

ORIGINAL ARTICLE

The neuropeptide calcitonin gene-related peptide affects allergic airway inflammation by modulating dendritic cell function

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Summary

Background The neuropeptide calcitonin gene-related peptide (CGRP) is released in the lung by sensory nerves during allergic airway responses. Pulmonary dendritic cells (DC) orchestrating the allergic inflammation could be affected by CGRP.

Objective To determine the immunomodulatory effects of CGRP on DC function and its impact on the induction of allergic airway inflammation.

Methods CGRP receptor expression on lung DC was determined by RT-PCR and immunofluorescence staining. The functional consequences of CGRP receptor triggering were evaluated *in vitro* using bone marrow-derived DC. DC maturation and the induction of ovalbumin (OVA)-specific T cell responses were analysed by flow cytometry. The *in vivo* relevance of the observed DC modulation was assessed in a DC-transfer model of experimental asthma. Mice were sensitized by an intrapharyngeal transfer of OVA-pulsed DC and challenged with OVA aerosol. The impact of CGRP pretreatment of DC on airway inflammation was characterized by analysing differential cell counts and cytokines in bronchoalveolar lavage fluid (BALF), lung histology and cytokine responses in mediastinal lymph nodes.

Results RT-PCR, immunofluorescence and cAMP assay demonstrated the expression of functionally active CGRP receptors in lung DC. RT-PCR revealed a transcriptional CGRP receptor down-regulation during airway inflammation. CGRP specifically inhibited the maturation of *in vitro* generated DC. Maturation was restored by blocking with the specific antagonist CGRP_{8–37}. Consequently, CGRP-pretreated DC reduced the activation and proliferation of antigen-specific T cells and induced increased the numbers of T regulatory cells. The transfer of CGRP-pretreated DC diminished allergic airway inflammation *in vivo*, shown by reduced eosinophil numbers and increased levels of IL-10 in BALF.

Conclusions and Clinical Relevance CGRP inhibits DC maturation and allergen-specific T cell responses, which affects the outcome of the allergic airway inflammation *in vivo*. This suggests an additional mechanism by which nerve-derived mediators interfere with local immune responses. Thus, CGRP as an anti-inflammatory mediator could represent a new therapeutic tool in asthma therapy.

Keywords allergic asthma, calcitonin gene-related peptide, dendritic cells, neuro-immune interactions, sensory nerves

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Introduction

The immune response generated by inhaled allergens is crucially dependent on the action of lung dendritic cells (DC). DC mediate sensitization to airborne allergens and induce allergen-specific Th2-driven immune responses leading to eosinophil-prone airway inflammation [1–3]. In addition to their critical role in sensitization, lung DC are substantially involved in established airway inflammation [4, 5]. The inflammatory allergic response induces neural dysregulation in sensory fibres, leading to typical symptoms of the disease, such as wheezing, cough and bronchospasm [6–8]. This response is mediated by sensory fibres located beneath the airway epithelium [9]. Upon activation, these neurons locally release neuropeptides due to the axon reflex [10]. It is thought that allergen provocation promotes this process, leading to elevated levels of neuropeptides like calcitonin gene-related peptide (CGRP) and tachykinins. These neuropeptides subsequently act directly on airway structural cells, thereby eliciting characteristic features of asthma like bronchoconstriction, vasodilation and mucus hypersecretion, known as 'neurogenic inflammation' [6, 10, 11].

Because of increased production and facilitated release triggered by inflammatory mediators, sensory neuropeptides are present in allergic asthma at elevated levels [12–18]. Specifically, neurotrophins such as nerve growth factor and brain-derived neurotrophic factor are released by inflammatory and epithelial cells and modulate sensory nerve activity. They trigger neuropeptide production and thereby enhance neurogenic inflammation and airway hyperreactivity [19]. Growing evidence suggests that the neuropeptides released may also directly affect immune cells [20]. Of particular interest is the potential modulation of DC because of their key role in orchestrating the allergic airway response [1–5]. In a previous study, we observed that lung DC were frequently found in association with neuropeptide-containing sensory nerves [21]. This colocalization was enhanced during allergic airway inflammation and represented sites of T cell proliferation [22]. This finding raised the question of whether neuropeptides released from sensory nerves modulate the function of DC with which they are colocalized. In this regard, CGRP is of particular interest because of its anti-inflammatory effects observed on several immune cells, such as macrophages, B cells and T cells and, particularly, DC [20, 23].

CGRP is a 37 – amino acid peptide, widely distributed in the nervous and cardiovascular system. It acts mainly as a potent vasodilator. CGRP is also present in afferent C-fibres throughout the respiratory tract [23]. The high-affinity receptor for CGRP is a heterodimer of calcitonin receptor-like receptor (CRLR) and receptor activity modifying protein 1 (Ramp1). There are also low-affinity receptors for CGRP, namely adrenomedullin receptor heterodimers CRLR/Ramp2 and CRLR/Ramp3 [24, 25].

CGRP has been described to exhibit diverse immunomodulatory effects on DC, such as the chemoattraction of immature DC and the inhibition of antigen presentation in human monocyte-derived DC or peripheral blood mononuclear cells [26–28]. In contrast, CGRP enhances antigen presentation in Langerhans cells, promoting the induction of Th2-type immune responses [29]. Recently, CGRP has been shown to exert both immunosuppressive and stimulatory effects on skin DC function, depending on the type of immune response induced by different contact sensitizers [30].

For murine bone marrow-derived dendritic cells (BMDC), similar to those used in this study, CGRP has been shown to inhibit toll-like receptor (TLR) ligand-induced TNF- α secretion [31, 32].

The effects of CGRP in modulating DC function in allergen-specific immune responses, especially with regard to the allergic airway inflammation *in vivo*, have not been addressed until now.

Thus, in this study, we investigated the effect of CGRP on BMDC maturation and the induction of allergen-specific T cell responses *in vitro*. To address the *in vivo* relevance, we used a transfer model of asthma to evaluate how DC-driven allergic airway inflammation is altered by CGRP.

Methods

Animals

OVA TCR transgenic BALB/c DO11.10 mice (The Jackson Laboratory, Bar Harbor, ME, USA) and CD11c-EYFP-transgenic mice (kindly provided by Michel C. Nussenzweig, The Rockefeller University, New York) were bred at the animal facility of the Fraunhofer ITEM and used at 10–15 weeks of age. Wild-type BALB/c (6–8 weeks old) were purchased from Charles River (Sulzfeld, Germany). All animals were held in regular 12 h dark/light cycles at 22 °C and received laboratory food and tap water *ad libitum*. The animals were acclimated for at least 1 week and randomized 6 days before the experiments started. The studies were approved by the appropriate governmental authority and performed in concordance with the German animal protection law.

Whole-mount tissue processing and immunostaining

Lung tissue whole mounts were dissected and processed as described earlier [21, 22]. Briefly, naïve CD11c-EYFP-transgenic mice were killed with an overdose of i.p.-administered pentobarbital and lungs were resected and inflation-fixed with 2% paraformaldehyde overnight at 4 °C. After washing in PBS, the main bronchi were dissected from the surrounding lung parenchyma. The airways were then washed and permeabilized using 0.3%

Triton X-100 in PBS. After repeated washing in PBS and 30 min blocking with 4% donkey serum, the whole-mount immunostaining was performed using the following primary antibody mix: chicken polyclonal antibody to green fluorescent protein (1 : 500) and guinea-pig polyclonal antibody to CGRP (1 : 400, both Acris, Hiddenhausen, Germany) as well as rat monoclonal antibody to MHC-II (1 : 50, Abcam, Cambridge, UK). For the isotype control, chicken IgY (1 : 50, Promega, Madison, WI, USA), guinea-pig IgG (1 : 400, Acris) and rat IgG2b (1 : 125, Abcam) were used. The primary antibody mix or isotype controls were incubated overnight at 4 °C. After repeated washing and blocking, the whole mounts were stained with the secondary antibodies donkey anti-chicken Cy2 (1 : 100), donkey anti-guinea-pig Cy5 (1 : 200) and donkey anti-rat Cy3 (1 : 400) (all from Dianova, Hamburg, Germany) overnight at 4 °C. After repeated washing, after the last washing step, the samples were mounted on a glass slide using Prolong Gold mounting medium (Molecular Probes, Eugene, OR, USA). Confocal images were acquired as three-dimensional image stacks using an LSM 510 META (Carl Zeiss, Jena, Germany).

Acute model of allergic airway inflammation induced by intraperitoneally sensitization

Acute allergic airway inflammation was induced as described before [21]. Briefly, BALB/c mice were sensitized i.p. with 10 µg OVA (grade VI, Sigma Aldrich, Taufkirchen, Germany) adsorbed to 1.5 mg aluminium hydroxide (Al(OH)₃, Imject Alum, Pierce, Rockford, IL, USA) on days 0, 14 and 21. The negative control group was sham-sensitized using saline adsorbed to Alum, respectively. After sensitization, allergen provocation was performed on days 27, 28 and 35 animals by exposure to 1% OVA aerosol. The negative control was exposed to saline aerosol on days 27 and 28. The last challenge was performed with 1% OVA aerosol as well. On day 36, animals of each group of $n = 12$ were killed by an overdose of i.p. administered pentobarbital and analysed. This treatment resulted in a pronounced eosinophilic airway inflammation in the positive group in contrast to the negative group, according to the differential cell count of the BALF (data not shown). A satellite group of each three animals for the positive and negative group was treated likewise and used for immunofluorescence staining.

Lung dendritic cell isolation

Lungs were excised from animals of the OVA-sensitized and challenged or the negative group, respectively. For each of the three independent preparations, the lungs of four animals of both groups were used and pooled for the further processing. The lung tissue was minced using small scissors and digested with 2 mg/mL collagenase type

III (Worthington Biochemical Corp., Lakewood, NJ, USA) and 0.1% DNase I (Roche Diagnostics, Mannheim, Germany) by incubation at 37 °C, shaking for 30 min. The digested tissue was further homogenized by thoroughly mixing with a transfer pipette to obtain a single-cell suspension. The cells were passed through a stainless-steel sieve (100 µm pore size) and, eventually, the remaining red blood cells were removed using a hypotonic lysis buffer. The cell suspension was then enriched for CD11c⁺ cells by magnetic separation using the CD11c⁺ isolation kit according to the manufacturer's instructions (MACS[®], Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequently, after washing and blocking with goat serum (1 : 25), the cells were stained with an APC-labelled hamster monoclonal antibody to CD11c (BD Biosciences, Heidelberg, Germany) and a PE-labelled rat monoclonal antibody to MHC class II (eBioscience, Nattuetec, Frankfurt, Germany) or the appropriate isotype controls hamster IgG1 and rat IgG2b for 30 min at 4 °C. Afterwards, cells were washed again and further analysed and processed by fluorescence-activated cell sorting using a FACS Aria (BD Biosciences). DC were identified and sorted as CD11c⁺ MHC-II^{high} cells, whereas contaminating tissue macrophages were separated by MHC-II^{low} expression. The sorted cells were directly used for further experiments or stored in RLT buffer for PCR analysis.

Real-time polymerase chain reaction and quantification

Lung DC were isolated from the OVA-sensitized and -challenged mice and the negative group, respectively. RNA was extracted from the sorted lung DC using the RNeasy kit and reverse transcribed using the Omniscript RT kit (both Qiagen, Hilden, Germany). cDNA was analysed for the expression of CGRP receptor molecules using specific primers for CRLR (5'-CAG CAG GGA CGG AAT CAA-3' and 5'-GCC TCC ACA GAG CAT CCA G-3'), Ramp1 (5'-GGA CCC TGA CTA TGG GAC TCT-3' and 5'-GCT TGC TCC TCC AGA CCA C-3'), Ramp2 (5'-TCC CTG AAC CAA TCT CTT CC-3' and 5'-TTG TGT CGT GAG TCC CCT TT-3') and Ramp3 (5'-TGA AGA CCC CAG CAC AGC-3' and 5'-CCA AAG CAA ACC AGA CAG AAG-3') as well as the housekeeping gene PBGD (5'-ATC CAG GTC CCT GTT CAG CAA GAA-3' and 5'-CAT TAA GCT GCC GTG CAA CAT CCA -3') by real-time PCR using Light-Cycler[®] FastStart DNA Master SYBR Green (Roche). PCR products were verified via sequencing and gel electrophoresis. Quantification of expression between OVA-sensitized/-challenged animals and naïve animals was performed using the delta delta C_t method. Target gene expression was normalized to PBGD and a ratio of sensitized : naïve group was calculated. Compared with the unchanged expression of PBGD, a ratio higher than 2 or lower than 0.5 was regarded to be biologically significant and was verified by statistical analysis.

Cyclic adenosine monophosphate assay

Freshly isolated lung DC were resuspended in phenolred-free DMEM (Gibco Invitrogen, Darmstadt, Germany) containing 500 μ M of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (Sigma Aldrich) and 1×10^5 cells were each stimulated with 10^{-6} M CGRP (Phoenix Pharmaceuticals, Burlingame, CA, USA) for 2, 5 and 10 min or vehicle (DMEM). The cells were washed twice with ice-cold PBS and treated with 0.1 M HCl for 10 min at room temperature (RT). Cell lysate supernatants were frozen at -20°C overnight and analysed using a cyclic AMP kit according to the manufacturer's instructions (Assay Designs, Ann Arbor, MI, USA). The lower detection limit of the assay was 0.78 pmol/mL.

Immunofluorescence staining

Lungs from mice of the satellite groups of the acute OVA-model were filled with a 1:3 dilution of OCT cryotek medium (Sakura Finetek, Zoeterwoude, the Netherlands) in PBS instilled through the trachea. The lungs were removed and embedded in OCT to be immediately frozen in liquid nitrogen. Five micrometre thick sections were immunostained after blocking with PBS/10% donkey serum/1% BSA for 30 min with the following primary antibodies at 4°C overnight: AlexaFluor[®] 488-labelled hamster polyclonal to CD11c (1:1000, Caltag, Invitrogen), goat polyclonal to Ramp1 (1:30, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-CRLR (1:20, kindly provided by A. Fischer, Charité, Berlin, Germany). CD11c staining was amplified by staining with rabbit anti-AlexaFluor[®] 488 (1:400, BD Biosciences) for 1 h. After washing all slides repeatedly with PBS/0.05% Tween-20 for a total of 1 h and PBS for 20 min, the secondary antibody staining was performed for 45 min at RT with AlexaFluor[®] 488-labelled donkey anti-rabbit (1:400) and AlexaFluor[®] 546 donkey anti-goat IgG (1:400) (both Invitrogen) or Cy3-labelled donkey anti-goat antibody (1:200, Jackson ImmunoResearch, Dianova, Hamburg, Germany). Nuclei were stained using To-Pro-3 (Invitrogen). Confocal images were analysed using an LSM 510 META (Carl Zeiss).

Generation of bone marrow-derived dendritic cells

BMDC generation was performed according to a protocol adapted from Lutz et al. [59]. Briefly, the tibia and femur of naïve BALB/c mice were dissected and flushed with RPMI. After red blood cell lysis, cells were seeded at 5×10^5 per well in tissue culture medium (TCM: RPMI-Glutamax (Gibco Invitrogen) with 1% penicillin-streptomycin (Lonza, Verviers, Belgium), 5% FCS and 50 μ M β -mercaptoethanol (both Sigma) with 20 ng/mL GM-CSF (ImmunoTools, Friesoythe, Germany) into six-well tissue culture plates. Cells were cultured for 10 days, with refreshing

of TCM on days 3 and 6. On day 9, cells were transferred to a serum-free medium before stimulation to avoid the degradation of CGRP.

Analysis of bone marrow-derived dendritic cell maturation

To induce maturation, BMDC were incubated on day 9 with 100 ng/mL LPS (Sigma) with or without costimulation with 10^{-9} M CGRP for 24 h. On day 10, cells were harvested and stained with the following monoclonal antibodies: hamster anti-CD11c-APC (BD Biosciences) and MHC class II-A1488 (BioLegend, Uithoorn, the Netherlands) to identify DC as well as rat anti-CD80-PE, rat anti-CD86-PE and rat anti-CD40-PE (all eBioscience) or the appropriate isotype controls (hamster IgG1-APC, rat IgG2b-A1488, hamster IgG1-PE and rat IgG2a-PE (isotype for both CD86/CD40) for 30 min. The flow cytometric analysis was performed using an FC500 (Beckmann Coulter, Krefeld, Germany).

T cell proliferation assay

BMDC were stimulated with 100 μ g/mL OVA, 10 ng/mL LPS and CGRP (10^{-10} – 10^{-6} M) overnight on day 9 in serum-free medium. On day 10, cells were harvested and washed in RPMI. BMDC were seeded at 10^4 /well in 96-well plates in triplicate and cocultured for 3 days with 10^5 /well OVA-specific T cells. These were isolated previously from the spleens of OVA-specific TCR transgenic D011.10 mice using the MACS[®] CD4⁺ negative isolation kit (Miltenyi Biotec) and labelled with CFSE (Sigma). After 3 days, supernatants were collected for cytokine determination. Cells were washed and blocked with goat serum (1:25) in PBS and stained with anti-CD4 PE and anti-KJ1-26 (transgenic T cell receptor) or the appropriate isotype controls (rat IgG2 a PE and mouse IgG2a, respectively). Flow cytometric analysis of the CFSE division profile of CD4⁺KJ1-26⁺ cells was performed using an FC500 (Beckmann Coulter).

Analysis of T cell activation and T reg

In some coculture experiments, the cells were additionally analysed for the proportion of T reg cells. Therefore, the cells were stained with CD4-PE, CD25-FITC and foxp3-APC or the appropriate isotype controls using the anti-mouse/rat foxp3 APC staining kit according to the manufacturer's instructions (eBioscience).

Acute model of asthma induced by intrapharyngeal adoptive transfer of bone marrow-derived dendritic cells

Allergic airway inflammation was induced in mice as described previously [3, 34, 35]. Briefly, BMDC were stimulated with 100 μ g/mL OVA (Worthington

Table 1. Study design for an adoptive transfer-induced model of asthma

Study group	n	BMDC pulse <i>in vitro</i>			Aerosol provocation <i>in vivo</i>
		OVA ($\mu\text{g/mL}$)	LPS (ng/mL)	CGRP	
NEG	7	100	1	0	saline
POS	11	100	1	0	1% OVA
CGRP	11	100	1	10^{-9} M	1% OVA
Dexa	5	100	1	0	1% OVA Dexa i.p. – 18/–2 h

On day 0, BMDC were pulsed with either an OVA/LPS/vehicle or OVA/LPS/CGRP and transferred intrapharyngeally without a further adjuvant to induce sensitization. On days 10 to 12, mice were exposed to 1% OVA aerosol or saline for 30 min. The Dexa treatment group received an additional i.p. injection of dexamethasone 18 and 2 h before each aerosol challenge.

BMDC, bone marrow-derived dendritic cells; NEG, negative; POS, positive; CGRP, calcitonin gene-related peptide; Dexa, dexamethasone; OVA, ovalbumin; LPS, lipopolysaccharide; i.p., intraperitoneally; i.ph., intrapharyngeally.

Biochemical, Lakewood, NJ, USA), LPS (1 ng/mL) and CGRP (10^{-9} M) or vehicle solution overnight. BALB/c mice were anaesthetized with Ketamin/Rompun i.p. and 1×10^6 BMDC resuspended in 80 μL PBS were instilled intrapharyngeally (i.ph.) by pipetting the inoculum to the larynx and preventing inhalation through the nose, leading to the inhalation of the inoculum. Animals that swallowed the instilled cells instead of inhaling them were not included in the study. On days 10–12, mice were exposed to 1% OVA aerosol for 30 min; the negative control group (NEG) received saline aerosol. The positive treatment control group (Dexa) additionally received i.p. treatment with 0.5 mg/kg Dexa (Ratiopharm, Ulm, Germany) in 200 μL saline 18 and 2 h before each challenge. The different groups and treatment are summarized in Table 1.

Animal dissection

Mice were killed 24 h after the last aerosol challenge by an overdose of pentobarbital (0.1 mg Narcoren[®] in 200 μL NaCl i.p.). Samples were taken and processed as described before [60]. Briefly, mice were bled via the vena cava caudalis, and serum was conserved at -80°C . Bronchoalveolar lavage was performed twice with 0.8 mL of ice-cold sterile PBS. After the determination of absolute cell counts in BALF using an ACT-8 cytometer (Beckmann Coulter), cytopots of BALF cells were prepared on a cyto-centrifuge (Shandon, Frankfurt/Main, Germany), stained according to Pappenheim and differential cell counts were assessed. BALF supernatants were collected for cytokine measurements. Lungs were filled with OCT as described above. Lung histology was assessed by staining cryosections with haematoxylin/eosin. MLN were collected in ice-cold RPMI for further *in vitro* restimulation with OVA.

Lymph node restimulation

MLN were homogenized and filtered using a 70 μm cell strainer (BD Biosciences) to yield a single-cell suspension. 1×10^5 cells per well were seeded in 96-well round-bottom

plates and restimulated with 100 $\mu\text{g/mL}$ endotoxin-free OVA (Worthington) for 5 days. Supernatants were collected for cytokine measurement.

Cytokine measurement

Cytokine concentrations in BALF and supernatants of MLN restimulation were determined using a mouse Th1/Th2 9-plex Ultra-Sensitive Kit according to the manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD, USA). IL-10 from T cell cocultures was measured by ELISA according to the manufacturer's instructions (R&D Biosciences, Wiesbaden-Nordenstadt, Germany).

Quantification of serum levels of ovalbumin-specific antibodies

To monitor sensitization processes in the transfer model of experimental asthma, the serum titre of OVA-IgE, -IgG1 and -IgG2a was assessed using OVA-specific antibody ELISA kits according to the manufacturer's instructions (Assay Designs, Ann Arbor, MI, USA). The detection limit for OVA-specific IgE, IgG1 and IgG2a was 16 U/mL, 31.25 U/mL and 31.25 U/mL, respectively. The IgE and IgG2a levels were below the detection limit in all groups; IgG1 was detectable but no alteration between groups was observed (data not shown).

Statistical analysis

Data are displayed as means $\pm$ standard error of the mean for the indicated number of experiments. To detect significant differences between groups, one-way ANOVA, followed by Dunnett's multi-comparison test in case of multiple groups, or Student's *t*-test for comparing two groups, were applied using the Graph Pad Prism 4 software. Results of $P < 0.05$ were considered to be significant.

Results

Major histocompatibility complex (MHC)-II-expressing lung dendritic cells express functional calcitonin gene-related peptide receptors

Lung DC that colocalize with CGRP-containing nerve fibres were identified as cells with a typical dendritic morphology expressing both CD11c and the antigen-presentation molecule MHC class II (Fig. 1).

These CD11c⁺MHC-II^{high} lung DC were isolated from lung tissue and analysed for the expression of CGRP receptor molecules. Sorted lung DC expressed mRNA for both the high- and the low-affinity receptor molecules CRLR, Ramp1, Ramp2 and Ramp3. A representative picture of the gel electrophoresis of the amplified polymerase chain reaction (PCR) products is shown in the supporting information Figure S1. During the acute allergic airway inflammation, the mRNA expression levels of lung DC revealed a transcriptional down-regulation of CRLR, Ramp1 and Ramp2 as indicated by the decreased expression ratio of the ovalbumin (OVA)-sensitized and challenged group to the negative control group (Fig. 2).

The expression of the high-affinity CGRP receptor (CRLR/Ramp1) was further confirmed by immunofluorescence staining of CRLR and Ramp1 in lung cryosections. CD11c⁺ DC localized beneath the airway epithelium express CRLR (Fig. 3a). Ramp1 was detected in DC and also in CD11c⁺ macrophages. These were distinguished from DC by the characteristic macrophage round morphology (Fig. 3b). Because the Ramp1 staining signal was rather weak, cytospin preparations of sorted lung DC were stained and Ramp1 staining was observed in a minor proportion of the cells and appeared to be vesicular (Fig. 3c).

The activity of the CGRP receptor expressed was demonstrated by the induction of the signalling molecule adenosine 3', 5'-cyclic adenosine monophosphate (cyclic AMP) after CGRP stimulation of isolated lung DC. The levels of cyclic AMP were enhanced after 2 min by CGRP stimulation vs. vehicle control (9.28 vs. 0 pmol/mL) and decreased rapidly (Fig. 3d).

Calcitonin gene-related peptide inhibits lipopolysaccharide (LPS)-induced dendritic cell maturation

To investigate whether CGRP modulates the functional properties of DC and to obtain an adequate number of cells to perform functional analyses, we used an *in vitro* model using BMDC. Inflammatory DC, seen in the airways of allergen-challenged mice, arise from the same precursors as BMDC and furthermore express a CD11c⁺MHC-II^{high} phenotype if cultured *ex vivo*, comparable to *in vitro* generated BMDC [33]. Moreover, BMDC also express CGRP receptor molecules that signal via cyclic AMP [31].

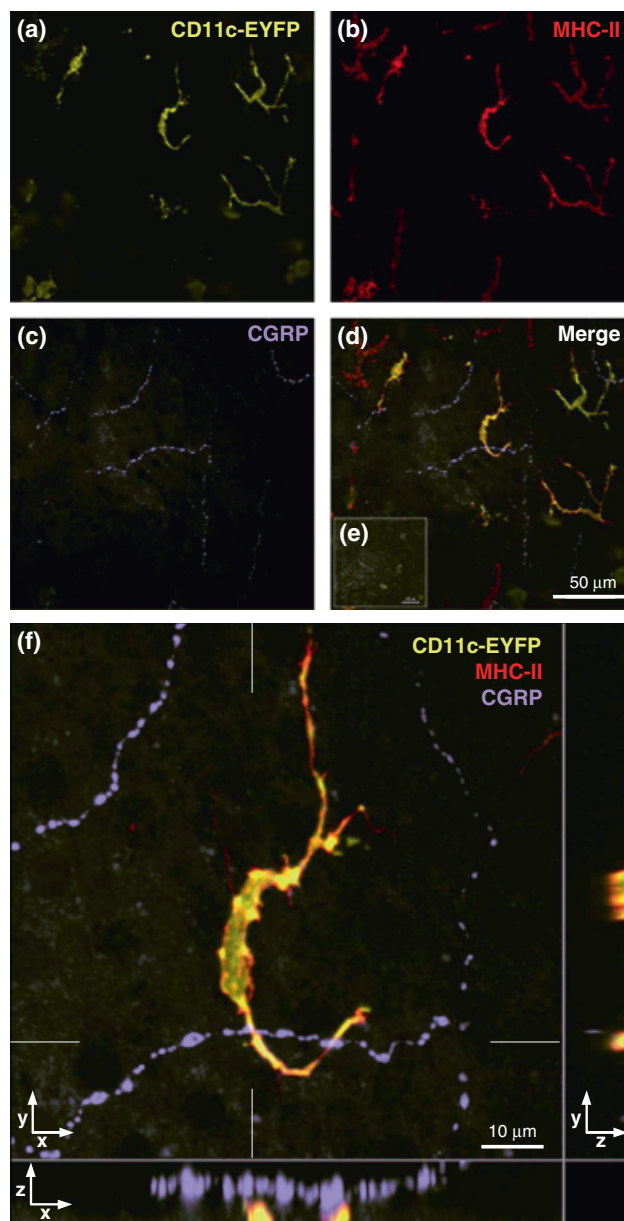


Fig. 1. Colocalization of lung dendritic cells (DC) and calcitonin gene-related peptide (CGRP)-positive sensory nerve fibres. Lung whole-mount specimens from CD11c-EYFP (yellow) transgenic mice were counterstained for CGRP (purple) and MHC class II (red) and analysed by confocal microscopy in the subepithelial region of the main bronchi. CD11c⁺ lung DC express MHC-II and colocalize with CGRP-positive sensory fibres. (a–c) shows the distribution of CD11c-positive cells, MHC-II-positive cells and CGRP-positive neurons, respectively, and the overlay of all channels in (d). The inset (e) represents the overlay of the isotype control. (f) Provides three dimensional information of a DC colocalized with a CGRP-positive neuron. Scale bar = 50 µm or 10 µm in a–e or f, respectively.

Flow cytometric analysis of the classical DC activation markers CD80, CD86 and CD40 demonstrated that CGRP has no effect on the steady-state expression of these costimulatory molecules (data not shown). However, if DC maturation was induced by LPS, leading to the

up-regulation of CD86 (Figs 4a and c) and CD40 (Figs 4b and d), CGRP significantly inhibited this process (10^{-9} M). The specificity of this effect was demonstrated by pre-treatment with the antagonistic peptide CGRP_{8–37}

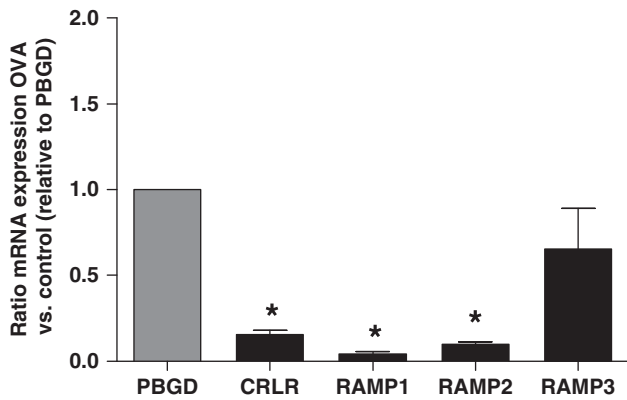


Fig. 2. mRNA expression of the calcitonin gene-related peptide (CGRP) receptor in isolated lung dendritic cells (DC). Quantitative analysis of CGRP receptor expression in isolated lung DC. Expression levels were normalized to the housekeeper gene PBGD and are shown as the ratio of ovalbumin (OVA)-lung DC to control DC (ratio 1.0 if no different expression). For each experiment, DC were isolated from a pool of four lungs from a total of 12 animals each per OVA and saline group analysed in $n = 3$ independent experiments. * $P < 0.01$ compared with PBGD.

(10^{-6} M), which restored the LPS-induced maturation (Figs 4a–d). The expression level of CD80 was not affected by CGRP in either immature or mature DC (data not shown).

Calcitonin gene-related peptide treatment of inflammatory dendritic cells reduces ovalbumin-specific T cell response

To evaluate how the CGRP-induced alterations influence the allergen-specific T cell response, BMDC were pulsed with OVA/LPS±CGRP and cocultured with naïve transgenic T cells from DO11.10 mice, which express OVA-specific T cell receptors. CGRP treatment of DC resulted in a dose-dependent inhibition of OVA-specific T cell proliferation (Fig. 5a), with a significant 28% reduction in proliferating OVA-specific T cells ($CD4^+KJ26^+$) at 10^{-6} M CGRP (Figs 5a and b). Similarly, IL-2 levels were reduced in a dose-dependent manner, reaching significance at 10^{-6} M CGRP (see supporting information Figure S3). The inhibition of T cell proliferation was not associated with a shift in typical Th1/Th2-cytokines, while the pro-inflammatory cytokine TNF- α was reduced in a dose-dependent manner as described previously [32] (see supporting information Figure S3).

As immature DC have been shown to induce the generation of regulatory T cells that express the foxp3

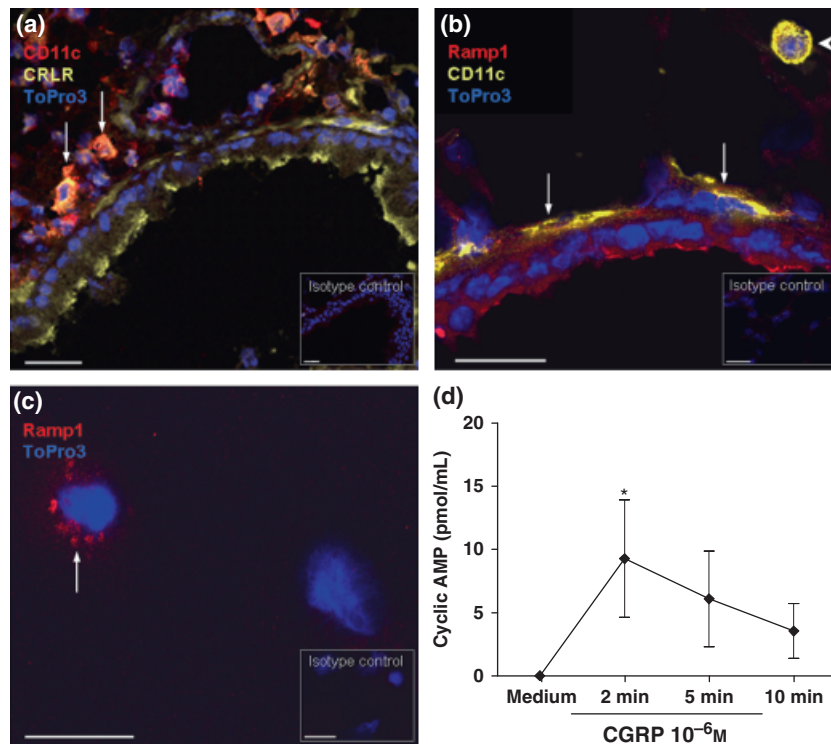


Fig. 3. Immunofluorescence staining and functionality of the calcitonin gene-related peptide (CGRP) receptor. Lung cryosections were stained for CD11c and CRLR (a) or Ramp1 (b) combined with To-Pro-3 nuclei staining. Arrows highlight the double-positive dendritic cells (DC) beneath the airway epithelium. The arrowhead indicates a tissue macrophage. (c) Sorted lung DC were stained for Ramp1 and To-Pro-3. Inserts show the appropriate isotype staining controls. Scale bars are 50 μ m in (a) and 20 μ m in (b) and (c). (d) cyclic adenosine monophosphate release after *ex vivo* stimulation of isolated lung DC with 10^{-6} M CGRP for the indicated time-points. Data show mean $\pm$ SEM for two independent experiments. * $P < 0.05$.

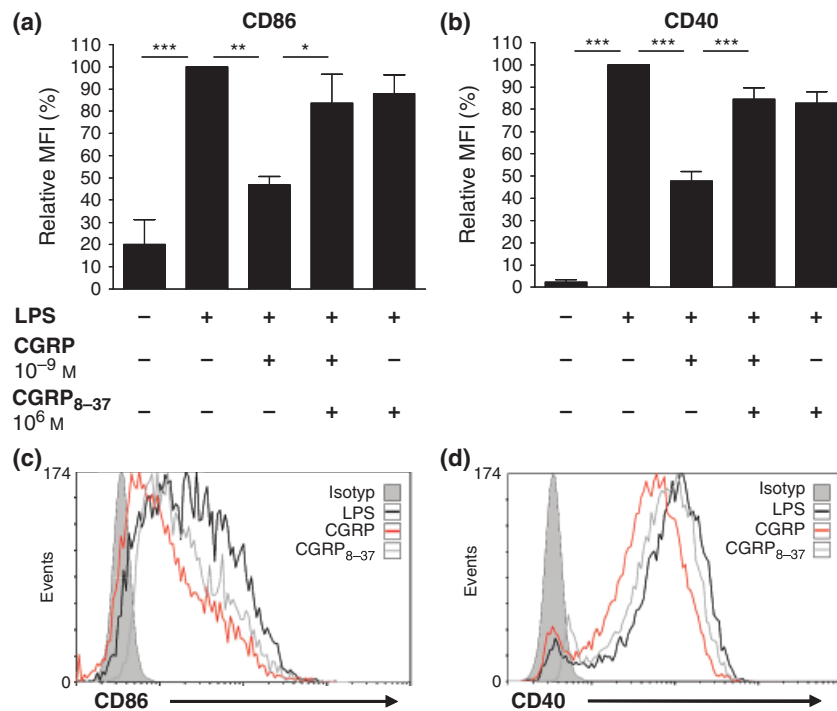


Fig. 4. Calcitonin gene-related peptide (CGRP) effects on lipopolysaccharide (LPS)-induced expression of costimulatory molecules bone marrow-derived dendritic cells were stimulated with LPS (100 ng/mL) to induce maturation and costimulated with or without CGRP (10^{-9} M) overnight. The antagonist CGRP₈₋₃₇ (10^{-6} M) was incubated for 15 min before LPS/CGRP stimulation when indicated. (a–b) Bars represent mean $\pm$ SEM of the mean fluorescence intensity (MFI) normalized to the LPS-treated control of $n = 3$ experiments. *, **, *** indicate significance, with $P < 0.05$, 0.01 and 0.001, respectively. (c–d) Representative overlay histograms for the expression of CD86 (c) and CD40 (d) after LPS stimulation without (black line) or with 10^{-9} M CGRP (red line) and before blocking with 10^{-6} M CGRP₈₋₃₇ (grey line).

transcription factor and are able to produce IL-10, cocultures were further analysed for foxp3⁺ T reg and IL-10 secretion. IL-10 levels increased by about 56% at 10^{-8} M CGRP, but declined again at higher concentrations. This increase did not reach statistical significance (120.6 ± 17.64 pg/mL at 10^{-8} M CGRP vs. 77.44 ± 12.46 pg/mL in the medium control) (Fig. 5c). To evaluate whether CGRP-treated DC secrete IL-10, supernatants from BMDC cultures were analysed as well. However, IL-10 secretion was not altered (see supporting information Figure S2).

In the cocultures, the proportion of foxp3⁺-regulatory T cells of CD4⁺CD25⁺ T cells was slightly increased in the presence of DC treated with the highest dose of CGRP (10^{-6} M), leading to a significant inhibition of T cell proliferation (Fig. 5d). T cell activation was also reduced, as evidenced by a clear reduction in the number of CD25⁺CD4⁺ cells in cocultures with CGRP-treated DC (Fig. 5e).

Calcitonin gene-related peptide treatment of inflammatory dendritic cells impairs their potential to induce eosinophilic airway inflammation *in vivo*

Finally, to assess whether the immunomodulation of DC by CGRP has an impact in the development of allergic

airway inflammation, a transfer model of allergic asthma was used [3, 34, 35]. In this model, sensitization is induced by the transfer of *in vitro* generated OVA-pulsed DC to naïve mice. Consecutive exposure of the mice to OVA aerosol induces a Th2-type immune response with characteristic eosinophilic airway inflammation [3]. By stimulating DC *ex vivo* before transfer, the impact of CGRP immunomodulation on the *in vivo* allergic immune response could be evaluated. Using this approach, we avoided inducing neurogenic effects of CGRP in cell types other than DC. Therefore, our results were dependent on the allergen-specific immune response and were not confounded by other features of asthma [6, 11].

As described previously [3], the transfer of OVA-pulsed BMDC to the lungs and the subsequent OVA aerosol challenge induced allergic airway inflammation with eosinophilic infiltration ($11.17 \pm 4.68 \times 10^5$ /mL) (Fig. 6a). The observed influx of neutrophils ($10.09 \pm 3.22 \times 10^5$ /mL) could have resulted from the additional LPS costimulation of BMDC that we used. The transfer of CGRP-pretreated BMDC resulted in a reduction of eosinophil numbers by about 40%, whereas neutrophil, lymphocyte and macrophage cell counts were not altered. The treatment control group receiving dexamethasone (Dexa) displayed a strong

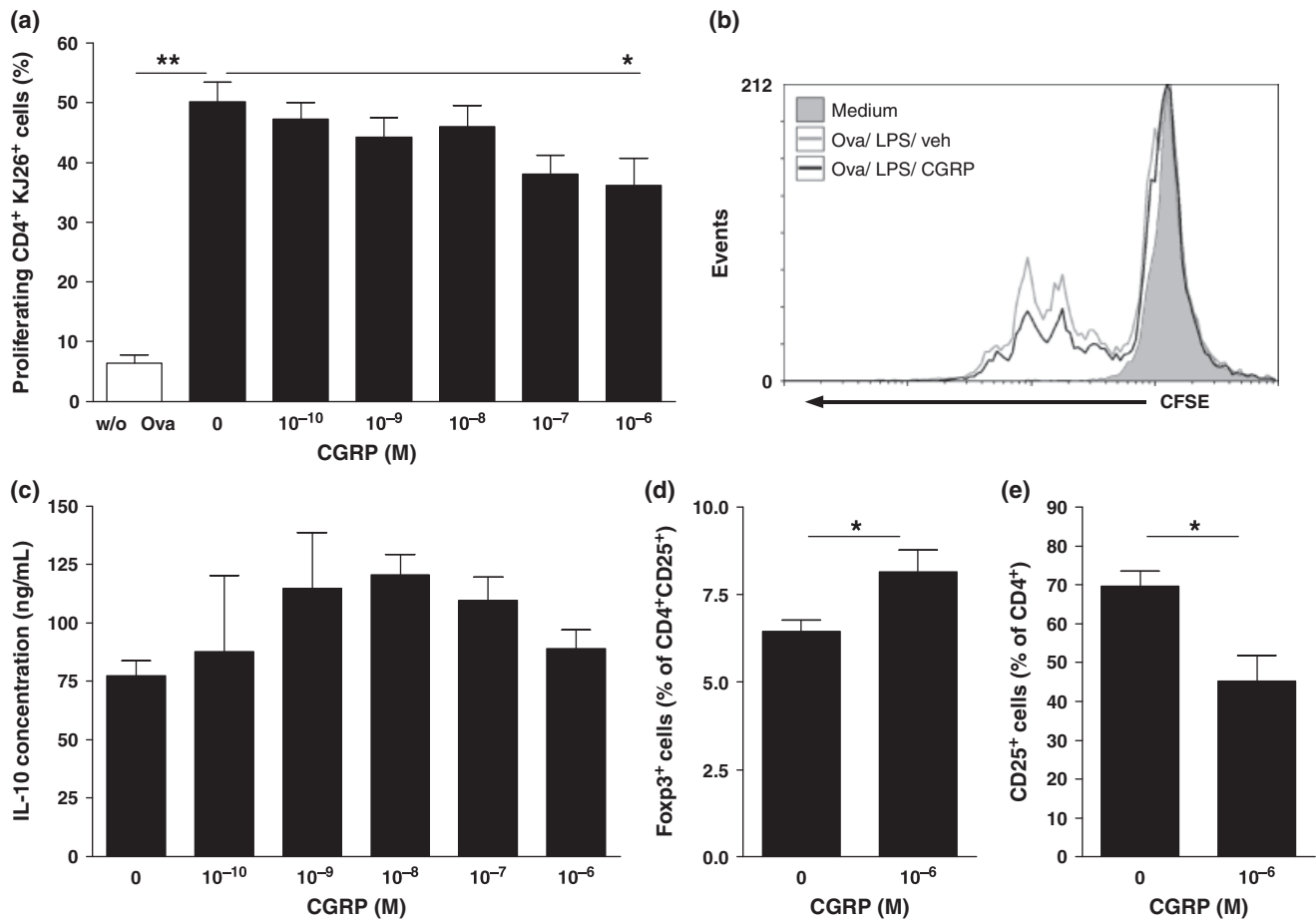


Fig. 5. Calcitonin gene-related peptide (CGRP) modulation of dendritic cells (DC)-induced T cell immune response. OVA/lipopolysaccharide (LPS)/CGRP-stimulated bone marrow-derived dendritic cells were cocultured with CFSE-labelled OVA-specific CD4⁺ T cells for 3 days. (a) Percentage of proliferating T cells as analysed by the CFSE dilution signal intensity. The overlay histogram in (b) shows a representative division profile of OVA-specific T cells (identified as CD4⁺KJ26⁺ cells) cocultured either with OVA/LPS/vehicle-stimulated DC (grey line) or OVA/LPS/CGRP-stimulated DC (black line) or medium control (filled grey line). (c) IL-10 cytokine levels in the supernatants of T cell cocultures. Percentage of foxp3⁺ cells (of CD4⁺CD25⁺) (d) and CD25⁺ (of CD4⁺) cells (e). Bars represent mean + SEM of $n = 6$ (a) or $n = 3$ (c–e) experiments. *, ** $P < 0.05$; 0.01.

reduction of infiltration compared with the positive control group (Fig. 6a).

The reduced degree of airway inflammation by CGRP was additionally reflected by the lung histology. The positive group (POS), which received OVA-pulsed DC and OVA aerosol challenges, displayed profound inflammatory infiltration, whereas the negative control group (NEG), which received saline aerosols instead, showed normal lung histology (Fig. 6b). In the CGRP group, the infiltration was reduced, but the reduction was not as strong as in the Dexa group.

In the bronchoalveolar lavage fluid (BALF), an induction of several cytokines such as IL-4, -5, -12, -2, IFN- γ and eotaxin-2 was observed in the positive control group compared with NEG. The levels of these Th1- and Th2-associated cytokines were not affected by CGRP (Fig. 6c and data not shown). Interestingly, the IL-10 level was more than sixfold enhanced in the CGRP group compared with POS (Fig. 6c). Restimulation of mediastinal lymph

node (MLN) cells induced a prominent Th2 cytokine secretion pattern, but the levels were not altered by CGRP. The increase in IL-10 levels did not reach statistical significance (Fig. 6d and data not shown).

Discussion

In this study, we have provided the first evidence of the modulation of DC function by CGRP to affect their central role in the onset of allergic airway inflammation. Based on our previous finding that sensory nerves and lung DC are colocalized [21, 22], this study addressed the question of how CGRP might modulate DC-driven allergic immune responses.

Our data reveal that lung DC express functional CGRP receptors, providing the basis for a functional modulation by neuronal-released CGRP. In a mouse model of experimental asthma, however, a down-regulation of the high-affinity CGRP receptor was observed in the allergic group.

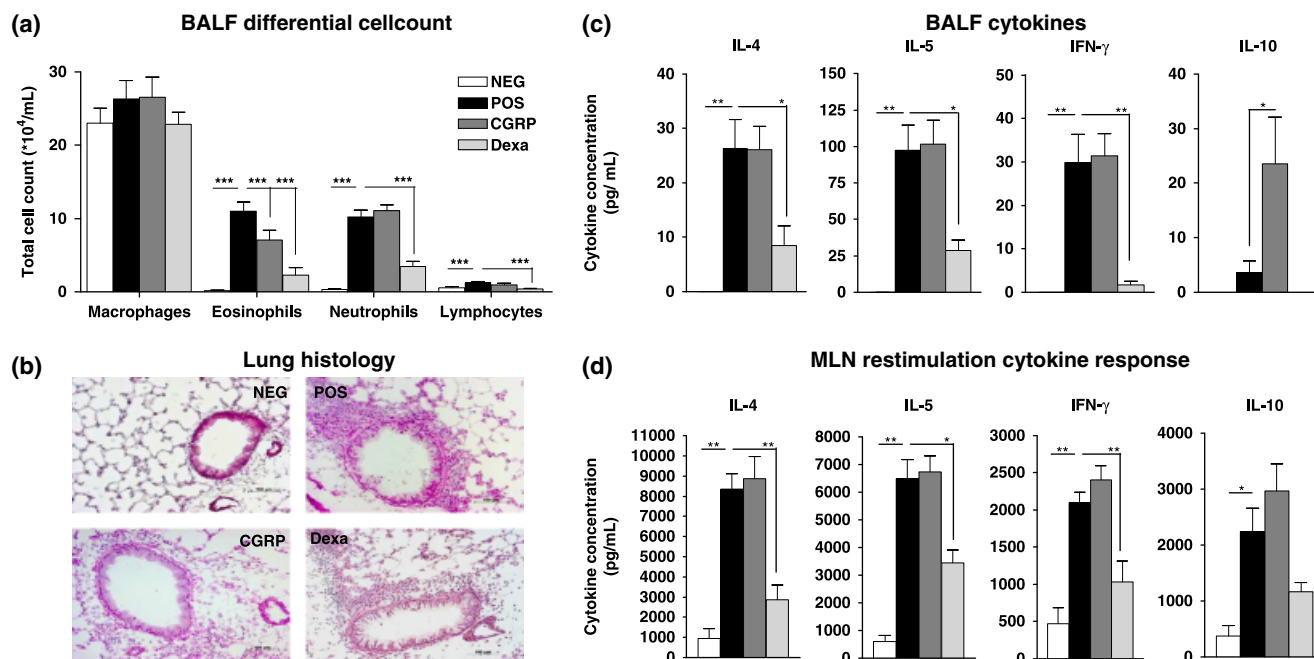


Fig. 6. Effects of calcitonin gene-related peptide treatment of dendritic cells on their potential to induce eosinophilic airway inflammation *in vivo*. On day 0, bone marrow-derived dendritic cells were pulsed according to Table 1 and adoptively transferred intratracheally to naïve mice, followed by aerosol provocation on days 10–12 and analysis 24 h later. (a) Differential cell count in bronchoalveolar lavage fluid (BALF). (b) Histology of lung cryosections after haematoxylin and eosin staining. (c) BALF cytokines and (d) cytokine pattern of MLN after restimulation with OVA for 5 days measured with MSD. Data show mean $\pm$ SEM for the number of animals per group indicated in Table 1. *, **, *** indicate significance, with $P < 0.05$, 0.01 and 0.001, respectively.

As CGRP levels are elevated in asthma, a ligand-activated desensitization of CGRP receptors might be present [36, 37]. Triggering of CGRP receptors on DC impaired the allergen-specific immune response. Thus, the down-regulation of CGRP receptors in inflammatory conditions might cause an unresponsiveness of DC to the tolerogenic effects of CGRP. Whether the loss of CGRP receptors on lung DC indeed contributes to the allergic airway inflammation remains to be elucidated. However, the down-regulation of the receptor molecule Ramp1 during allergic airway inflammation in epithelial cells [38] as well as decreased cAMP levels in a ragweed mouse model of asthma [39] have been described. These observations support the hypothesis of a desensitization of CGRP receptors involving a diminished CGRP receptor expression as well as a reduced subsequent activation of the signalling cascade.

Regarding DC maturation, our data agree with studies describing the inhibitory effects of CGRP on CD86 expression on human monocyte-derived DC [27] and peripheral blood mononuclear cells [28], while CD80 simultaneously was not affected by CGRP [28]. The up-regulation of costimulatory molecules during DC maturation is essential for the induction of Th2 responses [40]. However, for the development of Th2 immune responses, CD86 seems to be more important than CD80 [41]. The treatment of mice with anti-CD86 but not anti-CD80 was shown to reduce

the characteristic features of allergic asthma such as eosinophilic infiltration [42]. Likewise, CGRP differentially affected CD86 and CD80 expression, resulting in a reduction of eosinophils *in vivo*.

Our data suggest that this inhibition of DC maturation is the key mechanism of the inhibitory effects of CGRP. Immature DC have been described to induce tolerance rather than a T cell effector response. This might be mediated by IL-10-producing T regs [43, 44], which are able to reduce eosinophilia in experimental asthma [45]. Our data indicate a role for T reg in mediating the tolerogenic effects of CGRP. The differing IL-10 secretion observed in DC, cocultures and BALF might reflect the kinetics of IL-10, which is thought to be more important in the maintenance rather than the induction of tolerance [46, 47]. Further studies will have to address in detail the exact role of T regs in mediating the anti-inflammatory effects of CGRP *in vivo*.

Here, we presented an *in vivo* study focused on determining how the classical features of allergic airway inflammation, especially eosinophilia and Th2 cytokines, are altered by CGRP modulation of DC function. Previous studies, using CGRP knock-out mice, suggested that CGRP affects the lung function but not the eosinophilic airway inflammation. More precisely, the use of an acute OVA model of experimental asthma in CGRP-deficient mice resulted in an improved lung function but sustained

airway inflammation [48]. This approach, however, uses intraperitoneally (i.p.) sensitization and is not suitable to study the local CGRP triggering of DC responding to inhaled allergens. Conversely, in another study, *in vivo* application of CGRP in OVA-sensitized and -challenged mice abolished airway hyperresponsiveness. Effects on airway inflammation, however, were not shown [49]. Thus, we used the DC-transfer model of experimental asthma to specifically address the effects of CGRP on the immunological response rather than on structural cells mediating airway hyperresponsiveness. Within this model, co-administration of low-dose LPS during the OVA stimulation of DC was needed to induce an immune response rather than tolerance (data not shown). This is consistent with previous reports showing that a Th2-directed sensitization to inhaled allergens is dependent on TLR signals like LPS [50, 51]. This might, however, account for the neutrophilic influx we observed [52]. CGRP treatment of DC during allergen uptake resulted in a reduced eosinophilic infiltration *in vivo*. The Th2/Th2 cytokine pattern was not altered by CGRP both *in vivo* and in the *in vitro* coculture. This suggests that CGRP inhibits the overall T cell response without affecting the Th1/Th2 balance.

In summary, our data show that CGRP inhibits DC maturation, thereby leading to a reduced effector T cell response. In DC-driven experimental asthma, this results in a reduced eosinophilic airway inflammation *in vivo*. This

provides a new mechanism for a possible neuroimmune interaction in allergic airway inflammation, which is a promising target for therapeutic intervention. Interestingly, the phosphodiesterase 4 inhibitor roflumilast, a recently licensed drug, directly blocks cAMP degradation [53, 54]. Whether targeting of CGRP receptors is a therapeutic option in allergic airway inflammation has to be elucidated in further studies. However, asthma exacerbations are not only induced by allergen provocation but also stimuli known to trigger neuronal mechanisms, like stress or virus infections [54–58]. Thus, a therapeutical intervention targeting signal molecules induced by both immune and neuronal pathways seems to be most promising.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. mRNA Expression of CGRP receptor in isolated lung DC Representative agarose gel picture of PCR products from isolated lung DC. Receptor molecules CRLR, Ramp1, Ramp2 and Ramp3 are expressed.

Figure S2. Effect of CGRP stimulation on IL-10 secretion in BMDC cultures BMDC were stimulated with LPS 10 ng/mL and CGRP in the indicated doses for 24 h. Supernatants were collected and IL-10 measured in duplicates by ELISA. Bars represent mean + SEM of $n = 4$ experiments. $*P < 0.05$.

Figure S3. Effect of CGRP stimulation of BMDC on cytokine secretion in T cell cocultures. OVA/LPS/CGRP-stimulated BMDC were cocultured with CFSE-labelled OVA-specific CD4⁺ T cells for 3d. Cytokine levels in supernatants of T cell cocultures were measured using MSD multiplex. Bars represent mean + SEM of $n = 6$ experiments. $*P < 0.05$.

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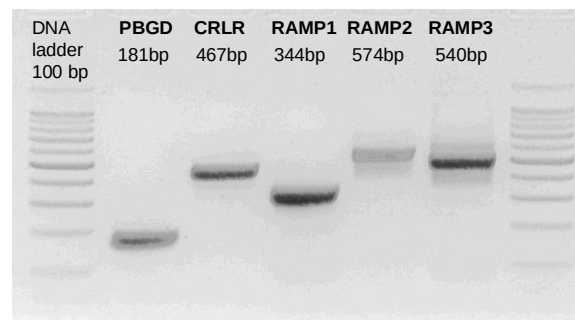


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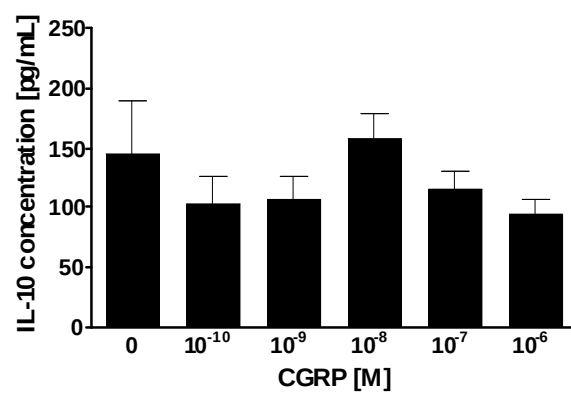


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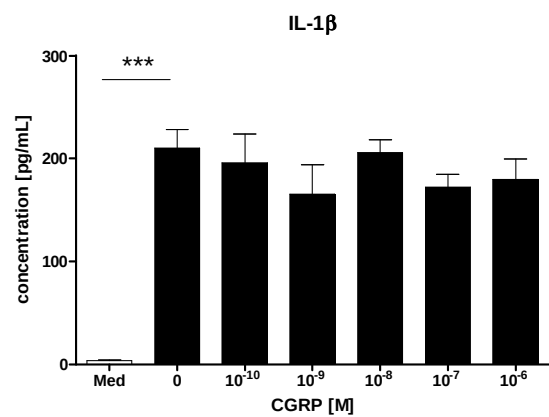
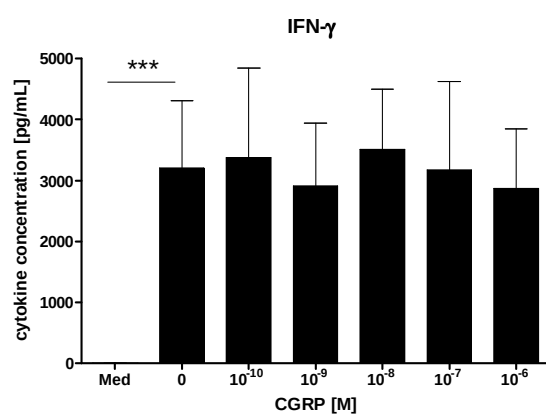
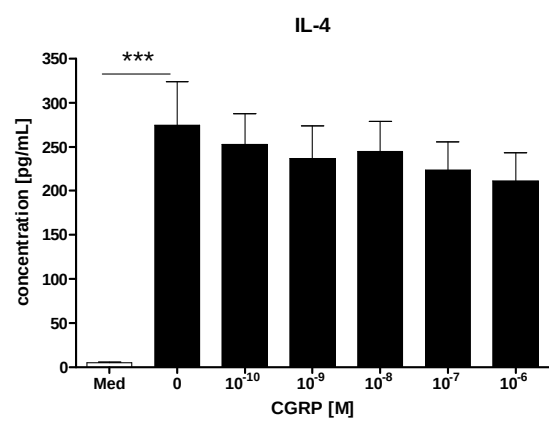
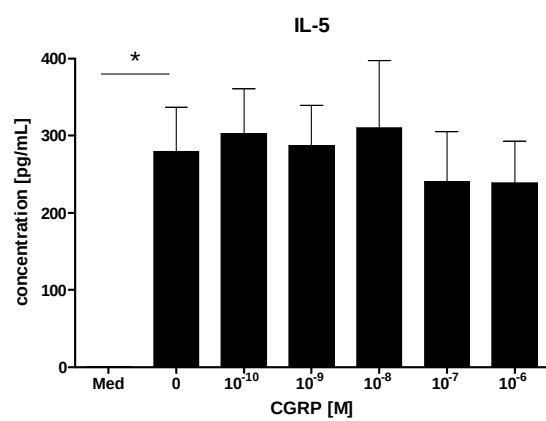
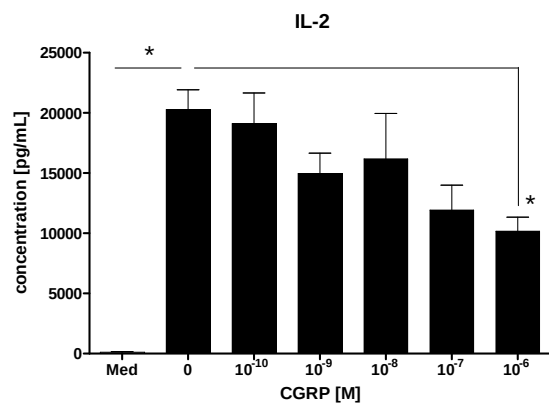
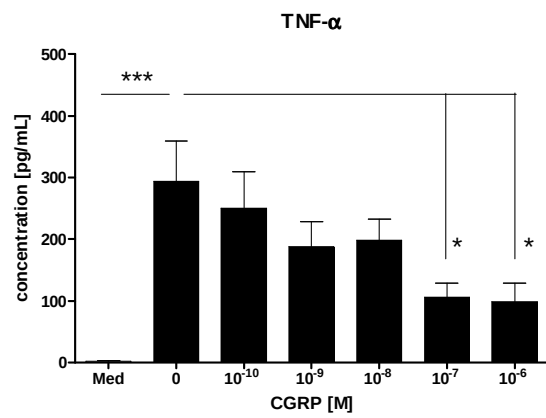


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