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Activity and components of the granulocyte colony-stimulating factor pathway in hidradenitis suppurativa*

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Summary

Background Hidradenitis suppurativa (HS) is a chronic inflammatory disease, characterized by painful, purulent and destructive skin alterations in intertriginous areas.

Objectives We investigated the expression and role in HS of granulocyte colony-stimulating factor (G-CSF), the regulator of neutrophil biology, as clinical signs of a neutrophilic granulocyte-driven inflammation are distinctive in the disease.

Methods Skin and blood samples obtained from different cohorts of patients with HS and control individuals were assessed by RNA sequencing, quantitative polymerase chain reaction on reverse transcribed mRNA, and/or enzyme-linked immunosorbent assay. Mechanistic studies using keratinocytes, dermal fibroblasts, immune cell populations and skin biopsies were performed.

Results G-CSF was abundant in HS skin, particularly in inflamed nodules and abscesses. Its levels even exceeded those found in other inflammatory skin diseases. Interleukin (IL)-1 and IL-17, respectively, induced G-CSF production by fibroblasts and keratinocytes. These effects were enhanced by tumour necrosis factor (TNF)-α and IL-36. Accordingly, fibroblasts separated from HS lesions expressed G-CSF, and IL-1 receptor antagonist reduced G-CSF levels in explanted HS skin. G-CSF blood levels positively correlated with severity of HS. Elevated lesional G-CSF receptor levels were linked to upregulation of molecules that contribute to prolonged activation of neutrophils by components of bacteria and damaged host cells [formyl peptide receptor 1 (FPR1), FPR2 and free fatty acid receptor 2 (FFAR2)], neutrophil survival [TNF receptor superfamily member 10C (TNFRSF10C/TRAIL-R3) and TNF receptor superfamily member 6B], kinases (tyrosine-protein kinase HCK and hexokinase 3), and skin destruction [MMP25 (matrix metalloproteinase 25) and ADAM8 (disintegrin and metalloproteinase domain-containing protein 8)]. G-CSF elevated the expression of FPR1, FFAR2, and TNFRSF10C/TRAIL-R3 in neutrophils and synergized with bacterial components to induce skin-destructive enzymes.

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Conflicts of interest
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Conclusions The G-CSF pathway engages both tissue and immune cells, is strongly activated in HS lesions, and offers the opportunity to target the neutrophil-driven inflammation.

What is already known about this topic?

- Hidradenitis suppurativa (HS) is a chronic debilitating skin disorder with a very high, unmet medical need.
- The diseased skin in patients with HS shows distinct features of a neutrophil-driven inflammation (e.g. abscess formation, purulent discharge).
- Granulocyte colony-stimulating factor (G-CSF) is the major regulator of neutrophil development, survival and function.

What does this study add?

- HS lesions show highly increased levels of G-CSF and its receptor.
- Major G-CSF inducers are interleukin (IL)-1β and IL-17.
- In neutrophils, G-CSF upregulates receptors for components of bacteria and damaged host cells, decoy receptors for apoptosis inducers and proteases.
- The production of skin-destructive enzymes induced by bacterial components is strengthened by G-CSF in neutrophils.
- G-CSF inducers and molecules upregulated by G-CSF in neutrophils *in vitro* are abundant in HS lesions.

What is the translational message?

- G-CSF is the central element of a pathogenetic pathway in HS.
- The G-CSF pathway may contribute to the persistence of abscesses, purulent secretion and progressive skin structure destruction.
- Targeting G-CSF or its pathway elements may represent an approach for the treatment of HS and other conditions with neutrophil-driven inflammation and skin destruction.

Hidradenitis suppurativa (HS, also referred to as acne inversa) is a common chronic inflammatory disease, which affects the intertriginous skin and is associated with numerous systemic comorbidities.[1] Skin lesions typically develop in axillary, inguinal, gluteal and perianal body areas, and include deep-seated inflamed nodules, abscesses and epithelialized tunnels.[1] First alterations usually appear in young adulthood, and the estimated prevalence of HS is approximately 1% worldwide.[2,3] The cutaneous alterations are highly painful and discharge malodorous purulent secretions,[4–6] which may be the main factors leading to discomfort, mental depression, anxiety, sexual distress and impairment of self-body image of patients with HS.[7–11] This has a negative impact on patients' sleep quality, professional activity, and even the quality of life of their family members.[12–15] The comorbidities patients with HS frequently suffer and which greatly reduce their life expectancy[16] include metabolic syndrome,[17] atherosclerosis,[18] spondyloarthritis[19,20] and inflammatory bowel disease.[21,22]

Despite its high prevalence and severe physical and mental burden, treatment options for HS remain very limited,[1] with the anti-tumour necrosis factor (TNF)-α antibody adalimumab being the only approved systemic treatment.[23] The paucity of therapy options is mainly due to our limited understanding of the molecular pathways underlying the persistence of the cutaneous inflammation in HS.

The aetiology of HS involves a largely unexplained genetic contribution as well as lifestyle attributes such as obesity and smoking.[24] In intertriginous skin, these factors lead to subclinical inflammation around terminal hair follicles with infundibular acanthosis and hyperkeratosis. This results in follicular plugging, stasis and subsequent rupture with a release of the immune-stimulatory content into the dermis. In the course of this, cell damage-associated molecules and components of locally growing bacteria, especially anaerobic Gram-negative types, are hypothesized to stimulate tissue-resident immune cells to secrete proinflammatory cytokines.[24] These mediators in turn activate tissue and immune cells, leading to production of chemokines and subsequent massive infiltration of various immune cell types. Several cytokines, including interleukin (IL)-1, IL-17, IL-26, IL-36, TNF-α, and interferon-γ, were found to be strongly expressed in HS lesions.[25–31] Eventually, the mediators of the heterogeneous immune cell infiltrate lead to the destructive skin alterations.

Given the fact that HS shows features of a disease driven by neutrophilic granulocytes (further referred to as 'neutrophils' in this article), including purulent suppuration, we hypothesized a role of granulocyte colony-stimulating factor (G-CSF). G-CSF is the major regulator of neutrophil survival and function. Although a phase Ib clinical trial testing a G-CSF inhibitor in HS is under way (ClinicalTrials.gov identifier: NCT03972280), no data demonstrating regulation and function of G-CSF in HS are available. Therefore, this study focuses on the expression, cellular sources and effects of G-CSF in the context of HS.

Materials and methods

Study populations and samples

This case–control study was performed with a total of 62 patients with HS (mean ± SD age 40·0 ± 11·0 years, 56·5% female, Hurley stages I–III), 45 healthy donors (mean ± SD age 38·3 ± 10·4 years, 48·9% female), 26 patients with psoriasis (mean ± SD age 43·9.1 ± 13·4 years, 46·2% female), six patients with atopic dermatitis (AD) (mean ± SD age 27·1 ± 7·4 years, 66·7% female) and five patients with pityriasis rubra pilaris (mean ± SD age 44·1 ± 15·4 years, 40·0% female) (further details in Appendix S1; see Supporting information).

Demographic, anamnestic and clinical data were collected; whole skin biopsies were taken for quantitative polymerase chain reaction on reverse transcribed RNA (RT-qPCR) analysis, RNA sequencing and *ex vivo* culture; and venous blood was collected for plasma recovery and isolation of immune cell populations (see below). Body areas of healthy donors chosen for skin biopsies were the axillae (when exclusively comparing with HS intertriginous skin) or nonintertriginous sites such as limbs (when comparing with lesional skin from patients with further inflammatory conditions). Lesional/perilesional skin biopsies from patients with HS were obtained as described in Appendix S1.

The study was conducted according to Helsinki Declaration principles. Data collection, sampling and respective analyses were approved by the institutional review board, and written informed consent was obtained from all participants.

Skin biopsy culture

Skin punch biopsies of 3-mm diameter taken from the axillae of healthy donors and from perilesional and lesional regions of patients with HS were cultured without any stimulant for 4 h in KGM™ Gold Keratinocyte Growth Medium (Lonza, Basel, Switzerland), before culture supernatant was taken for enzyme-linked immunosorbent assay (ELISA) analysis. Paired 3-mm skin biopsies taken from lesional areas of patients with HS were cultured with/without 2 μg mL^{-1} of recombinant IL-1 receptor antagonist (IL-1RA) in KGM Gold medium for 24 h. Afterwards, cells were lysed for RT-qPCR analysis and culture supernatant was taken for ELISA analysis.

Isolation and culture of cells

Epidermal keratinocytes, isolated from skin biopsies from healthy donors, and human dermal fibroblasts (Provitro, Berlin, Germany) were cultured as described previously[32] and stimulated as indicated in Appendix S1. Isolation and culture of total peripheral blood mononuclear immune cells, neutrophils, monocytes and macrophage-like cells from healthy donor blood is described in Appendix S1. Isolation of $CD4^+$ T cells and fibroblasts from HS lesional skin is also described in Appendix S1.

Quantitative polymerase chain reaction on reverse transcribed mRNA

Snap-frozen skin biopsies were homogenized and total cellular RNA was isolated and reverse transcribed as described previously.[33] Samples were then analysed in triplicates by TaqMan®-based real-time PCR as described and used earlier.[34,35] Expression levels were calculated relative to that of a housekeeping gene.[34] For details, see Appendix S1.

Enzyme-linked immunosorbent assay

DuoSet® ELISA kits (R&D Systems Bio-Techne, Minneapolis, MN, USA) were used to determine cytokine and protease concentrations in the supernatant of stimulated cells and cultured skin biopsies. G-CSF concentrations in blood plasma were quantified by the Quantikine® ELISA system (R&D Systems Bio-Techne).

RNA sequencing and evaluation

For RNA sequencing analysis, mRNA libraries were prepared using the TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA, USA) and sequenced on a HiSeq 2000 or 4000 platform (Illumina). For details, see Appendix S1.

Statistical analyses and presentation

For comparison of unpaired and paired groups, the Mann–Whitney U-test (two tailed) and the Wilcoxon matched-pairs signed-rank test (two tailed), respectively, were used (SPSS 14·0; IBM SPSS Statistics, Armonk, NY, USA). Correlations based on RT-qPCR data were assessed by Spearman's rank correlation test using SPSS 14·0 software. For RNA sequencing data, $\log_{10}$-transformed transcripts per million values were used to calculate Spearman's rank correlation coefficients, and Benjamini-Hochberg adjustment of P-values was performed using the 'psych' package in R (https://CRAN.R-project.org/package=psych). Graphs were prepared using GraphPad Prism version 8·0 (GraphPad Software, San Diego, CA, USA) and volcano plot of differentially expressed genes (Figure 5f) was prepared using Array Studio software (OmicSoft/Qiagen, Hilden, Germany). For detailed data analyses and presentation, see Appendix S1.

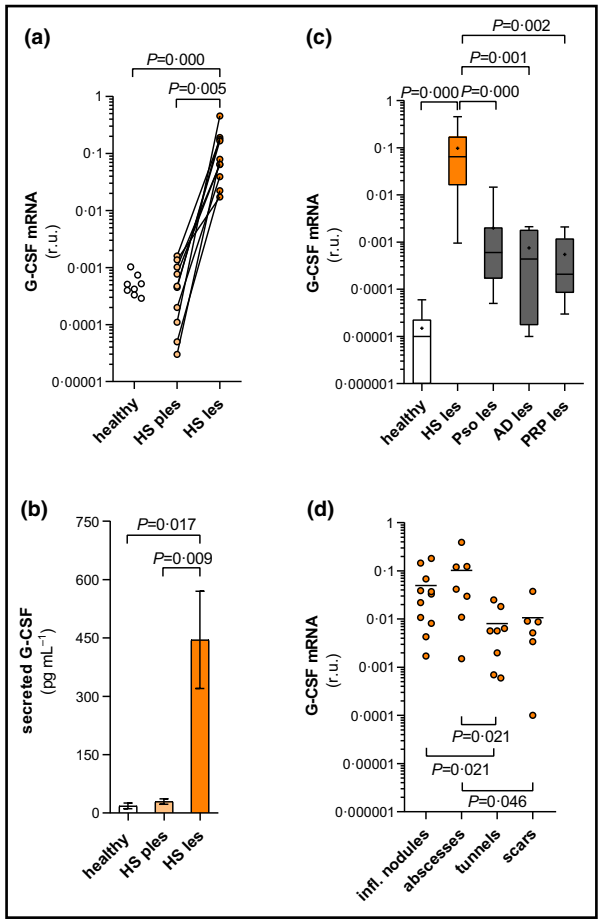


Figure 1 Granulocyte colony-stimulating factor (G-CSF) is highly expressed in lesional skin of patients with hidradenitis suppurativa (HS). (a) Skin biopsies obtained from eight healthy donors and 10 patients with HS [both paired perilesional (ples) and lesional (les) skin] were subjected to quantitative polymerase chain reaction on reverse transcribed mRNA (RT-qPCR) analysis to assess G-CSF mRNA expression. Differences between sample groups were analysed using the Mann–Whitney U-test (comparison with healthy donor skin) or Wilcoxon matched-pairs signed-rank test (paired HS samples), and P-values are presented. (b) Skin biopsies obtained from four healthy donors and four patients with HS (total of eight perilesional and eight lesional samples) were cultured without any stimulation for 4 h. Concentration of G-CSF diffused into culture supernatant was quantified using enzyme-linked immunosorbent assay. Differences between sample groups were analysed using the Mann–Whitney U-test. Mean ± SEM data indicated as column heights and P-values are presented. (c) Biopsies obtained from skin of eight healthy donors and lesional skin of 14 patients with HS, 13 patients with psoriasis (Pso), six with atopic dermatitis (AD) and five with pityriasis rubra pilaris (PRP) were subjected to RT-qPCR analysis to assess G-CSF mRNA expression. Differences between groups were analysed using the Mann–Whitney U-test. Data indicated by box plots (with the box extending from the 25th to 75th percentiles of values, the line in the middle of the box and '+' representing median and mean of the data, respectively, the maximum length of box whiskers corresponding to the most extreme values) and P-values are presented. (d) Skin biopsies obtained from different HS lesion types [11 inflammatory (infl.) nodules, seven abscesses, eight tunnels and six scar tissue sites] were subjected to RT-qPCR analysis to assess lesion type-specific G-CSF mRNA expression. Differences between lesion types were analysed using the Mann–Whitney U-test, and P-values are indicated. r.u., relative units, i.e. relative to hypoxanthine-guanine phosphoribosyltransferase expression.

Results

Granulocyte colony-stimulating factor is highly expressed in lesional skin of patients with hidradenitis suppurativa

To explore the potential role of G-CSF in HS skin inflammation, we first quantified the expression of this cytokine in lesional and perilesional skin of patients with HS and in intertriginous skin samples obtained from healthy control donors. As demonstrated in Figure 1a, G-CSF mRNA levels in lesional HS skin were strongly elevated compared with both paired perilesional samples and healthy donor skin (~210- and ~240-fold upregulation, respectively). Similarly, we detected strongly elevated G-CSF protein levels, spontaneously diffused from briefly cultured lesional skin biopsies into the medium (Figure 1b).

To investigate if elevated G-CSF levels are HS-specific, we then compared HS lesional G-CSF levels with G-CSF levels in lesional skin of patients with other chronic inflammatory conditions including psoriasis, AD and pityriasis rubra pilaris. Analysis of healthy donor skin, this time taken from nonintertriginous body areas, was used as a control. Expression in HS clearly exceeded those in the other diseases by ~50- to ~180-fold, suggesting a role of this cytokine in the HS-specific pathogenetic processes (Figure 1c). Interestingly, G-CSF mRNA expression in healthy donors' nonintertriginous skin was much lower than that in intertriginous skin (healthy donor groups in Figure 1c vs. 1a, $P = 0{\cdot}001$).

Diseased HS skin areas are not homogeneous but usually contain different types of alterations. To analyse potential lesion type-specific variations in G-CSF expression, skin samples, also including weakly inflamed specimens, were collected from a second, independent patient cohort. As demonstrated in Figure 1d, G-CSF levels were clearly raised in lesion types including scars, with highest levels found in inflamed nodules and abscesses.

Interleukin-1β and interleukin-17 are the major inducers of granulocyte colony-stimulating factor in skin tissue cells

The detected G-CSF abundance in HS lesions raised the question of the mode of production of this mediator. Therefore, we investigated G-CSF production in healthy-donor skin tissue cells and immune cells, stimulated with a range of HS-relevant cytokines, for 24 h. As shown in Figure 2a, keratinocytes secreted G-CSF mainly in the presence of IL-17A, while IL-1β

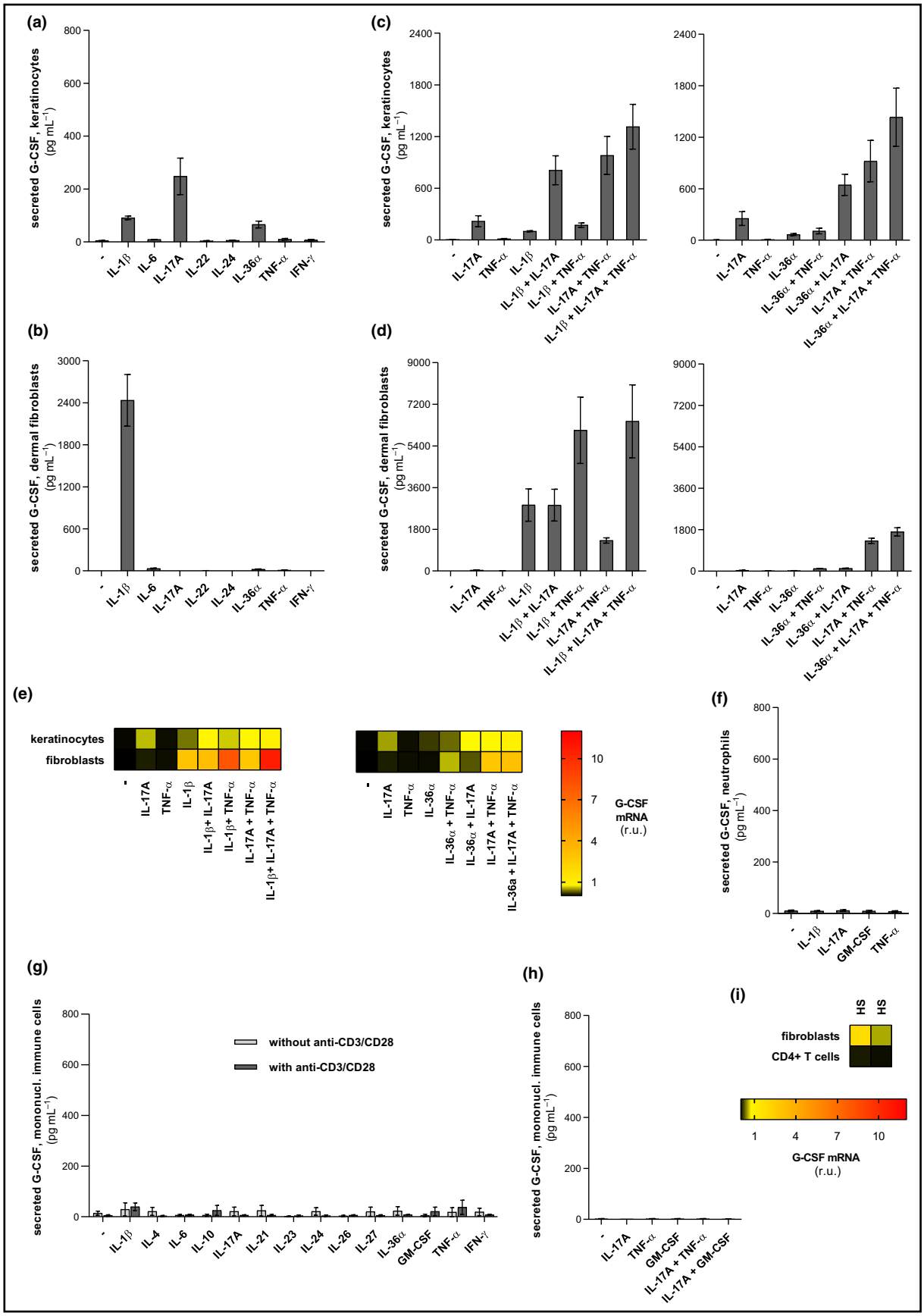


Figure 2 Interleukin (IL)-1β and IL-17 are the major inducers of granulocyte colony-stimulating factor (G-CSF) in skin tissue cells. (a–d) Keratinocytes (a,c) and fibroblasts (b,d) isolated from skin of healthy donors were cultured in the presence or absence of the indicated cytokines/cytokine combinations for 24 h. Concentration of G-CSF secreted by these cells in culture supernatant was analysed using enzyme-linked immunosorbent assay (ELISA). Mean ± SEM data from 3–6 experiments are presented as column heights. (e) Keratinocytes and fibroblasts isolated from the skin of healthy donors were treated as in (c) and (d). Cellular G-CSF mRNA expression was quantified using quantitative polymerase chain reaction on reverse transcribed mRNA (RT-qPCR) analysis. Data from one experiment each are displayed as heat maps. (f) Neutrophils isolated from blood of healthy donors were cultured in the presence or absence of the indicated cytokines for 24 h. Concentration of G-CSF secreted by these cells in culture supernatant was quantified using ELISA. Mean ± SEM data from four experiments are presented as column heights. (g–h) Mononuclear immune cells isolated from blood of healthy donors were cultured with or without the indicated cytokines in the presence and absence of T-cell-stimulating agents (anti-CD3/anti-CD28-coated beads) for 24 h (g) or treated with the indicated cytokines/cytokine combinations as indicted for 72 h (h). Concentration of G-CSF secreted by these cells in culture supernatant was quantified using ELISA. Mean ± SEM data from three experiments are presented as column heights. (i) Fibroblasts and $CD4^+$ T cells isolated from lesional skin of a patient with hidradenitis suppurativa (HS) using flow-cytometric cell sorting were subjected to RT-qPCR analysis to quantify cellular G-CSF mRNA expression. Data are presented as heat map. TNF, tumour necrosis factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; r.u., relative units, i.e. relative to hypoxanthine-guanine phosphoribosyltransferase expression.

and IL-36α had a smaller but also inducing effect. In contrast, G-CSF production by fibroblasts was mainly induced by IL-1β, and resulting levels highly exceeded those found in IL-17A-stimulated keratinocytes (Figure 2b). The IL-17A effect on keratinocytes was strengthened by IL-1β, TNF-α and IL-36α; the highest induction was detected when IL-17A was combined with TNF-α and, additionally, with IL-1β or IL-36α (Figure 2c). In fibroblasts, the effect of IL-1β was strongly strengthened by TNF-α, but there was no amplifying effect of IL-17A on either IL-1β or the combination of IL-1β/TNF-α (Figure 2d). A similar pattern was observed for G-CSF mRNA levels in respectively stimulated keratinocytes and fibroblasts (Figure 2e), suggesting transcriptional regulation.

In contrast to tissue cells, activated immune cells did not secrete G-CSF. In fact, none of the various cytokines tested led to G-CSF production in neutrophils (Figure 2f) or mononuclear immune cells (Figure 2g). The same was true in mononuclear immune cells stimulated in the presence of T-cell activation (Figure 2g) or for an extended time (72 h) (Figure 2h).

In line with results of the *in vitro* experiments, we detected strong expression of G-CSF in fibroblasts but not $CD4^+$ T cells isolated from HS skin (Figure 2i).

Granulocyte colony-stimulating factor-inducing cytokines appear to be relevant in lesional hidradenitis suppurativa skin

Matching the G-CSF-inducing effect of IL-1β and IL-17A observed *in vitro*, mRNA levels of these cytokines showed a clear positive correlation with G-CSF levels in lesional HS skin

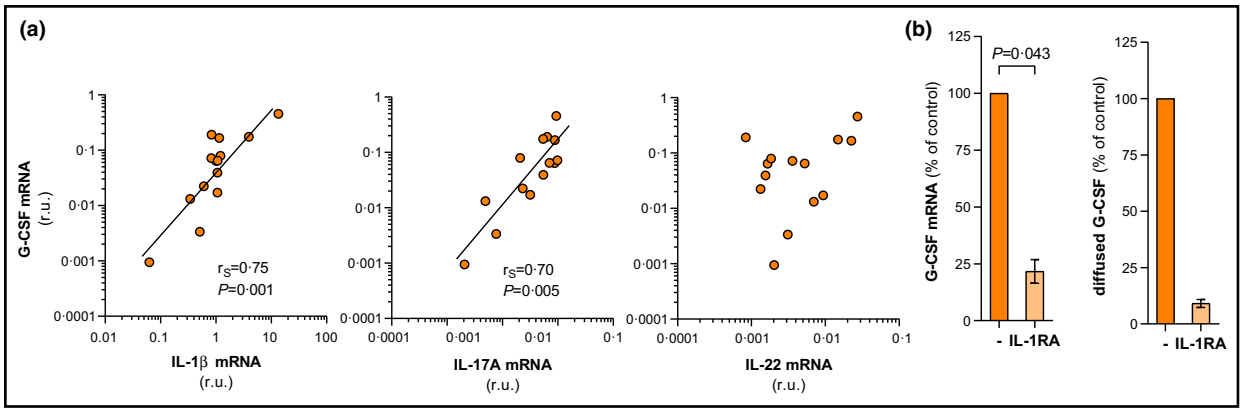


Figure 3 Granulocyte colony-stimulating factor (G-CSF)-inducing cytokines appear to be relevant in lesional hidradenitis suppurativa (HS) skin. (a) Lesional skin samples obtained from 14 patients with HS were subjected to quantitative polymerase chain reaction on reverse transcribed mRNA (RT-qPCR) analysis to assess cytokine mRNA expressions. To confirm a relationship between G-CSF and interleukin (IL)-1β/IL-17A *in situ*, correlation of G-CSF expression with that of IL-1β, IL-17A and, as control, IL-22, was tested using Spearman's rank correlation test. Correlation coefficient r_S and P-values are indicated. (b) Paired biopsies of lesional skin obtained from five (mRNA) or four (protein) patients with HS were cultured for 24 h in the presence or absence of IL-1 receptor antagonist (IL-1RA), a molecule able to block the action of IL-1 within the inflamed lesion. Cultured biopsies were then subjected to RT-qPCR analysis to assess G-CSF mRNA expression (left). The concentration of G-CSF diffused from the biopsies was analysed in culture supernatant using enzyme-linked immunosorbent assay (right). Difference between treatment groups was analysed using Wilcoxon matched-pairs signed-rank test (for mRNA data, sufficient replicate experiments to allow statistical analysis). Mean ± SEM percentages of control indicated as column heights and P-values are presented. r.u., relative units, i.e. relative to hypoxanthine-guanine phosphoribosyltransferase expression.

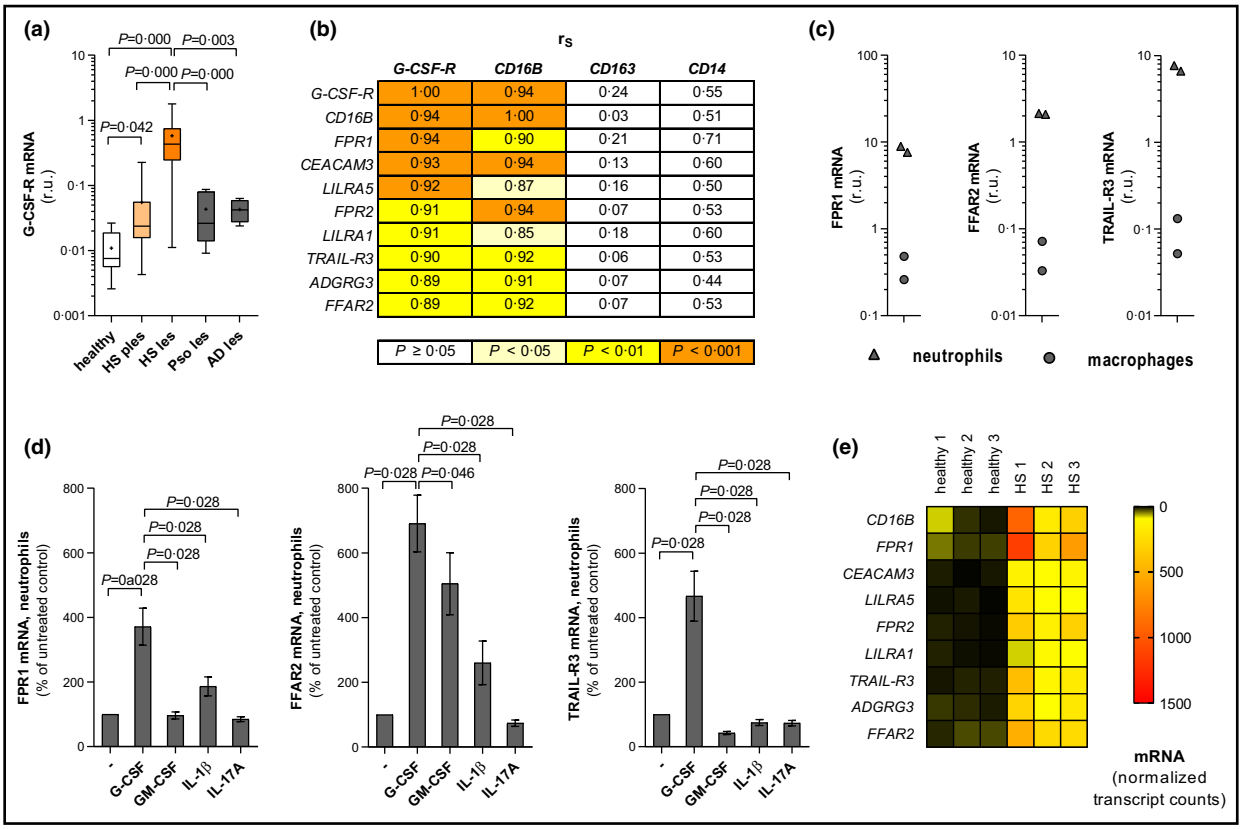


Figure 4 Granulocyte colony-stimulating factor (G-CSF) strengthens expression of several transmembrane receptors in neutrophils. (a) Biopsies obtained from skin of seven healthy donors, skin of 11 and 15 patients with hidradenitis suppurativa (HS) [perilesional (ples) and lesional (les), respectively], and lesional skin of 13 patients with psoriasis (Pso) and six with atopic dermatitis (AD), were subjected to quantitative polymerase chain reaction on reverse transcribed mRNA (RT-qPCR) analysis to assess G-CSF receptor (G-CSF-R) mRNA expression. Differences between groups were analysed using the Mann–Whitney U-test. Data indicated by box plots (the box extends from 25th to 75th percentiles of values; the line in the middle of the box and '+' representing median and mean of data, respectively; the maximum length of box whiskers corresponds to the most extreme values) and P-values are presented. (b) Eighteen lesional skin biopsies obtained from patients with HS were subjected to whole transcriptome analysis by RNA sequencing. Using normalized, $\log_{10}$-transformed transcripts per million values, correlations of G-CSF-R and low-affinity immunoglobulin gamma Fc region receptor III-B (*CD16B*) (neutrophil markers) as well as *CD163* and *CD14* (monocyte/macrophage markers) vs. all other transcripts were analysed using Spearman's rank correlation test. Correlation coefficient r_S and P-values adjusted using the Benjamini–Hochberg method are indicated for those nine transcripts showing the strongest correlations with the neutrophil marker G-CSF-R (P-values < 0·01), suggesting their involvement in the G-CSF pathway. (c) Neutrophils and macrophage-like cells isolated from blood of healthy donors were subjected to RT-qPCR analysis to assess the cellular mRNA expression of three molecules identified in (b): formyl peptide receptor 1 (*FPR1*), free fatty acid receptor 2 (*FFAR2*), and tumour necrosis factor receptor superfamily member 10C (*TRAIL-R3*). Individual data each obtained from two donors are presented. (d) Neutrophils isolated from blood of healthy donors were cultured in the presence or absence of the indicated cytokines for 24 h. Cellular mRNA expression of *FFR1*, *FFAR2*, and *TRAIL-R3* was then quantified using RT-qPCR. Differences between the G-CSF-treated group vs. the other treatment groups were analysed using the Wilcoxon matched-pairs signed-rank test. Mean ± SEM data obtained from six donors indicated as column heights and P-values are presented. (e) Biopsies obtained from axillary skin of three healthy donors and lesional (axillary) skin samples obtained from a second cohort of three patients with HS were subjected to whole transcriptome analysis using RNA sequencing. The individual Deseq2-normalized counts of the nine molecules identified in (b) are presented as heat maps. ADGRG3, adhesion G protein-coupled receptor G3; CEACAM3, CEA cell adhesion molecule 3; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; LILRA, leukocyte immunoglobulin-like receptor subfamily A member; r.u., relative units, i.e. expression relative to hypoxanthine-guanine phosphoribosyltransferase (a) or glyceraldehyde-3-phosphate dehydrogenase (c,d) expression.

(Figure 3a). To confirm the importance of IL-1β for lesional G-CSF expression, we cultured biopsies, taken from HS lesions in a pairwise manner, in the presence or absence of IL-1RA for 24 h. As demonstrated (Figure 3b), prevention of endogenous IL-1 action decreased levels of both G-CSF mRNA and its diffused protein to an average of 22% and 9%, respectively.

Blood granulocyte colony-stimulating factor levels correlate with hidradenitis suppurativa disease severity

Soluble mediators produced in inflamed HS skin may become available in the circulation and exert systemic roles.[36–38] G-CSF was shown to act on haematopoietic precursor cells,

stimulating neutrophil production and mobilization from the bone marrow.[39] Therefore, we quantified the G-CSF concentration in the blood plasma of patients with HS and of control individuals. An increase, which did not reach statistical significance, was detected in the mean and median levels in HS compared with healthy donors and patients with psoriasis (Figure S1a; see Supporting Information). Nevertheless, plasma G-CSF concentrations in HS correlated with HS disease severity expressed by the Sartorius score ($r_s = 0{\cdot}57$, $P = 0{\cdot}007$; Figure S1b). Detailed analyses showed specific association of plasma G-CSF with the number of skin regions containing nodules (Figure S1c). These data suggested involvement of G-CSF in HS in mainly local processes, while its systemic effect is greatly limited, especially to cases with rather severe disease.

Granulocyte colony-stimulating factor strengthens the expression of several transmembrane receptors in neutrophils

G-CSF acts on its target cells via the G-CSF receptor (G-CSF-R), which forms a homodimeric complex.[39] To elucidate the local role of G-CSF, we first analysed the expression of G-CSF-R in lesional and perilesional skin of patients with HS and compared it with levels in lesional skin of patients with psoriasis and with AD, and in healthy donor skin. As demonstrated in Figure 4a, G-CSF-R mRNA expression in HS lesional skin was clearly upregulated compared with other skin conditions. This might reflect the abundance of G-CSF target cells (neutrophils) in HS, and supported relevant local effects of G-CSF.

To identify neutrophil-expressed molecules potentially regulated by G-CSF in HS lesions, we conducted RNA sequencing of 18 samples taken from different HS lesion types and analysed the correlation between expression of *G-CSF-R* and levels of ~51 000 transcripts. In these analyses, we additionally included low affinity immunoglobulin gamma Fc region receptor III-B (*FCGR3B*/*CD16B*), a low-affinity membrane receptor for IgG, as a further marker for neutrophils, as well as *CD14* and *CD163*, two markers for monocytes/macrophages (Table S1; see Supporting Information). We detected a highly significant (P-value adjusted by the Benjamini–Hochberg method < 0·01) correlation of G-CSF-R levels with levels of *CD16B* and eight further transcripts (Figure 4b). All of them encode membrane receptors: formyl peptide receptor 1 (*FPR1*) and *FPR2*, free fatty acid receptor 2 (*FFAR2*), CEA cell adhesion molecule 3 (*CEACAM3*), *ADGRG3* (adhesion G protein-coupled receptor G3), leukocyte immunoglobulin-like receptor subfamily A member 1 (*LILRA1*) and *LILRA5*, and TNF receptor superfamily member 10C (*TNFRSF10C*/*TRAIL-R3*). The specificity of these molecules for neutrophils was supported by the fact that their levels also correlated with *CD16B* but not with monocyte/macrophage markers (Figure 4b). Quantifying the expression of some of these receptors by RT-qPCR, we confirmed much higher expression levels in neutrophils compared with monocytes/macrophages (Figure 4c). Importantly, when isolated neutrophils were stimulated with different cytokines, G-CSF turned out as the most potent inducer of the receptors, emphasizing their involvement in the G-CSF pathway (Figure 4d). These molecules were also strongly upregulated in HS lesional skin, as demonstrated in samples from an independent patient cohort (Figure 4e).

Identification of further elements of the granulocyte colony-stimulating factor pathway in hidradenitis suppurativa lesions

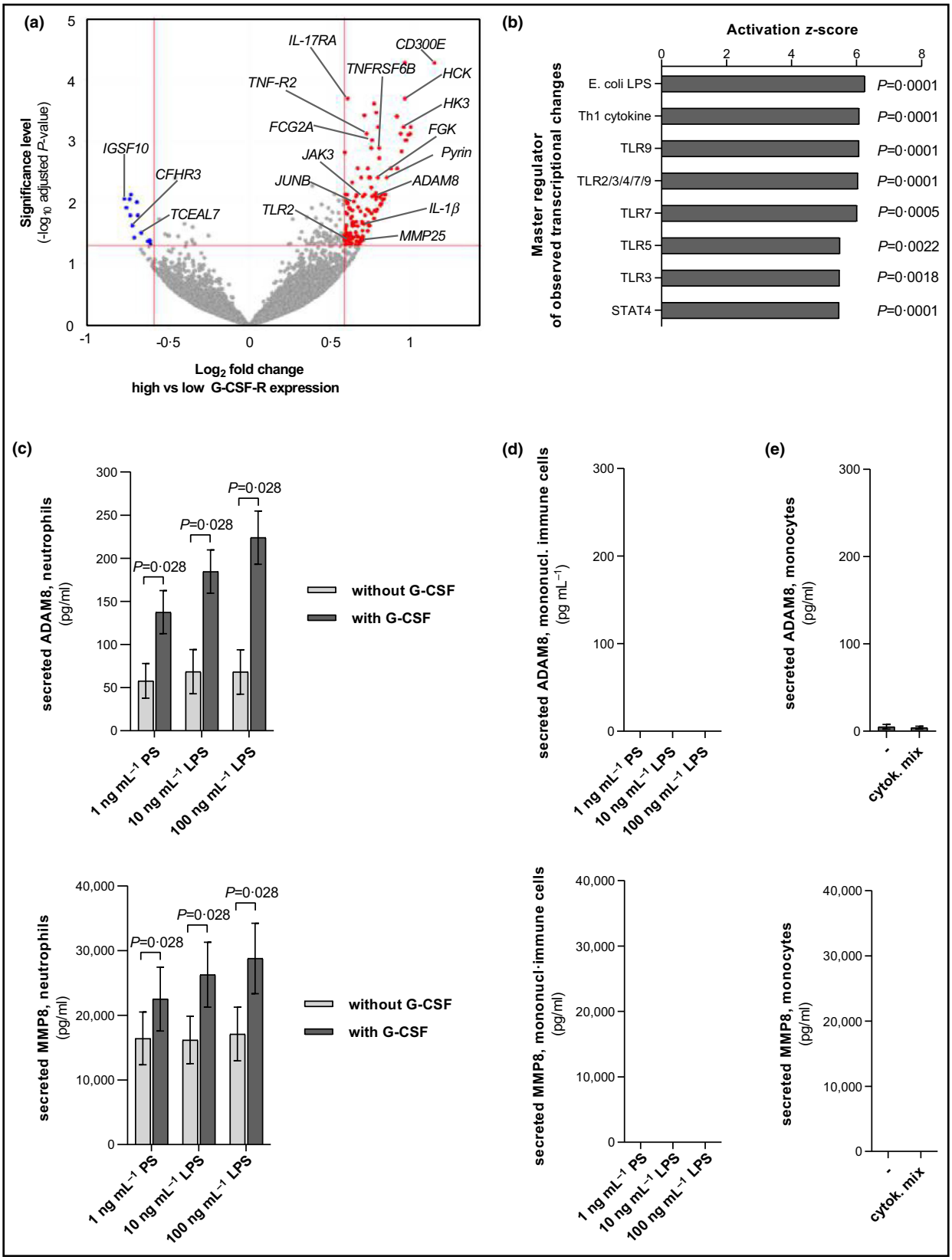
To identify further potential elements of the G-CSF pathway in HS skin, the HS lesional samples used in Figure 4b were divided into two groups based on G-CSF-R expression: one with the highest and one with the lowest levels. Evaluation of RNA sequencing data identified 137 protein-coding genes that were differentially expressed (124 upregulated, 13 downregulated) in the high vs. the low-G-CSF-R group [Figure 5a; Table S2 (see Supporting Information)]. All eight receptors identified by the correlation approach (Figure 4b; Table S1) were also found by this analysis to be upregulated (Table S2). Molecules additionally identified included further receptors, kinases, proteases, and cytokines, such as: pyrin (*MEFV*), IL-1β (*IL1B*), IL-17 receptor A (*IL17RA*), cytokine receptor common subunit beta (*CSF2RB*), TNF receptor 2 (*TNFRSF1B*/*TNFR2*), TNF receptor superfamily member 6B (*TNFRSF6B*), janus kinase 3 (*JAK3*), and tyrosine-protein kinase HCK (*HCK*). Moreover, this analysis revealed the metalloproteinases *MMP25* (matrix metalloproteinase 25) and disintegrin and metalloproteinase domain-containing protein 8 (*ADAM8*), which may represent most downstream elements of the G-CSF pathway.

To get hints to the master regulators of G-CSF pathway elements, we conducted ingenuity causal regulator analysis of the 137 differentially expressed transcripts (Table S2/Figure 5a). This analysis revealed microbial components and their innate receptors [toll-like receptors (TLRs)] as well as T helper 1-related stimuli (Figure 5b).

According to this pathway construction, production of ADAM8 (Figure 5c), MMP8 (Figure 5c), and MMP9 (data not shown) by neutrophils was elevated by G-CSF specifically after TLR4 stimulation with bacterial components [lipopolysaccharides (LPS)]. Interestingly, these metalloproteinases were not induced in monocytic immune cells stimulated with LPS (Figure 5d) or a mix of proinflammatory cytokines (Figure 5e). Taken together, these data suggest that G-CSF enhances the response of neutrophils towards bacterial components/metabolites, which carries the risk of skin destruction in HS.

Discussion

The features of diseased skin in HS (inflamed nodules, abscesses, purulent discharge) indicate a neutrophil-driven inflammation.[1] Accordingly, HS lesions harbour numerous neutrophils. These cells can perform diverse functions: phagocytosis, production of reactive oxygen species, hydrolytic enzymes and antimicrobial proteins (released into phagosomes or the extracellular space), formation of neutrophil



extracellular traps, and secretion of cytokines and lipid mediators. These mechanisms allow neutrophils to crucially participate in the elimination of tissue-invading microbial pathogens.[40,41] However, in chronic settings, neutrophils might contribute to persistent inflammation and tissue damage.[40,41]

As G-CSF is the major regulator of neutrophils,[42,43] this study investigated the activity and elements of G-CSF pathway in patients with HS. We demonstrated for the first time abundance of G-CSF in diseased HS skin at both the mRNA and protein levels, most pronounced in inflamed nodules and abscesses. The extent of G-CSF upregulation appeared to be HS specific as it was undoubtedly greater than the upregulation in skin of patients suffering from other chronic inflammatory dermatoses. Our study postulates the existence of the G-CSF pathway in HS, and demonstrates numerous potential elements of this pathway. While several of these elements can be placed up- or downstream of G-CSF in the pathway, further studies are needed to elucidate the position of others.

The elements upstream of G-CSF might include IL-1β, IL-17A, TNF-α and IL-36 as well as transmembrane receptors for these cytokines. In fact, we observed that IL-1β and IL-17A induced G-CSF production by fibroblasts and keratinocytes, respectively. TNF-α and IL-36 strengthened this production. Furthermore, evaluation of skin RNA–sequencing data revealed pathway involvement of the following molecules that play a role in the production/action of these inducers: pyrin (an intracellular inflammasome component involved post-transcriptional activation of IL-1β; encoded by *MEFV*), IL-17RA (a component of cellular receptor complexes for IL-17 family members), and TNF-R2 (one of the cellular TNF-α receptors; encoded by *TNFRSF1B*/*TNFR2*).[44–47]

G-CSF acts via the transmembrane receptor complex formed by two G-CSF-R subunits, mainly expressed on neutrophils and its precursor cells.[39] Pathway elements downstream of G-CSF/G-CSF-R revealed by our analyses include transmembrane receptors, such as FPR1, FPR2, FFAR2, TNFRSF10C/TRAIL-R3, TNFRSF6B, CSF2R, CD16B and CEACAM3. Most of them may be responsible for the increased activation and/or extended lifespan of the neutrophils in HS lesions. For example, FPR1 and FPR2 are G protein-coupled receptors, which recognize products of bacteria and formylated peptides derived from the mitochondria of disrupted host cells.[48,49] Their ligands might be abundant in HS lesions.[1] FFAR2 is activated by short-chain fatty acids such as butyrate, acetate or propionate.[50] The decoy receptor TNFRSF10C/TRAIL-R3 protects cells from TRAIL-induced apoptosis,[51,52] whereas TNFRSF6B, another decoy receptor, prevents the effect of three apoptosis inducers including TNFSF6 (TNF ligand superfamily member 6/Fas ligand).[53] The Fc-receptor CD16B delays neutrophil apoptosis when becoming cross-linked.[54] CSF2RB is the so-called receptor common β chain, which associates with cytokine-specific receptor chains to mediate the effects of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3 and IL-5.[55] GM-CSF in turn also delays apoptosis[56] and elevates production of proinflammatory cytokines by neutrophils.[56] Importantly, the receptors for formylated peptides and short-chain fatty acids were found to be highly expressed by neutrophils in the presence of G-CSF in our study and, to our knowledge, this is the first report to show that. Another of these transmembrane receptors, CEACAM3, is known for its prominent role in the opsonin-independent detection and elimination of individual Gram-negative, human-restricted bacteria.[57]

Besides the transmembrane receptors, several kinases and transcription factors [e.g. JAK3, HCK, hexokinase 3 (HK3) and transcription elongation factor A protein-like 7 (TCEAL7)] might belong to the G-CSF pathway in HS lesions. However, it should be mentioned that their exact position in the pathway needs to be

Figure 5 Identification of further elements of the granulocyte colony-stimulating factor (G-CSF) pathway in hidradenitis suppurativa (HS) lesions. (a) The 18 lesional HS skin samples investigated by whole transcriptome analysis described in Figure 4b were divided into two groups based on their G-CSF receptor (*G-CSF-R*) mRNA expression, so that nine with the highest *G-CSF-R* levels were compared against nine samples with the lowest levels. The volcano plot shows differentially expressed genes between both groups, with significance ($-\log_{10}$ of P-values adjusted by the Benjamini–Hochberg method) plotted against the $\log_2$ of fold-change in expression. Grey points indicate transcripts with no significant difference in expression between both groups. Red and blue points ($n = 137$) indicate transcripts with significantly increased and decreased expression, respectively (adjusted $P < 0{\cdot}05$ and absolute value of fold change $> 1{\cdot}5$). Names of selected molecules are indicated. (b) The significantly regulated transcripts shown in (a) were subjected to ingenuity pathway and regulator analysis. The top eight master regulators of the activated pathway, selected based on calculated activation z-scores, are shown. Network bias-corrected P-values are indicated. (c) Neutrophils isolated from the blood of healthy donors were cultured in the presence and absence of G-CSF for 24 h. After 20 h, lipopolysaccharide (LPS) was added at the indicated concentrations. The concentrations of disintegrin and metalloproteinase domain-containing protein 8 (ADAM8) and matrix metalloproteinase 8 (MMP8) in culture supernatant were analysed using enzyme-linked immunosorbent assay (ELISA). Differences between G-CSF-treated and not treated groups were analysed using Wilcoxon matched-pairs signed-rank test. Mean ± SEM data from six experiments indicated as column heights and P-values are presented. (d) Mononuclear (mononucl.) immune cells isolated from the blood of healthy donors were cultured for 24 h. After 20 h, LPS was added at the indicated concentrations. Concentrations of ADAM8 and MMP8 in culture supernatants were analysed using ELISA. Data obtained in one experiment are shown as column heights. (e) Monocytes isolated from the blood of healthy donors were cultured with or without a cytokine (cytok.) mix consisting of tumour necrosis factor (TNF)-α, interferon-γ, and granulocyte-macrophage colony-stimulating factor for 72h. Concentrations of ADAM8 and MMP8 in culture supernatants were analysed using ELISA. Mean ± SEM data from three experiments are presented as column heights. IL, interleukin; HCK, tyrosine-protein kinase HCK; HK3, hexokinase; JAK, janus kinase; TCEAL, transcription elongation factor A protein-like; Th, T helper; TLR, toll-like receptor; TNFRSF6B, TNF receptor superfamily member 6B; STAT, signal transducer and activator of transcription.

clarified. HCK (a Src family tyrosine kinase) acts downstream of receptors that bind immunoglobulin Fc domains, and plays a role in extravasation and phagocytosis of neutrophils.[58,59] HK3 catalyses the first rate-limiting step of glycolysis, namely, phosphorylation of glucose.[60] As the resulting glucose 6-phosphate cannot leave the cell and is used for energy metabolism, elevated HK3 levels found in HS lesions might ensure the energy supply of the permanently activated neutrophils. JAK3 is involved in signal transduction of cytokine receptor complexes that consist of the common γ-chain, and is a member of the type I cytokine receptor family including IL-2R and IL-21R. TCEAL7, one of the few downregulated transcripts linked to the G-CSF pathway in HS lesions, is a negative regulator of NF-κB signalling.[61]

The most downstream elements of the G-CSF pathway may include proteases (e.g. ADAM8, MMP8, MMP9, MMP25). ADAM8 is present both on the cell surface and in intracellular granules of human neutrophils.[62] ADAM8 cleaves extracellular matrix components and bioactive molecules,[63,64] and promotes neutrophil chemotactic response and transmigration through endothelial and epithelial layers.[65] MMP8 mainly degrades type I collagen that forms large fibres in the dermis. Degradation of this extracellular matrix component might lead to development of the skin cavities (abscesses and draining sinus tracts) seen in HS skin.[66,67] MMP9 mainly cleaves type IV and V collagens and gelatin, and also contributes to extracellular matrix degradation.[67] The membrane-bound MMP25 inactivates α1-proteinase inhibitor, the major tissue protector against neutrophil elastase, cathepsin G and proteinase 3, and, thereby, also favours neutrophil-mediated proteolytic tissue damage.[68] MMP25 additionally activates MMPs including MMP2.[69]

Apart from its local action, G-CSF can exert systemic effects: it strengthens granulopoiesis and mobilizes haematopoietic stem cells, immature neutrophils, and plasma cells/blasts from bone marrow.[39,70] Interestingly, HS lesions harbour a plasma cell/blast signature.[71] However, based on the observation that only a trend of an increase in blood G-CSF levels was detected in patients with HS, we assume that the systemic role of G-CSF is only obvious in individual patients, especially those with extensive skin alterations. In fact, strengthened granulocytosis was predominantly detected in patients with severe HS.[42,72] By analogy, G-CSF probably does not contribute significantly to the presence of plasma cells/blasts in HS skin.

Taken together, our work describes a pathogenetic pathway in HS, whose central element is G-CSF, a key regulator of neutrophils. The pathway may start by TLR activation by components of bacteria or damaged cells present in HS skin. This results in production of the direct inducers of G-CSF, in particular IL-1β and IL-17, as well as TNF-α and IL-36. Downstream of G-CSF appear receptors for bacterial metabolites and peptides derived from disrupted host cells, decoy receptors for apoptosis inducers, kinases and signal transduction elements, as well as MMPs. Importantly, G-CSF is one of the few cytokines whose upregulation and the consequence thereof appears to be rather specific to HS lesions. The G-CSF pathway might contribute to the persistence of inflammatory nodules and abscesses, to purulent secretion, and to tissue destruction. The future will show if blocking G-CSF's action is an effective approach for HS treatment.

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Supporting Information

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

Appendix S1 Supporting materials and methods: Study populations and samples; isolation and culture of skin tissue cells and immune cells; RT-qPCR and RNA sequencing and evaluation; statistical analyses and visualization.

Appendix S2 Supporting Table legends.

Figure S1 Blood G-CSF levels correlate with HS disease severity.

Table S1 List of transcripts correlating with G-CSF receptor expression in lesional HS skin.

Table S2 List of protein-coding genes differentially expressed in HS lesional skin samples with high versus low G-CSF receptor expression.

Powerpoint S1 Journal Club Slide Set.