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PH.D. THESIS

A NEW TH17 CYTOKINE IN HIDRADENITIS SUPPURATIVA: ANTIMICROBIAL AND PRO-INFLAMMATORY ROLE OF IL-26

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Summary

Interleukin (IL)-26 is a member of the IL-10 cytokine family which includes IL-10, IFN-λs (IL-28A/B and IL-29), and the IL-20 subfamily (IL-19, IL-20, IL-22, IL-24, and IL-26). It is mainly expressed in Th17 cells and plays a dual role. Indeed, IL-26 is a pro-inflammatory cytokine that exerts antimicrobial response producing direct bactericidal action through affecting the formation of membrane pores. IL-26 acts through IL-10R2/IL-20R1 heterodimeric receptor. While IL-10R2 is broadly expressed, IL-20R1 is mainly expressed by epithelial cell types, including keratinocytes, suggesting its possible role in the immune response of the skin. To date, IL-26 has been implicated in psoriasis and allergic contact dermatitis (ACD). In particular, IL-26 contribute to the cytotoxic mechanism, proper of ACD. This mechanism is responsible for the tissue injury of immune-mediated skin diseases, including Hidradenitis Suppurativa (HS). The latter is characterized by recurrent, painful, nodules and abscesses that break, leading to the formation of sinus tracts and scarring. Lesions usually affect apocrine gland-bearing anatomical areas of the body involving build-up of keratin within the hair follicle that plugs the follicle causing the occlusion and subsequent rupture of pilosebaceous unit. Morever, colonies of specialized bacteria (mainly, staphylococci and streptococci) bind irreversibly to sinus tract epithelium and hair follicles, further sustaining chronic inflammation. In this study, we have evaluated IL-26 in HS exploring its antimicrobial activity.

CHAPTER I

IL-26 cytokine

Interleukin (IL)-26 is an emerging member of IL-10 family cytokines. It was initially named as AK155 following its identification in herpesvirus saimiri-transformed human T cells (Knappe *et al*., 2000). T helper (Th)17 cells are predominant sources of IL-26 (Donnelly *et al.*, 2010; Stephen-Victor *et al*., 2016). The *IL-26* gene is located on chromosome 12q15 and is flanked by genes encoding for two important cytokines, IL-22 and IFN-γ (Stephen-Victor *et al*., 2016). Secreted IL-26 is a 19-kDa protein containing 171 amino acids with approximately 25% homology and 47% similarity to human IL-10. The protein is largely cationic (~20%), resulting in a positive charge (Stephen-Victor *et al*., 2016). IL-26 comprises six helices with a capacity to form dimers and higher-order multimers (Knappe *et al*., 2000). IL-26 signals via the heterodimeric IL-20R1/IL-10R2 receptor and induces Janus kinase-signal transducer and activator of transcription (JAK-STAT) activation, resulting in STAT1 and STAT3 phosphorylation (Hör *et al*., 2004; Sheikh *et al*., 2004). Current evidence suggests that IL-26 recognizes IL-20R1 directly, whereas IL-10R2 helps in the proper assembly of functional IL-26 receptor complex (Hör *et al*., 2004; Stephen-Victor *et al*., 2016). Although the distribution of IL-10R2 is broad, only a subset of cells, particularly epithelial cells and keratinocytes, expresses IL-20R1 (Hör *et al*., 2004). The restricted expression pattern of IL-20R1 suggests a possible role of IL-26 in the immune response of the skin.

Biological functions of IL-26

Despite the structural similarity with the archetype anti-inflammatory cytokine IL-10, and the sharing of one of its heterodimer receptor subunits, functional studies are compatible with IL-26 driving or sustaining inflammation rather than suppressing it (Tengvall *et al*., 2016).

Recent important studies have demonstrated that IL-26 is not strictly a pro-inflammatory cytokine but can also act as a carrier for extracellular DNA (Meller *et al*. 2015; Poli *et al*., 2017) and as an antimicrobial molecule (Meller *et al*., 2015), thanks to its particular biochemical properties. As shown by Larochette *et al*., 2019, the binding of IL-26 to the IL-20R1 and IL-10R2 chains induces the production of inflammatory cytokines. Moreover, IL-26 can also act as a carrier molecule allowing extracellular DNA to get access to intracellular nucleic sensors. Both pathways induce the production of inflammatory cytokines, chemokines, and type I and type II interferons by selected immune and non immune cells. IL-26 also acts as an antimicrobial protein (AMP) through its capacity to form pores in bacterial membranes (Figure 1).

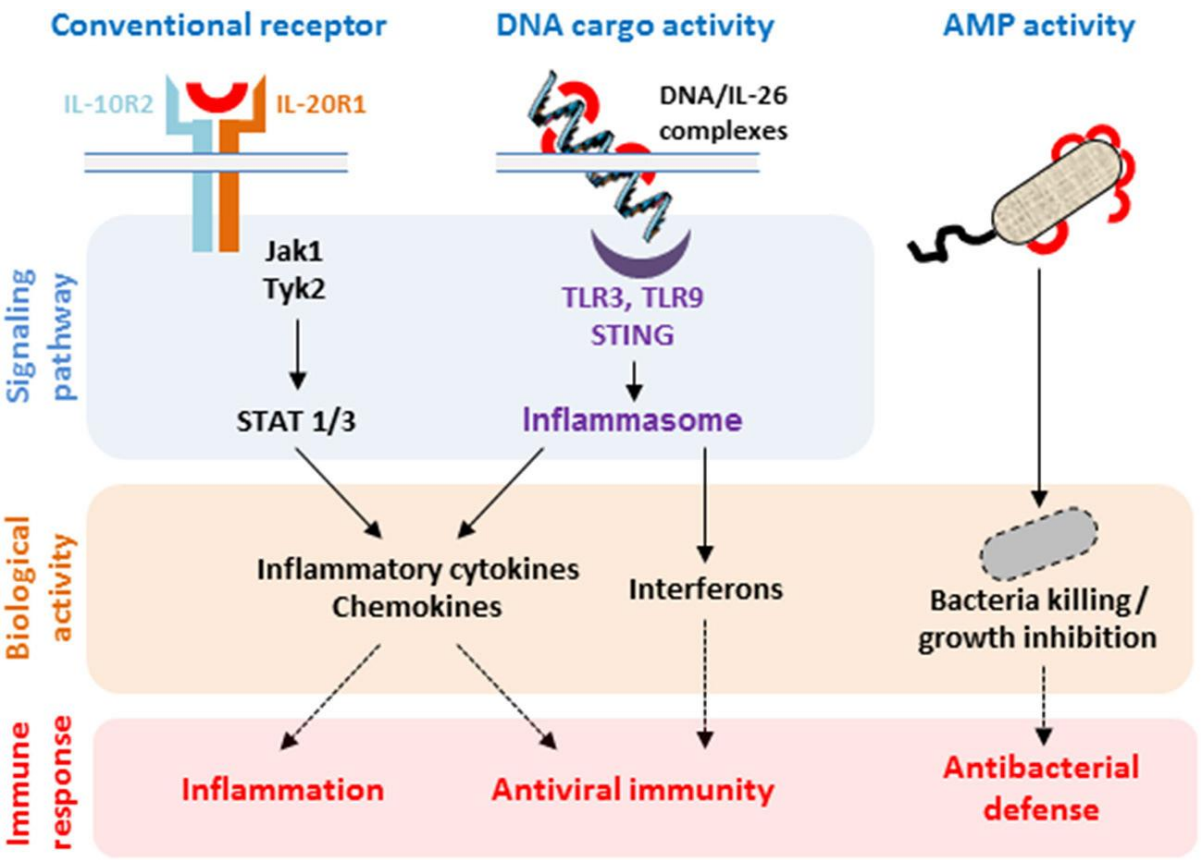

Figure 1. Biological functions of IL-26. Retrieved from "IL-26, a Cytokine With Roles in Extracellular DNA-Induced Inflammation and Microbial Defense" by Larochette *et al*., 2019.

IL-26 and human diseases

Several studies have suggested a potential role for IL-26 in the pathophysiology of chronic inflammatory disorders (Dambacher *et al*., 2009; Corvaisier *et al*., 2012; Meller *et al*., 2015; Miot *et al*., 2015; Fujii *et al*. 2017). *IL26* gene polymorphisms are associated with an increased risk for developing multiple sclerosis (Goris *et al*., 2002) or rheumatoid arthritis (RA) (Vandenbroeck *et al*., 2003). Moreover, elevated levels of circulating and tissue-associated IL-26 are reported in Crohn's disease (Dambacher *et al*., 2009; Fujii *et al*. 2017), psoriasis (PSO) (Meller *et al*., 2015) and allergic contact dermatitis (ACD) (Caiazzo *et al*., 2018). In particular, we have previously shown that IL-26 contribute to the cytotoxic mechanism, proper of ACD (Caiazzo *et al*., 2018). This mechanism is responsible for the tissue injury of several immune-mediated skin diseases including hidradenitis suppurativa (HS) (Boer et al., 1996; Napolitano *et al*., 2017).

CHAPTER II

Hidradenitis Suppurativa

Hidradenitis Suppurativa (HS) is a chronic, inflammatory, debilitating skin disease characterized by recurrent, painful, nodules and abscesses that rupture, leading to the formation of sinus tracts and scarring (Kurzen *et al*., 2008; Napolitano *et al*., 2017; Kridin *et al*., 2019). Lesions usually affect apocrine gland-bearing anatomical areas of the body such as axillae, inguinal and anogenital regions (Figure 2).

HS typically occurs after puberty, with the average age of onset in the second or third decades of life and with a female predominance (Napolitano *et al*., 2017). The exact prevalence of HS remains unknown: in Europe, several studies have estimated a prevalence of 1% in the general population (Jemec *et al*., 2006; Revuz *et al*., 2008) and of 4% in young adult women (Jemec, 1988), whereas epidemiological data from American surveys reported a prevalence between 0.05% and 0.20% (Vazquez *et al*., 2013; Cosmatos *et al*., 2013). Due to its chronic nature and frequently occurring relapses, HS has a great impact on the patient's quality of life, deeply affecting social, working, and psychological aspects (Napolitano *et al*., 2017).

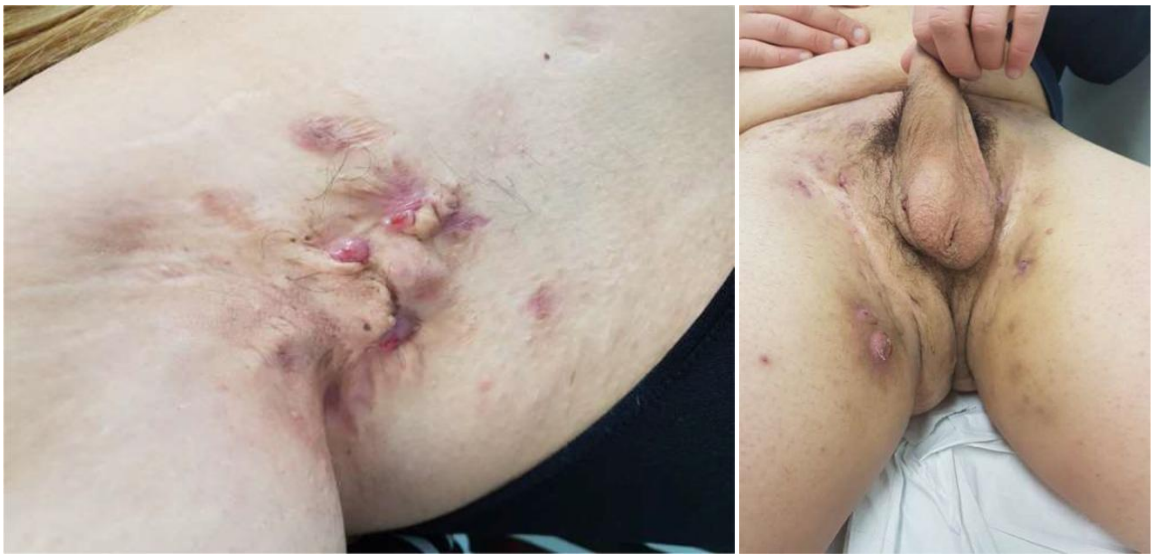

Figure 2. Hidradenitis suppurativa: clinical aspects. Retrieved from "Hidradenitis suppurativa: from pathogenesis to diagnosis and treatment" by Napolitano *et al*., 2017.

Etiology and pathogenesis

The exact etiology of HS is still unproven. In the last few years, numerous studies (Kurzen *et al*., 2008; de Winter K *et al*., 2012; Pink *et al*., 2012; Vazquez *et al*., 2013; Schrader *et al*. 2014; Zouboulis *et al*., 2015) hypothesized that the disease is triggered by genetic and environmental factors (Figure 3).

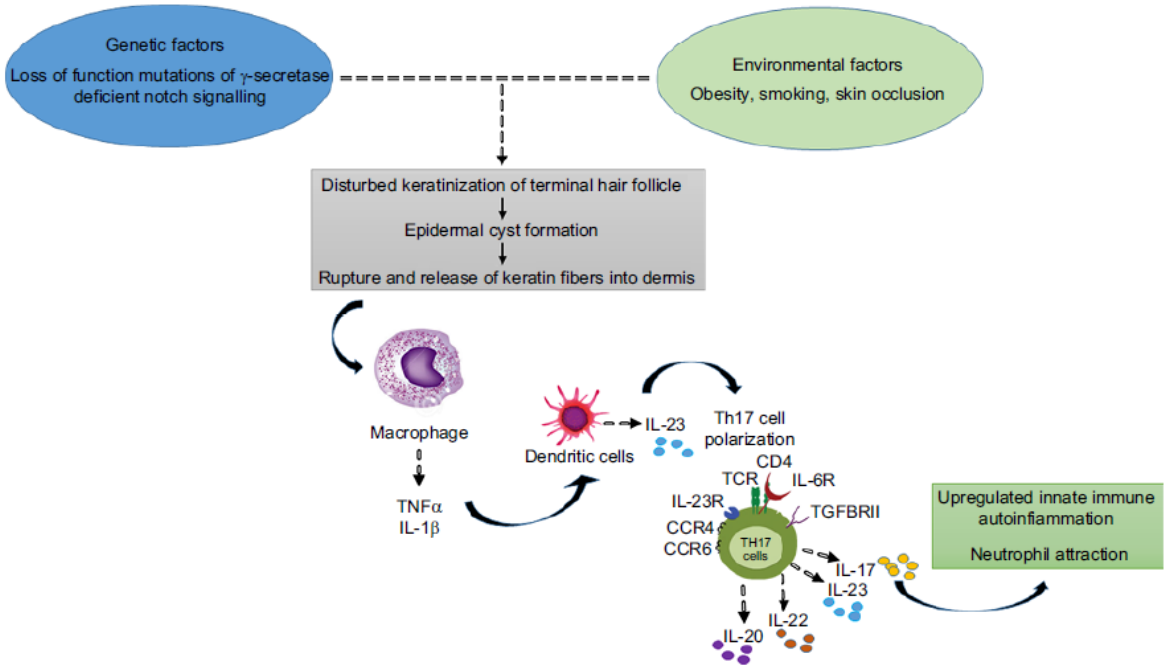

Figure 3. Hidradenitis suppurativa pathophysiology: a schematic overview. Retrieved from "Hidradenitis suppurativa: from pathogenesis to diagnosis and treatment" by Napolitano *et al*., 2017.

Cigarette smoking, obesity, and overweight are eminent environmental risk/trigger factors in HS development (Napolitano *et al*., 2017). At the same time, the importance of genetic factors is highlighted by studies showing that 30%–40% of HS patients reported a family history of HS (Fitzsimmons *et al*., 1985; Jemec *et al*., 2006; Pink *et al*., 2012). Moreover, familial forms of HS following an autosomal dominant pattern of inheritance with 100%

penetrance have been described in different populations, being linked to mutations in subunits of the gamma-secretase proteins (up to 5% of HS cases) (Wang *et al*. 2015; Pink *et al*., 2013; Scheinfeld, 2013). Deficiency in the gamma-secretase/Notch pathway results in conversion of hair follicles to keratin-enriched epidermal cysts, compromises apocrine gland homoeostasis, and leads to the stimulation of toll-like receptor (TLR)-mediated innate immunity, supporting and maintaining chronic inflammation (Rangarajan *et al*., 2001; Pan *et al*., 2004; Napolitano *et al*., 2017). Altered TLRs signaling on macrophages and dendritic cells (DCs), the most abundant cells in HS lesions, produces increased amounts of these cytokines, leading to activation of DCs, which secrete IL-23 promoting Th17 cell polarization (IL-17-producing T helper cells were found to infiltrate the dermis in chronic HS lesions) (Re *et al*., 2001; Giamarellos-Bourboulis *et al*., 2007; Hunger *et al*. 2008; Schlapbach *et al*. 2001; van der Zee *et al*., 2012; Zhang *et al*., 2012).

Although HS is not primarily an infectious disease, the role of bacteria seems to be very important in HS pathophysiology (Ring *et al*., 2015; Napolitano *et al*., 2017; Kridin *et al*., 2019). Follicular hyperkeratinization and occlusion result in the rupture of pilosebaceous units, releasing bacteria within the dermis and triggering a local inflammatory response and thereby sustaining chronic inflammation (Napolitano *et al*., 2017; Ghias *et al*., 2019). In addition, colonies of specialized bacteria that are difficult to eradicate form bacterial biofilms that bind irreversibly to sinus tract epithelium and hair follicles, further sustaining chronic inflammation (Kathju et al., 2012; Jahns *et al*., 2014). A microbiological study of HS lesions showed S*taphylococcus aureus* (*S. aureus*) is one of the most prevalent species (Guet-Revillet *et al*., 2014; Ring *et al*., 2015; Kridin *et al*., 2019). Other common species dominating HS lesions included polymicrobial anaerobic microflora consisting of strict anaerobes, milleri group streptococci, and actinomycetes (found in 24% of abscesses or

nodules and in 87% of chronic suppurating lesions) (Guet-Revillet *et al*., 2014; Ring *et al*., 2015; Napolitano *et al*., 2017; Kridin *et al*., 2019).

Therapeutic weapons against HS include antibiotics (i.e., clindamycin, rifampicin, and tetracycline), non biological systemic therapy (i.e., cyclosporine), and biological therapy with adalimumab (human anti-TNF-α monoclonal antibody) which is the only FDA-approved biologic for moderate to severe HS (Bettoli *et al.*, 2016; Napolitano *et al*., 2017; Saunte *et al*., 2017).

CHAPTER III

Experimental Design

Objective

Investigation of IL-26 in HS, through its involvement in the antimicrobial activity.

Materials and Methods

Study population

The overall study population included 30 patients [8 affected by atopic dermatitis (AD), 12 affected by HS and 10 affected by PSO] and 30 age- and sex-matched healthy control (HC) volunteers. All participants were recruited from the database of patients attending the dermatology unit of the University of Naples Federico II, Italy. Inclusion criteria were diagnosis of moderate-to-severe AD (Scoring Atopic Dermatitis ≥ 25), HS (Sartorius score ≥ 30) or psoriasis (Psoriasis Area and Severity Index ≥ 10); disease duration of at least 6 months; a topical and/or systemic treatment washout period of ≥ 3 weeks and age ≥ 18 years. Patients underwent lesional skin biopsies (3 mm) and blood extraction. For controls, blood and skin samples were taken from healthy donors. The inclusion criterion for healthy volunteers was age ≥ 18 years, and exclusion criteria were a present or past positive history of inflammatory skin disorders. The experimental protocol was performed according to the current version of the Declaration of Helsinki, and each participant gave written informed consent before the onset of the study. The clinical and demographic characteristics of the patients with HS are summarized in Table 1. The disease severity of the HS population was defined according to Sartorius score (Sartorius *et al*., 2003) and International Hidradenitis Suppurativa Severity Score System (iHS4) scale (Zouboulis *et al*., 2017). None of the recruited patients presented a Sartorius score below 50 or higher than 95. In addition, the

patients had an IHS4 score ranging from 4 to 10 points. Thus, our study is focused on patients with moderate HS.

Table 1. Clinical and demographic characteristics of HS patients (n=12)

Male, n %	3 (25)
Age (years)	27.3 ± 8.32
Disease duration (years)	7.42 ± 4.19
Family history (yes), n (%)	2 (17)
Body mass index (kg/m^2)	28.4 ± 3.6
iHS4	6.25 ± 2.49
Sartorius score	61.1 ± 10.1

Data are given as the mean ± SD unless stated otherwise, iHS4, International Hidradenitis Suppurativa Severity Score System.

PBMC preparation and stimulation by toxic shock syndrome toxin-1

Blood samples were used for plasma and peripheral blood mononuclear cell (PBMC) isolation. IL-26 plasma levels from patients with HS (n=12) and healthy controls (n=12) were measured by enzyme-linked immunosorbent assay (ELISA) (Cusabio, Wuhan, China). PBMCs from healthy controls (n=8) and patients with HS (n=8) were separated by densitygradient centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). For silencing experiments, PBMCs were transfected with 3 µmol/L Riboxx small interfering (si)RNA oligo (Qiagen, Venlo, the Netherlands) using electroporation (Amaxa Inc., Gaithersburg, MD, U.S.A.). IL-26 siRNA target sequence or control siRNA oligonucleotide with scrambled sequence was used. More than 80% of cells remained viable after the transfection procedures, as determined by trypan blue exclusion.

PBMCs silenced or not were placed in 12-well plates (1x10^6 per well) and cultured in Roswell Park Memorial Institute (RPMI) medium (Thermo Fisher Scientific Inc., Waltham,

MA, U.S.A.) supplemented with 10% fetal bovine serum in the presence or absence of toxic shock syndrome toxin (TSST)-1 (Sigma-Aldrich, Oakville, ON, Canada) at a final concentration of 100 ng/mL. Subsequently, mRNA was extracted from PBMCs for examination through quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) of selected genes. Culture supernatants were harvested and stored at -80 °C, and the presence of IL-26 was checked by ELISA and antimicrobial assay.

Antimicrobial assay

To evaluate the bactericidal effect of the collected supernatants, they were placed into a 96-well microtitre plate at an initial concentration of 4000 µg/mL. Then they were serially diluted using Luria Bertani (LB) medium (twofold dilution). The final concentrations of samples were 2000, 1000, 500, 250 and 125 µg/mL. A single colony of the selected pathogen strains, *S. aureus* ATCC 6538 or *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853, was used to inoculate 3 mL of liquid LB medium in a sterile bacteriological tube. Dimethyl sulfoxide (2% v/v) was used as a negative control. After 5 h of incubation, growth was measured by monitoring the absorbance at 600 nm, and about 0.04 optical density per mL in 100 µL was dispensed into each well of the prepared plate, reaching a final volume of 200 µL. Plates were incubated at 37 °C for 24 h and growth was measured with a Cytation3 Plate Reader (Biotek, Winoosky, VT, U.S.A.) by monitoring the absorbance at 600 nm. The antimicrobial activity of the samples is reported as the IC_{50}, the half maximal inhibitory concentration.

Cytotoxicity assay

HaCaT target cells (immortalized human keratinocytes) were plated (2500 per well) in 96-well flat-bottom plates in the presence or absence of *S. aureus* as described previously (Fusco

et al., 2017). They were co-cultured with PBMCs (healthy controls, n=8; HS, n=8) transfected or not at various target–effector ratios for 5 h at 37 °C. Next, supernatants were checked for the presence of lactate dehydrogenase (Pierce LDH Cytotoxicity assay; Thermo Fisher Scientific Inc.) according to the manufacturer's protocol.

Phagocytosis assay

PBMCs, transfected or not (healthy controls, n=8; HS, n=8), were cultured to adhere to 96-well plates maintaining the same effector ratios used for the cytotoxicity tests. After 30 min, non-adherent cells were removed by repeated washes in RPMI medium, as described previously (Wang *et al*., 2017) Then, 10^8 colony forming units (CFU) per mL of *S. aureus* was added to the plates before incubation at 37 °C for 1 h. A control test in the absence of PBMCs was carried out. Adherent cells were lysed with 0.1% Triton X-100. Solubilized bacteria were counted by spreading serial dilutions on trypticase soy agar plates and incubation at 37 °C overnight. The bacterial survival index was calculated with the equation: CFU number in the experimental test divided by CFU number in the control test. A survival index ≥ 1.0 represented bacteria that were not killed by

PBMCs.

***In vivo* skin analysis**

Skin biopsies (3 mm, each) from involved sites of patients affected by AD (n=8), HS (n=10) or PSO (n=10), as well as from normal skin of healthy controls (n=20), were used for mRNA examination through qRT-PCR (LightCycler; Roche, Indianapolis, IN, U.S.A.) of *IL26*, *DEFB4A* [encoding human β-defensin (HBD)2] and *DEFB103A* (encoding HBD3). The amount of mRNA for a given gene in each sample was normalized to the amount of mRNA

of the 18S reference gene. Fold induction of gene expression was calculated using the ΔΔCT method.

Ex vivo skin organ cultures

Three skin biopsies (3 mm, each) obtained from involved sites of each patient with HS (n=9), as well as from normal skin of healthy controls (n=9), were placed in 12-well culture plates (Becton Dickinson Co., Franklin Lakes, NJ, U.S.A.) with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 2 mmol/L L-glutamine (all Thermo Fisher Scientific Inc.). Biopsies were treated in the presence or absence of IL-26-neutralizing antibody or respective isotype IgG control (R&D Systems Inc., Minneapolis, MN, U.S.A.) at a final concentration of 5, 10 or 15 µg/mL. Specimens were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. mRNA was extracted from each sample for qRT-PCR analysis of selected genes.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, U.S.A.). Data were analyzed with Wilcoxon matched-pairs test or Mann–Whitney test to calculate statistical differences, and correlations were evaluated with Pearson's rho test. Values of $P < 0.05$ were considered significant. Data are expressed as the mean ± SD.

Results

Il-26 acts as a pro-inflammatory and antimicrobial mediator in healthy controls

Healthy control PBMCs were stimulated with TSST-1, a superantigen secreted by *S. aureus*. This stimulation was able to induce an increase of IL-26 at both the gene and protein level in control PBMCs ($P < 0.05$ and $P < 0.001$, respectively) (Figure SM1; see Supplementary Material). To confirm IL-26 induction after infection, healthy control PBMCs were transfected with IL26 siRNA or empty vector and stimulated with TSST-1. The transfection was able to reverse IL-26 induction (Figure SM1; see Supplementary Material). Expression of the genes encoding IL-1b, IL-23 and IL-17A was significantly decreased in IL-26-transfected cells after TSST-1 stimulation compared to nontransfected cells ($P < 0.05$) (Fig. SM2; see Supplementary Material). No significant changes were observed in terms of gene modulation when healthy control PBMCs were transfected with empty vector (data not shown).

Considering the bactericidal role of IL-26 against several bacteria (Stephen-Victor *et al*., 2016; Meller *et al*., 2015) increasing concentrations of supernatants (from 125 to 1000 µg/mL) from healthy control PBMCs in resting, TSST-1-stimulated and IL-26-silenced conditions were incubated with *P. aeruginosa* or *S. aureus*. The results shown in Table 2 demonstrate that supernatants were totally inactive against *P. aeruginosa*, whereas *S. aureus* growth was inhibited by supernatants of healthy control PBMCs previously stimulated with TSST-1 (IC_{50} value 125 µg/mL). A slight bioactivity against *P. aeruginosa* was observed with supernatants from stimulated healthy control PBMCs (at higher concentrations ranging from 2000 to 4000 µg/mL), but without reaching an IC_{50} value (data not shown). Supernatants from resting samples, and silenced healthy control PBMCs with or without TSST-1 stimulation showed no inhibitory effects on *S. aureus* growth (Table 2).

Table 2. Antimicrobial activity of supernatants from resting, silenced HC or HS PBMCs with or without TSST-1 (100 ng/mL) stimulation against *P. aeruginosa* and *S. aureus*

	IC_{50} value	
Samples	*P. aeruginosa*	*S. aureus*
HC resting	NI	NI
HC + TSST-1	NI	125 µg/mL
HC IL-26 siRNA	NI	NI
HC IL-26 siRNA + TSST-1	NI	NI
HS resting	NI	NI
HS + TSST-1	NI	NI
HS IL-26 siRNA	NI	NI
HS IL-26 siRNA + TSST-1	NI	NI

Bacterial growth was measured as the optical density at 600 nm per mL. HC, healthy controls; HS, hidradenitis suppurativa; IL, interleukin; NI, no inhibition; si, small interfering.

IL-26 is increased in HS patients

We quantified IL-26 by ELISA in the plasma of patients with HS and healthy controls. IL-26 was significantly increased in patients with HS compared with controls ($P < 0.001$) (Figure 4A). As shown in Figure SM3 (see Supplementary Material), increasing IL-26 concentration significantly correlated with disease severity ($r=0.6$, $P=0.02$). Cultured PBMCs of patients with HS produced higher basal levels of IL-26 than PBMCs from healthy controls ($P < 0.05$) (Figure 4B). TSST-1 stimulation induced a comparable increase of IL-26 in both HS and control PBMCs (Figure 4B). IL-26 silencing was able to shut down IL-26 production in HS PBMCs (TSST-1 stimulated or not) and in TSST-1-stimulated healthy control PBMCs (Fig. 4B).

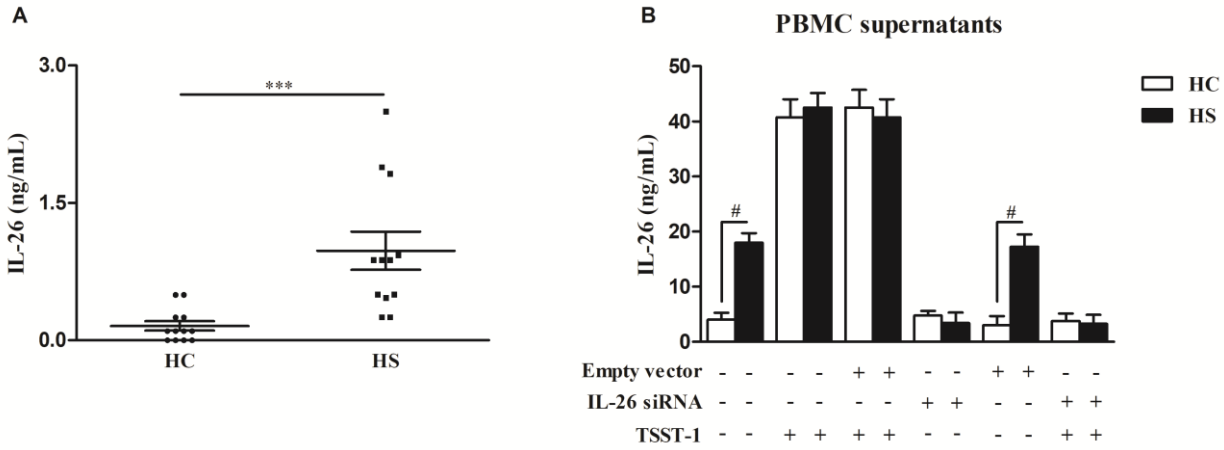

Figure 4. (A) IL-26 plasma levels in 12 patients with hidradenitis suppurativa (HS) and 12 healthy controls (HC). Data are displayed as the mean ± SD. Statistical significance was assessed using the Mann–Whitney test. ***P <0.001. (B) IL-26 protein detection in supernatants derived from cultured HC (n=8) and HS (n=8) peripheral blood mononuclear cells (PBMCs) without or with IL26 small interfering (si)RNA, empty vector or toxic shock syndrome toxin (TSST)-1 (100 ng/mL). Data are displayed as the mean ± SD. #P <0.05 calculated using the Mann–Whitney test.

Antimicrobial activity of IL-26 in HS

P. aeruginosa or *S. aureus* were incubated with increasing concentrations of supernatants (from 125 to 1000 µg/mL) from HS PBMCs in resting, TSST-1-stimulated and IL-26-silenced conditions to assess IC_{50} values. The results in Table 2 show that supernatants from resting and silenced HS PBMCs were totally inactive for both bacterial strains. Likewise, HS PBMC supernatants previously stimulated with TSST-1 did not inhibit *S. aureus* growth as previously observed in healthy controls (Table 2). It is noteworthy that the results from silencing experiments performed with empty vector were comparable with those using unsilenced conditions (data not shown).

Cytotoxic role of IL-26 in healthy controls and HS patients

HC and HS PBMC cytotoxicity mechanisms were evaluated *vs*. HaCaT cells. The activity of HC PBMCs was comparable with that of HS PBMCs (Figure 5A). IL-26 transfection was able to reduce significantly the capacity of HC PBMCs to kill the target cells (Figure 5A). Conversely, no significant differences were detected in silenced HS PBMCs compared with unsilenced ones (Figure 5A). Cytotoxicity mechanisms in HC and HS PBMCs were evaluated also against HaCaT cells infected by *S. aureus*. HS PBMC cytotoxicity was significantly decreased compared with control PBMCs at almost all analysed ratios (Figure 5B). Silenced control PBMCs dramatically lost the ability to kill target cells compared with unsilenced control PBMCs. On the other hand, silenced HS PBMCs showed no significant difference compared with HS unsilenced cells (Figure 5B). No significant difference was observed between the cells receiving the empty vector and those not transfected (data not shown). The killing activity of healthy control and HS PBMCs *vs*. *S. aureus* was thoroughly evaluated by phagocytosis tests. At a target–effector ratio of 1:8, HS PBMCs showed a lower phagocytic activity than PBMCs from healthy donors ($P < 0.05$) (Figure 6). Interestingly,

silencing experiments were able to reduce significantly the phagocytosis of control PBMCs ($P < 0.05$), whereas no significant differences were observed between silenced and unsilenced HS PBMCs (Figure 6). The same outcomes were obtained at all examined target–effector ratios (data not shown). No difference was found between the cells receiving the empty vector and those not transfected (data not shown).

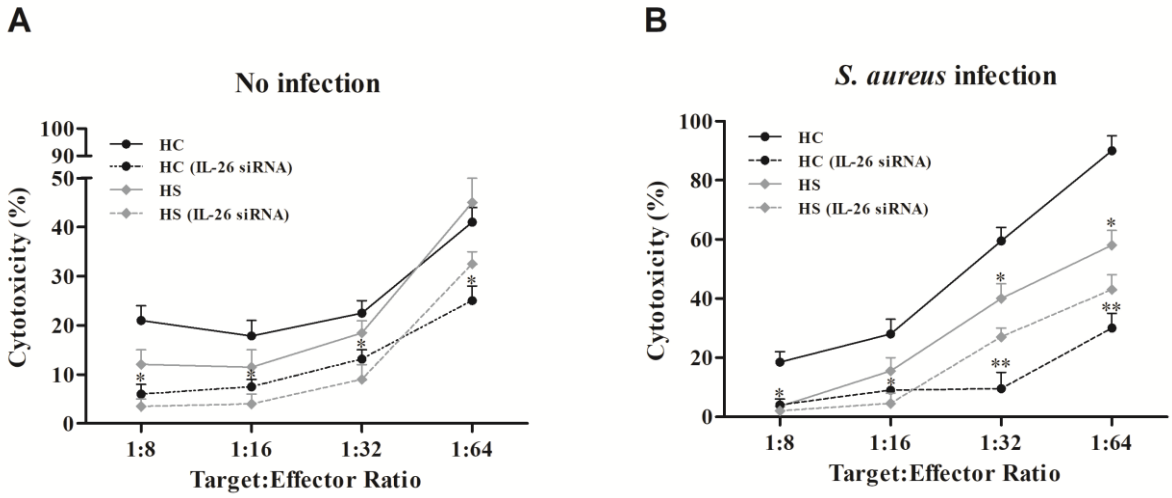

Figure 5. Lactate dehydrogenase release to measure the percentage of cytotoxicity of peripheral blood mononuclear cells (PBMCs) from healthy controls (HC) (n = 5) and patients with hidradenitis suppurativa (HS) (n = 5). PBMCs were transfected or not with IL26 small interfering (si)RNA *vs*. HaCaT cells (A) without or (B) with *Staphylococcus aureus* infection. Data are displayed as the mean ± SD. Statistical significance was assessed using the Wilcoxon matched-pairs test (transfected vs. nontransfected) or Mann–Whitney test (HS vs. HC nontransfected). *P <0.05, **P <0.01.

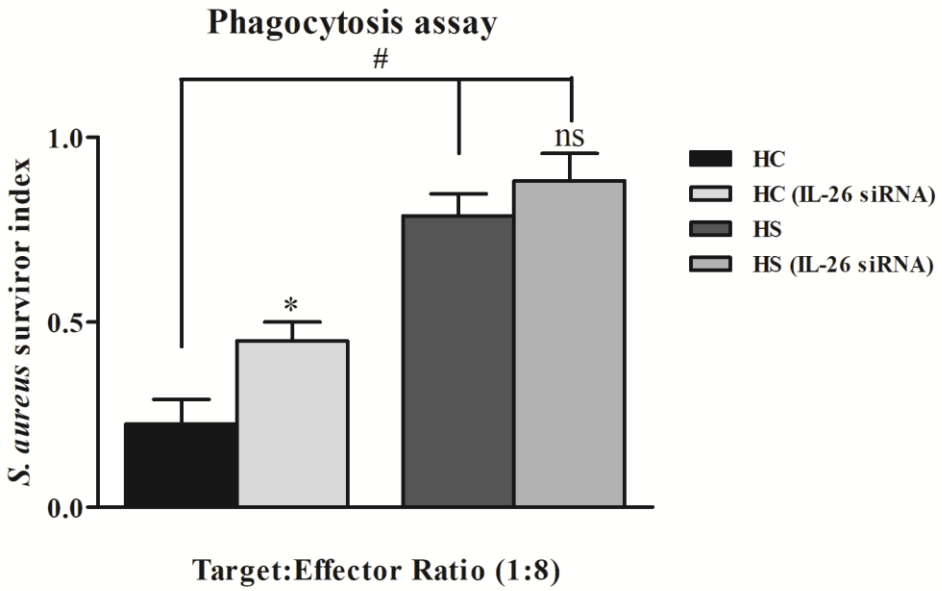

Figure 6. Survival index of *Staphylococcus aureus* in the presence of peripheral blood mononuclear cells (PBMCs) transfected or not with IL26 small interfering (si)RNA from healthy controls (HC) (n=5) and patients with hidradenitis suppurativa (HS) (n=5). Data are displayed as the mean ± SD. Statistical significance was assessed using the Wilcoxon matched-pairs test (transfected *vs*. nontransfected) or Mann–Whitney test (HS *vs.* HC). *P <0.05 (transfected *v*s. nontransfected), #P <0.05 (HS *vs*. HC nontransfected). ns, not significant.

Involvement of IL-26 in HS, PSO and AD skin

IL-26 gene expression was evaluated in clinically different skin conditions to uncover its involvement in chronic inflammatory skin disorders. IL26 expression was significantly enhanced in HS, PSO and AD lesional skin compared with healthy skin ($P < 0.05$) (Figure 7A). A slight increase, but not significant, of IL-26 expression was observed in HS compared with PSO and AD skin (Figure 7A). HBD2 and HBD3 were significantly enhanced in all diseases compared with healthy control skin (Figure 7A). Interestingly, a relative significant decrease was found for both HBD2 and HBD3 in HS *vs*. PSO and AD skin ($P < 0.01$), whereas this was not detected for IL-26 (Figure 7A).

Pro-inflammatory role of IL-26 in HS skin

HS lesional skin was used to perform an *ex vivo* organ culture in the presence and absence of IL-26-neutralizing antibodies or IgG isotype (5, 10 or 15 µg/mL). Gene expression of IL-1β and IL-6 was significantly decreased by treatment with IL-26 antibody (15 µg/mL) ($P < 0.05$), whereas IL23A and IL-17A were not affected (Figure 7B). Regarding antimicrobial peptides (AMPs), IL-26 inhibition was able to reduce significantly the expression of both HBD2 and HBD3 ($P < 0.05$) (Fig. 7C). No significant differences were observed when HS skin biopsies were treated with IgG isotype control or with lower concentrations of IL-26 antibody (data not shown).

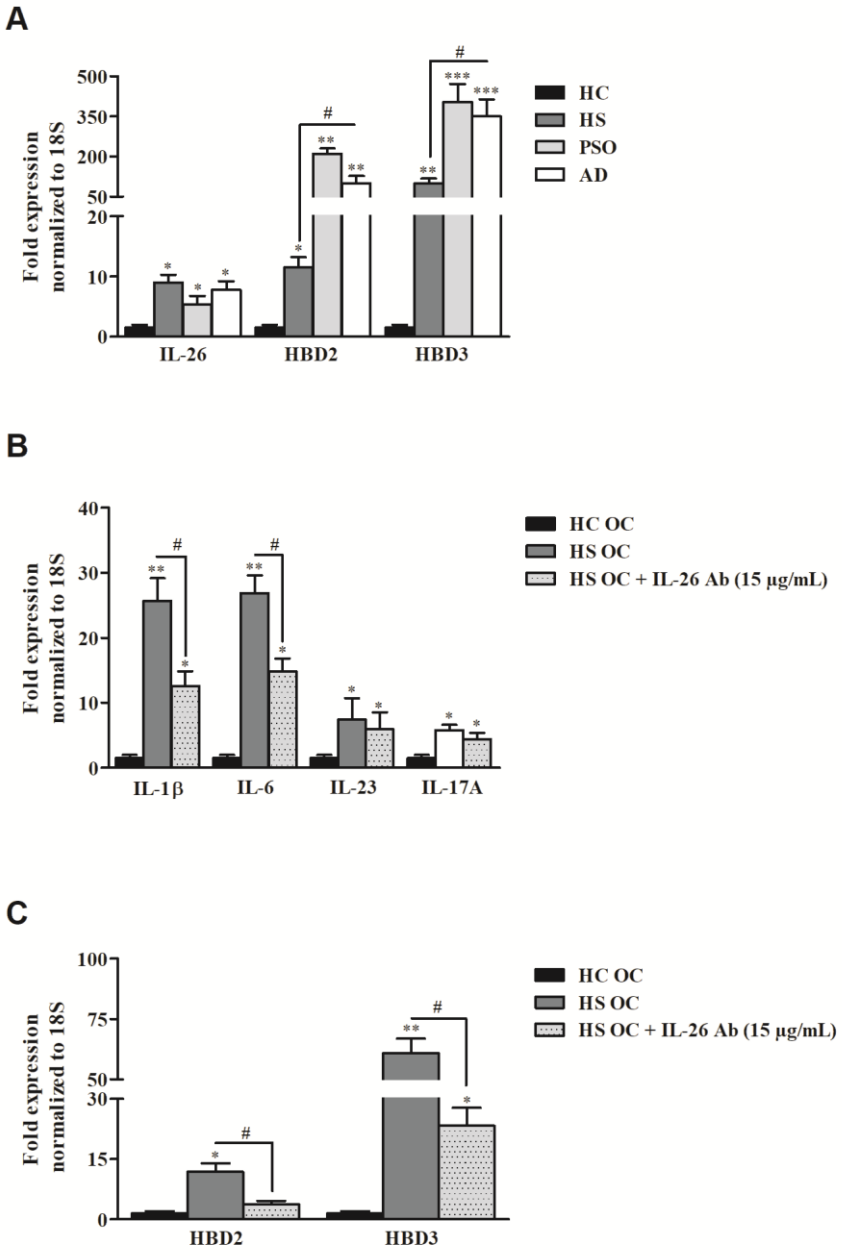

Figure 7. (A) Gene expression of IL-26, HBD2 and HBD3 in patients with hidradenitis suppurativa (HS) (n=10), psoriasis (PSO) (n=10) and atopic dermatitis (AD) (n=8) and in healthy controls (HC) (n=20). Data are displayed as the mean ± SD. Statistical significance was assessed using the Mann–Whitney test. *P <0.05, **P <0.01, ***P <0.001 for disease *vs*. HC; #P <0.05 between PSO, AD and HS. (B, C) Gene expression of (b) IL-1β, IL-6, IL-23 and IL-17A and (C) HBD2 and HBD3 in HS ex vivo skin organ culture (OC) treated or not with IL-26-neutralizing antibody (Ab) compared with HC OC. *P < 0.05, **P <0.01 *vs*. HC OC, calculated using the Mann–Whitney test. #P <0.05 for IL-26 Ab treatment *vs*. non-treatment, calculated using the Wilcoxon matched-pairs test.

CHAPTER IV

Discussion

IL-26 is a signature Th17 cytokine and a member of the IL-10 family, and has been described as both a potent antimicrobial and pro-inflammatory mediator. IL-26 has previously been reported in immune-mediated inflammatory diseases, (Dambacher *et al*., 2009; Corvaisier *et al*., 2012; Meller *et al*., 2015; Fujii *et al*. 2017) but its involvement in inflammatory skin disorders is unclear. Here we analyzed IL-26 in HS, PSO and AD, paying particular attention to its involvement in HS. Our results showed that IL-26 gene expression was enhanced in HS lesional skin, although not significantly, with respect to psoriasis and AD skin, whereas HBD2 and HBD3 levels were lower in HS skin than in PSO and AD skin, as reported previously (Wolk *et al.*, 2011; Hofmann *et al*., 2012).

HS is characterized by a deficiency of IL-20 and IL-22 (a close homologue of IL-26), which could cause AMP pauperization by epithelial cells, leading to severe and recurrent infections of the skin (Wolk *et al.*, 2011; Hofmann *et al*., 2012). In line with other chronic inflammatory diseases such as RA (Corvaisier *et al*., 2012), we found a higher plasma concentration of IL-26 in patients with HS than in healthy controls. Increasing concentrations of IL-26 significantly correlated with disease severity, even if the number of patients was limited and we studied only patients with moderate HS. Upon these observations, we expanded the significance of IL-26 as an inducer of pro-inflammatory and antimicrobial mediators. Our results suggest that inhibition of IL-26 reduces cutaneous expression of pro-inflammatory mediators essential for T-cell differentiation, and the expression of AMPs in HS. For the first time, we identified the functional relevance of IL-26 in the positive regulation of HBD2 and HBD3 in HS. To date, other pro-inflammatory cytokines such as IL-1α, IL-20 and IL-22 have been described as strong inducers of AMPs (Liang *et al*., 2006; Eyerich *et al*., 2010; Wolk *et al.*, 2011; Kanni *et al*. 2018). Interestingly, previous neutralization studies on IL-1α have

shown reduction of HBD2 in patients with HS (Kanni *et al*. 2018). Going deeper into the antimicrobial activity of IL-26, we compared the behaviour of healthy control and HS PBMCs towards *S. aureus* and *P. aeruginosa*. Although both colonize HS skin, *S. aureus* infections are the most frequently reported (Guet-Revillet *et al*., 2014; Ring *et al*., 2015). No bactericidal activity against *P. aeruginosa* was observed, whereas *S. aureus* growth was inhibited only by healthy control PBMCs previously stimulated with TSST-1. Curiously, TSST-1-stimulated HS PBMCs were unable to kill *S. aureus*. This difference could be explained by the fact that HS is characterized by the absence or reduction of several cytokines whose cooperation is responsible for antimicrobial processes (Eyerich *et al*., 2011; Wolk *et al*., 2011). In particular, Wolk *et al*. have reported that IL-22 and IL-20 downregulation is responsible for limited AMP levels in HS lesions. We wondered, could these facts correlate with the attenuation of IL-26 function in HS?

In view of this, we also explored the cytotoxic activity of IL-26 in HS. Similar to the antimicrobial results, cytotoxic activity during *S. aureus* infection was lower in patients with HS than in healthy volunteers. Interestingly, IL-26 silencing was able to reduce the cytotoxic activity in healthy controls but not in patients with HS, supporting the idea that the IL-26 cargo might be not efficacious in HS. Consistently with the bactericidal and cytotoxic activity phagocytosis against *S. aureus* was also lower in patients with HS than in healthy controls. Phagocytosis was strongly reduced by IL-26 silencing only in healthy controls. Increasing evidence suggests that dysfunctional immune responses occur in HS, leading to the bacterial colonization seen in HS lesional skin (van der Zee *et al*., 2012; Giamarellos-Bourboulis *et al*., 2017; Melnik *et al*., 2018). Nowadays, it is still debated whether bacterial colonization is a primary or secondary event in HS lesion evolution. It has been speculated that bacterial invasion leads to a series of pathogen-associated molecular pathways, which may trigger the initiation of inflammasomes (Matusiak *et al*., 2014). Alternatively, the deposition of keratin

fragments into the dermis may be the trigger for bacterial colonization (van der Zee *et al*., 2012). Consistent findings have been reported showing that the major bacterial species found in HS lesional skin are *S. aureus*, coagulase-negative staphylococci and Corynebacterium spp., which probably trigger local inflammation (Sartorius *et al*., 2012; Guet-Revillet *et al*., 2014; Ring *et al*., 2015). The efficacy of antibiotics like rifampicin, clindamycin or tetracycline in HS treatment further supports a microbial role in disease pathogenesis (Bettoli *et al*., 2016; Napolitano *et al*., 2017). Despite the role of bacteria in HS remaining controversial, this paper supports the theory that the antimicrobial response is defective in HS, shedding light on the possible involvement of IL-26 in this process along with inflammation.

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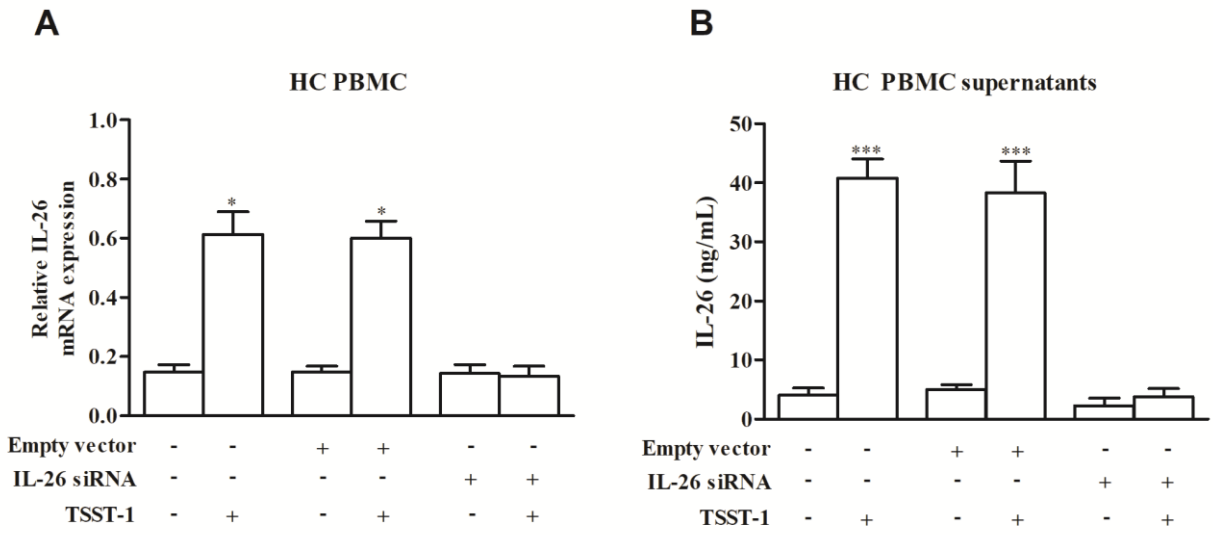
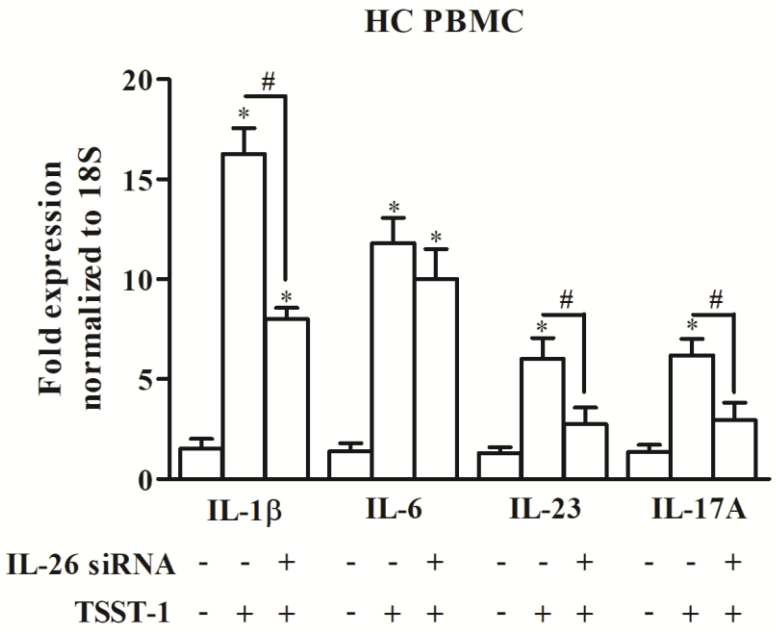
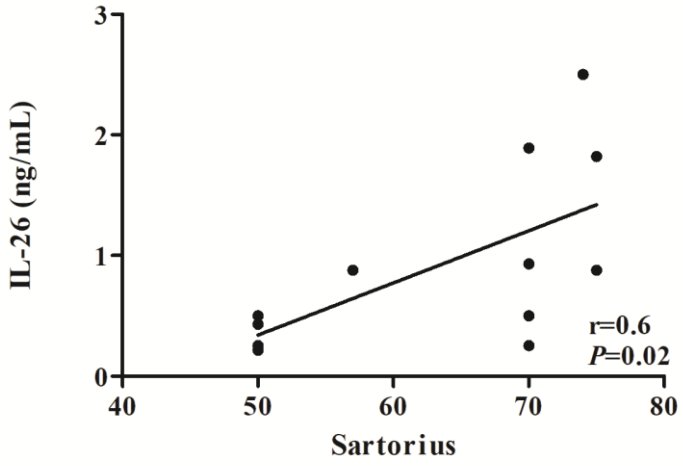
Supplementary Material



SM1. IL-26 is increased by TSST-1 *stimulus*. (A) HC (n=8) PBMC relative IL-26 mRNA expression; (B) IL-26 protein detection in supernatants derived from cultured healthy control (HC) PBMC (n=8) without or with IL-26 siRNA, empty vector and in presence or absence of TSST-1 (100 ng/mL). Data are displayed as mean ± SD. *P*-value symbols (*) indicate differences with HC PBMC not transfected and not stimulated with TSST-1 (*P<0.05, ***P<0.001) calculated using Wilcoxon matched pairs test.



SM2. TSST-1 *stimulus* enhances the production of inflammatory cytokines via IL-26 induction in PBMC. IL-1β, IL-6, IL-23 and IL-17A gene expression in healthy control (HC) PBMCs (n=8) transfected with IL-26 siRNA in presence or absence of TSST-1 (100ng/mL). Data are displayed as mean ± SD. Statistical significance was assessed using Wilcoxon matched pairs test. *P*-value symbols (*) indicate differences with HC PBMC not transfected and not stimulated with TSST-1 (*$P<0.05$). *P*-value symbols (#) indicate differences of TSST-1 stimulated HC PBMC transfected with IL-26 siRNA *vs* TSST-1 stimulated HC PBMC not transfected ($^{\#}P<0.05$).



SM3. IL-26 plasma levels of 12 HS subjects correlated with Sartorius score. *P*=Pearson correlation coefficient (rho=r); significant correlation (**P*=0.02).