Title: Elevated levels of the antimicrobial peptide LL-37 in hidradenitis suppurativa are associated with a Th1/Th17 immune response

Running Head: LL-37 in hidradenitis suppurativa

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SUMMARY

Hidradenitis suppurativa (HS) is an inflammatory skin disease with poorly understood immunopathogenic mechanisms. LL-37 is an antimicrobial peptide, which is transcribed from the CAMP (cathelicidin antimicrobial peptide) gene. Previous reports showed upregulated levels of CAMP and LL-37 in HS lesions and therefore the aim of this study was to compare levels of LL-37 in HS to other inflammatory skin diseases and to establish immunomodulatory functions of LL-37 in HS. We confirm an upregulation of the LL-37 peptide in lesional HS skin with comparable levels as in psoriasis patients and are able to positively correlate the presence of LL-37 in HS with the presence of T cells, macrophages, neutrophils, IFNγ, IL-17, IL-23, TNFα, IL-32 and IL-1β. Mechanistically, LL-37 boosts the proliferation of unspecifically activated $CD4^+$ T cells via an increased calcium signalling independent of antigen presenting cells. Targeting LL-37 may therefore represent a new therapeutic option for the treatment of this recalcitrant disease, but it has to be kept in mind that LL-37 also has an antimicrobial function.

INTRODUCTION

Hidradenitis suppurativa (HS), also termed acne inversa, is a chronic inflammatory disease of the apocrine gland-bearing skin. HS lowers the quality of life of affected patients to a higher degree than other inflammatory skin diseases and is characterised by painful subcutaneous nodules in the axillary, inguinal and anogenital regions of the body.[1, 2] These nodules can progress into deep dermal abscesses with sinuses and fistula, accompanied by malodorous suppuration.[2] Efficient therapy of this recalcitrant disease is highly challenging due to the poor understanding of the involved pathogenetic mechanisms. Nowadays, the trigger for HS is thought to be hyperkeratinisation of the hair follicle, which leads to distention and rupture of the pilosebaceous unit, leading to an infiltration of inflammatory cells with a subsequent dysregulation of the immune system.[3, 4]

LL-37 is the only mature human antimicrobial peptide (AMP) of the cathelicidin family and is transcribed and processed from the CAMP gene. It has an alpha-helical and amphipathic structure, which enables LL-37 to kill bacteria.[5, 6] LL-37 is produced by activated tissue resident cells such as keratinocytes and epithelial cells (ECs), but also by innate and adaptive immune cells, such as neutrophils, macrophages, T cells and B cells.[7-13] Next to its antimicrobial function, LL-37 is also a potent immunoregulator. LL-37 is a chemoattractant for $CD4^+$ T cells[10] and, *in vitro* LL-37-stimulated dendritic cells upregulate co-stimulatory molecules and increase the production of TNFα, IL-6 and IL-12, which leads to the induction of a Th1/Th17 phenotype in T cells.[14-16] Moreover, in human peripheral blood mononuclear cells (PBMCs), LL-37 enhances certain pro-inflammatory responses in synergy with IL-1β, such as an increased production of CCL2, CCL7, CXCL-8 and IL-6.[17] Furthermore, LL-37 has an impact on cell proliferation in angiogenesis and wound healing, and regulates hair growth.[18-20] LL-37 also promotes the establishment of several chronic inflammatory diseases such as obesity, insulin resistance and atherosclerosis.[21, 22] In PS, LL-37 is upregulated and some patients even possess autoreactive skin-homing T cells which can recognize LL-37. [9, 23] In contrast to psoriasis (PS) patients, atopic dermatitis (AD) patients have decreased serum levels of LL-37.[24] These low levels could be attributed to high IL-10 levels, which suppress the induction of LL-37.[25] In HS, elevated CAMP and LL-37 levels have been reported,[26, 27] which were found to be produced by ECs around inflamed hair follicles in the outer root sheath and in the adjacent apocrine sweat glands.[27]

Due to these results in the literature, we sought to further delineate the role of LL-37 in HS by comparing its levels to other inflammatory skin diseases and by deepening the understanding of the immunomodulatory function of LL-37 in HS. To this end, we investigated the expression of LL-37 and CAMP in HS, PS, AD and healthy skin by immunohistochemistry and semi-quantitative real time PCR and compared whether LL-37 peptide and CAMP mRNA levels correlate in lesional HS skin. In addition, we examined what

kind of cells (T cells, macrophages, and neutrophils) and cytokines (IFNγ, IL-17, IL-23, IL-32, IL-1β, TNFα and IL-10), which are known to be elevated in HS[28-35] correlate with the presence of LL-37. To complete our analysis, we investigated the impact of LL-37 on the proliferation of lectin stimulated $CD4^+$ and $CD8^+$ T cells isolated from the blood of healthy donors and HS patients and delineated by calcium assay, how LL-37 impacts proliferation of T cells directly.

MATERIAL AND METHODS

Samples from patients

The design of 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 study subjects signed a written informed consent. Skin biopsies were obtained from 24 HS patients (median age 36.5 years, range 21-51 years, mean Hurley stage 2.25±0.68). Furthermore, skin biopsies from 5 PS patients (median age 54 years, range 33-59 years) and 5 AD patients (median age 25 years, range 18-48 years) were collected. Biopsies were targeted in the central lesional area. Healthy control skin was obtained from intertriginous skin areas of 9 donors (median age 41 years, range 23-66 years). Skin samples were immediately embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, USA), snap frozen with liquid nitrogen and stored at -70°C. PBMCs were isolated by Ficoll density gradient centrifugation from 14 HS patients (median age 33 years, range 18-64 years) and 15 healthy controls (median age 33.5 years, range 23-74 years). After isolation, PBMCs were immediately frozen and stored at -80°C in fetal calf serum (FCS) (Biochrom, Berlin, Germany) + 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, USA).

Immunohistochemical staining

Immunostaining was performed as previously described.[28] In brief, air dried cryostat-cut tissue sections were fixed in 4% cold acetone (10 minutes) and rehydrated in 0.1% saponin containing TRIS buffered saline. After 15 minutes blocking (Roti-ImmunoBlock, Roth GmbH, Karlsruhe, Germany), the sections were incubated at room temperature with the primary antibody (rabbit-anti-human LL-37 [Abcam, Cambridge, UK], mouse-anti-human CD3 [Biolegend, San Diego, USA], mouse-anti-human CD66b [Biolegend] or mouse-anti-human CD68 [Dako, Santa Clara, USA]) for one hour. This was followed by a one hour incubation with a biotinylated swine-anti-rabbit or rabbit-anti-mouse antibody (Dako) and thereafter with the streptavidin-biotin complex/alkaline phosphatase method (BD Biosciences, San Diego, USA; Dako). Finally, a three minutes counterstaining with hematoxylin was performed. As a negative control, the tissue was only stained with the secondary antibody. To determine the score for the presence of LL-37, CD3, CD66b and CD68 sections were analysed for stained cell density and cell distribution across the biopsy. The allocation of a semi-quantitative score was attributed as previously described,[36] using the following scale (in steps of 0.5): 0 = absent, 1 = weak/low expression with little distribution, 2 = moderate expression with intermediate distribution, 3 = strong expression with wide distribution.

RNA isolation, complementary DNA synthesis and semi-quantitative real-time PCR analysis

As previously described,[28] OCT-embedded biopsies were cut into 10µm sections, before the RNA was extracted with the help of the RNeasy lipid tissue mini kit (Qiagen, Valencia, USA). A RNase-free DNase step removed contaminating DNA (Qiagen). Complementary DNA was then synthesised utilising the BD Reverse Transcriptase Kit (BD Biosciences). For semi-quantitative real-time PCR pre-made Taqman© gene expression assays were used with exon-exon spanning primers and probes (Applied Biosystems, Foster City, USA); target genes: CAMP (Hs00189038_m1), IFNγ (Hs00174143_m1), IL-17 (Hs00174383_m1), IL-23 (Hs00372324_m1), IL-1β (Hs01555410_m1), TNFα (Hs00174128_m1), IL-10 (Hs00174086_m1) and IL-32 (Hs00992441_m1); reference genes: β_2-microglobulin (B2M) (Hs99999907_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (Hs99999905_m1) and hypoxanthine phosphoribosyltransferase-1 (HPRT-1), (Hs99999909_m1). The PCR was performed on an ABI-Prism 7300 Sequence Detector System (Applied Biosystems) as described in Ref.[28] The final results are expressed as fold differences. These differences were calculated with 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.[37] In samples where no CT-value was detected, CT-value was set to 40, as previously described.[38]

Proliferation assay

PBMCs from healthy donors and HS patients were stained with 0.7µM Carboxyfluorescein succinimidyl ester (CFSE) for five minutes. Then PBMCs were plated in 96 round-bottom well plates in R9 medium (RPMI 1640 [Life Technologies, Basel, Switzerland], 2mM L-glutamine [Biochrom, Berlin, Germany], 50U/mL penicillin, 50µg/ml streptomycin [Bioconcept, Allschwil, Switzerland], 5% heat-inactivated human AB serum [Swiss Red Cross, Bern, Switzerland]) with 1µg/mL phytohaemagglutinin (PHA, [Brunschwig Chemie, Basel, Switzerland]) and three different concentrations of LL-37 (AnaSpec, Freemont, USA) (2µg/ml, 10µg/mL and 30µg/mL) and 30µg/mL of a scrambled control peptide LL-37 (Innovagen, Lund, Sweden). Flow cytometry analysis (FACS canto, BD biosciences) was performed after three days in culture with the following staining: CFSE (FITC), CD3 (PE-Cy7), CD4 (APC-Cy7) and CD8 (APC) (all biolegend). For analysis of T cells only, PBMCs were sorted via magnetic beads for the expression of CD3 (EasySep™ Human CD3 Positive Selection Kit (Stemcell, Vancouver, Canada) according to the manufacturer's protocol, before analysis after five days in culture. Proliferation rates of $CD4^+$ and $CD8^+$ T cells, were normalised to no LL-37 addition.

Calcium influx assay

Calcium influx measurements were assessed as described previously.[39] Briefly, PHA blasts, generated from healthy PBMCs in the presence of 50 IU/mL IL-2 (Roche, Basel, Switzerland) and PHA, were incubated with 2mg/ml fluo-4-AM (Invitrogen, Carlsbad, USA) and plated in half-area, clear-bottom, 96-well plates (VWR International, Dietikon, Switzerland) at 10^5 cells/well. The data were measured on a Synergy-4 instrument (BioTek, Highland Park, USA) with an excitation band of 485/20nm, and fluorescence was detected at 528/20nm. A baseline signal (F^0) was recorded for five minutes before the addition of PHA alone (2µg/ml) or PHA with three different concentrations of LL-37 (2µg/ml, 10µg/mL and 30µg/mL). Subsequently, fluorescence intensity (F) was monitored for 60 minutes. As negative control, PHA blasts were incubated with medium only. The results are presented as normalised fluorescence to the baseline level (F/F^0).

Statistical analysis

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

RESULTS

LL-37 peptide is expressed in lesional HS skin

LL-37 expression was immunohistochemically analysed on tissue sections of lesional HS skin. As comparison, lesional skin from AD and PS patients and healthy skin was assessed. Figure 1a-d shows representative examples of LL-37 staining in cryo-cut tissues and the semi-quantitative analysis of these stainings is presented in Figure 1e. Whereas lesional HS skin showed a dense infiltration of LL-37$^+$ cells in highly inflamed dermal areas and weak expression by keratinocytes, without any accumulation around the hair follicle, the dermal distribution of LL-37$^+$ cells is more uniform and brighter in the epidermis in PS lesions. In AD, occurrence of LL-37$^+$ cells is sparse and comparable to healthy skin. Examining the morphology of clearly positive LL-37$^+$ cells in lesional HS skin, we suggest that LL-37 is present in granulocytes, which was underlined with a positive double staining for neutrophils (data not shown).

In HS, CAMP mRNA levels do not always correspond to LL-37 peptide levels

After determining that LL-37 expression was increased in lesional HS skin, we sought to investigate whether these peptide levels correspond with the CAMP mRNA expression. Therefore, we used semi-quantitative RT-PCR to quantify relative changes of CAMP mRNA in lesional HS, PS, AD and healthy skin (Fig. 2a). In accordance with our IHC results, PS skin showed an upregulated presence of CAMP, while in AD no difference in mRNA presence compared to healthy skin could be demonstrated. In HS, a mean four times increase was observed, however, due to wide-spread individual sample values, this difference was not statistically significant compared to healthy skin. While some HS biopsies showed a clear increase in CAMP levels, others showed similar expression as in healthy skin or even a decreased presence of CAMP mRNA. This led us to the question, whether in HS LL-37 is transcribed and translated locally or partly also elsewhere. To this aim we correlated the semi-quantitative peptide score with the results of the CAMP mRNA analysis. This analysis (Fig. 2b) showed a non-significant correlation of the peptide and mRNA levels in lesional HS skin.

Positive correlation of LL-37 peptide presence with lesional inflammatory status

Next, we assessed the potential pathogenic role of LL-37 in HS by investigating both the correlation of LL-37 peptide levels with lesional parameters of disease severity, as well as with other pro- and also anti-inflammatory cytokines (IFNγ, IL-17, IL-23, IL-1β, TNFα, IL-32 and IL-10). Our data demonstrates that LL-37 peptide levels in lesional HS skin positively correlate with the presence of T cells, macrophages and neutrophils (Suppl. Fig. 1a-c).

Interestingly, the cytokines mentioned above also positively correlated with LL-37 levels (Fig. 3a-d, Suppl. Fig. 1d-f).

LL-37 drives the proliferation of T cells in the presence of an unspecific activation independent of antigen presenting cells via an increased calcium influx

To investigate the immunomodulatory role of LL-37 in HS patients, we focused on the impact of LL-37 on the proliferation of both $CD4^+$ and $CD8^+$ T cells. For this reason, PBMCs from healthy donors and HS patients were stained with CFSE and incubated for three days with PHA, a lectin, which leads to a polyclonal activation of T cells, and increasing concentrations of LL-37. In the presence of PHA, we report that LL-37 increases the proliferation rate of T cells, in particular of $CD4^+$ T cells (Fig. 4a-b). To determine whether the observed increased proliferation is due to a LL-37 mediated maturation of antigen presenting cells (APCs), we analysed magnetically purified $CD3^+$ T cells. After an incubation time of five days, similar, if a bit more pronounced, pro-proliferative effects of LL-37 on $CD4^+$ T cells in the presence of PHA were observed (Fig 4c-d). We could conclude that the proliferation enhancement was a direct effect of LL-37 on stimulated T cells. To figure out how this effect is mediated, we measured the calcium influx in PHA blasts, which were stimulated with PHA alone or with PHA in the presence of increasing concentrations of LL-37 (Fig. 4e). Indeed, we were able to detect a more prominent increase of the calcium influx in the presence of PHA and LL-37 compared to PHA only.

DISCUSSION

Here we show that LL-37 is upregulated on the peptide level in skin lesions of patients suffering from the recalcitrant skin disease HS, as already indicated by Emelianov et al.[27] On the CAMP mRNA level, we report an upregulation in PS, while no differences to healthy skin could be detected in AD skin. In HS, we cannot completely confirm Bechara et al.'s mRNA results,[26] as we only describe a mean four times increase in HS compared to healthy skin, but do not detect any statistically significant upregulation. By correlating peptide and mRNA levels, we were able to determine that LL-37 and CAMP do not correspond in all HS biopsies. This suggests that in some HS lesions LL-37 is either not produced locally and enters HS dermis in pre-stored granuli or CAMP mRNA is quickly degraded or translated after transcription. This might, on the one hand, explain the vast range of CAMP mRNA levels detected in HS lesions and, on the other hand, resolve the discrepancy between the peptide and mRNA levels found in HS biopsies.

LL-37 levels in lesional HS skin positively correlate with the presence of T cells, macrophages and neutrophils, as well as with the pro-inflammatory IFNγ, IL-17, IL-23, IL-1β, TNFα and IL-32 and also with the anti-inflammatory IL-10, which suggests that LL-37 correlates with the establishment of a Th1/Th17 phenotype and other pro-inflammatory loops in lesional HS skin. Moreover, we report that in the presence of PHA, LL-37 drives $CD4^+$ T cell proliferation independent of APC help. However, in contrast to PS patients, as it was described by Lande et al.,[23] we never detected any proliferation of T cells from HS patients in the presence of LL-37 alone (Suppl. Fig. 2). The proliferative effect was found to be mediated by an additional influx of calcium into the T cells after a co-stimulation with PHA and LL-37. Interestingly, the calcium influx after PHA+LL-37 starts earlier than with PHA alone, suggesting that LL-37 mediates its effect directly via the membrane and is not receptor mediated, which has already been a suggested mechanism in the literature.[40] The question why LL-37 has a more prominent impact on the proliferation of $CD4^+$ T cells remains. It was reported that LL-37 induces apoptosis in stimulated mouse $CD8^+$ T cells, which could counter-regulate this pro-proliferative effect.[41]

Concerning the role of LL-37 in HS pathogenesis, we hypothesise that LL-37 is present in early lesions, but then also remains high in the chronic phase, becoming a driver of the pro-inflammatory cascade. The arguments in favour of this hypothesis are that LL-37 is mainly produced by neutrophils, which enter the lesional skin early after initiation of inflammation where LL-37 mainly exerts its antimicrobial function. What is more, the presence of LL-37 also coincides with T cells and macrophages which enter HS lesions at a later stage and under the influence of LL-37 and other mediators, they may start producing Th1/Th17 cytokines, further driving the pro-inflammatory process.[42]

Taken together, we report that LL-37 is upregulated in HS patients and that its presence correlates with other pro-inflammatory mediators, such as Th1/Th17 cytokines. Further investigation of the involvement of LL-37 in immunomodulation showed that LL-37 and an unspecific activation lead to an APC independent boost of the proliferation of $CD4^+$ T cells via direct calcium signalling. More insight is needed to understand pathological mechanisms in HS and to understand the role of LL-37 in this process, which could pave the way for future treatment possibilities by blocking LL-37 in this recalcitrant skin disease.

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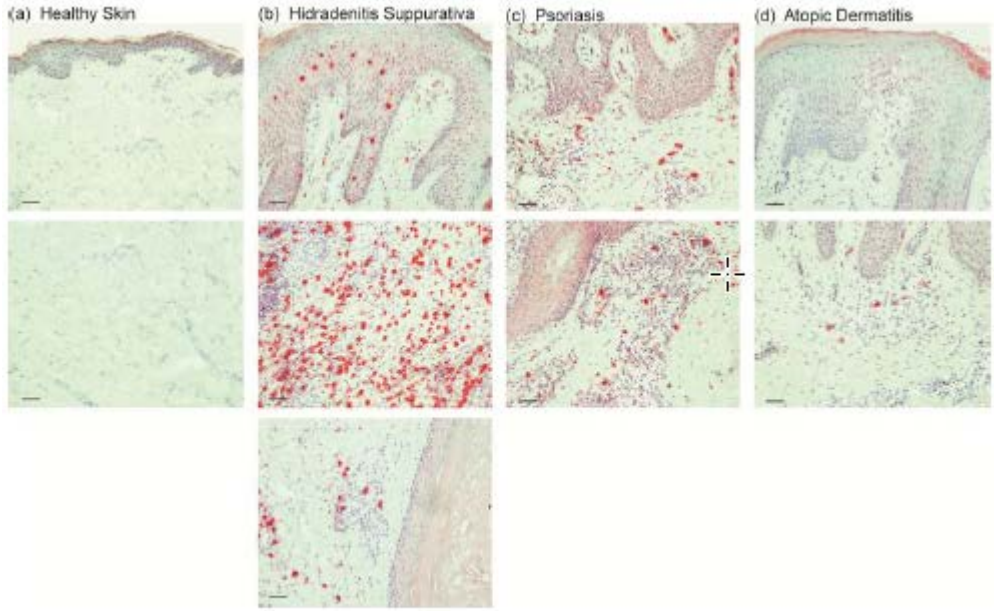
FIGURES



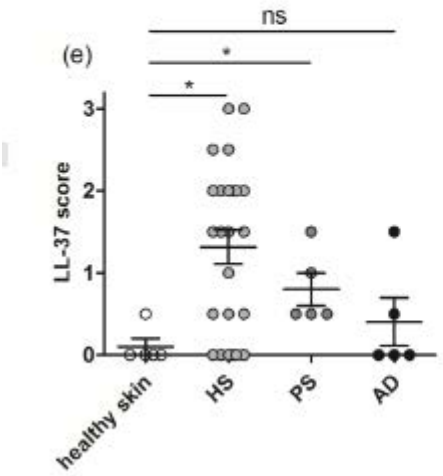


Figure 1

LL-37 is highly expressed on the peptide level in lesional hidradenitis suppurativa skin

a-d) Representative examples of frozen epidermal (above) or dermal (below) skin sections immunohistochemically stained for LL-37 in healthy skin and lesional skin of different inflammatory skin diseases (HS, PS, AD). The third HS picture represents a staining of LL-37 around the hair follicle. Sections were counterstained with hematoxylin; 200x magnification, scale bars indicate 50µm. e) Quantification of the density of LL-37^{+} cells in the four donor groups. Score ranges from 0–3. n(healthy skin) = 5, n(HS) = 24, n(PS) = 5, n(AD) = 5. Data

are presented as mean with SEM and were statistically analysed with the Mann-Whitney test, with * indicating a p-value <0.05.

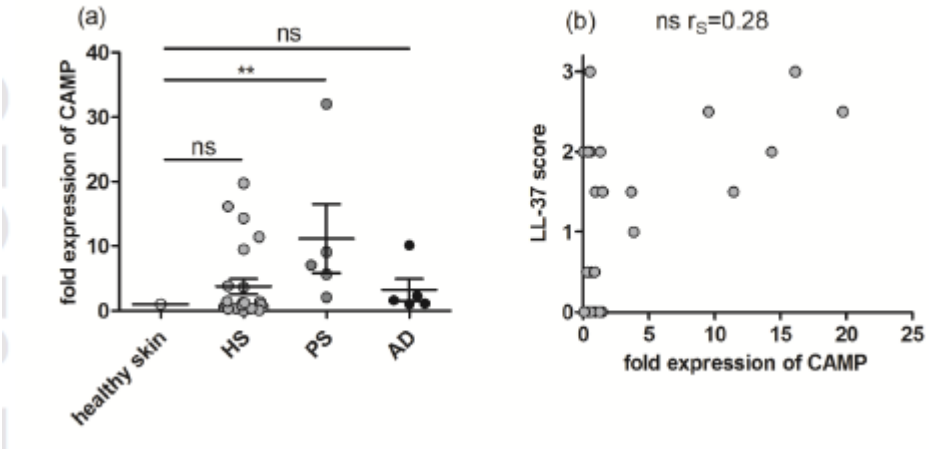


Figure 2

Messenger RNA levels of CAMP in hidradenitis suppurativa are not statistically elevated and do not correlate with LL-37 peptide levels

a) Analysis of CAMP mRNA expression in lesional HS skin normalised to healthy skin and compared to lesional PS and AD skin. Ct-values were normalised with housekeeping genes HPRT-1, GAPDH and B2M to healthy skin (=1); n(healthy skin)=9, n(HS)=24, n(PS)=5, n(AD)=5. Data are presented as mean with SEM and were statistically analysed with the Mann Whitney test with ** indicating a p-value <0.01. b) Correlation of LL-37 peptide levels with CAMP mRNA levels in lesional HS skin (r_S= 0.28). n(HS)=24. Significance of correlation was determined with the Spearman's rank correlation coefficient (r_S).

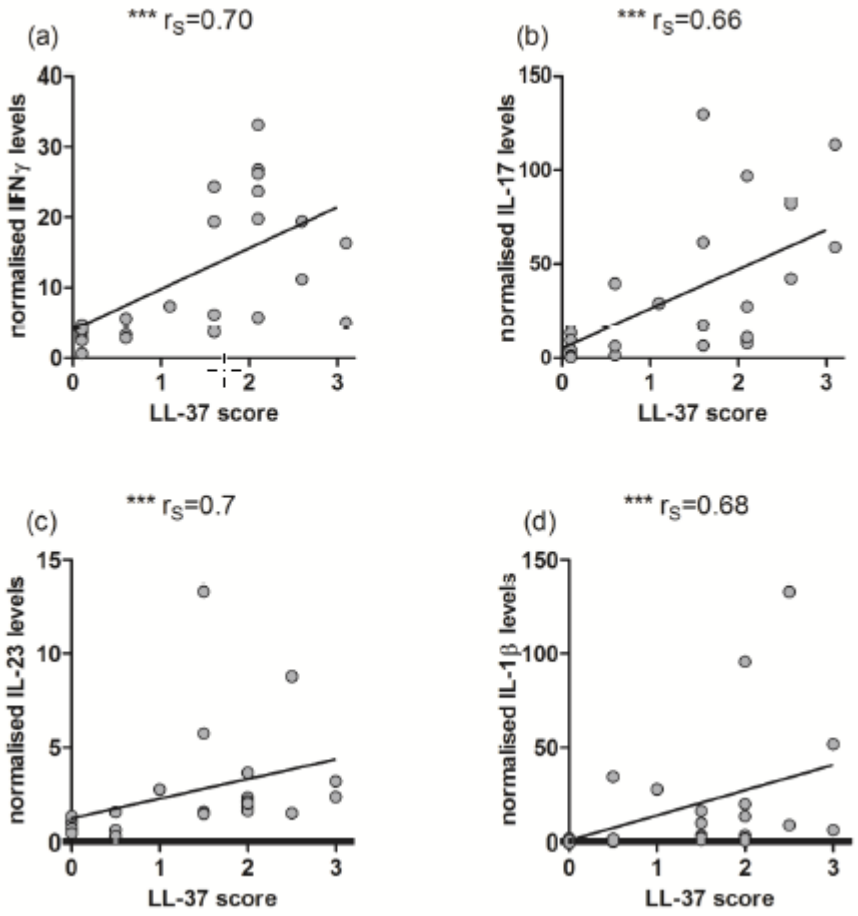


Figure 3

Peptide levels of LL-37 in hidradenitis suppurativa skin positively correlate with Th1/Th17 associated cytokines

a-d) LL-37 levels positively correlate with mRNA levels of the Th1/Th17 associated cytokines IFNγ (r_S=0.70), IL-17 (r_S=0.66), IL-23 (r_S= 0.70) and IL-1β (r_S= 0.68). Ct-values of mRNA analysis were normalised with housekeeping genes HPRT-1, GAPDH and B2M to healthy skin (=1); n(HS)=24. Significance of correlation was determined with the Spearman's rank correlation coefficient (r_S) with* indicating a p-value <0.05, ** indicating a p-value <0.01 and *** indicating a p-value <0.001.

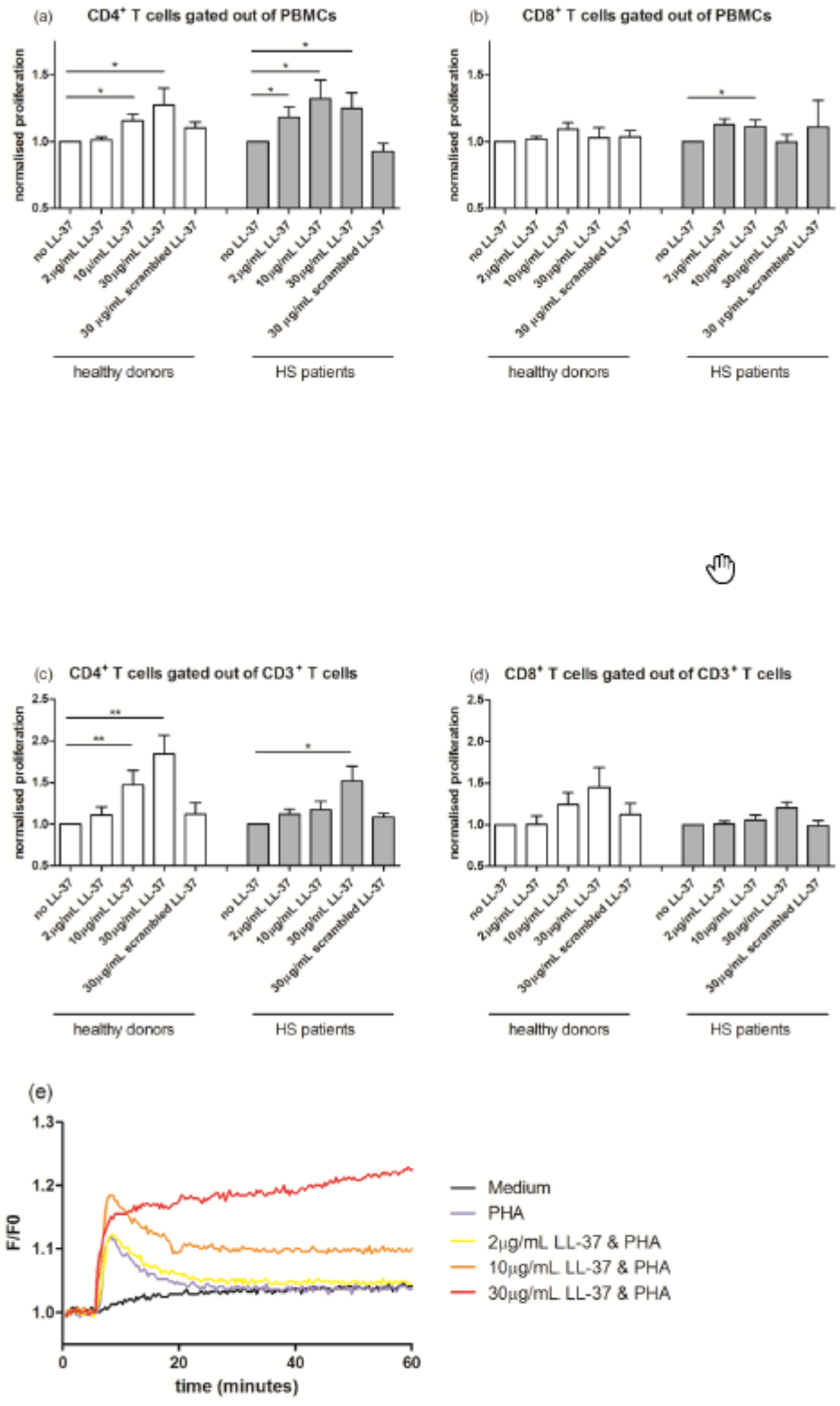


Figure 4

LL-37 together with an unspecific activation increases the proliferation of T cells by increasing the calcium influx independent of antigen presenting cells

a-b) Normalised proliferation ratios of $CD4^+$ and $CD8^+$ T cells in healthy donors and HS patients, which were incubated in a PBMC pool of cells in the presence of PHA and different concentrations of LL-37 and a scrambled control peptide. c-d) Normalised proliferation ratios of $CD4^+$ and $CD8^+$ T cells in healthy donors and HS patients, which were incubated in a pure T cell culture in the presence of PHA and different concentrations of LL-37 and a scrambled control peptide. Data of the proliferation of T cells are normalised to the percentage of CFSE low cells without LL-37 addition. Results are presented as mean with SEM and were statistically analysed with the non-parametric Wilcoxon rank sum test with * indicating a p-value <0.05 and ** indicating a p-value <0.01. PBMCs: n(healthy)= 9, n(HS)=10; T cells: n(healthy)= 10, n(HS)=11. e) A representative example of a calcium assay with PHA blasts derived from a healthy donor. Fluorescence intensity is normalised to baseline levels and was measured for one hour. Lines indicate relative fluorescence levels in the presence of medium (black), PHA (blue) and PHA and increasing concentrations of LL-37 (yellow, orange, red).

Supplementary Figure 1

Peptide levels of LL-37 in hidradenitis suppurativa skin positively correlate with lesional inflammatory status and mRNA levels of pro-inflammatory mediators

a-c) LL-37 expression positively correlates with the number of T cells (r_S= 0.72), macrophages (r_S= 0.68) and neutrophils (r_S= 0.86) in lesional HS skin. d&f) LL-37 levels also correlate with the presence of the mRNA of pro-inflammatory mediators TNFα (r_S= 0.55) and IL-32 (r_S= 0.48). e) mRNA expression of the anti-inflammatory IL-10 positively correlates with LL-37 peptide levels (r_S= 0.51). Ct-values of mRNA analysis were normalised with housekeeping genes HPRT-1, GAPDH and B2M to healthy skin (=1); n(HS)=24. Significance of correlation was determined with the Spearman's rank correlation coefficient (r_S) with* indicating a p-value <0.05, ** indicating a p-value <0.01 and *** indicating a p-value <0.001.

Supplementary Figure 2

LL-37 alone does not induce proliferation in T cells isolated from the blood of HS patients

Representative example of the proliferation of T cells in an HS patients after the incubation of three days with LL-37 alone.