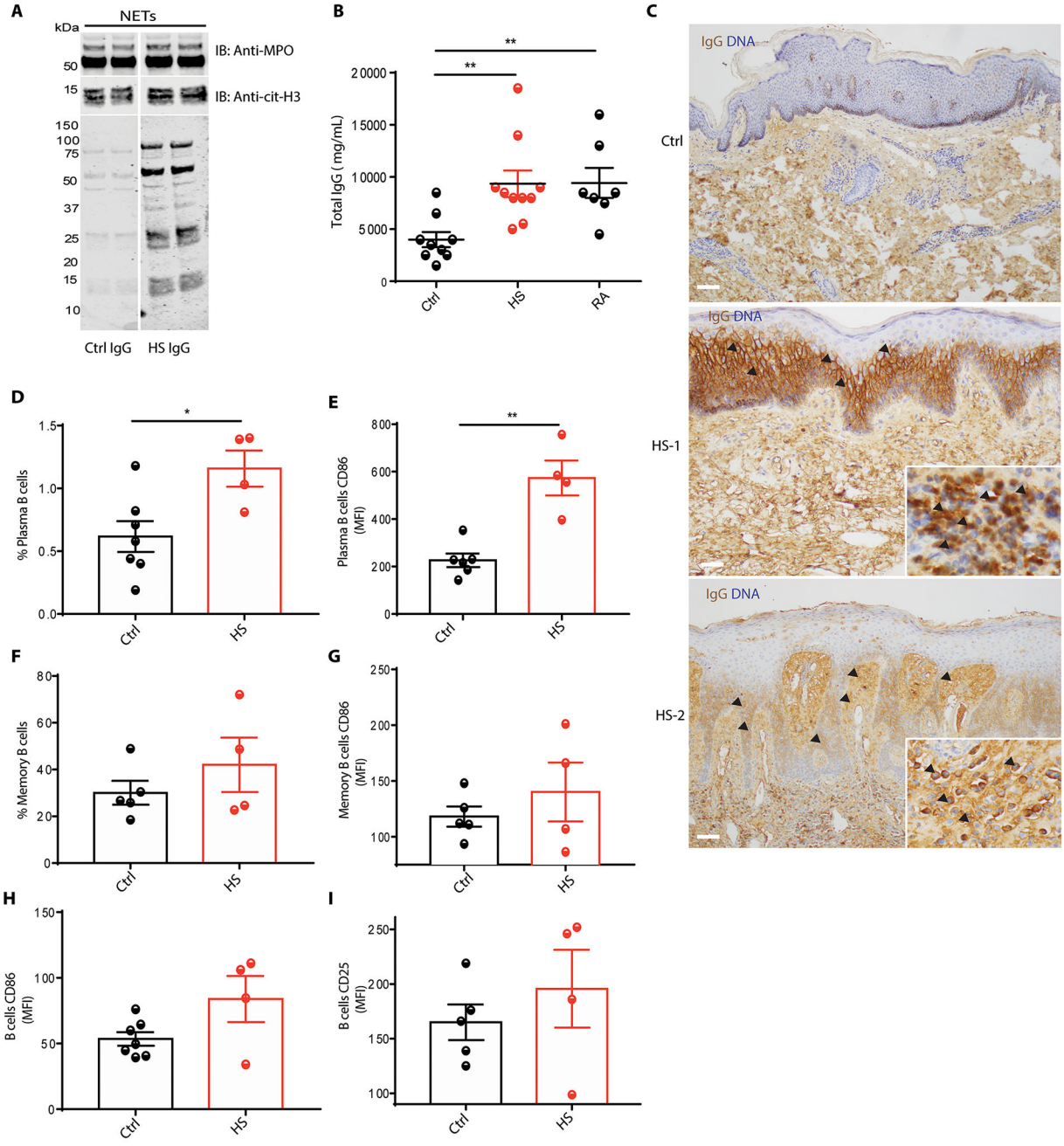

Figure 2. Antibodies recognizing NET antigens and citrullinated peptides are present in HS serum.

(**A**) NETs were resolved in a SDS-PAGE gel and transferred onto a nitrocellulose membrane. Membrane was probed incubated with serum from control or HS patients. Autoantibodies were detected using anti-IgG secondary antibody. Detection of MPO and cit-H3 were used as loading controls for NETs. (**B**) ELISA of total IgG in HS, RA, and control (Ctrl) samples. (**C**) Representative images of control and HS tissue stained for intercellular IgG IHC within the epidermis and IgG-positive plasma cells *arrows*; Nuclei/DNA were stained with hematoxylin *(blue)*. Scale bars, 50 μm. Peripheral blood from control and HS

patients were analyzed for the frequency and activation status of (**D,E**) plasma cells, (**F,G**) memory B cells, and (**H, I**) $CD19^+$ B cells by flow cytometry. Results are the mean +/− SEM, *p<0.05, **p<0.01, Mann-Whitney *U* test analysis was used.

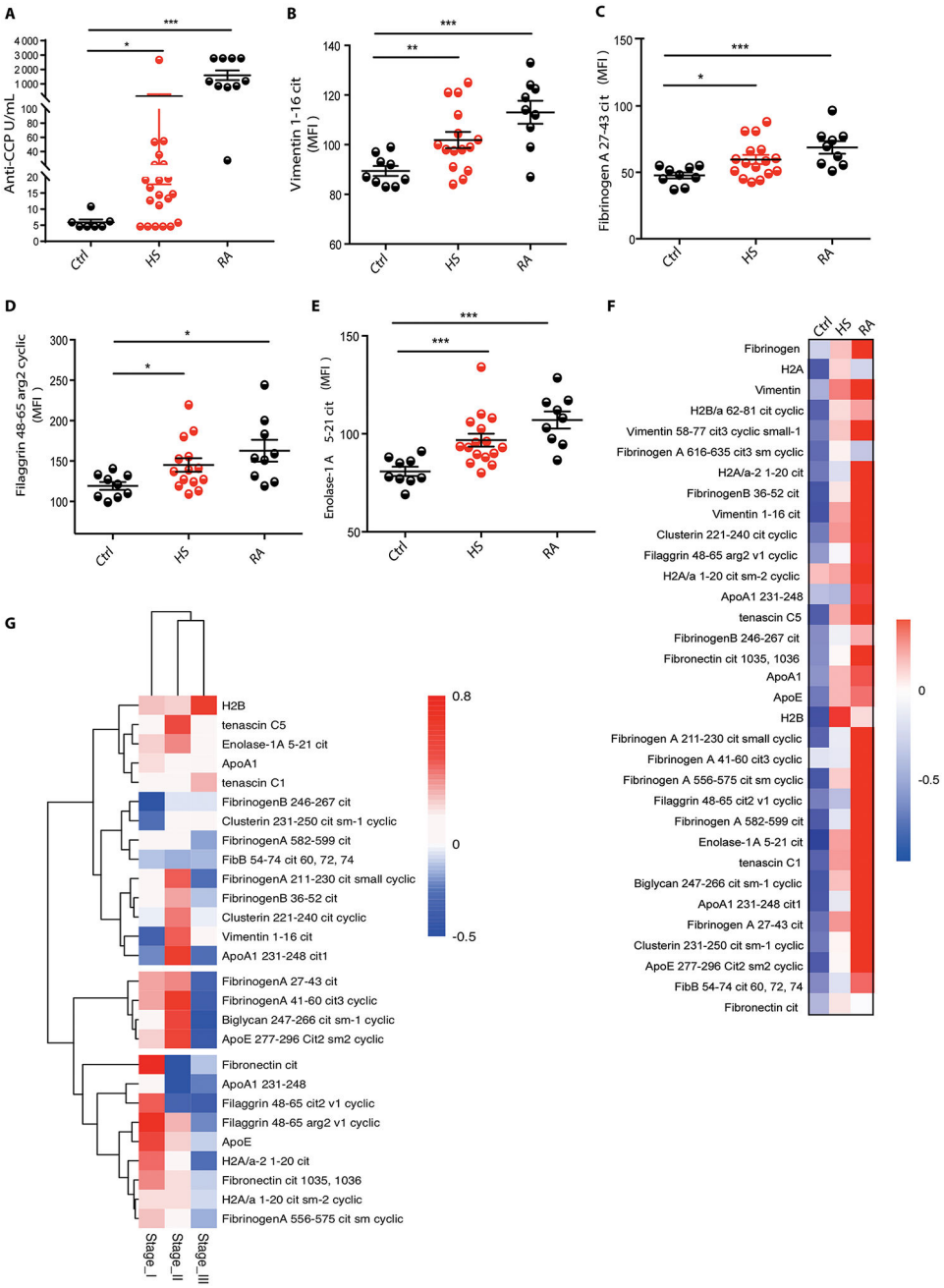

Figure 3. Autoantibodies against citrullinated antigens are present in HS patients.

(**A**) Ctrl (n=7), HS (n=21), and RA (n=10) sera were tested for the presence of autoantibodies against cyclic citrullinated peptides (anti-CCP1). Control (Ctrl) sera were used as negative control and Rheumatoid arthritis (RA) sera were used as positive control. Results are the mean +/− SEM, *p<0.05, ***p<0.001, Mann-Whitney *U* test analysis was used. (**B**) Ctrl (n=9), HS (n=16) and RA (n=9) sera were analyzed for the presence of antibodies recognizing citrullinated vimentin, (**C**) citrullinated fibrinogen, (**D**) citrullinated filaggrin, and (**E**) citrullinated enolase using a bead-based assay. Results are the mean +/− SEM, *p<0.05, **p<0.01, ***p<0.001, Mann-Whitney *U* test analysis was used. (**F**) Heat

map of the elevated antibodies recognizing citrullinated peptides present in HS sera (n=16) when compared to control sera (n=10). RA sera (n=9) were used as control. (**G**) Heat map of unsupervised clustering of citrullinated autoantibodies stratified by Hurley stage (Stage I n=4; Stage II n=5; Stage III n=8).

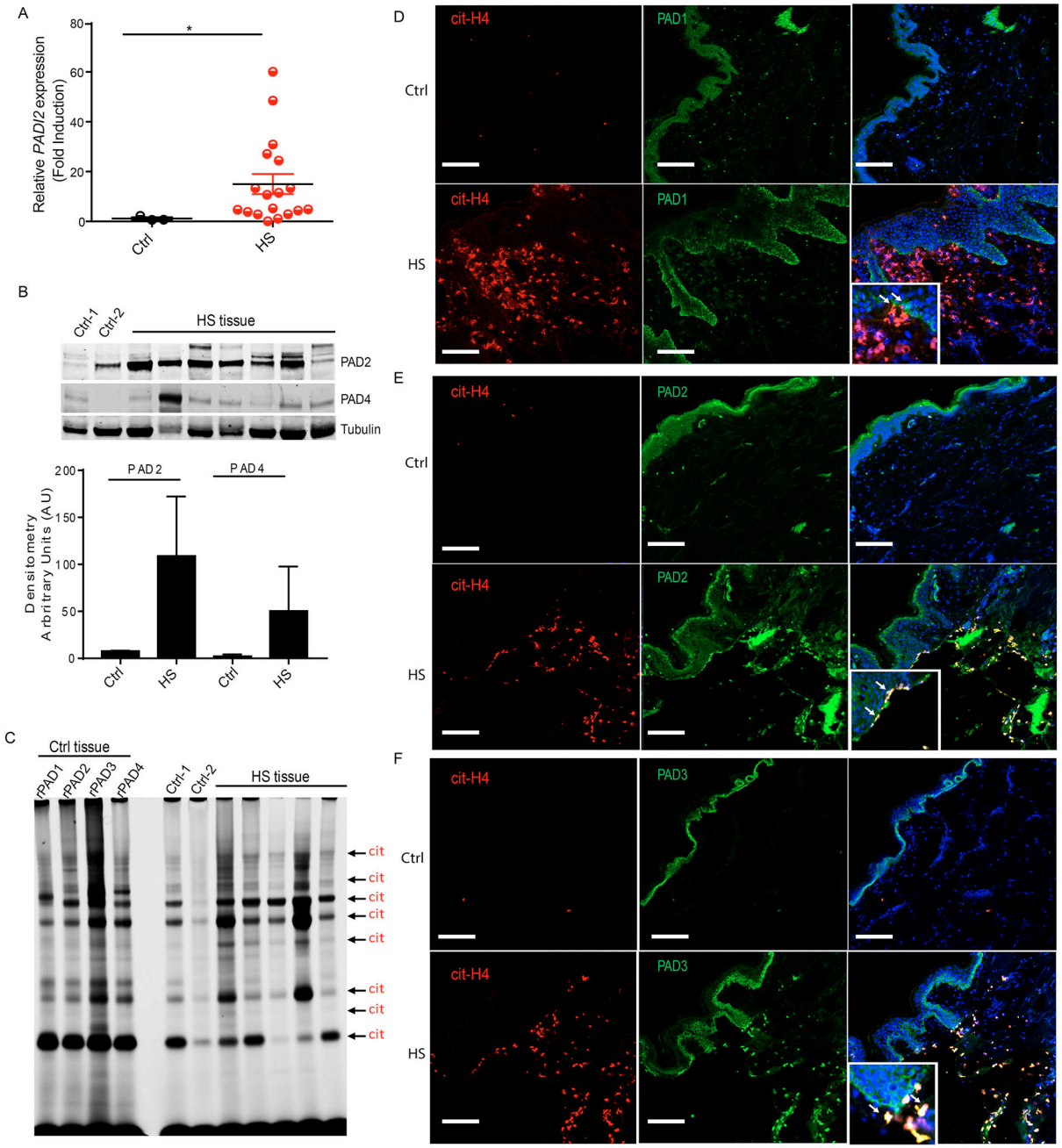

Figure 4. Peptidylarginine deiminase expression and activity are increased in HS lesions.

(**A**) *Padi2* qPCR analysis of lesions from HS patients and control tissue. *p<0.05; Mann-Whitney *U* test analysis was used. (**B**) PAD2 and PAD4 protein analysis in tissue from HS patients as assessed by Western blot. Tubulin was used as a loading control. Densitometry of PAD2/Tub and PAD4/Tub (lower panel) (**C**) PAD activity was measured using Rh-PG probe against citrulline in HS and control tissue. Ctrl tissue with recombinant PADs 1–4 were used as a positive control for substrates for citrullination pattern detection. Representative confocal images of NETs (*cit-H4, red*), and (**D**) PAD1 (*green*), (**E**) PAD2 (*green*), (**F**) PAD3

(*green*) in HS and control tissues. Arrows indicate co-existence of NETs and PADs. Hoechst was used to counterstain nuclei in blue. Scale bars, 100 μm.

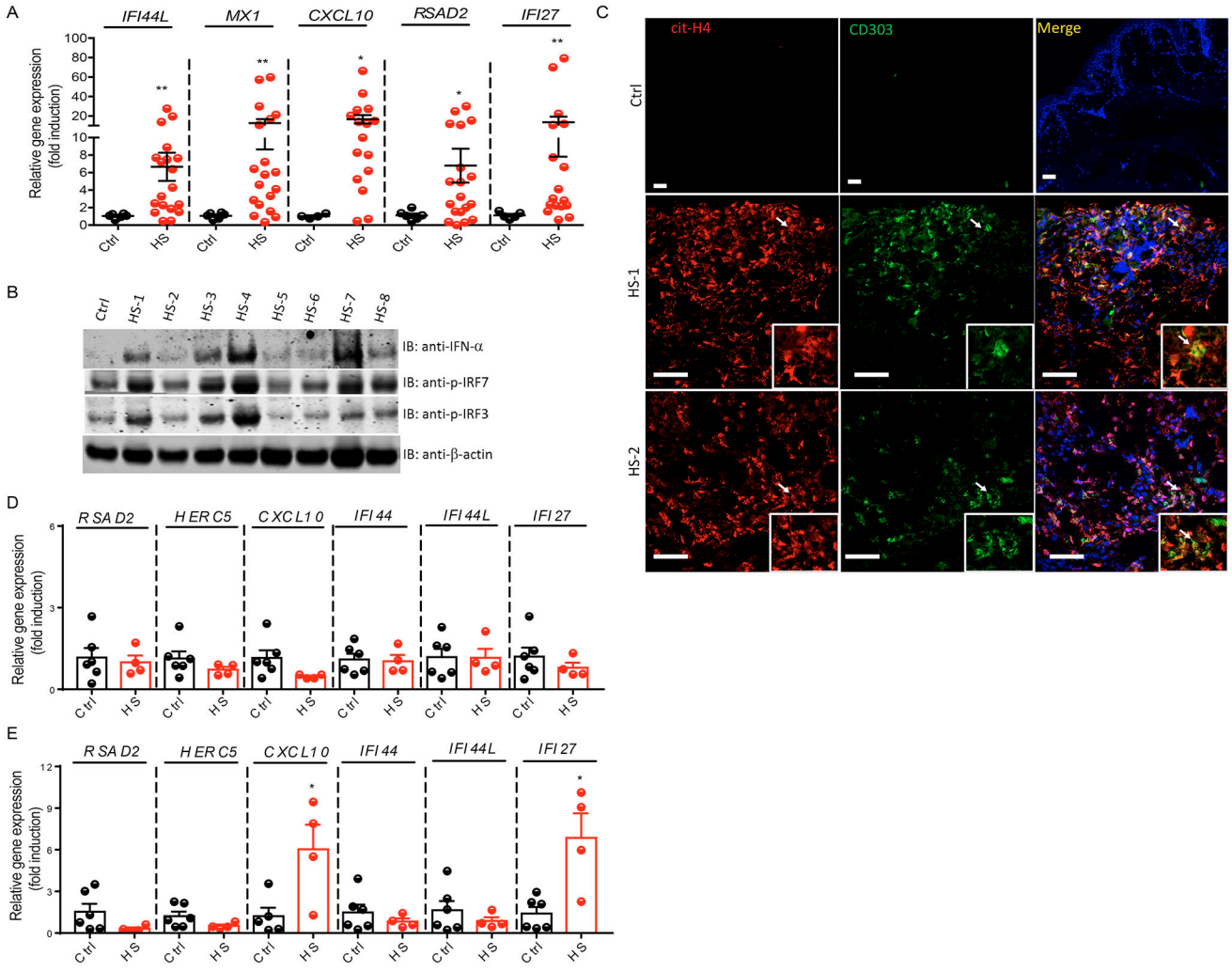

Figure 5. An enhanced type I interferon signature is detected in HS lesions.

RNA was isolated from homogenized HS lesional tissue or control tissue. (**A**) Quantitative PCR analysis of HS lesions (n=16) and control tissue (n=5) for type I interferon genes *IFI44L*, *MX1*, *CXCL10*, *RSAD2,* and *IFI27*. (**B**) Protein analysis of key interferon regulators, IRF3 and IRF7, in HS tissue and control tissue. Beta-actin was used as a loading control. (**C**) NETs and pDCs were visualized in HS tissue by staining cit-H4 (red) and CD303/BDCA-2 (green), respectively, showing co-existence of NETs and CD303 positive cells, *arrows*. Nuclei were stained blue. Scale bars, 100 μm. (**D**) PBMC and (**E**) neutrophils from HS patients (n=4) and control (n=6) were analyzed for type I IFN regulated genes. Results are the mean +/− SEM, *p<0.05, **p<0.01, Mann-Whitney *U* test analysis was used.