The anti-inflammatory potency of biologics targeting TNF-α, IL-17A, IL-12/23 and CD20 in hidradenitis suppurativa: an ex vivo study

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ABSTRACT

Background

Biologics targeting inflammatory mediators are able to clinically improve hidradenitis suppurativa (HS). However, their clinical efficacy shows great inter-patient variability in daily practice.

Objective

To investigate the anti-inflammatory potency of currently available biologics for the treatment of HS in an *ex vivo* skin culture system using lesional HS biopsies.

Methods

Lesional skin samples of ten HS patients and normal skin samples of five healthy controls were cultured *ex vivo* and exposed to prednisolone or biologics targeting TNF-α, IL-17A, IL-12/23p40, or CD20, respectively adalimumab, infliximab, secukinumab, ustekinumab, and rituximab. Real-time quantitative PCR and cytokine bead arrays were used to measure the inhibitory effect of the biologics on cytokines and antimicrobial peptides (AMPs).

Results

The relative mRNA expression of all tested cytokines and AMPs was significantly downregulated by all anti-inflammatory agents ($p < 0.0001$). The release of the pro-inflammatory cytokines TNF-α, IFN-γ, IL-1ß, IL-6, and IL-17A was significantly inhibited by adalimumab, infliximab, ustekinumab, prednisolone (all $p < 0.0001$) and rituximab ($p = 0.0071$) but not by secukinumab ($p = 0.0663$). In addition, adalimumab, infliximab, and prednisolone reduced the levels of a broader mix of individual cytokines than secukinumab, ustekinumab, and rituximab.

Conclusion

This *ex vivo* study suggests that TNF-α inhibitors and prednisolone are the most powerful inhibitors of pro-inflammatory cytokines and AMPs in HS lesional skin, which is in accordance with our clinical experience in patients with HS.

INTRODUCTION

Hidradenitis suppurativa (HS) is a chronic skin disease characterised by painful, deep-seated, inflamed nodules, abscesses, and in later stages sinus tracts.[1] Occlusion of the hair follicle with subsequent rupture, followed by a fierce local inflammatory response, are considered primary pathogenic events. The aberrant innate immune response is characterised by the upregulation of various pro-inflammatory cytokines and antimicrobial peptides (AMPs).[2]

Several studies have found elevated mRNA and protein levels of tumour necrosis factor (TNF)-α in lesional and perilesional HS skin.[3,4] Targeting TNF-α with adalimumab and infliximab has been shown to be clinically efficacious in HS in randomised placebo-controlled trials.[5-7] In addition, upregulation of interleukin (IL)-1ß, IL-17A, and IL-23 in HS lesions point to the importance of the IL-17 pathway in the pathophysiology of HS.[8-10] However, targeting IL-1 in HS gave ambiguous clinical outcomes. In a randomised controlled trial anakinra proved to be efficacious in moderate-to-severe HS, yet cases of failure on anakinra therapy have been reported.[11-13]

Most recently IL-17 has been targeted in two clinical trials in HS (NCT02421172, NCT03248531), but the results have not yet been released. Treatment with secukinumab or ustekinumab induced amelioration of HS in a few individual cases and case series.[14-16] In addition, enhanced levels of AMPs in lesional HS skin have been described for S100A7 (psoriasin), S100A8, S100A9, human ß-defensin (HBD)-2, HBD-3 and LL-37.[17-19] Upregulation of these AMPs in HS lesional skin is mainly driven by IL-17.[18]

Chronic HS lesions are characterised by a marked increase in the number of CD20+, CD79A+ B cells, and CD138+ plasma cells.[20] One case report described a clear improvement in HS after treatment with rituximab for concomitant idiopathic carpotarsal osteolysis syndrome.[21] This highlight the contribution of these B and plasma cells to the inflammatory process in HS.

Although biologics targeting inflammatory mediators are now widely used for the treatment of HS, their clinical efficacy shows great inter-patient variability. Our *ex vivo* skin culture system is a fast and simple method to simultaneously investigate and compare the effect of biologics in fresh human lesional skin samples.[22] It approaches the *in vivo* situation by maintaining the patients' skin architecture and allows close monitoring of the events following response to immunostimulators or suppressors in the same experiment.[23] Therefore, this study sought to investigate the anti-inflammatory potency of currently available biologics used for the treatment of HS in an *ex vivo* skin culture system using lesional HS biopsies.

MATERIALS AND METHODS

Ethics and informed consent

HS lesional skin was collected from excised skin after HS surgery conducted in the department of Dermatology of the Erasmus University Medical Center in Rotterdam, The Netherlands from October 2017 through February 2018. According to the opt-out principle used in the Erasmus University Medical Center no informed consent is required for the use of excised tissue for research purposes as this is considered waste material. Control skin samples were obtained from healthy individuals in the Sint Franciscus Hospital in Rotterdam, The Netherlands. All healthy volunteers provided written informed consent for the use of their excised skin in this study.

Hidradenitis suppurativa patients

Ten HS patients, five males and five females, with chronic, active disease (seven Hurley stage II, three Hurley stage III) requiring surgical excision under general or sedative anaesthesia were included. HS lesional skin samples were obtained from the inguinogenital area in 5 of these 10 patients, axillae in 4, and from the gluteal area in 1 patient. The mean (± SD) age was 43.7 ± 7.4 years, the mean body mass index was 30.3 ± 6.5 kg/m^2, and 7 of 10 patients were current smokers. Eight patients received a stable (≥ 28 days) dose of systemic antibiotics for their HS at the time of surgery. None of the patients had been using immunosuppressive or immunomodulatory therapies (e.g. biologics) for at least two months prior to surgery.

Healthy control skin

Healthy skin samples were obtained from the submammary and abdominal waste material of five women who underwent breast or abdominal reduction surgery. We argue that skin from these regions is suitable as a control samples because the inframammary and abdominal folds are predilection sites for HS. The control subjects were otherwise healthy and had no family history of HS, and were not using any immunosuppressive or immunomodulatory treatment at the time of surgery.

Biopsy procedure

A total of seven fresh 4-mm punch biopsies per subject were taken. HS lesional skin biopsies were obtained from the same palpable infiltrate, and at least 1 cm away from the excision border. Abscesses were not biopsied to avoid sampling only the roof of the abscess. Each biopsy was weighed prior to culture as described in an earlier publication.[4] HS biopsies had a mean weight of 32.4 ± 6.6 mg, which was significantly heavier than biopsies from healthy skin with a weight of mean 16.1 ± 3.0 mg ($p < 0.001$). This potential confounder was addressed by normalising all protein levels for the mg tissue.

Ex vivo skin culture

The 4-mm biopsies were immediately cultured in a transwell system (Netwell; Costar, Cambridge, MA) as described previously.[22,23] In brief, samples were placed in punched-out holes in the transwell membrane of a 12-well plate with the epidermis exposed to the air and the dermis immersed in 1 mL Iscove's modified Dulbecco's medium (Gibco, Paisley, U.K.) containing 0.5% human AB serum, penicillin (100 U/mL) and streptomycin (100 U/mL) with or without an anti-inflammatory agent. Biologics and prednisolone were separately added to the culture media resulting in the following seven conditions: (i) culture media as negative control; (ii) prednisolone 100 µg/ml as positive control; (iii) adalimumab 30 µg/ml; (iv) infliximab 20 µg/ml; (v) secukinumab 30 µg/ml; (vi) ustekinumab 10 µg/ml; (vii) rituximab 200 µg/ml. The concentrations of the monoclonal antibodies were a multiple of the reported trough levels in patients with plaque psoriasis (adalimumab[24,25], infliximab[25], ustekinumab[26], secukinumab[27]), and CD20+ B cell malignancies (rituximab[28]). Skin biopsies were incubated for 24h at 37°C in an atmosphere of 5% CO_2 and 98% humidity. Subsequently, the biopsies were placed in 250 µL lysis buffer containing 1% ß-mercaptoethanol. Both the supernatants and the biopsies were transferred to a polypropylene tube and stored at –20°C until further analysis.

Gene expression analysis

Total mRNA was extracted using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MA). RNA was treated with 0.1 U/µL DNAse (Invitrogen) and cDNA was subsequently synthesised using 1 µg total RNA template, with SuperScript II reverse transcriptase, random hexamer primers (Invitrogen) and oligo(dT)15 (Promega). Primers and probes were designed and chosen using ProbeFinder Sofware and the Universal Probe library (Roche Applied Science, Indianapolis, IN). *ABL1* was chosen as a reference housekeeping gene.[29] Real-time quantitative PCR (qPCR) was performed for 12 genes using the ViiA7 sequence-detection system (Applied Biosystems): *TNF-α*, *IL-1ß*, *IL-6*, *CXCL-8/IL-8*, *IL-12p19*, *IL-17A*, *IFN-α/MxA*, *S100A7*, *S100A8*, *S100A9*, *LL37*, and *HBD-2*. Gene expression was analysed with QuantStudio Real-Time quantitative PCR Software version 1.3 (Applied Biosystems, Waltham, MA).

Protein quantification

Eighteen inflammation-related cytokines were simultaneously measured in the supernatant using a customised bead-based Multi-Analyte Profiling assay (Luminex, R&D systems, Minneapolis, MN): CCL-20, TNF-α, IFN-γ, IL-1ß, IL-1R1, IL-5, IL-6, IL-10, IL-12/23p40, IL-17A, IL-17E, IL-18, IL-19, IL-22, IL-27, IL-31, IL-33, and IL-36ß. Assays were used according to the manufacturers' protocol. A dilution factor of 2 was used for all supernatants.

Statistical analyses

The relative mRNA expression and protein production per sample compared with culture media (negative control) was calculated for every condition. Protein levels were normalised according to input weight and expressed in picogram (pg)/mL/mg tissue weight. The lower limit of quantification was imputed when protein concentrations in the supernatant (pg/mL) were below limit of detection. The Kruskal-Wallis test, i.e. a one-way ANOVA on ranks, was used to compare the variance in mRNA and protein levels per inflammatory marker. If another condition stochastically dominated one other condition, the Dunnett's post-test was subsequently used to separately test conditions versus culture media (pairwise comparisons). We chose this approach in order to increase power. A two-sided *p*-value lower than 0.05 was considered significant. The level of significance for the relative mRNA expression and protein production was separately adjusted by the Benjamini Hochberg procedure for multiple comparisons. A correlation between relative mRNA and protein levels per cytokine was calculated using the Spearman rho test for non-normally distributed continuous variables. GraphPad Prism version 6 (GraphPad Software, La Jolla, CA) was used for all statistical analyses.

RESULTS

Sample flow for analysis

In total, samples of ten HS patients and five healthy controls were analysed by real-time qPCR. Supernatants of one HS patient were excluded from protein analysis because of erroneous sample processing, resulting in samples of nine HS patients with protein data. For control skin, supernatants of three healthy volunteers were analysed by Luminex assay. However, protein levels were below level of detection or in the very low range of the calibration line in these control samples (data not shown). Therefore, samples of the other two patients were not analysed.

Significant downregulation of gene expression of cytokines and AMPs by prednisolone and different biologics

The relative changes in mRNA expression of the 12 genes in HS samples including significance levels are shown in Table 1. The overall median inhibitory effect on the mix of cytokines and AMPs per condition was: prednisolone 0.57 (interquartile range [IQR] 0.34-1.03); adalimumab 0.51 (0.33-0.92); infliximab 0.53 (0.32-0.78); secukinumab 0.56 (0.29-0.99); ustekinumab 0.64 (0.44-1.00), rituximab 0.73 (0.50-1.19). Remarkably, the inhibitory impact on AMPs was stronger for the biologics than for prednisolone (Table 1, Figure 1). Prednisolone, adalimumab and infliximab

Table 1. Relative mRNA expression and their modulation by biologics in HS lesional skin.

	HS lesional skin (n = 10)											
	Prednisolone		Adalimumab		Infliximab		Secukinumab[1]		Ustekinumab		Rituximab	
	Median (IQR)	Unadj. *p*-value	Median (IQR)	Unadj. *p*-value	Median (IQR)	Unadj. *p*-value	Median (IQR)	Unadj. *p*-value	Median (IQR)	Unadj. *p*-value	Median (IQR)	Unadj. *p*-value
IL-1β	0.27 (0.15-0.48)	**<0.0001***	0.53 (0.23-0.74)	**0.0073***	0.60 (0.50-0.73)	**0.0306**	0.67 (0.53-0.87)	0.2377	0.76 (0.39-1.06)	0.6194	0.49 (0.35-0.72)	0.0597
IL-6	0.42 (0.36-0.51)	**<0.0001***	0.62 (0.52-0.89)	0.0956	0.65 (0.41-0.71)	**0.0047***	0.70 (0.44-0.90)	0.1063	0.55 (0.37-0.90)	**0.0122***	0.75 (0.55-0.97)	0.4396
CXCL-8 (IL-8)	0.44 (0.34-0.60)	0.1675	0.30 (0.22-0.49)	**0.0042***	0.29 (0.26-0.38)	**0.0002***	0.28 (0.16-0.47)	**0.0015***	0.39 (0.32-0.48)	**0.0296**	0.69 (0.41-0.86)	0.9779
IL-17A	0.13 (0.07-0.37)	**0.0049***	0.93 (0.59-1.37)	1.0000	0.55 (0.43-0.84)	1.0000	0.95 (0.62-1.42)	1.0000	0.96 (0.56-1.29)	1.0000	0.99 (0.43-1.61)	1.0000
IL-23p19	0.92 (0.33-1.20)	1.0000	0.48 (0.34-0.66)	0.3934	0.49 (0.31-0.88)	0.4901	0.48 (0.14-1.01)	0.3836	0.72 (0.46-1.79)	1.0000	0.97 (0.51-1.99)	1.0000
TNF-α	1.09 (0.64-1.58)	1.0000	0.92 (0.52-1.02)	1.0000	0.74 (0.37-1.03)	0.7250	0.53 (0.28-1.15)	0.5127	0.83 (0.53-2.56)	1.0000	0.71 (0.60-1.11)	1.0000
IFN-MXA	0.75 (0.60-1.23)	1.0000	0.56 (0.35-1.08)	0.7914	0.84 (0.59-1.19)	1.0000	0.67 (0.43-0.81)	0.3345	0.83 (0.55-1.17)	1.0000	0.91 (0.57-1.17)	1.0000
S100A7	0.80 (0.51-1.02)	0.3934	0.56 (0.50-0.70)	**0.0266**	0.56 (0.45-0.72)	**0.0116***	0.50 (0.34-0.88)	**0.0105***	0.64 (0.54-0.68)	**0.0223**	0.56 (0.46-0.74)	**0.0180**
S100A8	0.74 (0.55-1.11)	1.0000	0.50 (0.39-0.54)	**0.0060***	0.52 (0.44-0.60)	**0.0248**	0.67 (0.52-0.73)	0.2826	0.79 (0.59-1.12)	1.0000	0.69 (0.56-0.93)	0.7091
S100A9	0.80 (0.51-1.07)	0.8442	0.35 (0.30-0.53)	**0.0005***	0.29 (0.27-0.42)	**<0.0001***	0.36 (0.25-0.68)	**0.0010***	0.52 (0.42-0.78)	**0.0296**	0.66 (0.52-0.93)	0.9779
LL-37	0.67 (0.14-1.28)	0.8997	0.40 (0.37-0.83)	0.5915	0.64 (0.38-1.10)	1.0000	0.51 (0.32-1.84)	1.0000	0.77 (0.48-1.37)	1.0000	0.89 (0.67-1.22)	1.0000
HBD-2	0.48 (0.19-0.87)	**0.0416**	0.43 (0.29-0.65)	**0.0306**	0.32 (0.20-0.91)	**0.0445**	0.69 (0.13-1.07)	0.1763	0.49 (0.43-0.76	0.1538	0.76 (0.68-1.13)	1.0000
All (12)	0.57 (0.34-1.03)	**<0.0001**	0.51 (0.33-0.92)	**<0.0001**	0.53 (0.32-0.78)	**<0.0001**	0.56 (0.29-0.99)	**<0.0001**	0.64 (0.44-1.00)	**<0.0001**	0.73 (0.50-1.19)	**<0.0001**
Cytokines[2] (7)	0.41 (0.20-0.77)	**<0.0001**	0.52 (0.32-0.95)	**<0.0001**	0.54 (0.34-0.75)	**<0.0001**	0.60 (0.37-0.98)	**<0.0001**	0.61 (0.39-1.08)	**0.0002**	0.76 (0.44-1.21)	0.4780
AMPs[3] (5)	0.75 (0.44-1.11)	**<0.0001**	0.48 (0.33-0.68)	**<0.0001**	0.47 (0.29-0.73)	**<0.0001**	0.52 (0.30-0.96)	**<0.0001**	0.64 (0.45-0.89)	**<0.0001**	0.73 (0.53-0.98)	**0.0001**

p-values marked in bold indicate analytes that are significant at the 0.05 level. * Significant after correction with the Benjamini Hochberg test ($p < 0.0156$). [1] One HS sample for secukinumab was excluded for Real-Time qPCR analysis as a result of a human error during the process of cDNA synthesis (n = 9). [2] Cytokines: pooled effect of IL-1ß, IL-6, IL-8, IL-17A, IL-23p19, TNF-α, IFN-MXA. [3] AMPs: pooled effect of S100A7, S100A8, S100A9, LL-37, HBD-2. AMPs: antimicrobial peptides. HS: hidradenitis suppurativa. IQR: interquartile range. NT: not tested. Unadj: unadjusted.

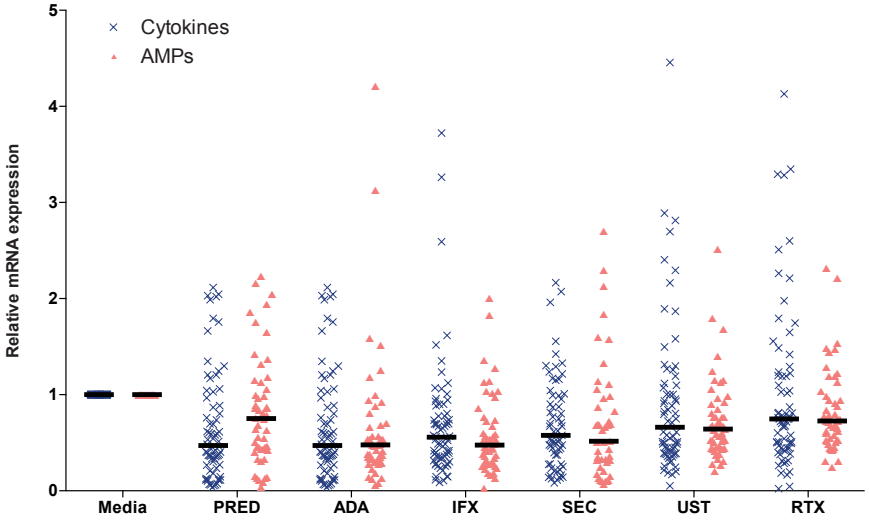

Figure 1. Anti-inflammatory impact on the relative mRNA expression of the mix of cytokines and AMPs in HS lesional skin, n = 10. All agents displayed significant inhibition of both cytokine and AMP mRNA expression except for the effect of RTX on cytokine mRNA expression. One data point is out of the y-axis range. Horizontal bars display the median. Media, culture media; PRED, prednisolone 100 µg/mL; ADA, adalimumab 30 µg/mL; IFX, infliximab 20 µg/mL; SEC, secukinumab 30 µg/mL; UST, ustekinumab 10 µg/mL; RTX, rituximab 200 µg/mL.

significantly inhibited *IL-1*β. Expression of *CXCL-8/IL-8* was significantly downregulated by adalimumab, infliximab, secukinumab, and ustekinumab. Messenger RNA levels of two other important pro-inflammatory cytokines, *TNF*-α and *IL-23p19*, were not significantly downregulated by any of the biologics or prednisolone. Gene expression of members of the *S100* family and *HBD-2* was significantly reduced by adalimumab, infliximab, secukinumab and ustekinumab (Table 1). In healthy control skin, only prednisolone, adalimumab and infliximab significantly downregulated mRNA expression of the mix of tested cytokines and AMPs (Supplementary Table 1, Supplementary Figure 1).

Significant *ex vivo* reduction in TNF-α, IFN-γ, IL-1ß, IL-6, and IL-17A protein levels of in HS lesional skin

In total, 18 cytokines were measured. Six cytokines were not detected in the majority of HS samples throughout all conditions: IL-1RI, IL-5, IL-12/23p40, IL-22, IL-31, and IL-33. Relative changes in protein production including significance levels for the other 12 cytokines are shown in Table 2. The overall median (IQR) effect on cytokines per condition was: prednisolone 0.51 (0.28-0.64); adalimumab 0.78 (0.54-0.91);

Table 2. Relative protein production and their modulation by biologics in HS lesional skin.

	HS lesional skin (n = 9)											
	Prednisolone		Adalimumab		Infliximab		Secukinumab		Ustekinumab		Rituximab	
	Median (IQR)	Unadj. *p*-value	Median (IQR)	Unadj. *p*-value	Median (IQR)	Unadj. *p*-value	Median (IQR)	Unadj. *p*-value	Median (IQR)	Unadj. *p*-value	Median (IQR)	Unadj. *p*-value
TNF-α	0.20 (0.15-0.29)	**0.0022***	0.09 (0.07-0.20)	**<0.0001***	0.03 (0.02-0.05)	**<0.0001***	0.89 (0.53-0.93)	1.0000	0.54 (0.23-0.76)	0.3305	0.70 (0.65-1.10)	1.0000
IFN-γ	0.37 (0.11-0.53)	**0.0068***	0.43 (0.30-1.17)	0.4169	0.46 (0.13-0.91)	0.0673	0.56 (0.51-0.66)	0.6285	0.39 (0.33-0.61)	0.0673	0.49 (0.41-0.60)	0.2596
CCL-20	0.50 (0.20-0.81)	**0.2845**	0.58 (0.28-1.80)	1.0000	0.71 (0.53-1.13)	1.0000	0.94 (0.53-1.19)	1.0000	0.73 (0.62-1.28)	1.0000	0.95 (0.70-1.38)	1.0000
IL-1ß	0.23 (0.17-0.45)	**0.0049***	0.41 (0.29-0.73)	0.3114	0.44 (0.31-0.75)	0.3305	1.08 (0.46-1.43)	1.0000	0.59 (0.45-2.06)	1.0000	0.70 (0.52-1.03)	1.0000
IL-6	0.59 (0.46-0.73)	**0.0006***	0.81 (0.68-0.85)	0.0724	0.76 (0.64-0.91)	0.1035	0.99 (0.76-1.08)	1.0000	0.76 (0.69-0.99)	0.1556	0.87 (0.71-0.92)	0.1035
IL-17A	0.09 (0.07-0.19)	**<0.0001***	0.83 (0.68-1.38)	1.0000	0.50 (0.32-0.52)	0.2085	0.70 (0.60-1.60)	1.0000	0.54 (0.25-0.81)	0.3022	0.96 (0.34-1.24)	1.0000
IL-18	0.86 (0.59-1.03)	1.0000	0.75 (0.66-3.48)	1.0000	1.05 (0.74-1.11)	1.0000	0.90 (0.71-1.04)	1.0000	0.82 (0.78-0.90)	0.7341	0.93 (0.78-2.75)	1.0000
IL-36ß	0.94 (0.48-1.45	1.0000	0.98 (0.81-2.04)	1.0000	1.04 (0.82-1.47)	1.0000	1.88 (0.94-2.34)	1.0000	1.05 (0.78-1.37)	1.0000	1.21 (0.85-1.65)	1.0000
IL-10[1]	0.29 (0.20-0.41)	**0.0062***	1.12 (0.22-1.94)	1.0000	0.93 (0.37-1.05)	1.0000	0.81 (0.62-2.02)	1.0000	0.91 (0.55-1.42)	1.0000	0.62 (0.43-0.91)	0.6975
IL-25/17E[1]	0.52 (0.33-0.64)	**0.0043***	0.59 (0.48-0.95)	0.2517	0.50 (0.44-1.01)	0.0931	0.68 (0.55-0.88)	0.3208	0.67 (0.55-0.90)	0.2932	0.74 (0.52-0.90)	0.2440
IL-19[1]	0.68 (0.64-0.99)	0.9862	0.91 (0.51-1.67)	1.0000	0.69 (0.56-1.31)	1.0000	0.79 (0.48-0.92)	1.0000	0.77 (0.65-1.12)	1.0000	0.92 (0.51-1.52)	1.0000
IL-27[1]	0.63 (0.26-0.85)	0.0751	0.92 (0.72-1.01)	1.0000	0.95 (0.61-1.12)	1.0000	0.66 (0.64-1.02)	0.963	0.69 (0.62-0.83)	0.3609	0.72 (0.63-0.79)	0.2365
All[2] **(12)**	0.51 (0.28-0.64)	**<0.0001**	0.78 (0.54-0.91)	**0.0006**	0.70 (0.49-0.94)	**<0.0001**	0.85 (0.69-0.95)	0.0684	0.71 (0.57-0.79)	**0.0010**	0.81 (0.70-0.94)	**0.0121**
Pro-inflammatory (8)	0.45 (0.19-0.82)	NT	0.69 (0.29-1.18)	NT	0.64 (0.30-1.04)	NT	0.91 (0.53-1.43)	NT	0.71 (0.42-0.99)	NT	0.80 (0.58-1.22)	NT
Anti-inflammatory (4)	0.54 (0.29-0.70)	NT	0.87 (0.51-1.42)	NT	0.70 (0.46-1.11)	NT	0.69 (0.54-1.13)	NT	0.75 (0.61-1.05)	NT	0.74 (0.50-0.98)	NT
Important[3] **(5)**	0.23 (0.20-0.37)	**<0.0001**	0.43 (0.41-0.81)	**<0.0001**	0.46 (0.44-0.50)	**<0.0001**	0.89 (0.70-0.99)	0.0663	0.54 (0.54-0.59)	**<0.0001**	0.70 (0.70-0.87)	**0.0071**

p-values marked in bold indicate analytes that are significant at the 0.05 level. * Significant after correction with the Benjamini Hochberg test ($p < 0.0156$). [1] Cytokines with an anti-inflammatory function, note that IL-17E and IL-27 also have an immunoregulatory/pro-inflammatory function. [2] All: pooled effect of 12 cytokines. [3] Important: 5 key pro-inflammatory cytokines in the pathophysiology of HS; TNF-α ,IFN-γ, IL-1ß, IL-6, IL-17A. AMPs: antimicrobial peptides. HS: hidradenitis suppurativa. IQR: interquartile range. NT: not tested.Unadj: unadjusted.

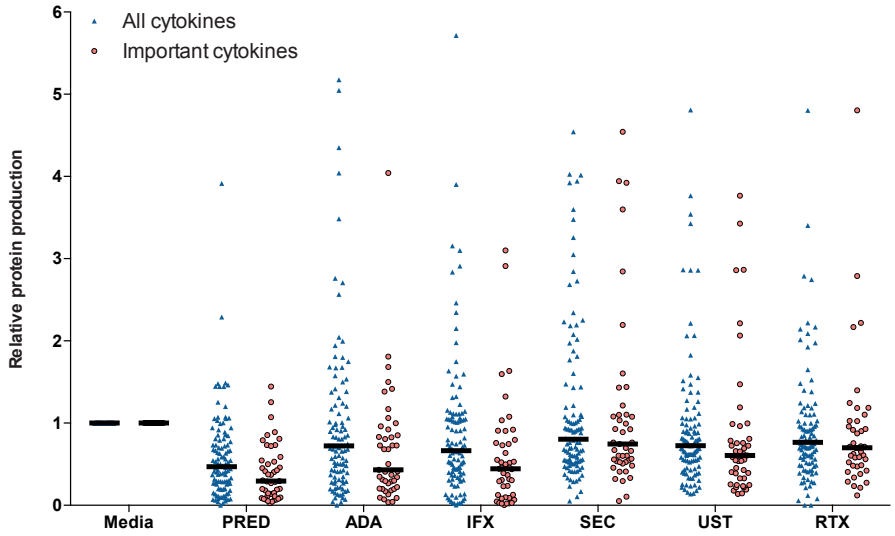

Figure 2. Anti-inflammatory impact on protein production of all cytokines and five important cytokines as measured by Luminex, n = 9. Important inflammatory cytokines: TNF-α, IFN-γ, IL-1ß, IL-6, IL-17A. Twelve data points are out of the y-axis range. Horizontal bars display the median. Media, culture media; PRED, prednisolone 100 µg/mL; ADA, adalimumab 30 µg/mL; IFX, infliximab 20 µg/mL; SEC, secukinumab 30 µg/mL; UST, ustekinumab 10 µg/mL; RTX, rituximab 200 µg/mL.

infliximab 0.70 (0.49-0.94); secukinumab 0.85 (0.69-0.95); ustekinumab 0.71 (0.57-0.79); rituximab 0.81 (0.70-0.94). Specifically the release of TNF-α, IFN-γ, IL-1ß, IL-6, and IL-17A was significantly inhibited by all tested drugs with the exception of secukinumab (Figure 2). In addition, prednisolone significantly inhibited the release of TNF-α, IFN-γ, IL-1ß, IL-6, IL-17A, IL-10, and IL-25/17E. As expected adalimumab and infliximab almost completely neutralised TNF-α levels in the supernatants (Figure 3). Of note, for the two important cytokines TNF-α and IL-17A we observed both a strong intra- and inter-patient variability among all biologic conditions (Figure 3).

Great variation in correlation between mRNA expression and protein production levels in HS samples

The cytokines IL-1ß, IL-6, IL-17A, and TNF-α, were measured by both real-time qPCR and Luminex. There was great variation in the correlation between mRNA and protein levels of these individual cytokines (Figure 4). A high correlation between mRNA and protein levels was found for IL-17A ($r = 0.86$; $p < 0.0001$), while the correlation for TNF-α was almost zero ($r = 0.04$; $p = 0.7480$).

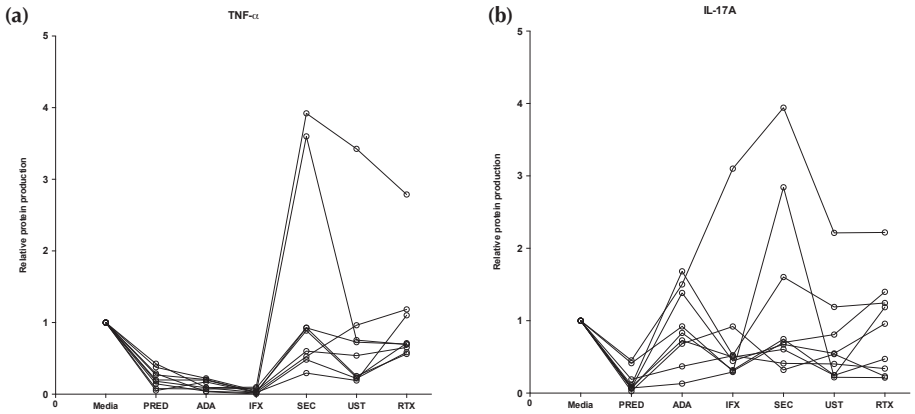

Figure 3. Inter- and intra-patient variability demonstrated by fluctuating protein levels of TNF-α and IL-17A, n = 9. **(a)** TNF-α. Connected dots represent one patient. High intra-patient variability as demonstrated by the fluctuating per patient protein levels between conditions. Low inter-patient variability for conditions PRED, ADA, IFX. **(b)** IL-17A. Connected dots represent one patient. Very high (more than TNF-α) intra-patient variability. Low inter-patient variability for conditions PRED. Media, culture media; PRED, prednisolone 100 µg/mL; ADA, adalimumab 30 µg/mL; IFX, infliximab 20 µg/mL; SEC, secukinumab 30 µg/mL; UST, ustekinumab 10 µg/mL; RTX, rituximab 200 µg/mL.

DISCUSSION

In this study we show that the commercially available biologics used in daily practice for the treatment of HS (adalimumab, infliximab, ustekinumab and rituximab), significantly inhibited mRNA and protein expression of various cytokines and AMPs in lesional HS skin, cultured *ex vivo* for 24 hours. Moreover, the anti-inflammatory effect of prednisolone and all biologics, except secukinumab, is the strongest on TNF-α, IFN-γ, IL-1ß, IL-6, and IL-17A, which are important cytokines in the pathogenesis of HS. In addition, prednisolone and TNF-α inhibitors seem the most effective in reducing the release of a broader range of pro-inflammatory cytokines and AMPs in lesional HS skin.

The strongest evidence in patients with moderate-to-severe HS has been documented for anti-TNF agents adalimumab and infliximab.[5,30-32] We confirmed the previously reported decrease of *ex vivo* IL-1ß protein levels after adalimumab treatment on the mRNA level.[7] As TNF-α is a multifunctional cytokine with numerous actions, a simultaneous downregulation of other cytokines, such as IL-6 and cytokines of the IL-1 family, could also explain the results of the anti-TNF agents in our study.[9,33] Our translational findings correspond with the observed efficacy of TNF-α inhibitors in HS daily practice. Inter-patient variability in the response to biologics could explain why some HS patients are clinically good responders while others show a lesser response.

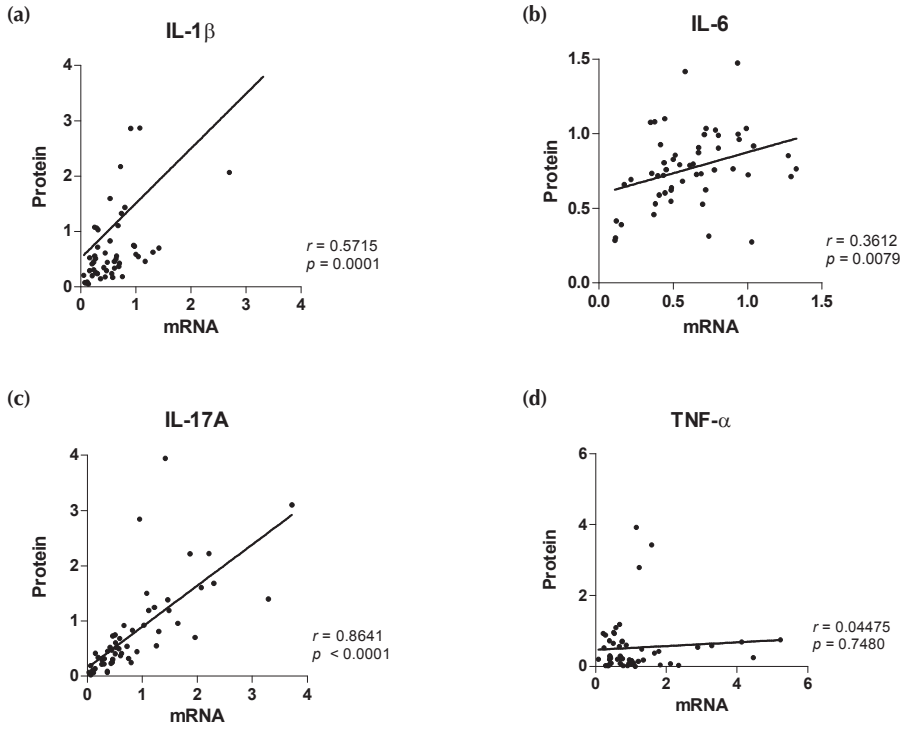

Figure 4. Correlation between the relative protein production (y-axis) and mRNA (x-axis) expression of HS lesional skin, n = 9. **(a)** IL-1ß. **(b)** IL-6. **(c)** IL-17A. **(d)** TNF-α. Values on x-axis and y-axis display the relative production and expression values of a cytokine. Abbreviations: r, correlation coefficient; *p*, *p*-value according to Spearman rho test.

Remarkably, secukinumab did not reduce the IL-17 protein levels in the same way as adalimumab and infliximab reduced that of TNF-α. Unfortunately, IL-12p40 protein, an important indicator of the IL-17 pathway, fell below the level of detection in the Luminex assay. It could be that other cytokines such as TNF-α and IL-1ß, not being blocked by secukimumab in this culture system were still able to induce production of IL-17. Moreover, levels of IL-17A were in the lower range of detection in most patients. Another explanation may be that the anti-IL-17A antibodies used in the Luminex assay detect a different epitope of IL-17A than recognised by secukinumab. The lower mRNA expression of AMPs, *IL-6* and *CXCL-8* may be considered as the result of blocking of IL-17A bioactivity by secukinumab.

The pan-cytokine inhibitory characteristics of prednisolone were demonstrated by inhibition of multiple cytokines on both the mRNA and protein level, which supports the efficacy of systemic and intralesional corticosteroids for acute HS flares in clinical practice.[34] However, prolonged high-dose systemic corticosteroids are not

recommended as HS rapidly flares after tapering, especially after a long course.[35] Nonetheless, low-dose systemic prednisolone could be a valuable adjunct therapy for recalcitrant HS.[36]

Rituximab was the only biologic without a significant inhibitory effect on individual inflammatory mRNA and protein levels. This is not surprising as B cell blockade in inflammatory diseases acts via inhibition of antibody production, antigen presentation and indirectly via cytokine reduction.[37] Therefore, the presence of the complete immune system is required for B cell blockade to be effective. Moreover, HS is considered primarily a disease of a deficient innate immunity. On the other hand, chronic HS lesions are full of B cells and plasma cells, indicating that adaptive humoral immunity is also activated in longstanding HS.[20,38]

The impact of biologics on AMP expression has never been investigated in lesional HS skin. Our findings indicate a potential supporting role for AMPs in HS pathophysiology as it is known that AMPs are capable of activating keratinocytes and attracting innate immune cells to amplify the local immune response.[39] Cytokines produced by innate and adaptive immune cells, such as TNF-α, IL-17, and IL-12, drive AMP production in the keratinocytes.[40-42] Moreover, AMPs can be activated by damage- and pathogen-associated molecular patterns (DAMPs/PAMPs) after follicle rupture with the release of keratin fibres and skin commensals in the dermis.

Although it is assumed that levels of mRNA and protein have a one-to-one correlation, the absence of correlation between TNF-α mRNA and its protein levels has been previously reported.[43-45] In addition, other factors such as the half-life of proteins and the degradation and stability of mRNA may vary widely.[43,46,47]

Major strengths of our study are the use of a standardised *ex vivo* transwell culture system and weighing each biopsy in order to normalise all protein levels for mg tissue weight. Cytokines were evaluated on both mRNA and protein level, including four cytokines that were assessed for validation purposes. Furthermore, the use of healthy control skin from regions that are suitable to function as control samples further increases the validity of our study. Possible limitations include the relatively small sample size, lack of dose-response relationships, and AMPs which have only been evaluated on the mRNA level.

In conclusion, prednisolone, adalimumab, infliximab, ustekinumab and rituximab significantly inhibited the expression levels of a selected panel of inflammatory cytokines and AMPs in *ex vivo* lesional skin of HS patients. The *ex vivo* model will enable studies with combinations of biologics, or targeting of novel important cytokines, alone or in combination with low-dose prednisolone.

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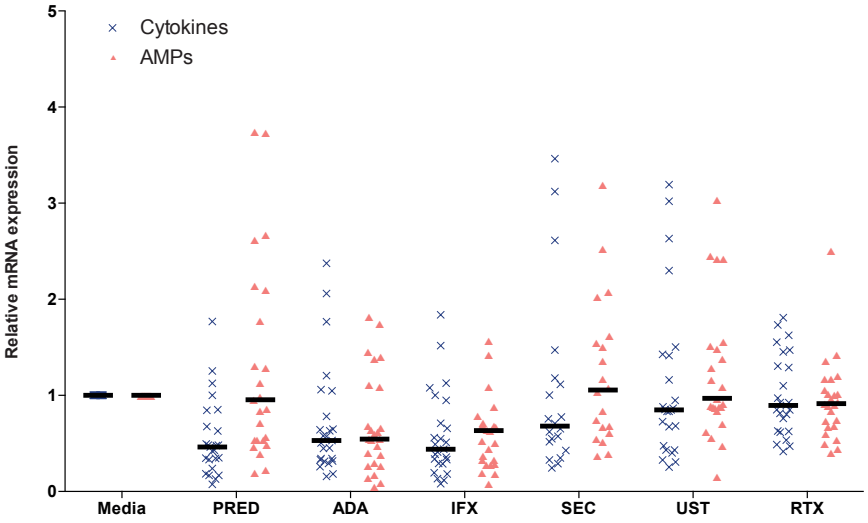
SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Relative mRNA expression of all cytokines (5) and all AMPs (5) in healthy control skin, n = 5. Two data points are out of the y-axis range. Horizontal bars display the median. Media, culture media; PRED, prednisolone 100 µg/mL; ADA, adalimumab 30 µg/mL; IFX, infliximab 20 µg/mL; SEC, secukinumab 30 µg/mL; UST, ustekinumab 10 µg/mL; RTX, rituximab 200 µg/mL.

Supplementary Table 1. Relative expression of mRNA and their modulation by biologics in healthy control skin.

	Healthy control skin (n = 5)											
	Prednisolone		Adalimumab		Infliximab		Secukinumab[1]		Ustekinumab		Rituximab	
	Median (IQR)	Unadj. *p*-value	Median (IQR)	Unadj. *p*-value	Median (IQR)	Unadj. *p*-value	Median (IQR)	Unadj. *p*-value	Median (IQR)	Unadj. *p*-value	Median (IQR)	Unadj. *p*-value
IL-1β	0.48 (0.43-0.63)	0.7616	0.50 (0.33-0.64)	0.9706	0.44 (0.34-0.66)	0.8102	0.90 (0.32-1.97)	1.0000	0.95 (0.43-2.63)	1.0000	0.62 (0.49-1.45)	1.0000
IL-6	0.18 (0.13-0.35)	**0.0168**	0.34 (0.31-1.05)	0.9146	0.36 (0.29-0.51)	0.4195	0.45 (0.32-0.61)	0.5504	0.43 (0.40-0.47)	1.0000	0.85 (0.63-1.10)	1.0000
CXCL-8 (IL-8)	0.35 (0.35-0.84)	0.2921	0.53 (0.45-0.57)	0.1324	0.39 (0.13-0.49)	**0.0187**	0.67 (0.54-0.85)	1.0000	0.85 (0.84-0.88)	1.0000	1.30 (0.97-1.55)	1.0000
IL-17A	NC		NC		NC		NC		NC		NC	
IL-23p19	NC		NC		NC		NC		NC		NC	
TNF-α	0.48 (0.33-0.48)	0.1324	0.65 (0.32-1.06)	1.0000	0.41 (0.33-0.71)	0.3639	0.86 (0.69-1.41)	1.0000	0.83 (0.73-1.43)	1.0000	0.93 (0.80-1.29)	1.0000
IFN-MXA	0.68 (0.49-1.13)	1.0000	0.63 (0.45-0.78)	0.4498	0.95 (0.56-1.00)	1.0000	0.92 (0.62-1.67)	1.0000	0.87 (0.85-1.50)	1.0000	0.85 (0.81-0.90)	1.0000
S100A7	1.28 (0.84-1.31)	1.0000	0.66 (0.63-1.11)	1.0000	0.79 (0.51-1.09)	1.0000	1.15 (0.73-1.79)	1.0000	1.09 (0.84-1.49)	1.0000	0.91 (0.74-1.01)	1.0000
S100A8	0.86 (0.72-0.99)	1.0000	0.55 (0.47-0.55)	0.3146	0.68 (0.29-0.88)	0.5516	1.15 (0.65-2.01)	1.0000	0.92 (0.88-1.39)	1.0000	1.20 (0.89-1.36)	1.0000
S100A9	0.95 (0.49-2.10)	1.0000	0.54 (0.30-0.60)	0.5695	0.81 (0.51-1.15)	0.5695	0.97 (0.89-1.52)	1.0000	1.02 (0.94-1.17)	1.0000	0.95 (0.49-2.10)	1.0000
LL-37	2.14 (0.40-3.73)	1.0000	0.64 (0.38-0.69)	0.5894	0.66 (0.65-0.68)	0.3146	1.11 (0.99-1.38)	1.0000	1.16 (0.88-1.28)	1.0000	0.74 (0.68-0.90)	1.0000
HBD-2	0.57 (0.54-1.13)	1.0000	0.40 (0.1-1.38)	0.7616	0.45 (0.38-0.53)	0.1989	1.06 (0.56-1.65)	1.0000	0.87 (0.47-2.46)	1.0000	0.69 (0.49-0.96)	1.0000
All[2] (10)	0.56 (0.40-1.13)	**0.0015**	0.55 (0.32-0.98)	**<0.0001**	0.51 (0.30-0.70)	**<0.0001**	0.77 (0.55-1.48)	0.7302	0.88 (0.69-1.47)	1.0000	0.91 (0.68-1.17)	0.9767
Cytokines (5)	0.46 (0.33-0.68)	NT	0.53 (0.33-0.78)	NT	0.44 (0.29-0.71)	NT	0.68 (0.49-1.13)	NT	0.85 (0.47-1.41)	NT	0.90 (0.63-1.30)	NT
AMPs[3] (5)	0.95 (0.54-2.10)	NT	0.55 (0.30-1.09)	NT	0.63 (0.33-0.68)	NT	1.06 (0.66-1.57)	NT	0.97 (0.87-1.52)	NT	0.91 (0.69-1.05)	NT

p-values marked in bold indicate analytes that are significant at the 0.05 level. None of the multiple tests performed with Benjamini Hochberg test yielded significant results. [1] One HS sample for secukinumab was excluded for Real-Time qPCR analysis as a result of a human error during the process of cDNA synthesis (n = 4). [2] Cytokines: pooled effect of IL-1ß, IL-6, IL-8, IL-17A, IL-23p19, TNF-α, IFN-MXA. [3] AMPs: pooled effect of S100A7, S100A8, S100A9, LL-37, HBD-2. IQR: interquartile range. NC: not calculated, non-reliable results due to low expression of these genes in healthy conditions. NT: not tested. Unadj: unadjusted.