

Calcitonin gene-related peptide modulates interleukin-13 in circulating cutaneous lymphocyte-associated antigen-positive T cells in patients with atopic dermatitis

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Summary

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Accepted for publication

18 March 2009

Key words

atopic dermatitis, calcitonin gene-related peptide, cutaneous lymphocyte-associated antigen, neuropeptides, T cells

Conflicts of interest

None declared.

DOI 10.1111/j.1365-2133.2009.09318.x

Background Neuropeptides (NPs) may play an important role in the pathogenesis of atopic dermatitis (AD) by regulating immune responses and contributing to the cross-talk between the immune and nervous systems.

Objectives To assess the ability of NPs to influence interleukin (IL)-13 and interferon (IFN)- γ production and the expression of the activation marker HLA-DR in skin memory T cells [cutaneous lymphocyte-associated antigen (CLA)+ T cells] from patients with AD with severe, chronic lesions and intense pruritus, and from nonatopic controls.

Methods Cells were cultured in the presence and absence of different NPs, calcitonin gene-related peptide (CGRP), somatostatin (SOM) and substance P (SP). IL-13 and IFN- γ production and HLA-DR expression were measured in both CLA+ and CLA- T-cell subsets by flow cytometry.

Results CGRP increased IL-13 production in peripheral blood mononuclear cells from patients with AD ($P < 0.05$), with no changes detected in the presence of SOM or SP. These patients with AD had a lower expression of CGRP receptor compared with controls ($P < 0.05$). Memory T cells incubated with CGRP also showed an increase in IL-13 ($P < 0.05$) and HLA-DR ($P < 0.05$) in CLA+ T cells from patients with AD compared with controls, but not in CLA- T cells. Patients with a higher production of IL-13 were those with higher total IgE and percentage of skin area involved. Furthermore, the IL-13/IFN- γ ratio was increased in patients with AD after cells were cultured with CGRP ($P < 0.05$).

Conclusions Our results suggest an immunomodulatory role of CGRP towards a Th2 pattern in CLA+ T cells, which may contribute to exacerbating clinical symptoms in patients with AD.

Atopic dermatitis (AD) is a chronic relapsing inflammatory pruritic skin disease with a prevalence of 10–20% in children and 1–3% in adults.¹ Clinical observations suggest the role of neural mechanisms in the pathogenesis of AD.² Pruritus represents a predominant symptom and a vicious itch–scratch cycle exists in which scratch damage enhances itch. Skin scratching induces the sprouting of cutaneous sensory nerve fibres in the skin,³ which synthesize and release neuropeptides (NPs) such as substance P (SP), somatostatin (SOM) and calcitonin gene-related peptide (CGRP).^{4,5} These NPs regulate cutaneous immune responses and play important roles in proinflammatory responses.^{4,5}

Previous reports have suggested that different NPs, such as SP, SOM and CGRP, can modulate cytokine production in T-cell

lines from mice,⁶ and an association has recently been detected between NP levels and Th1/Th2 balance in children with allergic sensitization.⁷ In AD, studies with peripheral blood mononuclear cells (PBMC) seem to indicate that NPs can also influence the course of the disease by modulating cytokine secretion and increasing the proliferative responses.^{8–12} However, further studies are necessary to define the role of NPs in a disease as complex as AD.

Skin-infiltrating T cells have been suggested to play a major role in the induction of pruritus in patients with AD by the production and release of mediators, including cytokines.^{13–15} In patients with AD, interleukin (IL)-31 has recently been shown to play a key role in itch¹⁶ and has been associated with T cells expressing the cutaneous lymphocyte-associated

antigen (CLA).¹⁷ CLA⁺ T cells are probably the most relevant T-cell population in the initiation and maintenance of dysregulated immune responses in AD.^{18,19}

In this study, we evaluated the capacity of three NPs (SOM, CGRP and SP) to induce IL-13, interferon (IFN)- γ and HLA-DR in patients with AD and nonatopic controls. We observed that CGRP induced T-cell activation and favoured the Th2 pattern of CLA⁺ T cells, suggesting an immunomodulatory role of this NP in skin-homing T cells in AD.

Materials and methods

Study subjects

The study included 10 patients (four men and six women; mean age 25 years, range 16–47) with AD according to the criteria of Hanifin and Rajka²⁰ and with chronic lesions (lesion onset more than 2 weeks before the study). The affected body surface area (BSA) was recorded and the clinical activity of the skin lesions was graded according to the severity of the AD (3–4, mild; 4.5–7.5, moderate; 8–9, severe).²¹ Ten nonatopic individuals (four men and six women; mean age 30 years, range 22–38) were recruited as a control group. None of the patients with AD or controls had respiratory symptoms or any other skin disease or had used any systemic steroids or immunomodulatory therapies for at least 6 months before the study.

Total serum IgE was quantified by fluoroenzyme immunoassay (UniCAP; Pharmacia Diagnostic AB, Uppsala, Sweden), according to the manufacturer's instructions. The study was approved by the institutional review board, and informed consent for all the diagnostic procedures was obtained from all patients and controls.

Peripheral blood mononuclear cell cultures with neuropeptides and cytokine production

PBMC from patients with AD and controls were isolated by density gradient centrifugation (Nycomed, Oslo, Norway) and cultured at 5×10^6 cells mL⁻¹ in complete RPMI with or without the different NPs, CGRP, SOM and SP (Sigma, St Louis, MO, U.S.A.) at 10^{-8} mol L⁻¹, for 16 h at 37 °C and 5% CO₂ in 48-well plates. Cells were cultured with ionomycin (Sigma) at 1 μ g mL⁻¹ and monensin (Sigma) at 10 μ g mL⁻¹ for the last 4 h. Intracellular IL-13 and IFN- γ production in both CLA⁺ and CLA⁻ subsets was determined by flow cytometry, as

described.¹⁹ Six parameters were analysed on a FACScalibur flow cytometer using CellQuest software (Becton Dickinson, Mountain View, CA, U.S.A.). Negative isotype controls were used to verify the staining specificity of the antibodies used.

Expression of calcitonin gene-related peptide receptor in peripheral blood mononuclear cells

Total RNA from PBMC was extracted in TriPure (Roche Diagnostics, Indianapolis, IN, U.S.A.), as described.²² Complementary DNA (cDNA) was obtained using 20 U Moloney murine leukaemia virus (Roche Diagnostics, Penzberg, Germany), 1 mmol L⁻¹ each of the deoxyribonucleoside triphosphates (Boehringer Mannheim; Roche, Basel, Switzerland), 10 U of RNase inhibitor (Roche Diagnostics), 4 μ g hexamer primer (Roche Diagnostics), 10 $\times$ polymerase chain reaction buffer (Boehringer Mannheim) and 5 mmol L⁻¹ MgCl₂ in a final volume of 20 μ L. The reverse transcription was performed at 42 °C for 60 min followed by 70 °C for 15 min with a thermocycler (Perkin-Elmer, Irvine, CA, U.S.A.). The cDNA was stored at -80 °C until use. Specific cDNA amplification of CGRP receptor (CGRP-R) was carried out with 2 μ L of cDNA by using FastStart DNA Master SYBR Green I (Roche Diagnostics). The specific primers and the housekeeping gene (porphobilinogen deaminase) used are shown in Table 1. The concentration was obtained by crossing-point extrapolation into a standard curve with known cDNA concentrations by using Light Cycler (Roche Diagnostics) system software and results were expressed as relative units that represent the ratio of concentration between the specific mRNA and the housekeeping mRNA.

Memory T-cell subset isolation from peripheral blood

Total T cells were purified with Human T Cell Enrichment Columns (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's instructions from patients with AD and controls. Separation efficiency was assessed by flow cytometry, and sample purity was > 95% T cells. To separate memory (CD45RO⁺) T cells from the naive (CD45RA⁺) T cells, the total T-cell population was incubated with anti-CD45RO magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 4 °C and isolated with a magnetic column, according to the manufacturer's instructions. The naive and memory T-cell populations were determined to be > 95% pure by means of flow cytometry.

Table 1 Specific primers used for detecting the expression of calcitonin gene-related peptide receptor (CGRP-R) and the housekeeping gene porphobilinogen deaminase (PBGD) in peripheral blood mononuclear cells

Gene	Sense	Antisense	Fragment size (bp)	T _m (°C)
CGRP-R	5'-TATTGAAAGATTGCTACCAC-3'	5'-CCAACTGAATTGAGTCCT-3'	261	80
PBGD	5'-TCCAAGCGAGCCATGTCTG-3'	5'-AGAATCTTGCCCTGTGGTGA-3'	204	88

T_m, melting temperature.

Memory T-cell cultures with calcitonin gene-related peptide and cytokine production

CD45RO+ T-cell subsets were dispensed in 96-well culture plates (Nunc, Roskilde, Denmark) at 2×10^6 cells mL⁻¹ in complete RPMI with or without CGRP at 10^{-8} mol L⁻¹ for 16 h at 37 °C and 5% CO₂. Cells were cultured with ionomycin (Sigma) at 1 µg mL⁻¹ and monensin (Sigma) at 10 µg mL⁻¹ for the last 4 h. Intracellular IL-13 and IFN-γ production and the HLA-DR activation marker in both CLA+ and CLA- subsets were determined as described above. To evaluate the specific effect of CGRP, CD45RO+ T-cell subsets were cultured with CGRP and the CGRP type 1 receptor antagonist, CGRP 8-37 (Sigma), at equimolar concentrations under the conditions previously described.

Statistical analysis

All the statistical analyses were done with nonparametric tests. Comparison of medians for the different variables was done by the Mann–Whitney test for nonrelated samples and by the Wilcoxon test for related samples. Spearman's nonparametric test was used to analyse correlations. Comparisons of individual data were made by a general linear model of repeated measures in two levels. All reported P-values represented two-tailed tests, and $P \leq 0.05$ was considered statistically significant. The statistical analysis was performed using the SPSS program, version 11.5 (SPSS, Chicago, IL, U.S.A.).

Results

Influence of neuropeptides on cytokine production: calcitonin gene-related peptide receptor expression in peripheral blood mononuclear cells

PBMC from patients with chronic AD lesions ($n = 10$) and nonatopic controls ($n = 10$) were cultured with three NPs (SOM, CGRP and SP) and the production of IL-13 and IFN-γ was analysed. Significant changes were induced only by CGRP in patients with AD and were restricted to CLA+ T cells, with an increase in IL-13 production [CGRP: median 5.17%, interquartile range (IQR) 4.70–5.70%; unstimulated: median 3.12%, IQR 2.76–3.18%; $P < 0.05$]. No changes were observed in IFN-γ production. No detectable changes were detected in nonatopic controls for any of the NPs assayed (Fig. 1).

Patients with AD had a lower expression of CGRP-R (median 0.14, IQR 0.075–0.23) compared with controls (median 0.41, IQR 0.23–1.37) ($P < 0.05$) (Fig. 2).

Influence of calcitonin gene-related peptide on cytokine production in cutaneous lymphocyte-associated antigen-positive CD45RO+ T cells

To rule out the influence of other subsets present in PBMC culture, CD45RO+ T cells from seven of the patients with AD and

nine of the controls were purified. The clinical characteristics of these patients are shown in Table 2. We found that IL-13 production, measured by mean fluorescence intensity (MFI), was significantly increased in CLA+ T cells (CGRP: median 12.38, IQR 6.83–13.81; unstimulated: median 5.60, IQR 4.62–8.815; $P = 0.035$) from patients with AD but not in CLA- T cells (Fig. 3). No changes were detected in the control group. Not all the patients with AD responded similarly to CGRP; the three patients (patients 1, 6 and 7) with the highest total serum IgE levels and the highest affected BSA were those in whom CGRP induced higher IL-13 production (Table 2).

Regarding IFN-γ production, CGRP induced a decrease, although not significantly, in some patients with AD, but no changes in control subjects. Again, this effect was restricted to CLA+ CD45RO+ T cells (data not shown).

In order to analyse the capacity of CGRP to modulate the Th1/Th2 pattern in CLA+ CD45RO+ T cells, we compared the IL-13/IFN-γ ratio (defined as the division of the MFI of IL-13 by the MFI of IFN-γ) in the presence or absence of this NP; a significant increase was found in the patients with AD after culture with CGRP (CGRP: median 0.46, IQR 0.41–0.57; unstimulated: median 0.32, IQR 0.22–0.43; $P < 0.05$), with no changes in the controls (Fig. 4). Moreover, to evaluate whether the effect caused by CGRP was mediated through their T-cell receptor, we added to the culture a CGRP C-terminal fragment (CGRP 8-37), which acts as a CGRP-R antagonist. The addition of CGRP 8-37 blocked the effect previously observed in IL-13 production by CGRP (CGRP: median 10.24, IQR 8.37–10.92; CGRP 8-37: median 7.86, IQR: 7.43–8.29; $P < 0.05$) (Fig. 5).

Surface expression of HLA-DR in CD45RO+ T cells

When we analysed the activation state of patients with AD we found, as expected, that the MFI of CD45RO+ HLA-DR+ T cells was significantly increased (median 12.17, IQR 8.47–21.22) compared with the controls (median 5.96, IQR 5.16–8.15) ($P = 0.024$). Analysing this marker in the CLA+ or CLA- subpopulation from patients with AD, we found that the MFI of HLA-DR+ cells was significantly higher in the CLA+ CD45RO+ T cells compared with the CLA- CD45RO+ T-cell subset (CLA+: median 38.36, IQR 22.91–51.60; CLA-: median 11.39, IQR 6.46–18.81; $P = 0.026$). Moreover, significant positive correlations between CLA+ CD45RO+ HLA-DR+ activated T cells and total IgE levels were detected ($R^2 = 0.68$; $P < 0.05$).

The addition of CGRP to the memory T-cell cultures induced a significant increase in HLA-DR surface expression in CLA+ CD45RO+ T cells from patients with AD (CGRP: median 43.67, IQR 27.28–61.62; unstimulated: median 38.36, IQR 22.91–51.60; $P = 0.028$) but not in controls ($P = 0.26$), with no significant effect on the CLA- CD45RO+ T cells in either patients or controls (Fig. 6).

Discussion

Increasing evidence suggests that the nervous system can influence inflammatory reactions in the skin through NP action on

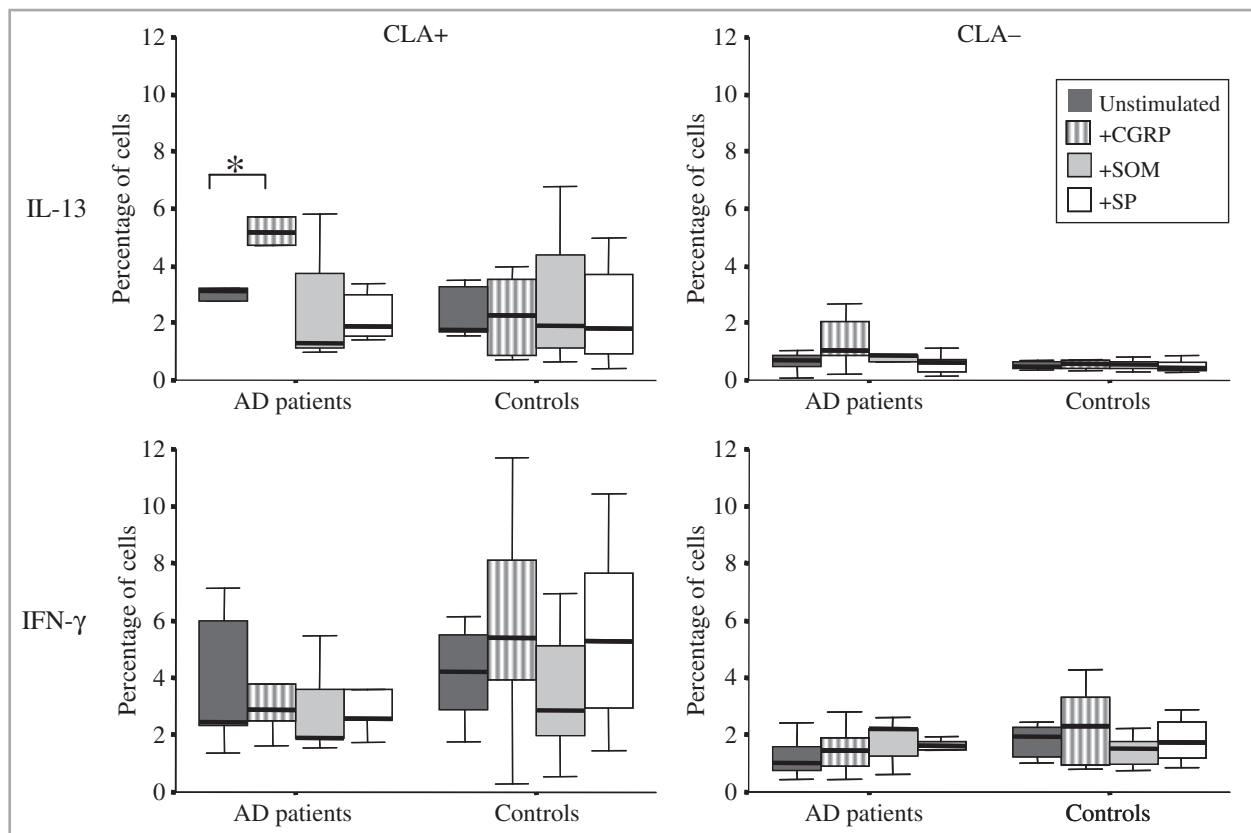


Fig 1. Effect of different neuropeptides [calcitonin gene-related peptide (CGRP), somatostatin (SOM) and substance P (SP)] on interleukin (IL)-13 and interferon (IFN)- γ production in peripheral blood mononuclear cells from patients with atopic dermatitis (AD) ($n = 10$) and nonatopic controls ($n = 10$), comparing cutaneous lymphocyte-associated antigen (CLA)+ and CLA- T cells. Differences (*) are considered significant at $P < 0.05$.

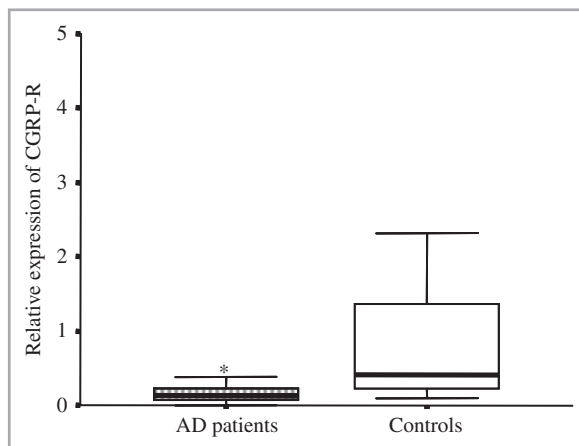


Fig 2. Relative expression of calcitonin gene-related peptide receptor (CGRP-R) in peripheral blood mononuclear cells from patients with atopic dermatitis (AD) ($n = 10$) and nonatopic controls ($n = 10$). Differences (*) are considered significant at $P < 0.05$. Relative units represent the concentration ratio between CGRP-R and porphobilinogen deaminase (PBGD) housekeeping mRNA.

immune system cells.^{4,5,8,23} NPs can directly affect T-cell function, either by stimulating or by silencing key immunological functions, such as cytokine secretion, proliferation and

cell trafficking.²⁴ The involvement of NPs has been specially suggested in AD, as the presence of different NPs, such as CGRP and SP, has been shown in skin lesions.^{8,25}

In the present study we analysed the effect of different NPs (CGRP, SOM and SP) on cytokine production in circulating T cells from patients with AD with chronic lesions and intense pruritus, where NPs may have a more relevant role. We selected these NPs because it has been previously shown that they can directly induce a marked secretion of the cytokines IL-2, IFN- γ , IL-4 and IL-10 from murine T-cell lines, changing the pattern of cytokine secretion from Th2 to Th1 and vice versa.⁶ In our experiments, of the three NPs studied only CGRP induced changes in PBMC, with increased IL-13 production. Interestingly, the effect observed occurred only in CLA+ T cells. The relevance of this T-cell subset in AD has been reported previously, with circulating CLA+ T cells responding to environmental stimuli and cutaneous allergens,^{18,26} as well as expressing T-cell receptor $\nu\beta$ specific for *Staphylococcus aureus* superantigens.²⁷ Moreover, an increase has been shown in the percentage of CLA+ cells in the circulation induced by psychological stress in patients with AD compared with healthy controls.²⁸ These data, together with our results, provide evidence for a possible functional link between CGRP and CLA+ T lymphocytes in AD.

Table 2 Severity, percentage body surface area (BSA) affected and total IgE of the patients with atopic dermatitis and interleukin (IL)-13 production induced by calcitonin gene-related peptide (CGRP)

Patient	Severity	BSA affected (%)	Total IgE (kU L ⁻¹)	IL-13 production induced by CGRP (MFI) ^a
1	Severe	60	13 160	12.53
2	Mild	30	862	1.76
3	Moderate	50	1574	-0.99
4	Moderate	20	2096	0.22
5	Moderate	30	1131	2.77
6	Severe	60	6650	6.86
7	Severe	60	3624	8.27

^aResults are expressed as increase in MFI, calculated as (MFI of IL-13 induced by CGRP) – (MFI of IL-13 of unstimulated cells). MFI, mean fluorescence intensity.

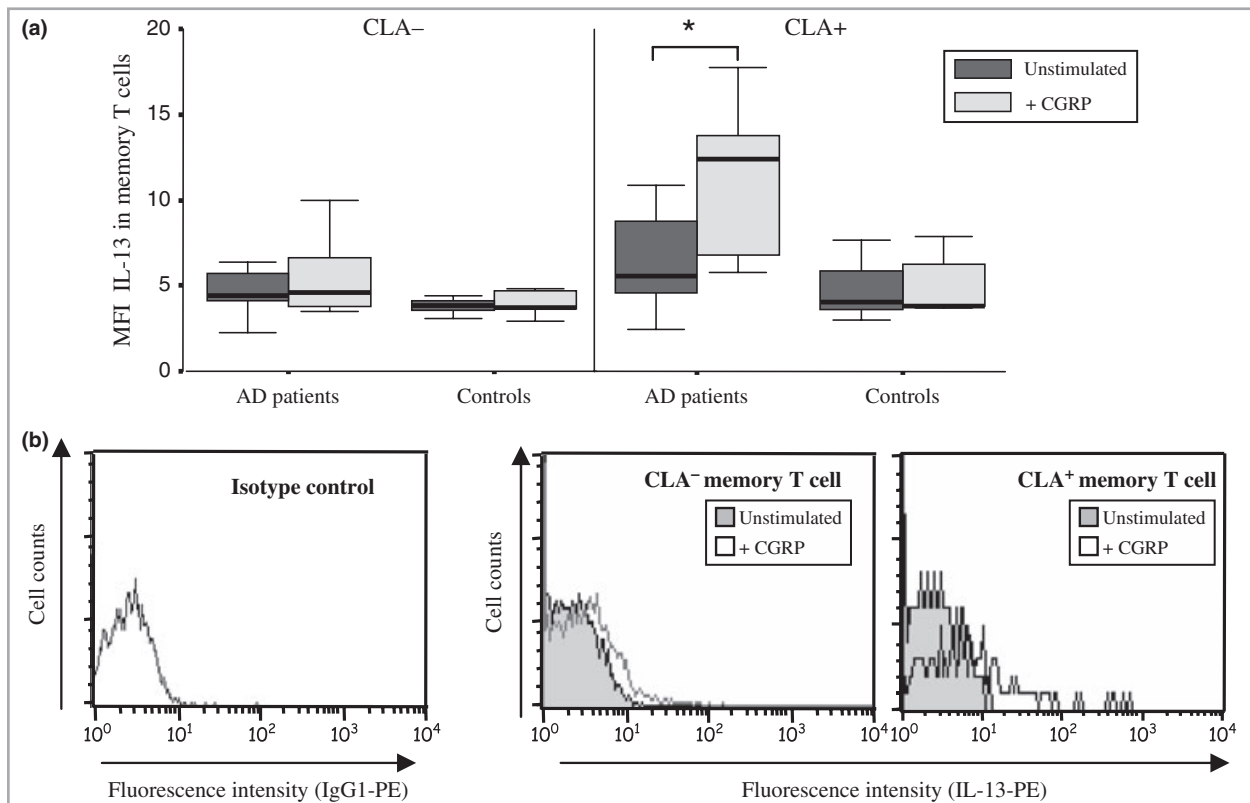


Fig 3. (a) Box plots of mean fluorescence intensity (MFI) of interleukin (IL)-13 in cutaneous lymphocyte-associated antigen (CLA)+ or CLA- memory T cells from patients with atopic dermatitis (AD) ($n = 7$) and nonatopic controls ($n = 9$) in the presence or absence of calcitonin gene-related peptide (CGRP) stimulation. Differences (*) are considered significant at $P < 0.05$. (b) Histograms representative of one patient with AD showing changes in IL-13 production after culturing with CGRP in CLA- or CLA+ memory T cells and isotype control. PE, phycoerythrin.

To investigate the basis of the apparent increased sensitivity of the T cells from patients with AD to respond to CGRP, we analysed the receptor levels of CGRP-R in PBMC, and found a significantly lower receptor level in patients with AD compared with controls. These results agree with those of other authors who have found lower CGRP plasma levels in patients with AD, which normalized after treatment,²⁹ and reflect a general downregulation of CGRP in patients with AD with higher susceptibility to inflammatory stimuli.

In order to rule out the influence of other subsets present in the PBMC culture, such as B lymphocytes, natural killer cells or monocytes, we isolated memory T cells and confirmed that CGRP directly influenced the T lymphocytes, significantly increasing IL-13 production and the surface expression of the activation marker HLA-DR, an effect that was again restricted to CLA+ T cells.

Th1 and Th2 imbalance has been thoroughly investigated in AD.^{9,19,30,31} However, the influence of NPs on cytokine production has not been sufficiently addressed. In T-cell lines

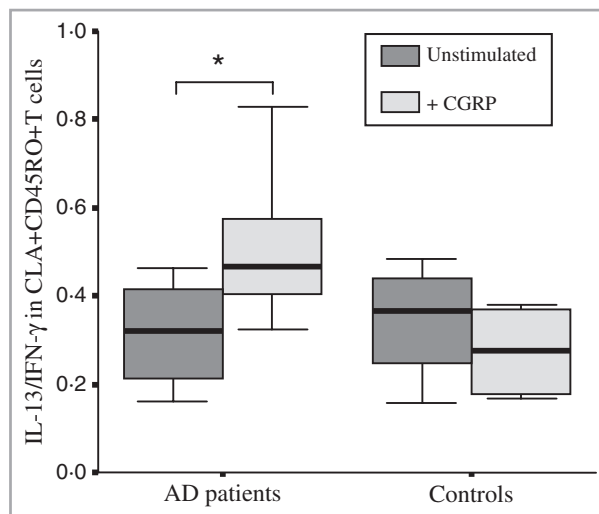


Fig 4. Effect of calcitonin gene-related peptide (CGRP) stimulation on interleukin (IL)-13/interferon (IFN)- γ ratio in cutaneous lymphocyte-associated antigen (CLA)+ memory T cells in patients with atopic dermatitis (AD) ($n = 7$) and nonatopic controls ($n = 9$). The ratio was calculated using the mean fluorescence intensity of both cytokines. Differences (*) are considered significant at $P < 0.05$.

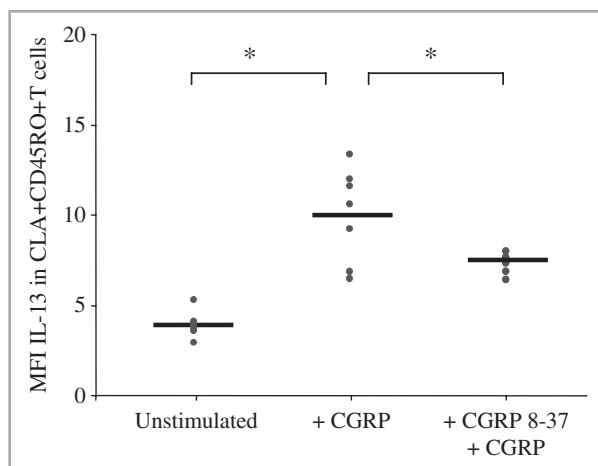


Fig 5. Effect of the calcitonin gene-related peptide (CGRP) 8-37 antagonist on interleukin (IL)-13 production induced by CGRP in cutaneous lymphocyte-associated antigen (CLA)+ memory T cells from patients with atopic dermatitis ($n = 7$). Differences (*) are considered significant at $P < 0.05$. MFI, mean fluorescence intensity.

obtained from mice and in PBMC from allergic subjects, CGRP has been shown to induce a shift from a Th1 to a Th2 cytokine profile.^{6,7} In the present study we evaluated the influence of CGRP on the cytokine profile in patients with AD by analysing the IL-13/IFN- γ ratio, which is considered a means of assessing the Th2/Th1 state.³² We observed that CGRP favoured the Th2-type skewing conditions, inducing an increase in the IL-13/IFN- γ ratio, indicating the ability of NPs to contribute to the modulation of the cytokine balance.

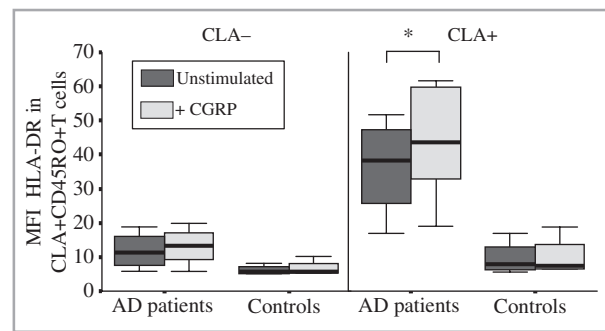


Fig 6. Analysis of induction of HLA-DR by calcitonin gene-related peptide (CGRP) in cutaneous lymphocyte-associated antigen (CLA)+ or CLA- memory T cells from patients with atopic dermatitis (AD) ($n = 7$) and nonatopic controls ($n = 9$). Data are presented as mean fluorescence intensity (MFI). Differences (*) are considered significant at $P < 0.05$.

Given the complexity of AD, we tried to select a homogeneous group of patients with extrinsic AD and chronic lesions. However, not all the patients had a similar response to CGRP. Those with more severe clinical symptoms, measured by percentage of skin area involved, had higher increases in IL-13 in response to CGRP, being selective to CLA+ memory T cells. Moreover, these patients had higher HLA-DR and IgE levels, indicating that the activation state of CLA+ T cells and baseline IgE levels can influence the response to CGRP. It has previously been reported that stress responsiveness of patients with AD depends on baseline serum IgE levels,^{33,34} and HLA-DR and IL-13 have been related with AD severity.^{35,36} Therefore, we postulate that in patients with AD CGRP might contribute to exacerbating the clinical symptoms, mainly CLA+ T cells. Previous reports have suggested the involvement of CGRP in the modulation of pruritus, as those patients with higher SCORAD scores showed significantly higher plasma CGRP levels compared with those patients with low SCORAD scores, independently of its downregulation in both groups compared with controls.²⁹ Whether this effect is mediated by CLA+ T cells is unknown, but IL-31 has recently been reported to be a cytokine involved in the induction of pruritus, and it is expressed in CLA+ T cells from patients with AD,¹⁷ supporting the validity of this hypothesis.

In this study, we present evidence that circulating CLA+ T cells can preferentially respond to CGRP by modulating cytokine production and increasing the activation state. Although the ability of NPs to modulate T-cell functions has previously been studied in T-cell lines⁶ and PBMC,^{9,11,37} this is the first study to show a preferential effect of an NP, CGRP, on CLA+ memory T cells from patients with AD, supporting the important role of CGRP in the pathogenesis of AD by regulating immune responses and contributing to the cross-talk between the immune and nervous systems. This information can be utilized for the development of future therapeutic approaches.

Acknowledgments

We thank Ian Johnstone for help with the final English language version of this manuscript. This study was partly supported by a grant from the Spanish Ministry of Health (01/0865) and from the Junta de Andalucía (198/04).

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