



Video S1 Follicular unit extraction: Surgical technique using a motorized 1 mm punch for harvesting follicular units from the scalp. These FUs can be stained with Neutral Red or Methylene Blue for eccrine gland identification under the stereomicroscope.

DATA S1 Methods of eccrine gland isolation:

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IL-26 in allergic contact dermatitis: Resource in a state of readiness

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Abstract

In this study, we investigated the role of IL-26 in allergic contact dermatitis (ACD), highlighting its' contribute in the cytotoxic mechanism responsible for the tissue injury. IL-26 is a signature Th17 cytokine, and immune cells are its predominant sources. Recently, it has shown that Th17 cell-derived-IL-26 functions like an antimicrobial peptide. Here, we hypothesized that IL-26 could be involved in cytotoxicity mechanism that underlies ACD. Indeed, we have attributed a role to IL-26 in this context, through PBMC cytotoxicity assays vs HaCat. To demonstrate that IL-26 was effectively involved in this activity, we performed the assay using transfected ACD PBMCs by siRNA for IL-26. Indeed, we demonstrated that these cells were less able to kill keratinocytes compared with ACD PBMCs ($P < .01$). In conclusion, our findings support the idea that this emergent cytokine, IL-26, is implicated in the killing mechanisms of KC observed during ACD.

KEYWORDS

allergic contact dermatitis, cytotoxicity, IL-26

1 | BACKGROUND

Despite considerable progress in understanding the development of hapten-specific immunity, less is known about mechanisms responsible for tissue injury during allergic contact dermatitis (ACD).^[1] Indeed, ultrastructural studies of ACD revealed damaged keratinocytes in close contact with mononuclear cells,^[1] suggesting a role for T-cell-mediated cytotoxicity.^[2] In particular, cytotoxic T cell activity becomes evident in the appearance of lesions, but subpopulations

of T helper (Th) cells also contribute to the release of specific cytokines.^[3] Today, there is experimental evidence to classify ACD amongst other inflammatory skin disorders as interleukin-17-producing T helper cell (Th17)-mediated disease.^[4] Indications for involvement of Th17, in particular the subpopulation cytotoxic T, in human skin allergy were reported for the first time in 2009.^[5] The expression of interleukin-26 (IL-26) by Th17 was first described in 2007.^[6] In humans, IL-26 is a signature Th17 cytokine, and its expression is regulated by IL-1 β , IL-23 and ROR γ t. Immune cells, including

Th1, Th17 and NK cells, are predominant sources of IL-26. It induces the secretion of pro-inflammatory cytokines and chemokines by myeloid cells and favours the generation and local recruitment of Th17 cells.^[6] IL-26, emerging member of IL-10 family cytokines, stands distinct as it exerts antimicrobial response not only by priming various innate immune cells and modulating antiviral responses but also by eliciting direct microbicidal action through affecting the formation of membrane pores.^[7] Indeed, it has been shown that Th17 cell-derived-IL-26 functions like an antimicrobial peptide killing bacteria, through its ability to form multimers.^[8] Taken together, all mentioned features confer a dual function to IL-26 both as potent natural antimicrobial and pro-inflammatory cytokine.

2 | QUESTION ADDRESSED

In this study, we investigated the role of IL-26 in ACD, highlighting its contribute in the cytotoxic mechanism responsible for tissue injury.

3 | EXPERIMENTAL DESIGN

The study population comprised 10 subjects affected by ACD; characteristics of these patients are listed in Table S1, 10 by atopic dermatitis (AD), 10 by psoriasis and 15 age and sex-matched healthy controls (HC). Skin biopsies as well as blood samples were taken from all patients. RNA and protein analysis was executed on skin biopsies and on peripheral blood mononuclear cells (PBMCs). Moreover, circulating plasma levels of IL-26 were assessed. In vitro assays were performed with PBMCs exposed to nickel sulphate (NiSO_4) and toxic shock syndrome toxin 1 (TSST-1). In addition, PBMC cytotoxicity silenced or not for IL-26 was tested against HaCat cells using the lactate dehydrogenase (LDH)-release assay. The sequences of the primers are listed in Table S2. Details in Data S1.

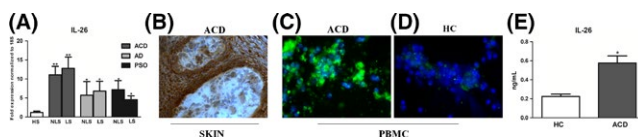


FIGURE 1 IL-26 is increased in ACD. (A) IL-26 gene expression in ACD, AD and PSO skin as well as healthy skin (HS). Fold expression was obtained from LS and NLS skin vs HS of controls and normalized to 18S. Data are displayed as mean $\pm$ standard deviation (SD). Statistical analysis was performed by Mann-Whitney test. * $P < .05$; ** $P < .01$. (B) Immunohistochemistry analysis of IL-26 in ACD lesional skin. (C-D) Immunofluorescence analysis of IL-26 (green) in ACD and HC PBMC. The nuclei were stained with DAPI (blue). Magnification 20 $\times$. (E) IL-26 circulating plasma levels in ACD and healthy control (HC) subjects. Data are displayed as mean $\pm$ standard deviation (SD). Statistical analysis was performed by Mann-Whitney test. * $P < .05$

4 | RESULTS

IL-26 gene expression was significantly enhanced in ACD skin both non-lesional (NLS) and lesional (LS) compared with healthy skin (HS) ($P < .01$) (Figure 1A). A minor increase was observed in atopic dermatitis (AD) and PSO skin ($P < .05$) (Figure 1A). Recently, it was reported that IL-19, another member belonging to the same family of IL-26, was significantly enhanced in inducible eczema (ACD) respect to naturally occurring eczema (AD, nummular or dyshidrotic eczema).^[9] Immunohistochemical analysis revealed a strong positivity for IL-26 in infiltrating cells of ACD lesional skin (Figure 1B). Consistent with this observation, to better characterize this positive staining, we assessed IL-26 in PBMCs from ACD subjects by immunofluorescence analysis (Figure 1C,D). A striking difference was observed in ACD vs healthy subjects. To confirm this finding, we determined IL-26 circulating plasma levels, and they were significantly increased in ACD

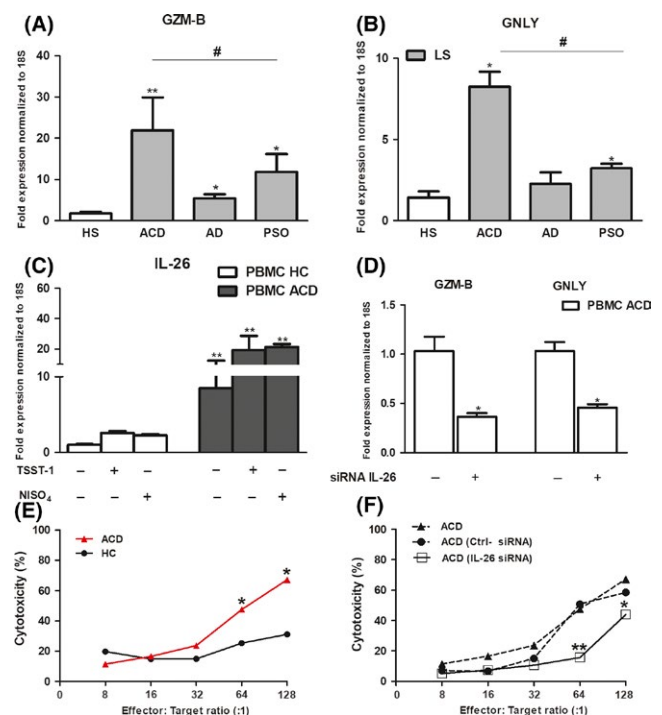


FIGURE 2 IL-26 involvement in cytotoxicity. (A,B) Granzyme B (GZM-B) and granulysin (GNLY) gene expressions in ACD, AD and PSO skin as well as HS. Statistical analysis was performed by Mann-Whitney test. (*) Comparison ACD, AD and PSO fold increase vs HS; (#) Comparison ACD fold increase vs AD and PSO fold increase. #/* $P < .05$; ** $P < .01$; (C) IL-26 gene expression in PBMC of HC and nickel-ACD subjects stimulated with toxic shock syndrome toxin (TSST)-1 and nickel (NiSO_4). Data are displayed as mean $\pm$ SD. ** $P < .01$ (D) GZM-B and GNLY gene expressions in ACD PBMC with or without siRNA for IL-26. Statistical analysis was performed by paired t test. Data are displayed as mean $\pm$ SD * $P < .05$. (E) LDH release to measure the percentage of cytotoxicity vs HaCat cells of ACD PBMC respect to HC PBMC. * $P < .05$ (F) LDH release to measure the percentage of cytotoxicity vs HaCat cells of ACD PBMC with or without siRNA for IL-26. As negative control siRNA was used an oligonucleotide with scrambled sequence (Ctrl-siRNA IL-26). * $P < .05$; ** $P < .01$

respect to HC subjects ($P < .05$) (Figure 1E). Moreover, gene expression of granzyme B and granulysin, main cytotoxicity mediators, was enhanced in all examined conditions respect to HS and in particular ACD showed the highest increase (Figure 2A,B). Data from literature show that the number of cytotoxic T cells was significantly increased in ACD and AD compared with PSO,^[10,11] suggesting that such cell-mediated cytotoxic mechanisms may play an integral part in the eczematous disorders. Regarding AD results, we found increased gene expression of the above-mentioned cytotoxicity mediators, even though these were not higher than ones in PSO; we hypothesize that this difference is ascribable to chronic phase of our AD subjects. To date, the mechanisms leading to keratinocyte damage and spongiosis in ACD have so far remained unclear. Indeed, to deepen this point, we have analysed IL-26 expression in PBMCs, stimulating these cells in vitro with toxic shock syndrome toxin 1 (TSST-1) and nickel sulphate (NiSO_4). IL-26 was highly expressed in PBMCs from ACD patients, and the stimulations were able to induce a further increase, even though not significant (Figure 2C). These findings showed that ACD PBMCs have a considerable amount of IL-26 at basal conditions, fitting with the elevated levels already found in immunofluorescence. Thus, it would seem that IL-26 levels, unlike previously analysed cytokines,^[12] are allergen independent in ACD PBMCs. It is not surprising that in vitro models do not always elicit adequate responses; indeed, it has been demonstrated that although present in ACD, Th17 cells are not responsive to nickel treatment in vitro.^[5] Given that it has been demonstrated a cytotoxic role for IL-26 vs different bacteria,^[8] we hypothesized that IL-26 could be involved in cytotoxicity mechanisms that underlie ACD. Firstly, ACD PBMCs were transfected with IL-26 siRNA, and cytotoxicity mediators were assessed. Both granzyme B and granulysin gene expressions were significantly decreased ($P < .05$) in IL-26 siRNA ACD PBMCs respect to ACD PBMCs without silencing (Figure 2D), suggesting a role for IL-26 in cytotoxic mechanisms. Subsequently, we deeply investigated this point through PBMC cytotoxicity assays vs HaCat. We observed that the cytotoxicity activity of ACD PBMCs was significantly enhanced respect to HC PBMCs ($P < .05$) (Figure 2E). Next, we performed the assay using IL-26 siRNA ACD PBMCs. Results derived from silenced ACD PBMCs showed that these cells were less able to kill keratinocytes compared with ACD PBMCs ($P < .01$) without silencing (Figure 2F).

5 | CONCLUSIONS

It is well established that CD4+ and CD8+ cytotoxic T lymphocytes (CTLs) in humans represent the key actors of cytotoxicity. It has been hypothesized that in ACD, activated keratinocytes present haptens to both CD8+ and CD4+ CTLs which subsequently elicit tissue damage through granzyme B exocytosis.^[10] Furthermore, given that Ag-specific T cells represent the minor fraction of skin-infiltrating T cells,

non-specific T-cell killing represents an efficient amplification mechanism of the initially Ag-specific allergic reaction, rendering virtually all T lymphocytes recruited at the site of skin inflammation capable to directly contribute to tissue damage.^[5] Taken together our findings suggest that IL-26 could play a crucial role in this amplification mechanism being an allergen independent cytokine highly expressed in ACD patients and constitutively ready to act in complex killing system proper of this disease.

CONFLICT OF INTEREST

The authors have declared no conflicting interests.

AUTHOR CONTRIBUTION

AB and GC designed the research study. GC, RDC and ES performed the research. GC wrote the manuscript. AR, CT, SL and AB analysed the research.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1 Materials and methods

Table S1 Characteristics of allergic contact dermatitis (ACD) recruited patients

Table S2 Primer sequence

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A short-term in vivo model for Merkel Cell Carcinoma

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Abstract

In vivo tumor models are essential for studying the biology of cancer, identifying tumor targets and evaluating antitumor drugs. Considering the request for the minimisation of animal experiments and following the “3R”-rule (“replacement,” “refinement,” “reduction”), it has become crucial to develop alternative experimental models in cancer biology. Several studies have already described the avian chorioallantoic membrane (CAM) model as an alternative to rodents, suitable to investigate growth, progression and metastasis of various types of cancer. In the present work, we grafted three Merkel cell carcinoma (MCC) cell lines onto the avian CAM and monitored tumor growth and development of solid tumor nodules. Morphology of xenograft was characterised histologically and immunohistochemically. Our results demonstrate CAM assay as a useful tool to study MCC pathophysiology.

KEYWORDS

Chorioallantoic membrane assay, MCPyV-LT antigen, Merkel cell carcinoma

1 | BACKGROUND

Merkel Cell Carcinoma (MCC) is a rare, highly aggressive neuroendocrine tumor of the skin with poor prognosis that typically occurs in elderly and immunosuppressed patients.^[1] The MCC is characterised by the presence of cytokeratin 20 (CK-20) and neuroendocrine granules. The

outcome of immune surveillance suggested viral carcinogenesis, which was indeed demonstrated in the majority of cases.^[2] UV radiation exposure is an additional epidemiologic risk factor for MCC.^[3] Due to the development of immune checkpoint inhibitors, a new therapeutic window opened for MCC patients.^[4-6] Recently, treatment with three humanized antibodies, namely avelumab, pembrolizumab and nivolumab

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Materials and Methods

Subjects

The overall study population included 30 patients with inflammatory skin diseases [10 affected by allergic contact dermatitis (ACD), 10 by atopic dermatitis (AD), 10 by psoriasis] and 15 age and sex matched healthy controls. ACD and AD patients were enrolled from the dermato-allergology center computer-based database of our Clinic. Inclusion criteria were age ≥ 18 years and diagnosis of ACD made by a dermatologist. Regarding ACD patients, the presence of at least one relevant positive reaction to patch test was required. The intensity of reactions were evaluated according to the guidelines of the Italian Society of Allergological, Occupational and Environmental Dermatology (SIDAPA). Results were recorded as negative, doubtful, and positive with 1+, 2+, 3+, respectively, for positive weak, moderate and strong reaction. The ACD patients characteristics are reported in Table S1. Psoriasis subjects were recruited from the database of patients attending the psoriasis center of University of Naples Federico II, Naples, Italy. Inclusion criteria were diagnosis of moderate to severe psoriasis [Psoriasis Area and Severity Index (PASI) > 10] (with or without psoriatic arthritis) made by a dermatologist, disease duration of at least 6 months, topical and/or systemic treatment washout period of at least 3 weeks and age ≥ 18 years. Regarding AD patients inclusion criteria were: diagnosis of atopic dermatitis by a high experienced dermatologist basing on clinical features, and/or medical history, and/or histological examination, and/or treatment response; disease duration ≥ 6 months and age ≥ 18 years. All enrolled AD subjects were in chronic phase of disease with Scoring Atopic Dermatitis (SCORAD) > 25 . They presented typical features of chronic form: lichenified, slightly pigmented, excoriated plaques. As regards healthy subjects, the enrollment was performed among chaperones of patients attending the general ambulatory service of our Clinic. Inclusion criteria included age ≥ 18 years whereas a present or past positive history of inflammatory skin disorders represented an exclusion criteria. The experimental protocol was approved by the Ethics Committee of our institution and conformed to the principles outlined

in the Declaration of Helsinki. Each subject gave written informed consent before entering the study. Skin biopsies (3 mm) as well as blood samples were taken from all patients. Regarding healthy subjects (HS) blood samples were taken, but skin specimens were obtained from plastic surgery remnants.

***In vivo* gene expression of IL-26 in the skin**

Skin biopsies (3 mm) were collected from uninvolved and involved sites of subjects affected by ACD (n=10), AD (n=10) and psoriasis (n=10). HS skin specimens were obtained from plastic surgery remnants (n=10) for *in vivo* mRNA examination through real-time PCR.

IL-26 protein level in ACD patients

IL-26 protein levels were assessed by immunohistochemistry in lesional ACD skin and by immunofluorescence analysis in peripheral blood mononuclear cells (PBMC) isolated from ACD as well as from healthy controls (HC)

Immunohistochemistry (IHC)

The immunohistochemical detection of IL-26 was carried out on lesional skin samples of 5 patients affected by ACD (#1,3,7,8,10). Healthy skin samples were used as controls. Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) was used according to the manufacturer's instructions. Sections were stained with polyclonal goat anti-human IL-26 (5µg/ml, R&D System Inc, Minneapolis, MN) antibody (overnight at 4°C) and then incubated with biotinylated secondary antibody. Peroxydase activity was revealed using diaminobenzidine (DAB) substrate (ImmPACT DAB, Burlingame, CA). In parallel, skin specimens were incubated with specific isotype control antibodies (Goat IgG, polyclonal - Isotype Control, Abcam Cambridge, UK) used at the same concentration as the corresponding primary antibody. Counterstaining was performed with hematoxylin

Immunofluorescence PBMC staining

PBMC were isolated from 5 healthy controls (HC) and 5 nickel-ACD (#1-5) patients by density gradient centrifugation over Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Cell number and viability were determined using Trypan blue test. 15×10^6 cells were divided in three equal parts, fixed with cold acetone 10 min at 4°C, washed three times with PBS 1X. 5×10^6 were used as control, and 5×10^6 were stained for IL-26 (polyclonal goat anti-human IL-26: 5µg/ml, R&D System Inc, Minneapolis, MN) diluted in PBS, BSA 1 %, incubated overnight at 4 °C. Then, cells were incubated with NorthernLights anti-goat IgG-NL493 secondary antibody (R&D Systems, Minneapolis, MN, USA) at 1/200 dilution for 1 h at 4 °C, while protected from light. In parallel, 5×10^6 were incubated with specific isotype control antibodies (Goat IgG, polyclonal - Isotype Control, Abcam Cambridge, UK) used at the same concentration as the corresponding primary antibody. The nuclei were stained with DAPI (GIBCO, Grand Island, NY, USA). Staining was observed using Leica DM2500 microscope (Leica Microsystems, Wetzlar, Germany).

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were centrifuged and the plasma was collected and used for Enzyme-linked immunosorbent assay (ELISA). IL-26 circulating concentrations were determined by ELISA assay in the control as well as allergic contact dermatitis subjects, using commercially available kit (Cusabio Biotech CO., LTD), according to the manufacturer's instructions.

***In vivo* gene expression of cytotoxicity mediators in lesional skin**

Gene expression of granzyme B and granulysin was assessed in involved sites of ACD (#1-10), AD (n=10) and psoriasis (n=10) as well as of normal skin of healthy control subjects (n=10) through mRNA examination with real-time PCR.

***In vitro* gene expression of IL-26 in stimulated PBMC**

PBMC were isolated from 5 subjects affected by nickel-ACD (#1-5) and psoriasis (n=5) as well as healthy control subjects (n=5) by density gradient centrifugation over Ficoll-Paque Plus (GE

Healthcare, Uppsala, Sweden). Then, PBMC were cultured in Roswell Park Memorial Institute (RPMI; GIBCO) medium, supplemented with antibiotics (100 IU/ml penicillin G and 100 lg/ml streptomycin; GIBCO) and 10% FBS (GIBCO), at a concentration of 2×10^6 in 12-well plates. They were stimulated 100ng/ml of toxic shock syndrome toxin (TSST-1) (Sigma-Aldirich, Oakville, ON, Canada) or 10^{-4} M of NiSO₄. They were incubated for 24 hours at 5% CO₂ at 37°C. Cell number and viability were determined using Trypan blue test. mRNA was extracted for examination through real-time PCR.

***In vitro* gene expression of cytotoxicity mediators in ACD PBMC**

PBMC from ACD-nickel-patients (#1-5) were transfected or not with 3μM/μL of Riboxx siRNA oligo (Qiagen) using electroporation (see paragraph below). They were incubated for 24 hours at 5% CO₂ at 37°C. Cell number and viability were determined using Trypan blue test. mRNA was extracted for examination of granzyme B and granulysin gene expression through real-time PCR.

Gene silencing via RNAi

5.0×10^6 PBMC from ACD-nickel-patients (n=5) were transfected with 3μM/μL of Riboxx siRNA oligo (Qiagen) using electroporation (Amaxa Inc., Gaithersburg, MD, USA). It was selected the appropriate nucleofector program in optimized protocol as manufacturer's instruction. IL-26 siRNA target sequence or control siRNA oligonucleotide with scrambled sequence was used. Greater than 80% of cells remained viable after transfection procedures as determined by trypan blue exclusion.

Cytotoxicity assay

PBMC cytotoxicity (ACD, HC) was tested against HaCat cells by using the lactate dehydrogenase (LDH)-release assay (Pierce LDH Cytotoxicity assay, Thermo Scientific) according to the manufacturer's protocol. HaCat target cells were plated at 2.5×10^3 cells/well in 96-well flat-bottom plates and co-cultured with PBMCs at various effector/target (E:T) ratios for 5 h at 37°C. The assay was performed using transfected or not PBMCs by siRNA for IL-26. After 5 h of effector and target

co-culture, supernatant was removed from each well for LDH release measurement. Cytotoxicity is quantified by calculating specific lysis using the following equation: $100 \times [(\text{experimental release} - \text{spontaneous release})/(\text{maximum release} - \text{spontaneous release})]$.

RNA extraction, cDNA synthesis and real-time polymerase chain reaction (qRT-PCR)

RNA was extracted (RNeasy Mini Protocol Qiagen, Valencia, CA) and cDNA was prepared (Transcriptor High Fidelity cDNA Synthesis, Roche, Indianapolis, IN) according to the manufacturer's instructions. qRT-PCR (LightCycler, Roche) was used to analyze IL-26, granzyme B and granulysin gene expression. The amount of mRNA for a given gene in each sample was normalized to the amount of mRNA of 18S reference gene in the same sample. Fold induction of gene expression was calculated using the $\Delta\Delta\text{CT}$ method.

Patient	Age/Sex	Hapten	Concentration in PET ¹ (%)	Score/Reading
#1	51/F	Nickel	5	1+/72 h
#2	26/F	Nickel	5	2+/72 h
#3	41/M	Nickel	5	1+/72 h
#4	47/F	Nickel	5	2+/72 h
#5	49/F	Nickel	5	2+/96 h
#6	43/M	Fragrance mix	8	2+/72 h
#7	51/F	Para-phenylenediamine	1	2+/72 h
#8	30/M	Potassium dichromate	0.5	2+/72 h
#9	39/F	Para-phenylenediamine	1	3+/72 h
#10	48/F	Potassium dichromate	0.5	2+/72 h

Table S1. Characteristics of allergic contact dermatitis (ACD) recruited patients.

¹PET= petrolatum

Table S2. Primer sequence

Gene	Primer sequence
18S	5'- AACCCGTTGAACCCATT - 3' 3'- CCATCCAATCGGTAGTAGCG - 5'
IL-26	5' -GGTTGCTGTTAGTCACTCTGTCTC-3' 3'- GCGGTCTTCTGGAATCGTTGC -5'
GZM-B	5'-TGGGGGACCCAGAGATTAAAA -3' 3'-TTTCGTCCATAGGAGACAATGC-5'
GNLY	5'- tggataagcccaccagaga -3' 3'-tgtgtaggacggggagggtca -5'