Title: Interleukin-32 is highly expressed in lesions of hidradenitis suppurativa

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BULLETED STATEMENTS

What's known? What's already known about this topic?
The immune system is dysregulated in lesions of hidradenitis suppurativa (HS). Interleukin-32 (IL-32) is a pro-inflammatory cytokine upregulated in various inflammatory diseases.

What's new? What does this study add?
The pro-inflammatory cytokine IL-32 is upregulated in lesional HS skin and is also elevated systemically. IL-32 levels positively correlate with IFNγ and IL-17 levels in lesional HS skin. No upregulation of IL-32 is detected in psoriasis and atopic dermatitis, two other inflammatory skin diseases.

What is the translational message?
The study suggests that IL-32 is involved in the pathogenesis of HS and thus is a potential therapeutic target.

SUMMARY

Background
Hidradenitis suppurativa (HS) is a chronic inflammatory skin disease. Its immunopathogenic mechanisms are still poorly understood. Previous studies could demonstrate that the pro-inflammatory cytokine Interleukin-32 (IL-32) is implicated in the pathogenesis of other inflammatory diseases.

Objectives
The aim of our study was to investigate the tissue expression and systemic levels of IL-32 as well as its cellular sources in HS patients in comparison to healthy donors and to patients suffering from two other inflammatory skin diseases: psoriasis (PS) and atopic dermatitis (AD).

Methods
Tissue samples were obtained from healthy skin and lesional HS, PS and AD skin to analyse the expression of IL-32 by immunohistochemistry and semi-quantitative real-time PCR. The cellular source of the cytokine was determined by double immunofluorescence staining. Serum of the four donors groups was used to measure systemic levels of IL-32 by enzyme-linked immunosorbent assay (ELISA).

Results
IL-32 was upregulated in HS patients in both lesional skin and serum when compared to healthy donors or AD and PS patients. In HS, IL-32 was found to be expressed by NK cells, T cells, macrophages and dendritic cells in highly infiltrated areas of the dermis. High IL-32 mRNA levels in lesional HS skin coincided with high amounts of T cells and macrophages present. Additionally, IL-32 mRNA levels in lesional HS skin correlate positively with IFNγ and IL-17 and negatively with IL-13.

Conclusions
Our findings suggest that IL-32 is overexpressed in HS. Targeting IL-32 may therefore represent a new therapeutic option for the treatment of this recalcitrant disease.

INTRODUCTION

Hidradenitis suppurativa (HS), also termed acne inversa, is an inflammatory disease of the skin. In early stages, HS manifests through painful subcutaneous nodules in the axillary, inguinal and anogenital regions of the body. Becoming chronic or recurrent, the lesions progress into deep dermal abscesses with draining sinuses and fistula, accompanied by malodorous suppuration.[1] HS is painful and lowers the quality of life of affected patients to a higher degree than other inflammatory skin diseases.[2] Therapy of this recalcitrant disease is highly challenging. One of the main reasons for the lack of efficient therapies is the poor understanding of HS pathogenesis. Nowadays, the trigger for HS is believed to be follicular keratinisation, inducing occlusion, dilation and rupture of the pilosebaceous unit, followed by an infiltration of inflammatory cells into the dermis with a subsequent dysregulation of the immune system.[3,4]

IL-32 is a pro-inflammatory cytokine, which is expressed by several immune cells.[5] It comprises eight exons and seven alternatively spliced isoforms have been proposed.[6-8] The four main isoforms are IL-32α, IL-32β, IL-32γ and IL-32δ, with IL-32α as the most abundant isoform and IL-32γ as the most biologically active one.[6,9] Several pro-inflammatory mediators initiate IL-32 production, such as IFNγ, IL-1β, IL-12 and IL-18.[6] In turn, IL-32 increases the production of the cytokines TNFα, IL-1β and IL-6,[6,9] but also of the chemokines IL-8, CCL2, CCL4 and CCL5.[6,10,11] Moreover, IL-32 was shown to upregulate the adhesion molecules ICAM-1 and sVCAM-1[10,12] and the antimicrobial peptide (AMP) cathelicidin.[13] In addition to its pro-inflammatory features, IL-32 negatively impacts on cancer cell growth[14] and has a protective role in viral and bacterial infections.[15-17]

In this study, we investigated the expression of IL-32 in HS and examined how HS inflammation differs from other inflammatory skin diseases. Numerous mediators, which are involved in the induction and function of IL-32 are known to be upregulated in HS, namely IFNγ, IL-1β, IL-6, IL-12, IL-18, TNFα, cathelicidin and CCL5.[18-23] Furthermore, IL-32 is dysregulated in diseases, which are associated with HS, such as inflammatory bowel disease and ankylosing spondylitis,[24-27] making IL-32 a possible linking factor in the pathogenesis of these diseases.

To elucidate the expression of IL-32 in HS lesions, we performed immunohistochemistry and semi-quantitative real time PCR and determined the cellular source of IL-32 by double immunofluorescence staining. Additionally, IL-32 mRNA levels in HS skin were correlated with lesional inflammatory status and the principal Th1, Th2 and Th17 cytokines (IFNγ, IL-13 and IL-17). To complete our analysis, we investigated systemic IL-32 levels. In order to show the relevance of IL-32 in HS pathogenesis, we compared expression of IL-32 in HS patients not only to healthy donors, but also to patients of two other inflammatory skin diseases, psoriasis (PS) and atopic dermatitis (AD).

MATERIAL AND METHODS

Samples from patients

This study followed the Declaration of Helsinki protocols and was approved by the Medical Ethics Committee of the Canton of Berne, Switzerland. Before enrolment, all patients gave written informed consent. Study subjects were not previously treated with biologics or systemic immunomodulatory drugs and systemic antibiotics were stopped at least 8 weeks before skin biopsy or blood donation. Skin biopsies were obtained from 20 patients with HS (12 women and 8 men, 50% obese and 92% smokers, median age 37.5 years, range 21-51 years). These patients suffered from HS with a mean Hurley stage of 2.3±0.66. Furthermore, skin biopsies from 8 PS patients (2 women and 6 men, median age 47.5 years, range 30-61 years) and 8 AD patients (5 women and 3 men, median age 20.5 years, range 18-47 years) were collected. Biopsies were targeted at the central area of the lesions. Healthy control skin was obtained from 10 donors (6 women and 4 men, median age 41.5 years, range 23-66 years). Tissue samples were immediately embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, USA), snap frozen and stored at -70°C. Serum was obtained from 17 HS patients (8 women and 9 men, 54% obese, 86% smokers, median age 37 years, range 21-55 years), 11 PS patients (4 women and 7 men, median age 54 years, range 29-61 years), 9 AD patients (5 women and 4 men, median age 32 years, range 20-63 years) and 5 healthy donors (2 women and 3 men, median age 24 years, range 23-40 years). Serum was stored at -20°C.

RNA isolation and complementary DNA synthesis

As previously described,[18] OCT-embedded tissue specimens were cut into 10µm sections. Then, RNA was extracted by means of the RNeasy lipid tissue mini kit (Qiagen, Valencia, CA, USA). Contaminating DNA was removed with a RNase-free DNase step (Qiagen). Complementary DNA was synthesised using the BD Reverse Transcriptase Kit (BD Bioscience, San Diego, CA, USA).

Semi-quantitative real-time PCR analysis

PCR primers and probes were purchased (Applied Biosystems, Foster City, CA, USA) as pre-made Taqman© gene expression assays. The following primer pairs, spanning exon-exon borders, were used: IL-32 (Hs00992441_m1), IL-32α (Hs04353657_gH), IL-32β (Hs04353658_gH), IL-32γ (Hs04353656_g1), IL-32δ (Hs04353659_gH), IFNγ (Hs00174143_m1), IL-17 (Hs00174383_m1), IL-13 (Hs00174379_m1); three reference genes: β_2-microglobulin (B2M) (Hs99999907_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (Hs99999905_m1) and hypoxanthine phosphoribosyltransferase-1 (HPRT-1), (Hs99999909_m1). The semi-quantitative real-time PCR was performed with ABI-Prism 7300 Sequence Detector System (Applied Biosystems) as previously described.[18] The results are expressed as fold difference. To determine these differences, relative units were calculated by the $2^{-\Delta\Delta CT}$ method. The threshold cycle (CT) for the reference genes was determined by using the geometric mean of the CT of the three reference genes, see Ref.[28] In samples where no CT-value could be determined, CT-value was set to 40, as previously described.[29]

Immunohistochemical staining

Immunostaining was performed as previously described.[18] In brief, cryostat-cut tissue sections were air dried, fixed in 4% ice-cold acetone (10 minutes) and rehydrated in 0.1% saponin containing TRIS

buffered saline. After blocking (Roti-ImmunoBlock, Roth GmbH, Karlsruhe, Germany) for 15 minutes, the sections were incubated with the primary antibody for 1 hour at room temperature, followed by a 1 hour incubation with a biotinylated secondary antibody (see Table 1) and thereafter with the streptavidin-biotin complex/alkaline phosphatase method (BD Biosciences). Finally, sections were developed with Fuchsin Substrate-Chromogen (Dako, Santa Clara, CA, USA) and counterstained with hematoxylin for 3 minutes. As a negative control, the same tissue was stained with the secondary antibody only. To score the inflammatory status of HS biopsies, slides were stained for CD3, CD68 and CD66b (Table 1). Stained cell density as well as cell distribution across the biopsy was assessed and included in the allocation of the score as previously described.[30] Briefly, CD3, CD68 and CD66b were assessed using a semi-quantitative scale: 0 = absent, 1 = weak/low expression with little distribution, 2 = moderate expression with intermediate distribution, 3 = strong expression with wide distribution. The inflammatory score was calculated by taking the mean of the scores for CD3, CD68 and CD66b.

Immunofluorescence double staining

Co-localization of IL-32 with NK, T cell, macrophage and DC markers (CD56, CD3, CD68, CD11c) (see Table 1) was assessed. Double immunofluorescence was performed as previously described.[18] Briefly, after pre-treatment (see above), sections were exposed to the primary IL-32 antibody for 1 hour, followed by an anti-rabbit AlexaFluor 594 conjugate (Invitrogen, Carlsbad, CA, USA). Sections were then incubated for 1 hour with one of the following antibodies (Table 1): (1) anti-CD56, (2) anti-CD3, (3) anti-CD68, (4) anti-CD11c before staining with isotype-specific Alexa Fluor 488 conjugated goat anti-mouse antibodies (Invitrogen). DAPI fluoromount-G (SouthernBiotech, Birmingham, AL, USA) was used to stain cell nuclei.

ELISA

To detect IL-32 in the serum, the IL-32 DuoSet ELISA kit (RnD Systems, Minneapolis, MN, USA) was utilised as per the manufacturer's instructions. The results are expressed in ng/mL.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (version 5.01 for Windows, San Diego, CA, USA). Semi-quantitative real time PCR and ELISA was evaluated by means of the non-parametric Mann-Whitney test. The Spearman's rank correlation coefficient (r_S) was used to statistically analyse correlations. A p-value less than 0.05 was considered statistically significant; $p<0.05$ = *, $p<0.01$ = **, $p<0.001$= ***.

RESULTS

IL-32 protein is expressed in lesional HS skin

Immunohistochemical analysis of IL-32 protein expression was performed on tissue sections of lesional HS biopsies. As control, lesional skin from AD and PS patients and healthy skin was analysed. Representative examples of IL-32 staining are shown in Figure 1a-d. Whereas lesional HS skin showed scattered IL-32$^+$ cells in highly inflamed regions of the deep dermis without any accumulation around the hair follicle, IL-32$^+$ cells were almost absent in lesional AD, PS and healthy skin.

Expression of IL-32 in lesional HS skin by T cells, macrophages, natural killer and dendritic cells

IL-32 has previously been shown to be expressed by T cells, macrophages, NK cells and dendritic cells (DCs).[5] We therefore performed a double immunofluorescence analysis to determine whether these cell types are producing IL-32 in lesional HS skin. We detected co-localisation of IL-32 with CD3, CD68, CD56 and CD11c (Fig. 2a-d). Hence our data suggests that IL-32 is produced by T cells, macrophages, natural killer cells and DCs in lesional HS skin.

IL-32 mRNA is upregulated in lesional HS skin

After determining that IL-32 protein expression was increased in lesional HS skin, we sought to investigate whether this is also true on the mRNA level and which IL-32 isoforms are contributing to this increase. Therefore, we used semi-quantitative RT-PCR to determine total IL-32 expression as well as the expression of the main isoforms IL-32α, IL-32β, IL-32γ and IL-32δ in lesional HS, PS, AD and healthy skin. Relative changes in gene expression between healthy and patient skin are shown in Figure 3a-e. In accordance with our IHC results, overall IL-32 expression was specifically elevated in HS skin. While IL-32α and IL-32β were significantly increased in HS compared to healthy skin, IL-32δ was only slightly elevated. In HS, IL-32γ was not found to be differently regulated than in healthy skin, but interestingly, a significant downregulation of IL-32γ was found in both lesional PS and AD skin compared to healthy skin.

Positive correlation of IL-32 mRNA expression with lesional inflammatory status, IFNγ and IL-17 in lesional HS skin

Next, we assessed the potential pathogenic role of IL-32 in HS by investigating both the correlation of IL-32 with lesional parameters of disease severity, as well as with the main cytokines produced by Th1, Th2 and Th17 cells (IFNγ, IL-13 and IL-17 respectively). Our data demonstrates that IL-32 mRNA expression levels in HS positively correlate with the lesional inflammatory status (mean number of T cells, macrophages and neutrophils, see methods) (Fig. 4a). In particular, IL-32 levels correlated with the amount of T cells (Fig. 4b) and macrophages (Fig. 4c). Interestingly, IFNγ and IL-17, the cytokines that characterise the Th1 and Th17 lineage, positively correlated with IL-32 levels (Fig. 4d,e), whereas the level of IL-13, which is a Th2 signature cytokine, decreased with increasing amounts of IL-32 in lesional HS skin (Fig. 4f).

Systemic IL-32 levels are elevated in HS patients

To investigate whether systemic levels of IL-32 are also elevated in HS patients, as observed in lesional HS skin samples, we determined IL-32 levels in serum samples from HS, AD and PS patients and healthy donors (see Fig. 5). Serum levels of IL-32 were only significantly elevated in patients with HS when compared to healthy donors.

DISCUSSION

Here we show that IL-32 is upregulated in skin lesions and in the serum of patients suffering from the recalcitrant skin disease HS. Levels of IL-32 mRNA in lesional HS skin positively correlate with the presence of T cells and macrophages, as well as with IFNγ and IL-17 levels, suggesting that IL-32 is induced as a secondary event in HS immunopathogenesis. In our hands, IL-32 is specifically upregulated in HS, but not in AD or PS. Even though there might be a trend towards elevated expression in AD, as proposed by Meyer et al.,[31] no significant differences in AD patients compared to healthy controls could be detected in this present study. Interestingly, we report no downregulation of the IL-32γ isoform in lesional HS skin compared to healthy skin, as it was the case for lesional PS and AD skin. As previously demonstrated, IL-32γ can be spliced into IL-32β as a measure to control its pro-inflammatory potential,[32] which suggests that this attenuation is reduced in HS compared to lesional PS or AD skin.

The functional relevance of IL-32 in HS lesions remains unclear and requires further investigation. We hypothesise that IL-32 with its strong pro-inflammatory potential is important in leading to and maintaining a state of chronic inflammation in lesional HS skin. Our arguments in favour of this hypothesis are that, first, previous studies showed that IL-32 drives Th1/Th17 polarisation both *in vitro* and *in vivo.*[33] Our data reveal a positive correlation between lesional IL-32 mRNA levels and IFNγ and IL-17 and a negative correlation between IL-32 and IL-13, indicating that this T cell polarising effect is also present in lesional HS skin. Additionally, IL-32 could be an important player in the appearance of AMP inducing IFNγ/IL-17 double positive cells in HS.[20,34] Second, IL-32 could upregulate other pro-inflammatory cytokines in lesional HS skin, similar to rheumatoid arthritis, where IL-32 exacerbates joint inflammation by upregulating IL-1β, IL-6, IL-8 and CCL20.[35] Third, IL-32 could potentially induce pro-inflammatory signalling pathways (NFκB, p38 MAPK).[6] Last, IL-32 could maintain inflammation in lesional HS skin by maturing and re-differentiating the local innate immune system, e.g. IL-32 is able to differentiate primary human monocytes into potent DCs[36] and it is able to re-differentiate DCs into actively phagocytosing macrophage-like cells.[37]

The most unexpected finding is that IL-32 is only upregulated in HS and not in AD or PS. Previous studies in PS show controversial results. While a large case study found no upregulation of IL-32 in PS,[38] another study found IL-32 elevated in lesional PS skin.[39] HS shares, in particular with PS, a lot of immunopathological pathways, such as the upregulation of the pro-inflammatory cytokines TNFα, IL-1β, IL-17, IL-12 and IL-23[18,22,40-44] and AMPs, such as cathelicidin, psoriasin and β-defensins 2 and 3.[23,45-50] We here identify IL-32 as a specific marker for HS, which may explain at least in part some distinct clinical features of this skin disease.

Epidemiological studies showed that HS is associated with obesity and a recent population-based cross-sectional study demonstrated that weight loss benefits HS patients.[51] This, taken together with the fact that IL-32 can be produced by adipocytes and that obese people have higher serum levels of IL-32[52] could explain why HS and obesity are so tightly related.

Treating HS by influencing the immune system is promising, but trials to block IL-1β in HS patients have yielded conflicting results.[53-55] As IL-32 can induce IL-1β, blocking IL-32 would reduce IL-1β induction and could therefore be a promising target to abrogate the pro-inflammatory cascade. This is supported by a study of Joosten et al.,[56] where it was reported that in mice injection of IL-32 alone is able to induce joint inflammation in an even more pronounced manner than IL-1β or TNFα.

Taken together, we report here that IL-32 is upregulated in HS patients, but not in PS and AD patients. We were able to correlate inflammatory status and Th1 and Th17 cytokines with IL-32 mRNA levels in lesional HS skin and were able to show that IL-32 is produced by T cells, macrophages,

NK cells and DCs in HS. Further investigation of the involvement of the IL-32 pathway will help to understand pathological mechanisms in HS and could pave the way for future treatment possibilities by blocking IL-32 in this recalcitrant skin disease.

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FIGURE LEGENDS

Figure 1

Interleukin-32 is expressed on the protein level in lesional hidradenitis suppurativa skin

Representative examples of frozen skin sections immunohistochemically stained for IL-32 in lesional skin of different inflammatory skin diseases (HS, PS, AD) and healthy skin. Sections were counterstained with hematoxylin; 200x magnification, scale bars indicate 50µm.

Figure 2

In lesional hidradenitis suppurativa skin Interleukin-32 is produced by NK cells, T cells, macrophages and dendritic cells

a-d) The first panel of each row in green shows the presence of NK cells (CD56), T cells (CD3), macrophages (CD68) and DCs (CD11c) in lesional HS skin. The second panel of each row in red shows the expression of IL-32. The overlay of the three colour immunofluorescence images (DAPI in blue) are presented in the third panel of each row; 400x magnification; scale bars indicate 25 µm.

Figure 3

Messenger RNA level of total Interleukin-32 and its four main isoforms are elevated in lesional hidradenitis suppurativa skin

a - e) Results of analyses of IL-32 mRNA expression in HS compared to PS and AD and healthy skin. Ct-values were normalised with housekeeping genes HPRT-1, GAPDH and B2M to healthy skin (=1); n(HS)=20, n(PS)=8, n(AD)=8, n(healthy skin)=10. Data are presented as mean with SEM and were statistically analysed with the Mann Whitney test with * indicating a p-value <0.05, ** indicating a p-value <0.01, *** indicating a p-value <0.001.

Figure 4

Messenger RNA levels of Interleukin-32 in lesional hidradenitis suppurativa skin positively correlate with inflammatory status, IFNγ and IL-17 levels

a-c) IL-32 mRNA expression positively correlates with lesional inflammatory status (r_S= 0.55) and in particular with the number of T cells (r_S= 0.58) and macrophages (r_S= 0.46) in lesional HS skin. d&e) IL-32 mRNA levels positively correlate with IFNγ (r_S=0.45) and IL-17 (r_S=0.47) levels. f) IL-32 mRNA levels correlate negatively with IL-13 (r_S=-0.53). Ct-values were normalised with housekeeping genes HPRT-1, GAPDH and B2M to healthy skin (=1); n(HS)=20. Significance of correlation was determined with the Spearman's rank correlation coefficient with* indicating a p-value <0.05 and ** indicating a p-value <0.01.

Figure 5

Interleukin-32 is upregulated in the serum of hidradenitis suppurativa patients

Sandwich ELISA was performed to detect systemic IL-32 levels in healthy donors, HS, PS and AD patients. Data are expressed in ng/mL. Results are presented as mean with SEM and were statistically analysed with the Mann Whitney test with * indicating a p-value <0.05. n(healthy)= 5, n(HS)=17, n(PS)=11, n(AD)= 9.

TABLES

Table 1

	Antigen	Isotype	Source
Primary Antibodies			
rabbit anti human	IL-32	polyclonal	NBP-76684, Novus (Littleton, CO, USA)
mouse anti human	CD3	IgG2a	317302, Biolegend (San Diego, CA, USA)
mouse anti human	CD11c	IgG2a	NCL-L-CD11c-563, Novocastra (Wetzlar, Germany)
mouse anti human	CD56	IgG1	M7074, Dako
mouse anti human	CD66b	IgM	305102, biolegend
mouse anti human	CD68	IgG1	M0718, Dako
Secondary Antibodies			
biotinylated swine anti rabbit	rabbit immunoblobulins	polyclonal	E0431, Dako
biotinylated rabbit anti mouse	mouse immunoglobulins	polyclonal	E0413, Dako
goat anti mouse AF488	IgG1	IgG	A21121, Invitrogen
goat anti mouse AF488	IgG2a	IgG	A21131, Invitrogen
goat anti mouse AF488	IgM	IgG	A21042, Invitrogen
goat anti rabbit AF594	IgG	IgG	A11072, Invitrogen