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## IL-1 and IL-36 are dominant cytokines in generalized pustular psoriasis

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### Abstract

**Background**—Generalized pustular psoriasis (GPP) is a rare, debilitating, and often life-threatening inflammatory disease characterized by episodic infiltration of neutrophils into the skin, pustule development, and systemic inflammation, which can manifest in the presence or absence of chronic plaque psoriasis (PV). Current treatments are unsatisfactory warranting a better understanding of GPP pathogenesis.

**Objective**—To understand better the disease mechanism of GPP to allow improved targeted therapies.

**Methods**—We performed a gene expression study on formalin-fixed paraffin-embedded GPP (n=28) and PV (n=12) lesional biopsies and healthy control (n=20) skin. Differential gene expression was analyzed using gene ontology and enrichment analysis. Gene expression was validated with qRT-PCR and immunohistochemistry, and a potential disease mechanism investigated using primary human cell culture.

**Results**—Compared with healthy skin, GPP lesions yielded 479 and PV 854 differentially expressed genes respectively, with 184 upregulated in both diseases. We detected significant contributions of IL-17A, TNF, IL-1, IL-36 and interferons in both diseases; although GPP lesions furnished higher IL-1 and IL-36 and lower IL-17A and interferon- $\gamma$  mRNA expression than PV. We detected prominent IL-36 expression by keratinocytes proximal to neutrophilic pustules and show that both neutrophils and neutrophil proteases activate IL-36. Suggesting another mechanism regulating IL-36 activity, the protease inhibitors serpin A1 and A3, which inhibit elastase and cathepsin G respectively, were upregulated in both diseases and inhibited activation of IL-36.

**Conclusions**—Our data indicate sustained activation of IL-1 and IL-36 in GPP, inducing neutrophil chemokine expression, infiltration and pustule formation, suggesting that the IL-1/IL-36 inflammatory axis is a potent driver of disease pathology in GPP.

## CAPSULE SUMMARY

Current treatments for generalized pustular psoriasis are unsatisfactory. We applied recently-developed techniques for transcriptomic analysis of archived FFPE biopsies revealing pro-inflammatory IL-1, IL-17, TNF and IL-36 activity, which provides a rationale for biologic targeting of these cytokines.

### Keywords

Psoriasis; generalized pustular psoriasis; DITRA; Inflammation; Interleukin

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## INTRODUCTION

Generalized pustular psoriasis (GPP, OMIM 614204) is a rare, debilitating and life-threatening disease, characterized by episodic infiltration of neutrophils into the skin, pustule development, generalized erythema and desquamation. The acute onset of GPP is frequently accompanied by chills, high-grade fever, fatigue and neutrophilia which can be potentially life-threatening and require hospitalization (1). Cases of GPP can occur either as a distinct entity or preceded by, concurrent with, or followed by chronic plaque psoriasis (*psoriasis vulgaris*, PV, OMIM 177900) which has complicated the study of the disease. GPP is often classified as a variant of PV yet striking clinical, histological and genetic differences suggest that the two diseases have distinct pathogenic mechanisms (1–5).

Many treatments for PV such as acitretin, cyclosporine A, and anti-TNF biologics are in common use for GPP but typically do not completely control the disease (6). This shortfall in the efficacy of these treatments likely stems from an incomplete overlap of the pathobiology of the two diseases. Resistance to existing treatments and disease recurrence are common with GPP thus there is a critical need to understand better the disease mechanism to develop new effective treatments.

To investigate the pathogenic mechanisms driving GPP, we harnessed a recently-developed technique to perform transcriptional profiling on archived formalin-fixed paraffin-embedded (FFPE) biopsies (7) of GPP skin to assess the transcriptome of GPP lesions, detect differentially expressed genes and identify which inflammatory pathways were activated with a view to the development of new therapeutic interventions tailored to GPP.

Herein we report that an IL36-chemokine-neutrophil axis appears to be central to the pathogenesis of GPP, with a greater utilization of innate immune mechanisms, preferential expression of IL-36 cytokines, KC expression of neutrophil chemokines, IL-36 activation by neutrophil proteases and induction of inflammatory keratinocyte responses. Our data provide a rational basis for targeted anti-cytokine biologic therapy for GPP.

## METHODS

### Subject recruitment

Archived formalin fixed paraffin embedded tissue was identified by search in our pathology database. Cases with diagnosis of pustular psoriasis were identified and diagnosis was verified by chart review. Healthy controls and patients with chronic plaque psoriasis were identified in our clinic and biopsy was obtained for formalin fixation and paraffin embedding prior to processing and analyses. Healthy volunteers were recruited for blood draws for neutrophil isolation after providing written informed consent. All protocols were approved by the institutional review board of the University of Michigan, Ann Arbor, and the study was carried out in accordance with the Declaration of Helsinki principles.

### Microarray analysis

RNA extraction was performed with an E.Z.N.A. FFPE RNA isolation kit (Omega Bio-tek, Norcross, GA) using the xylene-based extraction method as specified by manufacturer and five 20µm thick FFPE sections of biopsy. RNA was eluted into water and stored at -80°C until analyzed. Affymetrix Human Gene ST 2.1 microarrays (Affymetrix, Santa Clara, CA) were processed at the University of Michigan Microarray Core Facility according to the manufacturer's protocol. Raw microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo>) and are accessible through GEO series accession number GSE79704. The raw microarray data (.CEL files) were processed in the publicly available software R ([www.r-project.org](http://www.r-project.org)) using a modified version of the "affy" package and Human Entrez Gene custom CDF annotation version 19 (8) ([http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/genomic\\_curated\\_CDF.asp](http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/genomic_curated_CDF.asp)) using the Robust Multichip Average (RMA) method (9). Post-hybridization quality control checks were performed using RNA degradation score, relative log expression (RLE), and normalized unscaled standard errors (NUSE). Data were batch and age-corrected using an implementation of ComBat v3 (10) within the GenePattern pipeline (<http://www.GenePattern.org>). To remove background, we calculated the median values of all probe sets and removed those probes with expression values below the lowest median value + 1 standard deviation using a custom Perl script. This yielded 25,561 probe sets upon which subsequent analyses were based. Statistical analyses were performed using Significance Analysis of Microarrays method implemented in the MultiExperiment Viewer application (11). Hierarchical clustering and principal components analysis (PCA) were performed on the adjusted expression data using R and gene list comparisons done in Genomatix ([www.genomatix.de](http://www.genomatix.de)).

### Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed as described (12) with the following modifications. The top 250 genes expressed by KC, CD4+ T-cells, CD8+ T-cells, monocytes, neutrophils and dendritic cells (DC) as listed previously (13) or cytokine-treated KC (14) were matched against a ranked list of DEGs in averaged GPP and PV samples. The rank order of matching genes was used to construct a cumulative curve. Effect size was estimated by calculating the area under the curve and normalizing to the maximum possible effect size for each 250 member gene set. Statistical significance was determined by using a

phenotype-based permutation test, which preserves the complex co-expression relationships of gene expression data (12). Gene ontology (GO) analysis was performed using the GO\_ImmuneSystemProcesses ontology set using ClueGo v2.2.4 in the Cytoscape v.3.2.1 environment (15). A kappa score threshold of 0.4 was used to build networks and a two-sided hypergeometric test with Bonferroni step down correction to determine statistical significance.

### Quantitative PCR

Real time RT-PCR was performed after reverse transcribing RNA (High Capacity cDNA Transcription kit; Applied Biosystems, Foster City, CA) quantifying transcripts using a 7900HT Fast Real-Time PCR system (Applied Biosystems) using TaqMan primer sets purchased from Applied Biosystems. All values were normalized to the expression of the housekeeping gene RPLP0. Statistical significance between healthy, PV and GPP samples was determined using a Kruskal-Wallis test with Dunn's multiple comparison test in GraphPad Prism v6.

### Immunohistochemistry

Immunohistochemistry was performed on formalin fixed and paraffin embedded human skin biopsies sectioned at 5  $\mu$ m, and then immunohistochemically stained after antigen retrieval using polyclonal goat anti-IL-36 $\alpha$  (1 $\mu$ g/ml, AF1078, R&D Systems), IL-36 $\gamma$  (2.5 $\mu$ g/ml, rat, Y-12, Santa Cruz Biotech), polyclonal goat anti-CXCL8 (5 $\mu$ g/ml, AF2008, R&D Systems), polyclonal rabbit anti-cathepsin G (5 $\mu$ g/ml, NBP233498, Novus Biologicals), neutrophil elastase (1 $\mu$ g/ml, mouse, NP57, Dako), proteinase 3 (1 $\mu$ g/ml, EPR6277, LSBio), and matched isotype control antibodies. Immunoreactivity was visualized with 3, 3'-diaminobenzidine and counterstained with hematoxylin.

### IL-36 activation

Neutrophils were prepared from heparinized peripheral blood from healthy volunteers using ficoll and 3% dextran as described (16). NETs were generated on poly-L-lysine (Sigma-Aldrich) coated 12-well cell culture plates (Corning Costar) using 1 million neutrophils stimulated with 20nM PMA for 3 hours at 37°C. NET formation was confirmed by fluorescent microscopy with DAPI staining of DNA. After extensive washing with PBS, 500 $\mu$ l of 0.5 $\mu$ g/ml full-length IL-36 $\alpha$  or full-length IL-36 $\gamma$  in PBS were incubated with NETs for 1 hour at 37°C. After which, the processed IL-36 solutions were added to confluent cultures of primary human KC for 24 hours to determine IL-36 activity. Truncated IL-36 $\alpha$  (aa 6–158) and IL-36 $\gamma$  (aa 18–169) (R&D Systems) were used at 100ng/ml as positive controls. IL-36 activity was determined from its induction of CXCL1 and CXCL8 mRNA transcripts.

IL-36 activity assays were performed using 100ng/ml full-length IL-36 $\alpha$  or full-length IL-36 $\gamma$  (R&D Systems) in unsupplemented medium 154CF (Invitrogen), treated with 10mU neutrophil elastase (Enzo Life Sciences, Farmingdale, NY) or 1mU cathepsin G (Enzo), for 30 minutes at 37°C, then the reactions quenched with either cathepsin G inhibitor I (50 $\mu$ M, EMD), trypsin inhibitor (Sigma-Aldrich, 15 $\mu$ g/ml, inhibiting elastase activity), serpin A1 (1 $\mu$ g/ml, Enzo), or serpin A3 (1 $\mu$ g/ml, Enzo). The mixtures were then transferred onto

confluent cultures of primary human KC for 24 hours. IL-36 activity was determined as described above.

## RESULTS

### Plaque and pustular psoriasis are pathogenically distinct

To assess the pathophysiological differences between GPP and chronic plaque psoriasis (*psoriasis vulgaris*, PV) we analyzed archived FFPE biopsies of confirmed GPP (n=28) and PV (n=12) cases and healthy control (n=20) skin using Affymetrix Human Gene ST 2.1 microarrays. After batch correction, unsupervised principal component analysis (Figure 1a) of the GPP, PV and healthy control samples showed complete separation of the GPP and PV samples from the healthy samples, but there was substantial overlap between the plaque and pustular samples. Unsupervised clustering of transcripts confirmed separation of healthy control and disease samples as well as overlap of GPP and PV transcriptomes (Figure 1b). Interestingly, chart review revealed that 7/28 GPP cases had concurrent PV, and when analyzed by unsupervised clustering analysis, these 7 cases clustered with the GPP-only samples (Figure 1b). Using significance analysis for microarrays, gene expression in GPP and PV lesions were compared with healthy skin, finding 479 differentially expressed genes (DEGs) in GPP and 854 DEGs in PV with at least a 2-fold change in expression (FDR  $p < 0.05$ ) with 184 of these transcripts differentially expressed in both diseases (Figure 1c and Supplemental Table 1).

Of the 479 DEGs in GPP vs. healthy skin, 235 were up-regulated and 292 down-regulated. The up-regulated transcripts mapped to 13 gene ontology (GO) Immune System Process terms, with processes related to granulocyte chemotaxis mapping to the vast majority of transcripts (upper panel, Figure 2 and Supplemental Table 2). In contrast, of the 854 DEGs in PV, 169 mapped to 9 GO terms, which included several categories related to the adaptive immune response (lower panel, Figure 2 and Supplemental Table 2).

### Pustular psoriasis lesions have heightened IL-1/36 cytokine activity

DEGs in PV and GPP were interrogated using gene set enrichment analysis (GSEA) using gene sets comprised of the 250 most abundantly expressed genes in keratinocytes (KC), CD4<sup>+</sup> T-cells, CD8<sup>+</sup> T-cells, monocytes, neutrophils and dendritic cells (DC) (13). This revealed that a greater abundance of genes expressed by neutrophils and monocytes were detected in GPP compared with PV biopsies (Figure 3a and b). We extended this approach by using gene sets generated from KC cultures treated with a panel of recombinant cytokines to discern cytokine activity in the biopsies. We found evidence of IL-17A, IL-1 $\beta$ , IL-36 $\alpha$ , IL-36 $\gamma$ , IL-22, TNF- $\alpha$ , and IFN- $\gamma$  activity in the tissues, with much of this cytokine activity more prominent in pustular lesions than plaque biopsies (Figure 3c and d).

To confirm the increased cytokine activity in GPP skin biopsies, we performed qRT-PCR and found that while we could confirm increased expression of IL-1 $\beta$ , IL-1RA, IL-36 $\alpha$ , IL-36 $\beta$ , IL-36 $\gamma$  and IL-36RA in plaque psoriasis skin, we found a significantly greater abundance of transcripts for IL-1 $\beta$ , IL-36 $\alpha$  and IL-36 $\gamma$  in GPP versus PV lesions, with no significant changes in the expression levels of the respective receptor antagonists (Figure

4a). Conversely, and suggesting a departure from typical Th1/Th17 pathophysiology, we found that transcripts for IL-17A, IL-22, IL-23p19, IFN- $\gamma$ , IL-18 and myxovirus resistance 1 (MX-1, an interferon response gene) were all significantly more abundant in plaques compared with pustular lesions (Figure 4b). The elevated IL-36 $\alpha$  and IL-36 $\gamma$  expression in GPP was confirmed at the level of protein expression by immunohistochemistry, with an increased intensity of IL-36 $\alpha$  and IL-36 $\gamma$  expression, particularly by the KC surrounding the neutrophilic pustule, but not the neutrophils themselves (Figure 5).

### The neutrophilic environment of GPP lesions activates IL-36 $\alpha$ and IL-36 $\gamma$

GPP is characterized by periodic neutrophil infiltration into the skin and development of pustules. In concordance with this we observed strongly enhanced expression of the neutrophil chemokines CXCL1, CXCL2 and CXCL8 (IL8) in GPP, likely the factors driving the neutrophilic skin infiltration. Compared with plaque lesions, we found 15-, 5.8-, and 20-fold more transcripts for CXCL1, CXCL2 and CXCL8 in GPP lesions respectively (Figure 6a–c). The enhanced expression of CXCL8 was particularly striking also at the level of protein expression (Figure 6d and e). Given that we witnessed significantly more IL-36 expression in GPP compared with PV lesions and that neutrophils have been suggested to be a source of enzymes that are capable of processing IL-36 isoforms (17, 18), we assessed the expression of neutrophilic proteases in GPP, as well as the presence of networks of extracellular DNA suggestive of neutrophil extracellular traps (NETs), structures resulting from the programmed death of neutrophils (netosis) which expose the neutrophil proteases elastase, cathepsin G and proteinase 3 to the extracellular milieu. NETs were detected in both PV and GPP skin (Figure 6f) and immunohistochemistry confirmed the expression of cathepsin G, elastase and proteinase 3 by neutrophils in GPP skin (Figure 6g–i).

Given the close juxtaposition of heightened levels of IL-36 cytokines and the proteases implicated in their proteolytic activation, we then asked whether neutrophil NETs were capable of activating IL-36 $\alpha$  and IL-36 $\gamma$  *in vitro*. Full-length (FL)-IL-36 $\alpha$  and FL-IL-36 $\gamma$  were treated with NETs generated from PMA-activated neutrophils for 1 hour then exposed to cultures of primary human KC to assay their activity with respect to the induction of KC inflammatory responses. Exposure to NETs significantly increased the ability of FL-IL-36 $\gamma$  to induce CXCL1 and CXCL8 expression by KC more than 3-fold compared with untreated FL-IL-36 $\gamma$  (Figure 7a and c), suggesting that enzyme activity within the NETs caused FL-IL-36 $\gamma$  activation. Next we approached the IL-36 activation question using purified neutrophil elastase and cathepsin G to examine their influence on the activity of FL-IL-36 $\alpha$  and FL-IL-36 $\gamma$ . Treatment of FL-IL-36 $\alpha$  (1 $\mu$ g/ml) with 10mU neutrophil elastase before addition to cultures of primary KC lead to enhanced KC production of CXCL1, CXCL2, IL8 and CCL20 transcripts (Figure 7e–h). However, treatment of FL-IL-36 $\alpha$  with cathepsin G did not increase the cytokine's activity. On the other hand, no increase in FL-IL-36 $\gamma$  activity was seen with elastase treatment, but incubation of FL-IL-36 $\gamma$  with 1mU cathepsin G lead to a significant increase in its ability to induce chemokine transcript production by KC (Figure 7i–l)

### Physiologic protease inhibitors serpin A1 and A3 regulate IL-36 activation

Serpin A1 and A3 are extracellular protease inhibitors with activity against neutrophil elastase and cathepsin G respectively (19). Here we found that the induction of FL-IL-36 $\alpha$  activity was prevented by addition of serpin A1 (Figure 7e–h) and FL-IL-36 $\gamma$  activity prevented by treatment with the specific cathepsin G inhibitor serpin A3 (Figure 7i–l). Suggestive of attempts, albeit insufficient, to regulate IL-36 activity in skin, both of these protease inhibitors were found to be upregulated in our microarray dataset: serpin A1 was 1.86-fold ( $q=0.0048$ ) and 1.78-fold ( $q<0.001$ ) upregulated in PV and GPP lesions respectively, compared with healthy skin; whereas serpin A3 expression was increased 1.94-fold ( $q=0.020$ ) and 2.2-fold ( $q=0.035$ ) in PV and GPP lesions respectively.

## DISCUSSION

The adaptive immune system, particularly CD4+ and CD8+ T cells, has a critical, if not causal, role in the pathogenesis of PV and as such, agents specifically targeting elements of adaptive immunity have had great success in treating plaque psoriasis (20, 21). These therapies are generally less effective for GPP (6), suggesting a divergent pathogenic mechanism in the pustular disease. Using archived FFPE skin biopsy material, microarrays and a bioinformatics approach we have revealed that although the transcriptome of GPP overlaps with PV (Figure 1), the pustular disease has a much more pronounced utilization of the innate immune system than PV (Figure 2 and Supplemental Table 2). Our data suggest that GPP is a disease primarily involving the activities of KC, neutrophils and monocytes (Figure 3a), with inflammatory processes mainly driven by IL-36, IL-1, TNF- $\alpha$ /IL-17A (Figure 3b). Moreover, the increased expression (Figures 4 and 5), processing and activity (Figure 7) of IL-36 cytokines might be a central mechanism that promotes neutrophil accumulation in the epidermis.

The importance of IL-36 in pustular skin disease was recently highlighted when missense mutations in *IL36RN*, which affect the structure and function of the IL-36 receptor antagonist (IL-36RA), were found to be associated with GPP (3, 22). Loss of IL-36RA function leads to unrestrained IL-36 activity, as evidenced by induction of IL-1 $\beta$ , IL-6, and IL-8 production, and neutrophil infiltration. GPP can present either alone or preceded by, concurrent with, or followed by PV (1). Mutations in *IL36RN* have been shown to account for 46–82% of cases of GPP without associated PV (5, 23). The proportion of *IL36RN* mutant carriage is much lower (10–17%) in cases of GPP associated with PV (2), supporting the notion of divergent pathogenic mechanisms in the two diseases. A recent meta-analysis of 233 GPP cases revealed that carriage of 1 or 2 *IL36RN* mutant alleles conferred a more severe clinical phenotype with an earlier age of onset and increased risk of systemic inflammation than non-carriage (2). A gene-dosage effect was also apparent in that homozygous carriers had an earlier age of onset than heterozygotes. In addition, mutations in *AP1S3* (24) and *CARD14* (25) affecting the structure and function of the AP-1 complex subunit  $\sigma 1C$ , and the NF- $\kappa B$  activator CARD14 respectively, have also been associated with GPP, giving further insight into the mechanisms that may underlie this disease.

The three isoforms of IL-36, IL-36 $\alpha$ ,  $\beta$  and  $\gamma$ , form a trio of pro-inflammatory IL-1 family cytokines which we (26) and others (27, 28) have shown are overexpressed in plaque

psoriasis skin where they may drive keratinocyte inflammatory responses (26), synergize with other pro-inflammatory cytokines (26, 29), and promote dendritic cell activation (30, 31). The effects of IL-36 on skin have been modeled in mice (26, 27). Transgenic expression of IL-36 $\alpha$  in murine epidermis lead to epidermal thickening, hyperkeratosis, mixed inflammatory cell infiltrate, and elevated chemokine expression (CCL2, CXCL6, CCL7, and CXCL2) (27). Backcrossing to an *IL-36RN*-deficient mouse resulted in more severe skin lesions, reflecting the effect of nonsense *IL-36RN* mutations in GPP. This phenotype was partly reversed by the use of an anti-Gr1 neutrophil-depleting antibody or TNF-neutralizing antibodies, suggesting roles for both neutrophils and TNF in IL-36 $\alpha$ -induced skin hyperplasia. IL-36 $\alpha$  injected subcutaneously into mice induces acanthosis and a striking infiltration of granulocytes (CD11b<sup>+</sup>) concomitant with chemokine and growth factor induction (30). IL-36 family expression is also elevated in the skin of psoriasiform KC-Tie2 mice (26) and during imiquimod-induced skin inflammation (26, 32). One of the early features of imiquimod-treated mouse skin is neutrophil infiltration, and the inflammatory phenotype is ablated in IL-36 receptor deficient mice (32) further implicating a neutrophil/IL-36 axis as a central driver in the development of skin inflammation in GPP.

Like their IL-1 family counterparts IL-1 $\beta$ , IL-18 and IL-33 (33–35), the IL-36 cytokines require N-terminal peptide cleavage for activity (36) and it was recently demonstrated that IL-36 activation could be carried out by the neutrophil serine proteases elastase and cathepsin G (17). Here we show that neutrophils activated to form NETs have the ability to activate full-length IL-36 $\gamma$  (Figure 7a–d) and of the three proteases prominently expressed in neutrophilic pustules (Figure 6g–h), elastase could activate IL-36 $\alpha$  and cathepsin G was found to activate IL-36 $\gamma$  increasing their ability to induce KC to produce neutrophil chemokines (Figure 7e–l). Interestingly we detected an upregulation of two endogenous protease inhibitors, serpins A1 and A3 in both PV and GPP (Figure 7e–l) and which appear to be labile to induction by inflammatory cytokines (data not shown), suggesting a counter-regulatory mechanism to control IL-36 activity. However, the serpin mechanism of action is one of suicide inhibition and a 1:1 serpin:protease stoichiometry is required (19), thus the approximate 2-fold upregulation of serpins A1 and A3 we detected in lesional skin is unlikely to counter the formidable protease expression afforded by the neutrophilic infiltrate (Figure 6). This imbalance may suggest a yet unexplored therapeutic angle in pustular diseases.

We have shown that GPP utilizes pathways both overlapping and separate from PV, and because of their efficacy in moderate-severe PV, a number of therapies specifically targeting cytokines are in use for GPP. Several reports describe the use of the anakinra, an IL-1 receptor antagonist, to treat GPP (37–39). Anakinra appears to induce a rapid normalization of systemic inflammatory symptoms followed by improvement of the pustular skin eruption. The responses to IL-1 receptor inhibition in the skin tend to be incomplete however, with erythema and hyperkeratosis remaining in some cases, which suggests that IL-1 is not playing a central role in GPP but acts in a positive feedback loop inducing and being induced by IL-36 (26). The relevance of using IL-1 receptor antagonism to tackle a disease which may be primarily driven by deviations in IL-36 signaling, particularly when *IL36RN* mutations are present, has recently been questioned (40). TNF- $\alpha$  is a central mediator in chronic plaque psoriasis as evidenced by the effectiveness of therapies that block TNF- $\alpha$ .



activity. Of the three TNF blockers in use for PV, infliximab has most commonly been used for GPP (41, 42) and as such has become one of the recommended treatment options for severe acute GPP despite the lack of adequate clinical trials (6). Infliximab has been reported to have a rapid effect, with systemic inflammation and skin pustules starting to recede in as little as 2 days from the first infusion. In this context, the efficacy of infliximab likely stems from the rapid availability of the drug following infusion, and its inhibition of the synergy between TNF and multiple inflammatory cytokines including IL-36, IL-17A, IL-1 $\beta$  (26, 29, 43, 44). We detected increased IL-17A activity in GPP lesions using GSEA of microarray data (Figure 3) despite not being able to detect a significant increase in IL-17A transcripts by qRT-PCR (Figure 4); this discordance may be the result of the lack of sensitivity of the PCR assay for IL-17A in FFPE samples, contrasting with the ability of GSEA to detect the downstream effects of cytokines on transcriptional networks, or possibly similarities in IL-1/17/36-induced gene sets driving a perceived IL-17A signature in the data. Notwithstanding, biologics targeting IL-17A signaling have been used with success for GPP. An initial case report detailed the rapid improvement of both systemic symptoms and the skin disease of one GPP patient with secukinumab (45), followed by a clinical trial which provided encouraging, but because of its design not conclusive, data from 10 out of 11 GPP patients on secukinumab (46). Likewise brodalumab, a biologic targeting IL-17RA, was shown to induce clinical improvement in 11 out of 12 GPP patients (47).

The above mentioned therapies have largely been tested empirically for GPP, but now our data (Figures 3 and 4) provide evidence for the selection of these treatments as we demonstrate that responses to IL-1, IL-17A and TNF- $\alpha$  are heightened in GPP, with the IL-1 and IL-36 families of cytokines being the most robust. Our data provide a rational basis for the development of therapies specifically targeting these pathways, particularly the IL-36 system in GPP (48, 49).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>GPP</b>	generalized pustular psoriasis
<b>PV</b>	psoriasis vulgaris

<b>DEG</b>	differentially expressed gene
<b>FFPE</b>	formalin-fixed paraffin-embedded
<b>NET</b>	neutrophil extracellular trap
<b>GSEA</b>	gene set enrichment analysis
<b>PCA</b>	principal component analysis
<b>FL</b>	full-length
<b>FDR</b>	false discovery rate

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**CLINICAL IMPLICATIONS**

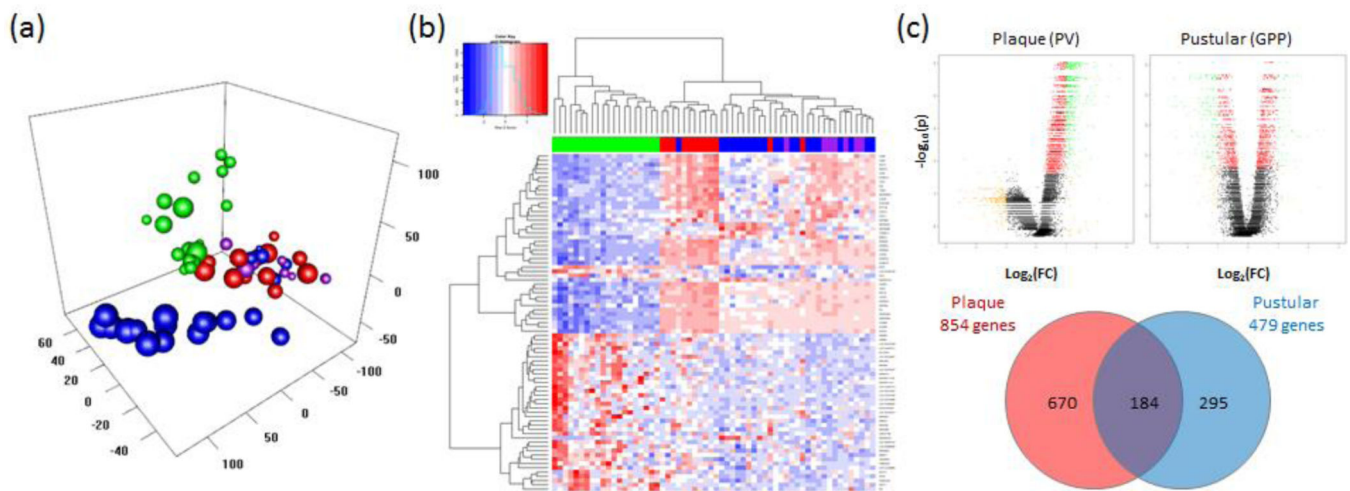
This work demonstrates the sustained activity of IL-1, IL-17, TNF and IL-36 cytokines in generalized pustular psoriasis lesions, which provides a rational basis for targeting these cytokines in this disease.

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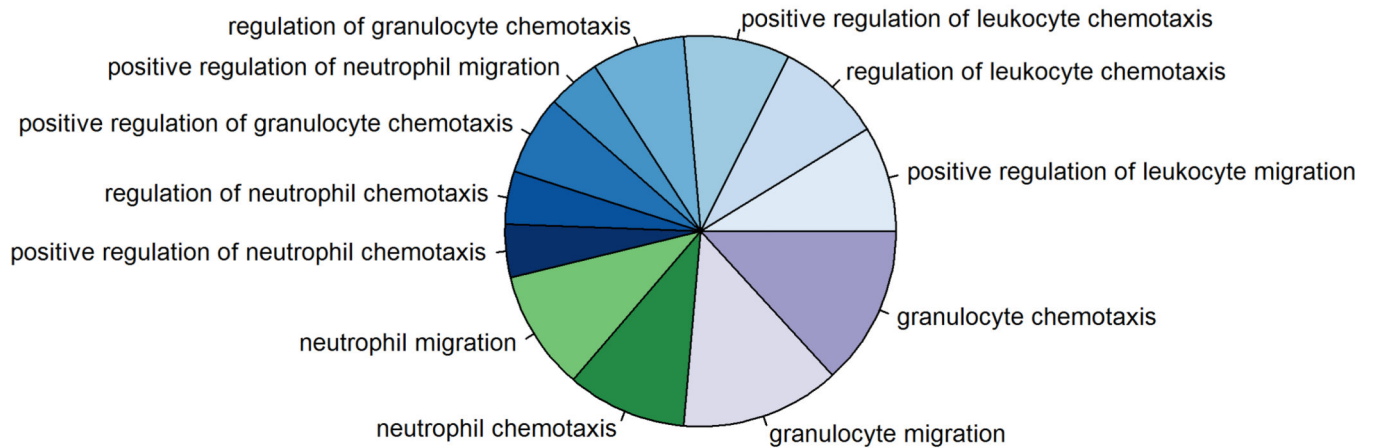
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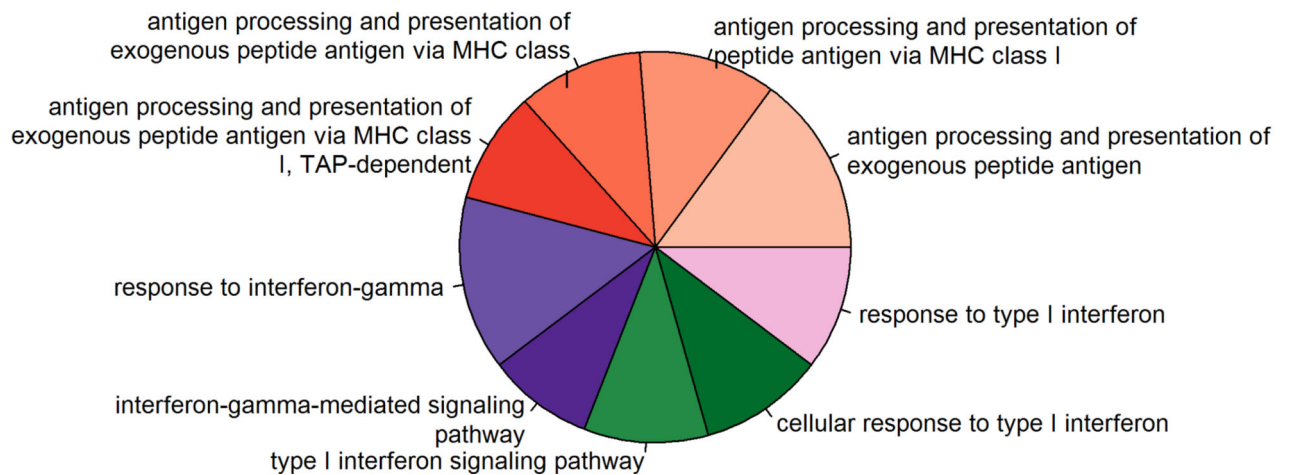
**Figure 1. The transcriptome of generalized pustular psoriasis shares features of plaque psoriasis but is skewed towards innate immune inflammation**

Formalin-fixed paraffin-embedded biopsies of chronic plaque psoriasis (PV, n=12), generalized pustular psoriasis (GPP, n=28) and healthy control skin (NN, n=20) were processed for RNA extraction and analyzed using Affymetrix ST 2.1 arrays. Principal component analysis of the GPP, PV and NN samples showed segregation of the GPP and PV samples from the healthy samples, with some overlap between the plaque and pustular samples. NN, PV, PV+GPP and GPP-only phenotypes are indicated by green, red, purple and blue spheres respectively (a). A heat map generated from unsupervised clustering of differentially expressed transcripts between GPP, PV and NN skin shows heterogeneity of healthy and disease samples. For clarity, the 79 transcripts with  $0.25 > FC > 4.0$ , and FDR  $p < 0.05$  were selected. Blue indicates low expression levels whereas red indicates high expression levels. The green, red, purple and blue bars above the heat map indicate NN, PV, PV+GPP and GPP-only skin samples respectively (b). Volcano plots of gene expression showing roughly equal numbers of up- and down-regulated genes in each disease although many of the down-regulated transcripts in PV did not reach statistical significance. Yellow indicates transcripts with  $0.5 > FC > 2$ , red denotes FDR  $p < 0.05$ , and green labels transcripts satisfying both criteria (upper panels, c). Despite overlap of differentially expressed genes between PV and GPP skin lesions, each disease had a set of uniquely-expressed transcripts (lower panel, c).

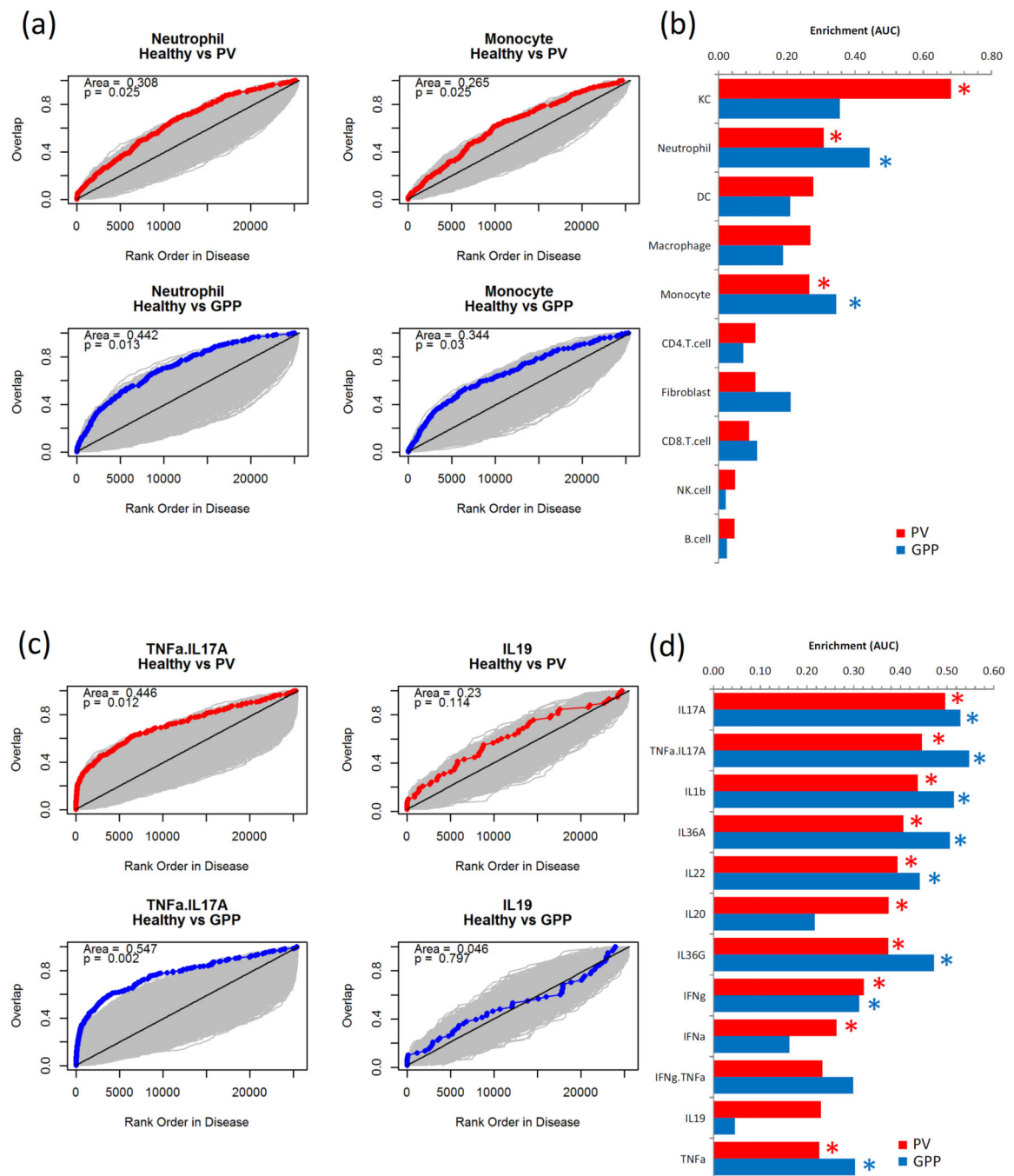
## Pustular (GPP)



## Plaque (PV)



**Figure 2. The transcriptome of pustular psoriasis lesions are enriched for innate immune genes** Gene ontology analysis of DEGs in GPP and PV using the GO\_ImmuneSystemProcess category. GO analysis highlighted an over-representation of innate immune genes in GPP (A) contrasting with more prominent expression of genes involved in acquired immunity in PV (B). Categories shown are those reaching statistical significance using a two-sided hypergeometric test. Details of individual GO terms are listed in Supplemental Table 2.



**Figure 3. Neutrophil and monocyte transcripts show greater enrichment in pustular psoriasis (GPP) lesions than plaque psoriasis (PV), whereas robust cytokine gene induction is evident in both diseases**

Microarray datasets for KC, CD4+ T-cells, CD8+ T-cells, monocytes, neutrophils and dendritic cells (DC) were compiled and used to generate signature gene lists (13). DEGs in PV vs NN and GPP vs NN comparisons were ordered by fold-change and the rank position each of the cell signature genes noted and cumulative overlap between the sets plotted (red/blue line), showing a greater up-regulation of neutrophil-specific genes in GPP than PV lesions. Grey areas indicate space generated by 1000 random permutations of NN, PV and



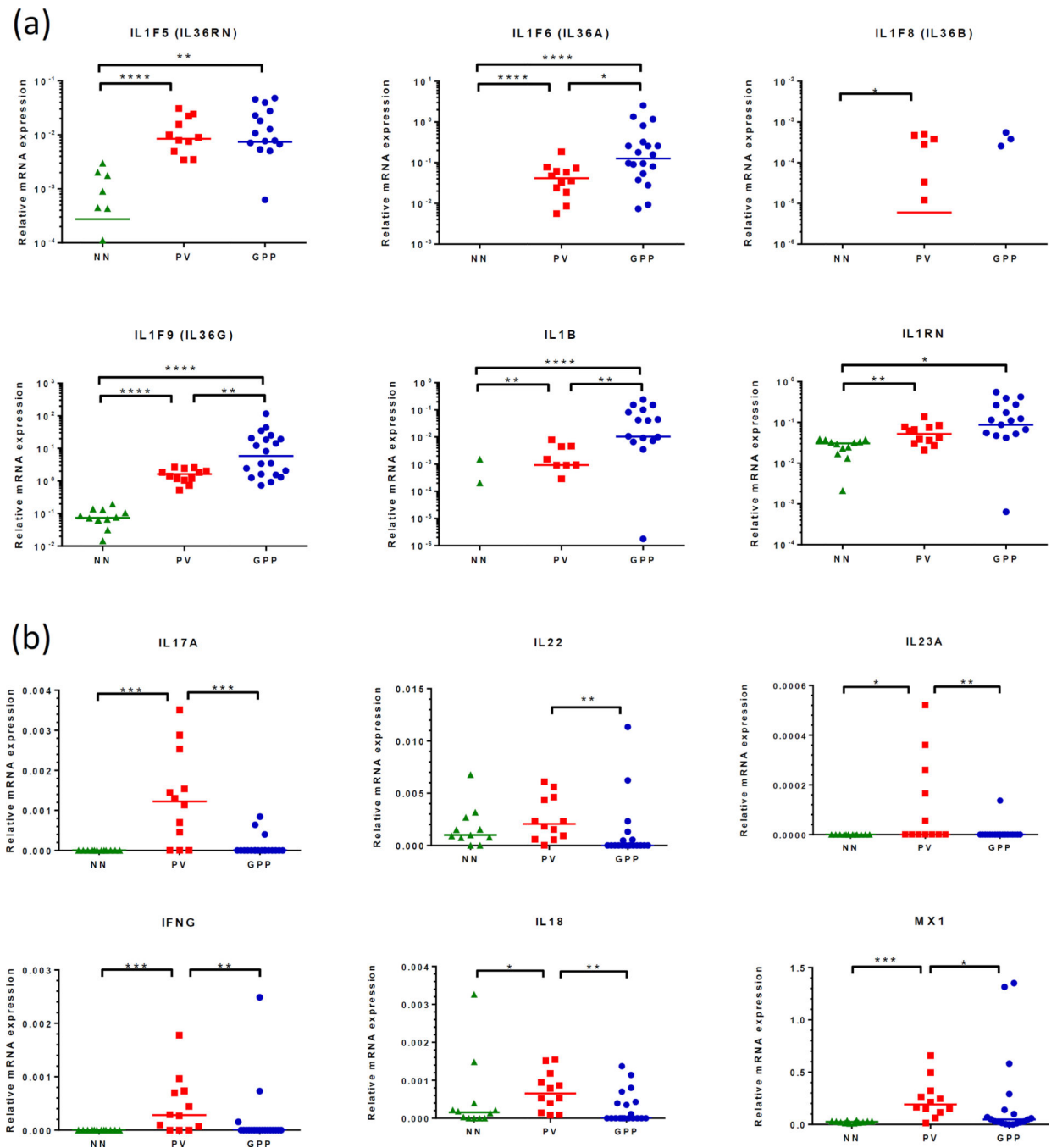
GPP datasets from which statistical significance is derived (a). Data from each of the cell signature comparisons is summarized, red bars PV, blue bars GPP, statistical significance indicated by \* $p < 0.05$  permutation test (b). Likewise gene signatures for cytokine activity on KC were generated and used to discern cytokine activity within the PV and GPP samples, showing prominent and significant presence of genes induced by TNF- $\alpha$ +IL17A in PV and GPP lesions, contrasted by the lack of enrichment in genes induced by IL-19 (c and d).

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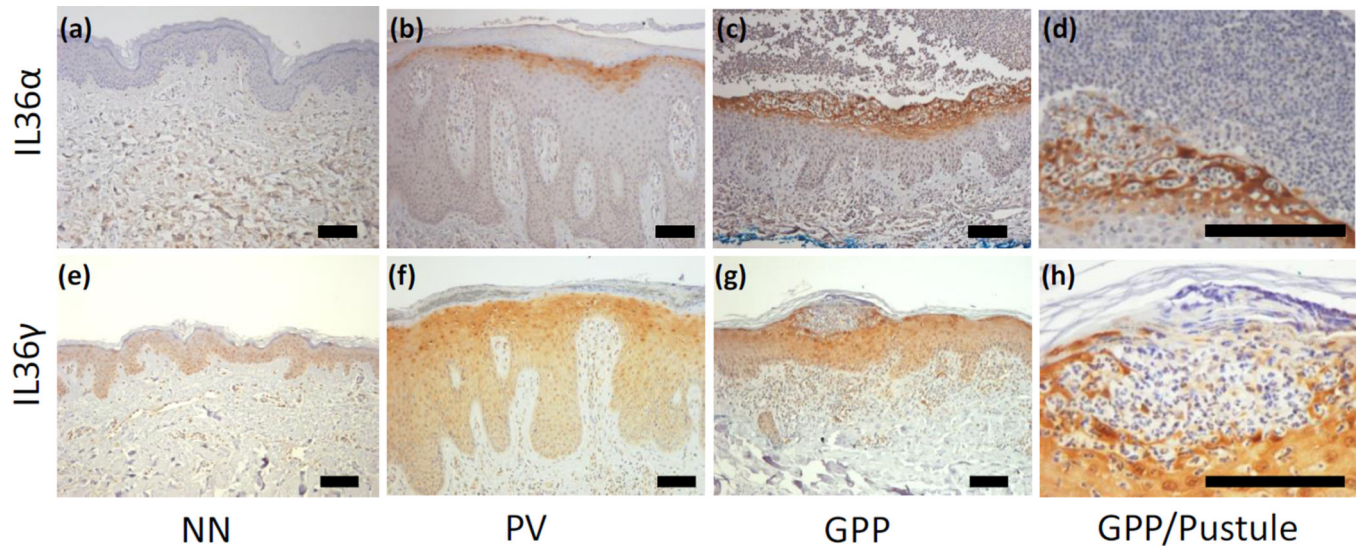
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**Figure 4. GPP lesions have a heightened IL-1/IL-36 cytokine axis but less pronounced Th1/Th17 gene expression than PV**

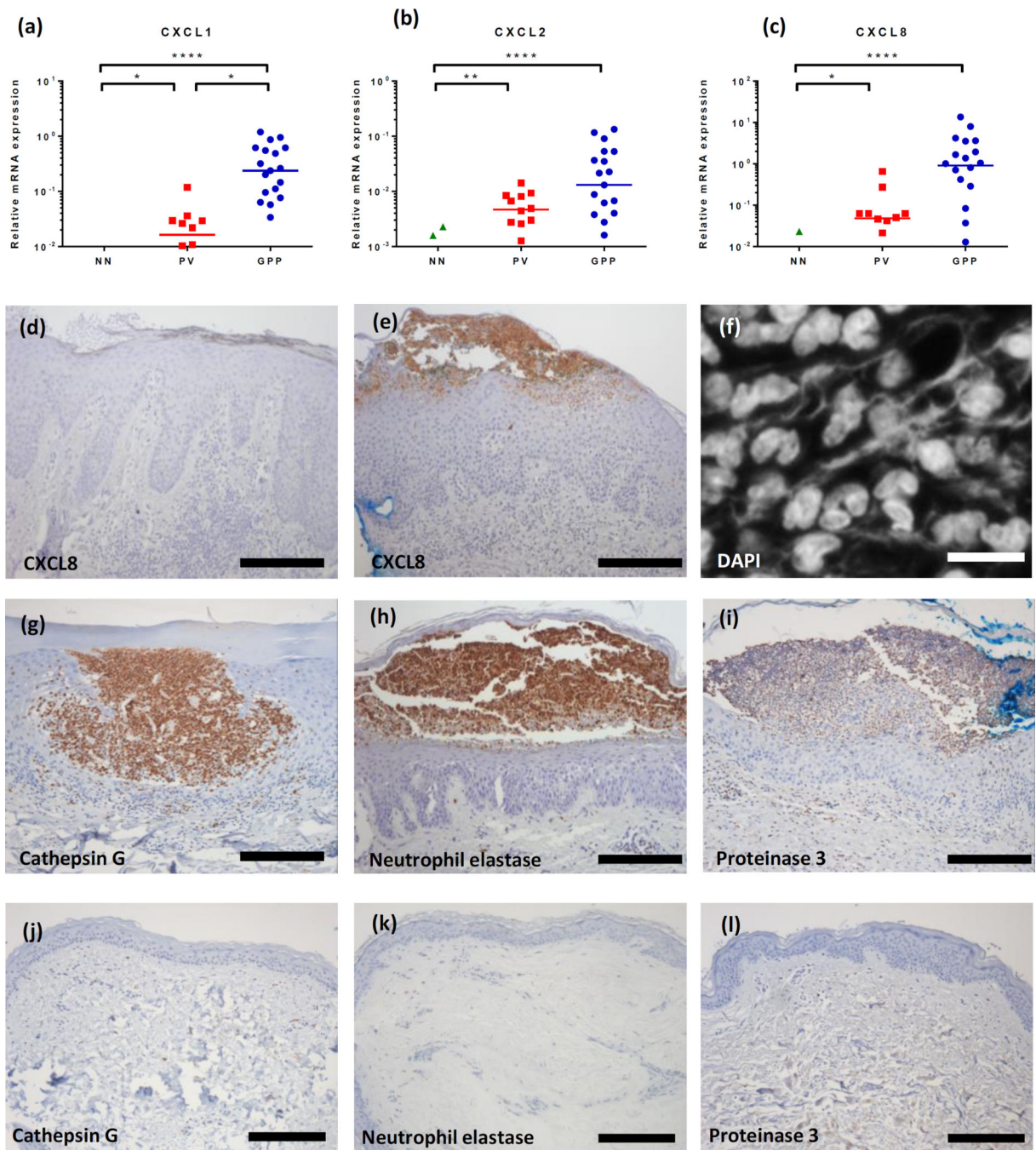
Quantitative RT-PCR revealed more abundant expression of IL36A, IL36G and IL1B transcripts in GPP compared with PV biopsies, this difference was not seen with respect to the receptor antagonists IL1RN and IL36RN (a). Suggesting a departure from typical Th1/Th17 pathophysiology, *IL23A*, *IL17A*, *IFNG*, *CXCL9*, *CXCL10* and *MX1* transcript expression was found to be significantly lower in GPP compared to PV lesions (b).

Statistical significance indicated \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . Kruskal-Wallis test with Dunn's multiple comparison test.



**Figure 5. Immunohistochemical detection of IL-36 $\alpha$  and IL-36 $\gamma$  in skin confirms elevated expression of IL-36 in GPP and localizes IL-36 expression to keratinocytes surrounding the neutrophilic pustule**

IL-36 $\alpha$  was not expressed by healthy skin (a), found in the cytoplasm of the uppermost layers of viable KC in PV lesions (b) and expressed at a higher intensity by a wider band of KC in GPP lesions (c). IL-36 $\alpha$  expression was strongest in the LC proximal to neutrophilic pustules, but not the neutrophils themselves (d). Faint expression of IL-36 $\gamma$  could be detected in the cytoplasm, but was most apparent in the nuclei of healthy skin KC (e). IL-36 $\gamma$  was intensified in PV lesions (f) yet this was more intense in GPP lesions (g), most pronounced in the KC adjacent to pustules (h). Representative images from 6 GPP, PV and NN donors used (Supplemental Figure 1). DAB with hematoxylin counterstain. Scale bar 100 $\mu$ m.



**Figure 6. The neutrophil chemokines CXCL1, CXCL2 and CXCL8 are more strongly expressed in GPP than PV lesions**

Using qRT-PCR we detected significantly higher expression of *CXCL1*, *CXCL2*, and *IL8* transcripts in GPP (n=20) compared with plaque psoriasis (n=12) or healthy control skin (NN, n=12) (a–c). CXCL8 immunoreactivity was barely evident in PV (d) but very strong in and around neutrophilic pustules in GPP biopsies (e). NET formation was prominent in GPP lesions as visualized with DAPI (f) as was the expression of the neutrophil proteases cathepsin G, elastase, and proteinase 3 in GPP (g, h, i) but not healthy control skin (j, k, l). Representative images of at least 6 GPP, PV, NN donors used (Supplemental Figure 1). Scale

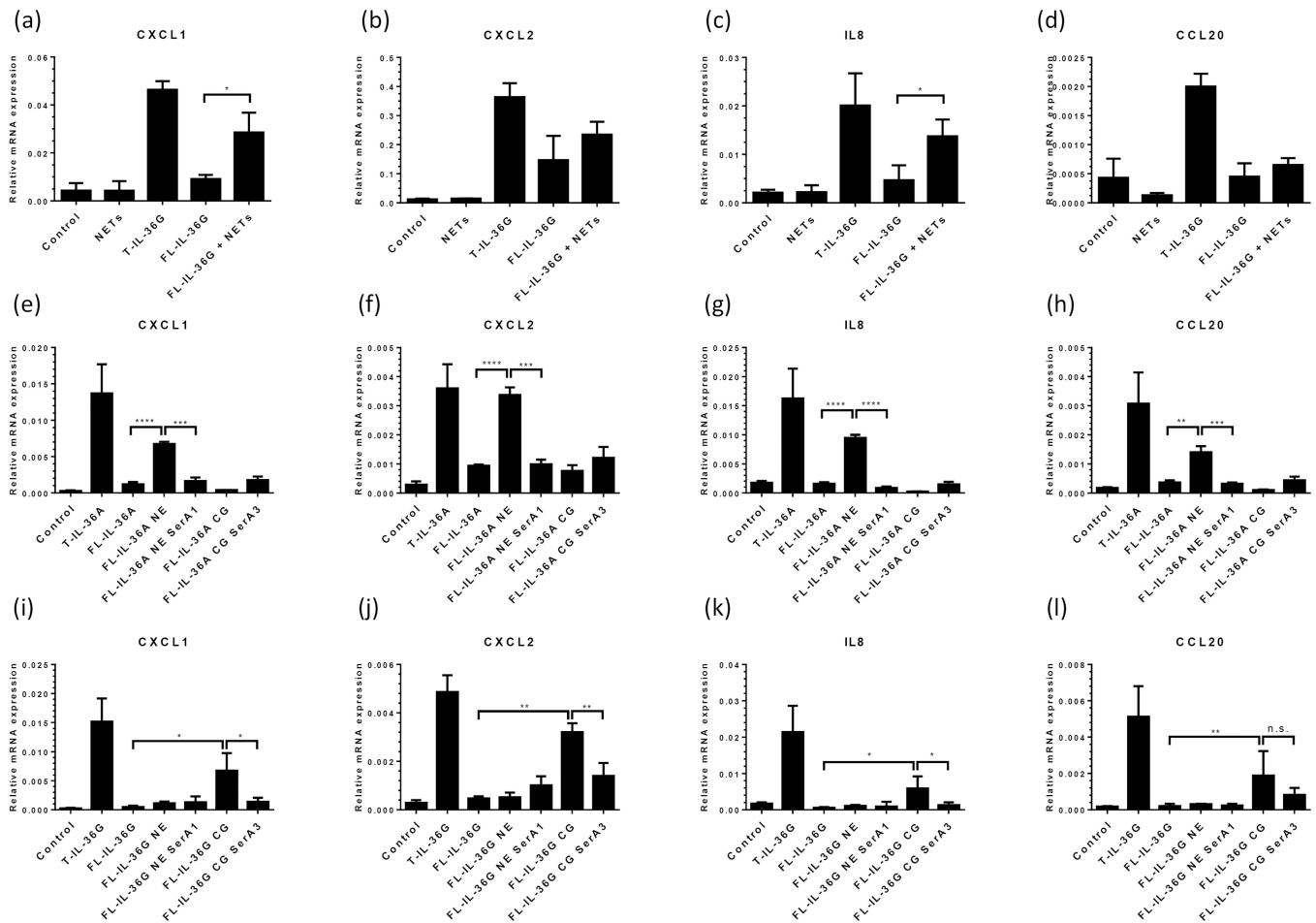
bar, d- e, and g-l: 200µm, f: 10µm. Statistical significance indicated \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001. Kruskal-Wallace test with Dunn's multiple comparison test.

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**Figure 7. Neutrophil extracellular traps (NETs) and neutrophil proteases activate IL-36**  
 Exposure to NETs for 1 hour (a–d) significantly increased the ability of full-length (FL)-IL-36 $\gamma$  to induce CXCL1 and IL8 expression by KC more than 3-fold compared with untreated FL-IL-36 $\gamma$  (a and c), suggesting that enzyme activity within the NETs caused FL-IL-36 $\gamma$  activation. Truncated (T)-IL-36 as positive control for IL-36 activity. Purified neutrophil elastase (NE, e–h) and cathepsin G (CG, i–l) activated FL-IL-36 $\alpha$  and FL-IL-36 $\gamma$  respectively which was inhibited by the addition of specific inhibitors of elastase (serpin A1) or cathepsin G (serpin A3). Statistical significance determined with Student’s t-test and indicated \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , n.s. non-significant.