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

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Abbreviations: allergic contact dermatitis (ACD), atopic dermatitis (AD), granulysin (GNLY), granzime B (GZM-B), healthy skin (HS), lesional (LS), nickel sulphate ($NiSO_4$), non lesional (NLS), psoriasis (PSO), T-helper (Th), toxic shock syndrome toxin 1 (TSST-1).

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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 of 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 versus Hacat. In order 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 to ACD PBMC ($p<0.01$). In conclusion, our findings support the idea that this emergent cytokine, IL-26, is implicated in the killing mechanisms of KC observed during ACD.

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-

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mediated cytotoxicity (2). In particular, cytotoxic T cells 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 cells (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-1β, IL-23 and RORγt. 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 favors 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.

Question addressed

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

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Experimental design

The study population comprised 10 subjects affected by ACD, characteristics of these patients are listed in supplementary table-1 (ST-1), 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 (PBMC). Moreover, circulating plasma levels of IL-26 were assessed. *In vitro* assays were performed with PBMC 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 by using the lactate dehydrogenase (LDH)-release assay.The sequences of the primers are listed in ST-2. Details in SM.

Results

IL-26 gene expression was significantly enhanced in ACD skin both non lesional (NLS) and lesional (LS) compared to healthy skin (HS) ($p<0.01$) (Fig.1a). A minor increase was observed in atopic dermatitis (AD) and PSO skin ($p<0.05$) (Fig.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 (Fig.1b). Consistent with this observation, to better characterize this positive staining, we assessed IL-26 in PBMC from ACD subjects by immunofluorescence analysis (Fig.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 respect to HC subjects ($p<0.05$) (Fig.1e). Moreover, gene expression of granzyme B and granulysin, main cytotoxicity mediators, was

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enhanced in all examined conditions respect to HS and in particular ACD showed the highest increase (Fig.2a,b). Data from literature show that the number of cytotoxic T cells was significantly increased in ACD and AD compared to 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, in order to deepen this point, we have analyzed IL-26 expression in PBMC, stimulating these cells *in vitro* with toxic shock syndrome toxin 1 (TSST-1) and nickel sulphate ($NiSO_4$). IL-26 was highly expressed in PBMC from ACD patients and the stimulations were able to induce a further increase, even though not significant (Fig.2c). These findings showed that ACD PBMC 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 analyzed cytokines (12), are allergen independent in ACD PBMC. It is not surprising that *in vitro* models don't always elicit adequate responses, indeed it has been demonstrated that although present in ACD, Th17 are not responsive to nickel treatment *in vitro* (5). Given that it has been demonstrated a cytotoxic role for IL-26 *versus* different bacteria, (8) we hypothesized that IL-26 could be involved in cytotoxicity mechanisms, that underlie ACD. Firstly, ACD PBMC were transfected with IL-26 siRNA and cytotoxicity mediators were assessed. Both granzyme B and granulysin gene expression were significantly decreased ($p<0.05$) in IL-26 siRNA ACD PBMC respect to ACD PBMC without silencing (Fig.2d), suggesting a role for IL-26 in cytotoxic mechanisms. Subsequently, we deeper investigated this point through PBMC cytotoxicity assays *versus* Hacat. We observed that the cytotoxicity activity of ACD PBMC

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was significantly enhanced respect to HC PBMC ($p<0.05$) (Fig.2e). Next, we performed the assay using IL-26 siRNA ACD PBMC. Results derived from silenced ACD PBMC showed that these cells were less able to kill keratinocytes compared to ACD PBMC ($p<0.01$) without silencing (Fig.2f).

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, nonspecific 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.

Figure legends

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 ± standard deviation (SD). Statistical analysis was performed by Mann-Whitney test. *$P<0.05$; **$P<0.01$. **(b)** Immunohistochemistry analysis of IL-26 in ACD lesional skin. **(c-d)** Immunofluorescence

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analysis of IL-26 (green) in ACD and HC PBMC. The nuclei were stained with DAPI (blue). Magnification 20x. **(e)** IL-26 circulating plasma levels in ACD and healthy control (HC) subjects. Data are displayed as mean ± standard deviation (SD). Statistical analysis was performed by Mann-Whitney test. **P*<0.05.

Figure 2. IL-26 involvement in cytotoxicity. (a,b) Granzime B (GZM-B) and granulysin (GNLY) gene expression 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<0.05; **p <0.01; **(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 ± SD.***P*<0.01 **(d)** GZM-B and GNLY gene expression in ACD PBMC with or without siRNA for IL-26. Statistical analysis was performed by paired *t*-test. Data are displayed as mean ± SD **P*<0.05. **(e)** LDH release to measure the percentage of cytotoxicity *versus* Hacat cells of ACD PBMC respect to HC PBMC. **P*<0.05 **(f)** LDH release to measure the percentage of cytotoxicity *versus* 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*<0.05; ***P*<0.01.

Author contribution:

AB and GC designed the research study

GC, RDC and ES performed the research

GC wrote the paper

AR, CT, SL an AB analysed the research

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