

Decreased Migration of Dendritic Cells into the Jugular-Nodose Ganglia by the CXCL12 Neutraligand Chalcone 4 in Ovalbumin-Sensitized Asthmatic Mice

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Keywords

Neutraligands · Dendritic cells · Asthma · Neuroimmune interactions · CXCL12 · CXCR4 · Chemokines · Ovalbumin

Abstract

Objective: The chemokine CXCL12 interacting with the CXCR4 receptor has been reported to play a role in the development and progression of bronchial asthma, but its mechanism of action is still unknown. The objective of this study was to assess the effect of the CXCL12 neutraligand chalcone 4 on the migration of dendritic cells (DCs) in a murine model of allergic airway inflammation. **Methods:** A 21-day ovalbumin (OVA)-induced allergic-airway T_H2 inflammation model in BALB/c mice was used. Four groups were sensitized with OVA adsorbed on alum and challenged either with OVA or saline for 4 days. Mice were treated intranasally with chalcone 4 (300 nmol/kg body weight) or solvent 2 h before each OVA or saline challenge; 24 h after the last challenge, CD11c⁺F4/80⁺ DCs were counted in the bronchoalveolar lavage. Jugular-nodose ganglion complex (JNC) sec-

tions were sampled, and for immunofluorescence staining, cryocut sections were prepared. MHC II⁺F4/80⁺ DCs as well as calcitonin gene-related peptide (CGRP)- and substance P (SP)-positive neuronal cell bodies were analyzed. **Results:** In OVA-challenged mice, chalcone 4 caused a significantly decreased DC/neuron ratio in the JNC from 51.7% in solvent-treated to 32.6% in chalcone 4-treated mice. In parallel, chalcone 4 also decreased the DC population in BALF from 11.5×10^3 cells in solvent to 4.5×10^3 cells in chalcone 4-treated mice. By contrast, chalcone 4 had no effect on the expression of the neuropeptides CGRP and SP in JNC. **Conclusion:** This study reported the CXCL12 neutraligand chalcone 4 to affect DC infiltration into the airways and airway ganglia as well as to decrease airway eosinophilic inflammation and, therefore, validated CXCL12 as a new target in allergic disease models of asthma.

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Introduction

Asthma is a widespread chronic disease which affects about 300 million people worldwide. This chronic inflammatory disease is characterized by morphological changes, including remodeling, mucus hypersecretion, and episodic obstructive events in the airways [1]. Bronchial asthma represents a very heterogeneous and multilayered disorder. Traditionally, it was classified as an intrinsic (nonallergic) or an extrinsic (allergic) phenotype. To further specify the subtype, the nomenclature was expanded by the T_H2 or non- T_H2 mediation of asthma. T_H2 -mediated forms of bronchial asthma were early-onset and late-onset phenotypes with their distinct configurations. Early-onset T_H2 -mediated asthma progression can be mild to severe, and it was often accompanied by many comorbid conditions. It manifests mostly in childhood. Other T_H2 -mediated asthma subtypes were exercise-induced and late-onset eosinophilic asthma [2]. Non- T_H2 -mediated asthma subtypes were defined as very late-onset asthma in women, and obesity-associated, smoking-associated neutrophilic, and smooth muscle-mediated asthma [1, 2]. The pathophysiological mechanisms of asthma are very complex, and many instances of innate and adaptive immunity with inflammatory cells are involved [3]. Chemokines and their corresponding chemokine receptors are known to operate the “steering wheel” of cell attraction to the effector tissue.

In the past years, studies on the chemokine CXC motif receptor 4 (CXCR4) and its ligand, the chemokine CXCL12, ascribed the CXCR4/CXCL12 axis a role in inflammatory processes, in particular asthma [4–7]. CXCR4 is widely expressed on immune and inflammatory cells, in particular dendritic cells (DCs) [8], which migrate from the tissue to lymph nodes after maturation and activation [9] to present antigen to T lymphocytes [10] and to neural ganglia to cross talk with neurons [11, 12]. The interplay of CXCR4 and CXCL12 in the migration of cutaneous DCs has also been pictured [13].

Chalcone 4 was identified as an inhibitor of the interaction between CXCR4 or CXCR7 and their ligand CXCL12 [6, 14] through binding to the chemokine in a neutralizing-like manner rather than on its receptors [15]. Chalcone 4 was therefore called a neutraligand. Administered intranasally, chalcone 4 decreased the recruitment of inflammatory cells, in particular eosinophils, in a mouse model of allergic eosinophilic airway inflammation [6, 14]. Furthermore, chalcone 4 derivatives, modified as highly soluble prodrugs, attenuated at very low

doses the inflammatory reaction in an allergic airway hypereosinophilia model [14].

The aim of this study was to approach the mechanism of the anti-inflammatory action of the CXCL12 neutraligand chalcone 4 by the evaluation of its impact on the presence of DCs in the jugular-nodose ganglion complex (JNC) in our experimental mouse model of allergic airway inflammation.

Methods

Animals

For this experiment, 9-week-old male BALB/c mice were purchased from Janvier Laboratories (Saint-Berthevin, France) and maintained under constant conditions at a temperature of $22 \pm 2^\circ\text{C}$ at a 12-h-light/12-h-dark cycle. Standard laboratory food (standard diet, 4RF21; Mucedola, Settimo Milanese, Italy) and tap water were provided ad libitum. All animal experiments were performed in strict accordance with the governmental regulations and legal requirements of France (APAFIS No. 1341-2015080309399690).

Ovalbumin Mouse Model, Treatment Protocol, and Tissue Preparation

A 21-day ovalbumin (OVA) mouse model of allergic airway inflammation was studied [16, 17]. Intraperitoneal sensitization with OVA (grade V, 50 μg /application; Sigma Aldrich, France) adsorbed on aluminum hydroxide (2 mg; Sigma Aldrich) was performed on days 0 and 7. Four groups of mice received either chalcone 4 (synthesized in our laboratory, [14]) intranasally (300 nmol/kg body weight) dissolved in 10% 2-hydroxypropyl- β -cyclodextrin (CDx; C0926; Sigma Aldrich) or solvent alone 2 h before intranasal OVA (10 μg in 25 μL saline) or saline challenge (controls) on days 17–20. Mice were killed on day 21, and bronchoalveolar lavage fluid (BALF) and JNC were collected as described [11, 18].

BALF and Flow Cytometry

Total CD45⁺ leukocytes were counted in BALF on a Muse[®] counter. For differential cell counts, BALF was supplemented with Fc Block (5 μL , 553142; BD Biosciences) in a black microplate. After a 20-min incubation at room temperature, the following marker antibodies were added: CD45-Alexa Fluor 700 (103128; BioLegend), CD11b-APC-Cy7 (557657; BD Biosciences), CD11c-FITC (557400; BD Biosciences), Gr-1-Pe-eFluor610 (61-5931-82; eBioscience, USA), and F4/80-PE (12-4801-82; eBioscience). Antibodies were incubated for 30 min before DAPI (5 μL ; BD Biosciences) was added. Flow cytometry was performed immediately thereafter. FlowJo[®] software was used to identify and count inflammatory cells.

OVA-Specific IgE and IgG1 ELISA

ELISA was conducted to measure OVA-specific IgE and IgG1 levels in plasma. Microtiter plates coated with capture antibodies were incubated at 4°C overnight before antimouse IgE and IgG1 (clones R35-118 and R19-15, respectively; BD Pharmingen) were added in carbonate buffer. After blocking with 10% bovine serum

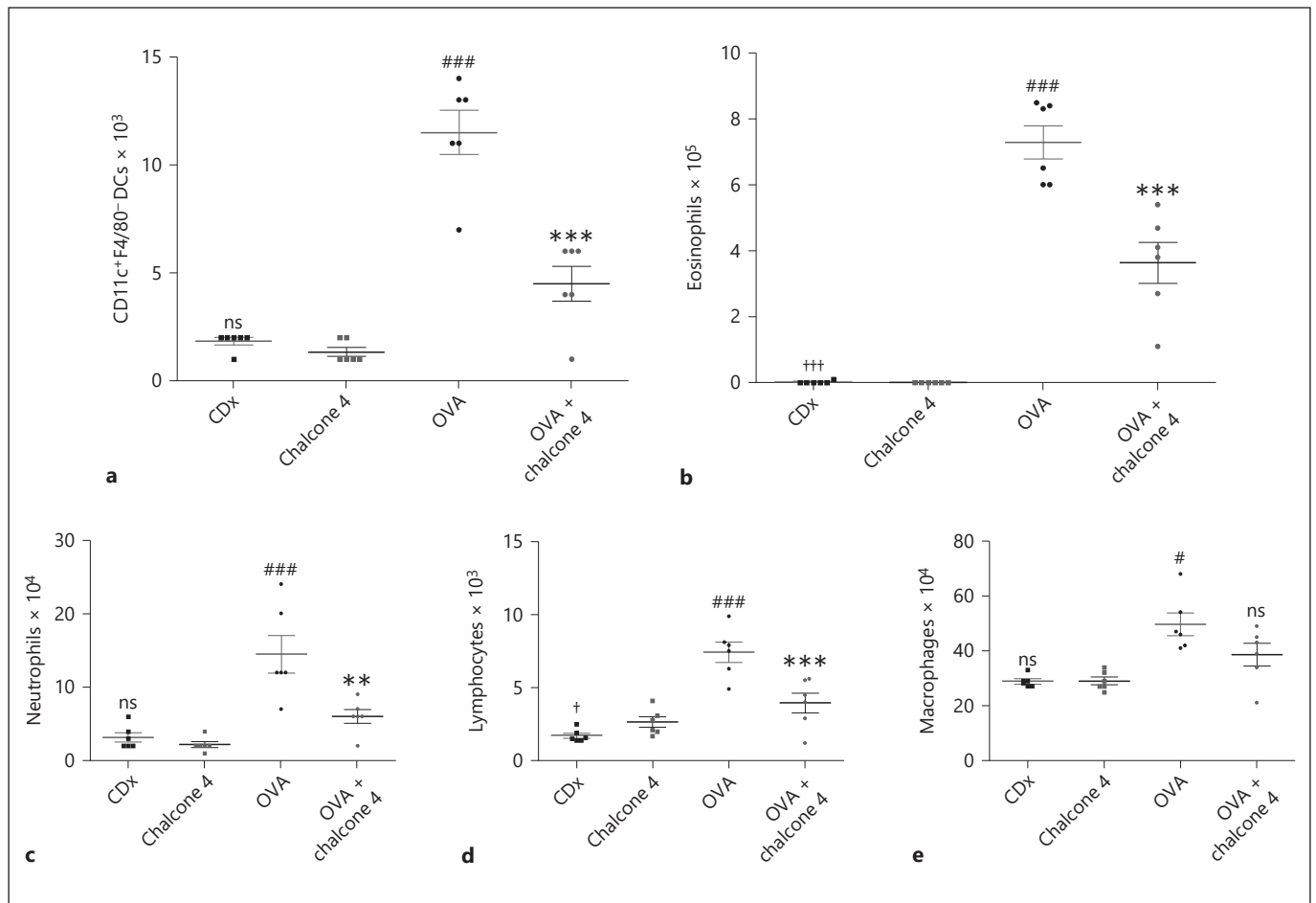


Fig. 1. Distinct inflammatory cell type composition in BALF: effects of chalcone 4 on DCs (CD11b⁺GR1⁻CD11c⁺F4/80⁻) (a), eosinophils (CD11b⁺CD11c⁻GR1⁻) (b), neutrophils (CD11b⁺CD11c⁻GR1⁺) (c), lymphocytes (SSC^{low}CD11b^{low}) (d), and macrophages (CD11b⁺GR1⁻CD11c⁺F4/80⁺) (e). One-way ANOVA with Bonferroni multicomparison post-test was conducted using GraphPad Prism5 software. OVA + chalcone 4 vs. OVA: ** $p \leq 0.01$, *** $p \leq 0.001$; OVA vs. CDx: # $p \leq 0.05$, ### $p \leq 0.001$, CDx vs. OVA + chalcone 4: † $p \leq 0.05$, ††† $p \leq 0.001$.

albumin, plasma samples were incubated at room temperature for 2 h. To detect IgE and IgG1, OVA-horseradish peroxidase (HRP) (BUF048; AbD Serotec) was added and incubated at room temperature for 1 h. After adding the HRP substrate TMB (tetramethylbenzidine; BD Pharmingen), the reaction was allowed to develop for 15–20 min and then stopped with 0.5 M H₂SO₄. Absorbance was measured at a wavelength of 450 nm, and OVA-specific IgE and IgG1 serum levels were presented as optical densities (OD).

Immunofluorescence Staining

Zamboni-fixed [19] JNC cryosections (8 μm) (CM1950; Leica Cryostat, Germany) were dried at room temperature. For immunolabeling, cryosections were rehydrated in saline. To reduce non-specific antibody binding, cryosections were blocked with 5% donkey serum (host of the secondary antibodies) prior to addition of the primary antibody incubated for 1.5 h at room temperature and then overnight at 4 °C. Immunolabeling was performed with the

APC marker MHC II (rat monoclonal antimouse MHC II, dilution: 1:200; eBioscience) to label DCs [11], the neuropeptides calcitonin gene-related peptide (CGRP) (goat polyclonal and rabbit monoclonal antimouse CGRP, dilution: both 1:500; Acris, Germany) and substance P (SP) (rat monoclonal antimouse SP, dilution: 1:200; Abcam, UK), and a marker of neuronal structures, PGP9.5 (rabbit monoclonal antimouse PGP9.5, dilution: 1:200; Abcam; chicken monoclonal antimouse PGP9.5, dilution: 1:500; Acris). After 3 washings, cryosections were incubated with the secondary antibodies for 2 h at room temperature, then for 15 min with DAPI, washed several times, and covered with Fluoroshield™ fluorescence mounting medium (Sigma Aldrich, Germany). Quantification of APC, and CGRP- and SP-positive cells in JNC was performed under epifluorescence microscopy (Axio Imager M2; Zeiss, Germany) and analyzed with AxioVision software (Zeiss). The following secondary antibodies were used: donkey antichick IgY⁺ Alexa Fluor® 488; donkey antichick IgY⁺ Al-

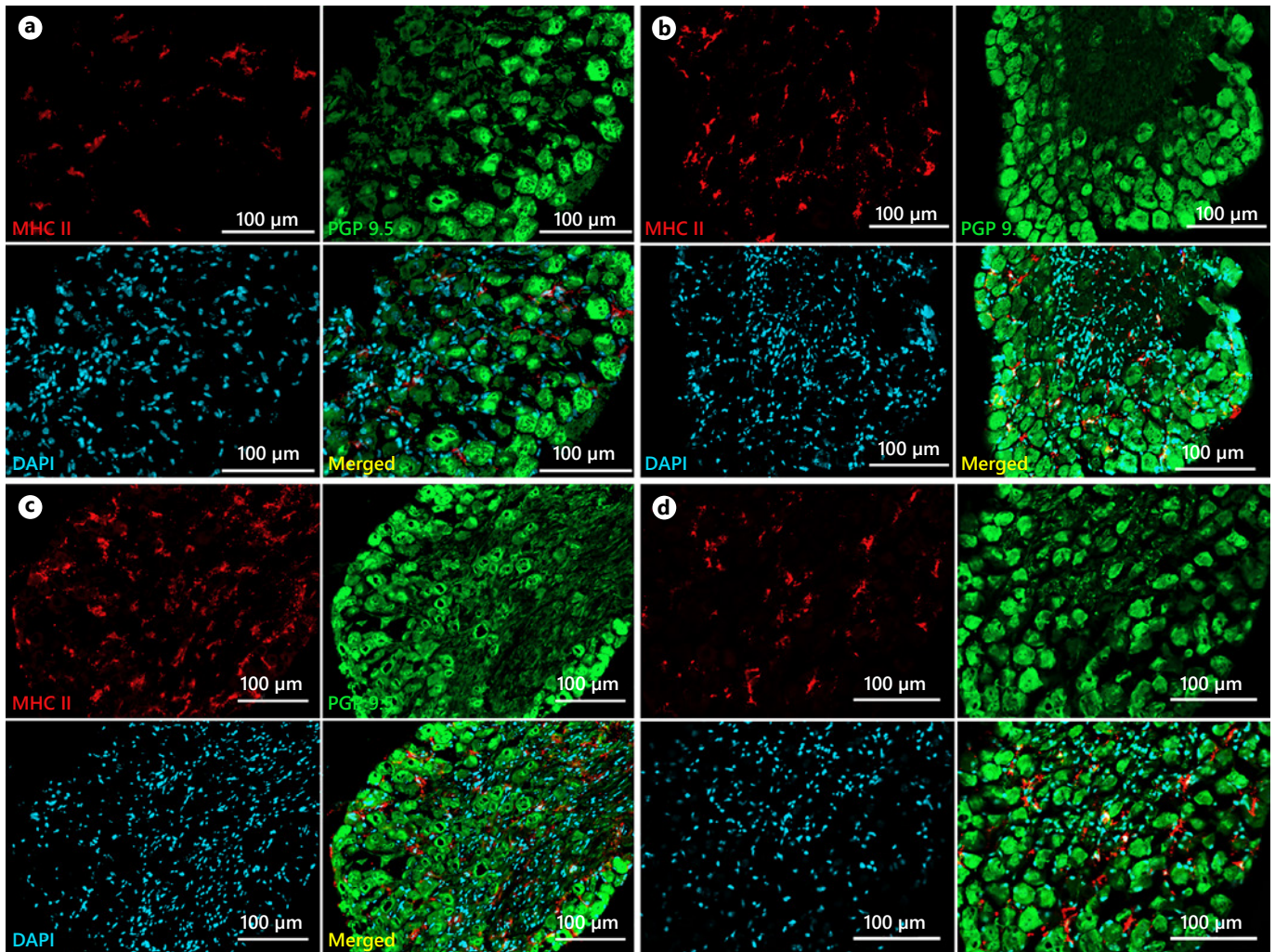


Fig. 2. Immunohistochemical analysis of the jugular-nodose ganglion complex (JNC). Cryosections of cyclodextrin-treated control mice (**a**), chalcone 4-treated mice (**b**), ovalbumin (OVA)-treated mice (**c**), and OVA- and chalcone 4-treated mice (**d**). JNC slides were stained with different antibodies to mark dendritic cells (an-

ti-MHC II antibody), nuclei (DAPI), and locate the ganglion (anti-PGP9.5 antibody). Quantification was performed by calculating the ratio of ganglion cells to MHC II⁺ cells. Images were acquired using epifluorescence microscopy (Axio Imager M2; Zeiss) and AxioVision. $\times 200$.

exa Fluor[®] 647, donkey antirat IgG Alexa Fluor[®] 488, donkey antirat IgG cyanine Cy3, donkey antirabbit IgG cyanine Cy3, donkey antirabbit IgG Alexa Fluor[®] 647, and donkey antigoat IgG cyanine Cy3. All secondary antibodies were provided by Jackson (USA) and dilution was 1:300.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, USA) via 1-way ANOVA with a Bonferroni protected multicomparison post hoc test conducted with Prism software (GraphPad). $p \leq 0.05$ was considered significant.

Results

Effect of Chalcone 4 on DCs in BALF

The number of CD11c⁺F4/80⁺ DCs in BALF summed up to 1.8×10^3 in saline-challenged control mice (controls) and increased in OVA-challenged and solvent-treated animals to 11.5×10^3 ($p \leq 0.0001$). Treatment with chalcone 4 induced attenuation of the number of DCs in BALF to 4.5×10^3 cells ($p \leq 0.0001$) (Fig. 1).

There were no eosinophils in BALF from controls; their number increased in OVA-challenged mice (7.3×10^5 ; $p \leq 0.0001$). Chalcone 4 treatment significantly de-

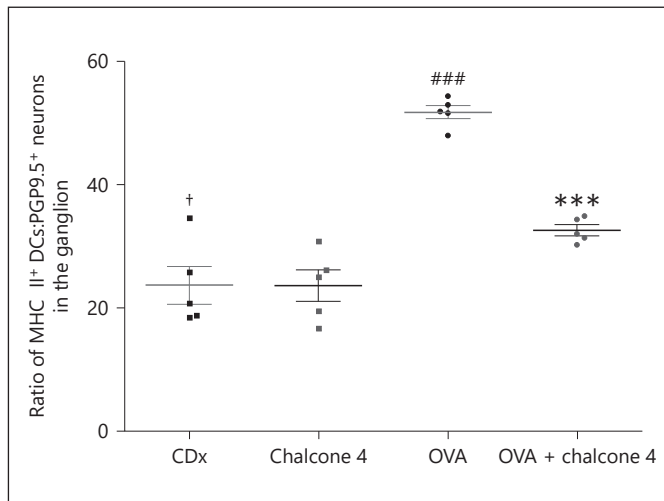


Fig. 3. Chalcone 4-dependent dendritic cell (DC) migration into jugular-nodose ganglion complex (JNC). Effects of chalcone 4 (gray) treatment in an ovalbumin (OVA; circles) mouse model of allergic asthma on DC migration into JNC. MHC II⁺ DCs were counted manually, and the DC/neuron ratio (mean $\pm$ SEM) was calculated. One-way ANOVA with a Bonferroni multicomparison post hoc test was conducted using GraphPad Prism5 software. *** $p \leq 0.001$, OVA + chalcone 4 vs. OVA; ### $p \leq 0.001$, OVA vs. cyclodextrin (CDx); † $p \leq 0.05$, CDx vs. OVA + chalcone 4.

creased their amount to 3.8×10^5 ($p \leq 0.0001$), which was still significantly higher compared to the CDx group ($p < 0.001$) (Fig. 1). The number of neutrophils in BALF in controls (3.2×10^4) increased to 14.5×10^4 ($p \leq 0.0001$) and, similarly, was significantly decreased by chalcone 4 (6×10^4 ; $p \leq 0.001$) (Fig. 1). Differences in the amount of lymphocytes in BALF showed the same profile, with 1.4×10^3 cells in controls, 7.4×10^3 ($p \leq 0.0001$) in OVA-challenged, and 4.0×10^3 (significant decrease: $p \leq 0.001$) in chalcone 4-treated, OVA-challenged animals (Fig. 1). Again, there were significantly less lymphocytes in CDx mice than in OVA-challenged chalcone 4-treated ones ($p < 0.05$). Concerning the number of macrophages in BALF, there was a significant increase ($p \leq 0.05$) compared to controls (49.7×10^4 in OVA-challenged mice vs. 28.8×10^4 in controls) but no significant difference in chalcone 4-treated, OVA-challenged animals (39.0×10^4 ; $p > 0.05$) (Fig. 1).

Chalcone 4 Caused an Attenuated Migration of MHC II⁺ Cells into the JNC

Immunohistochemical staining showed a significant increase in MHC II⁺ DC numbers in the JNC in OVA-challenged as compared to saline-challenged mice (Fig. 2).

The median DC/neuron ratio increased from 23.7% in controls to 51.7% in the OVA group ($p \leq 0.0001$). This effect was significantly decreased following chalcone 4 treatment (32.6%; $p \leq 0.05$) (Fig. 3) but still significantly higher than at baseline ($p < 0.05$). Interestingly, the DC/neuron ratio was not modified by chalcone 4 (23.7%) as compared to solvent (23.6%) in saline-challenged mice ($p > 0.05$) (Fig. 3).

MHC II⁺ Cells in Ganglia Are DCs

MHC II⁺ cells infiltrating the ganglia have been identified previously as DCs [11]. To ascertain this in the present study, double immunofluorescence staining with antibodies directed against the macrophage marker F4/80 and MHC II were conducted. MHC II⁺ cells present in the ganglia were negative for F4/80 (Fig. 4), indicating that the MHC II⁺ cells were not macrophages. To further refine the characterization of these MHC II⁺F4/80⁻ cells, double immunostaining for CD11c and MHC II was conducted, and colocalization of both markers was noted, indicating the DC nature of these cell phenotypes (Fig. 5).

MHC II⁺CD11c⁺F4/80⁻ DCs Do Not Express CXCR4 in JNC

To approach the mechanism of DC migration to the sensory ganglia, immunohistochemical analysis of CXCR4 expression on DCs was performed. CXCR4 was not expressed on ganglion cells (Fig. 6). Indeed, co-incubation of antibodies directed against MHC II, CD11c, and CXCR4 showed no colocalization on DCs, ascertaining that CXCR4 was not expressed at the surface of DCs migrating into JNC (Fig. 6).

Chalcone 4 Did Not Affect OVA-Specific IgE and IgG1 Levels in Plasma

Serum OVA-specific IgE and IgG1 levels were evaluated to determine whether chalcone 4 acted as an immunomodulator or not. Plasma samples were taken 24 h after the final OVA challenge, and IgE and IgG1 levels were assessed using ELISA. It could be demonstrated that chalcone 4 had no effects on the biosynthesis of OVA-specific IgE and IgG1 (Fig. 7).

Effect of Chalcone 4 on CGRP⁺ and SP⁺ Cell Bodies in JNC

The median percentages of SP⁺ cell bodies in JNC were 21.4% in controls, 20.3% in chalcone 4-treated controls, 29.8% in OVA-challenged mice, and 20.7% in chalcone 4-treated, OVA-challenged mice (Fig. 8). Although there is a slight increase in OVA-challenged animals, the effect

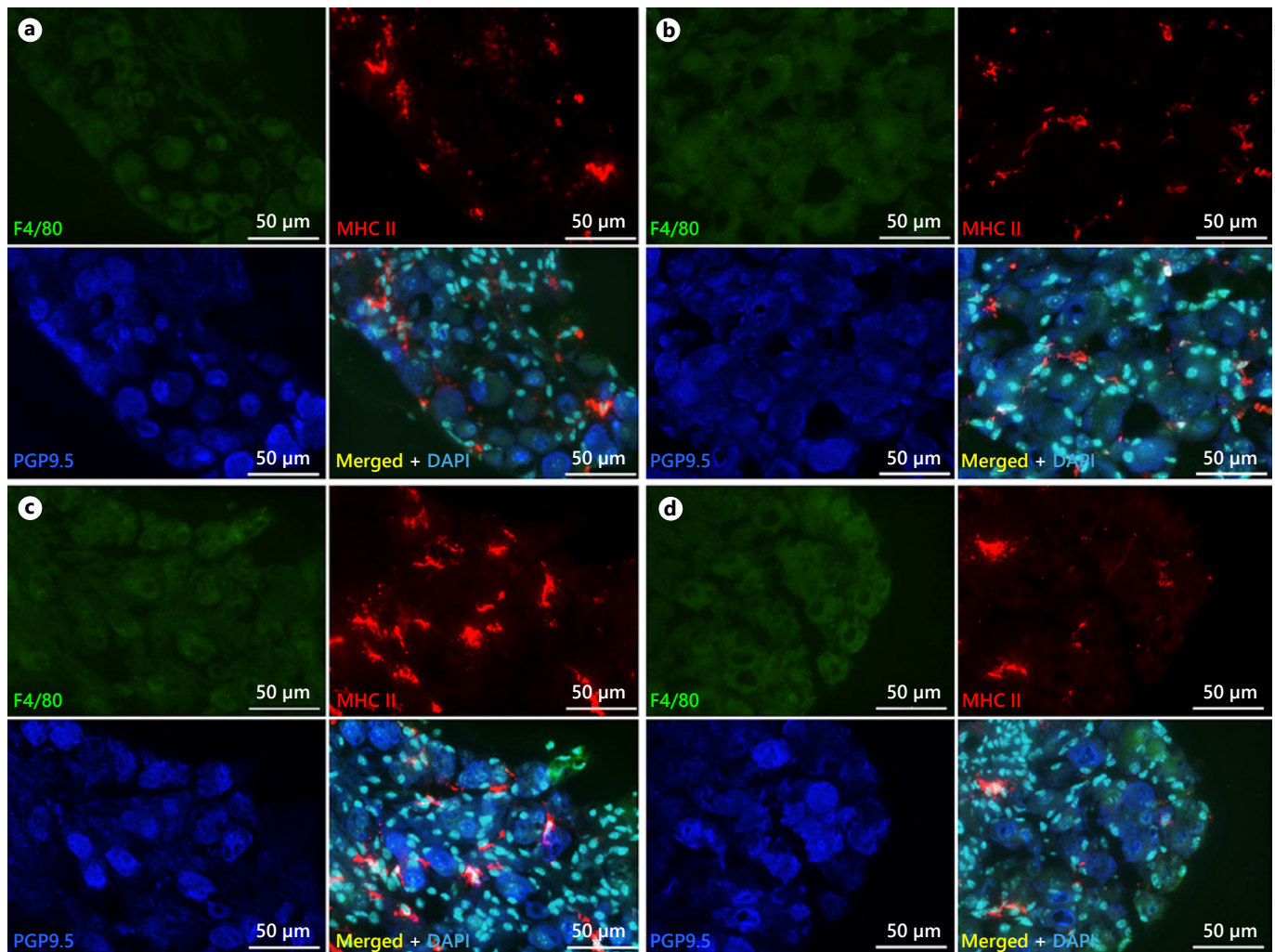


Fig. 4. Immunohistochemical characterization of DCs in JNC: cryosections of CDx-treated control mice (a), chalcone 4-treated mice (b), OVA-treated mice (c), and OVA- and chalcone 4-treated mice (d) were stained with F4/80 to show that MHC II⁺ cells in JNC were not macrophages but DCs. Images were acquired using epifluorescence microscopy (Axio Imager M2; Zeiss, Germany) and AxioVision software. $\times 200$.

was not significant. Similarly, there were no significant differences in CGRP⁺ cell bodies among the different treatment groups (control: 18.8%, control + chalcone 4: 16.3%, OVA-challenged mice: 25.6%, OVA-challenged, chalcone 4-treated mice: 16.8%; $p > 0.05$) (Fig. 9).

Discussion

DCs play a central role in the initiation and development of allergic airway inflammation and asthma [3, 9]. We have previously located DCs at the vicinity of CGRP

cell bodies in the JNC [11] and of peripheral CGRP-positive nerve fibers in the airways in a chronic house dust mite model of asthma [20]. In bronchial asthma, the orchestration of the reaction involves cytokines and chemokines attracting immune and inflammatory cells [21, 22]. Chalcone 4 is a nonpeptidic CXCL12 neutraligand, i.e., a small chemical molecule neutralizing the activity of CXCL12 through direct interaction, thus preventing CXCL12 binding to its receptors (CXCR4/CXCR7) [15]. The use of neutraligands is a very interesting therapeutic strategy because they do not bind the chemokine receptors and thereby do not modify cell homeostasis [21, 23].

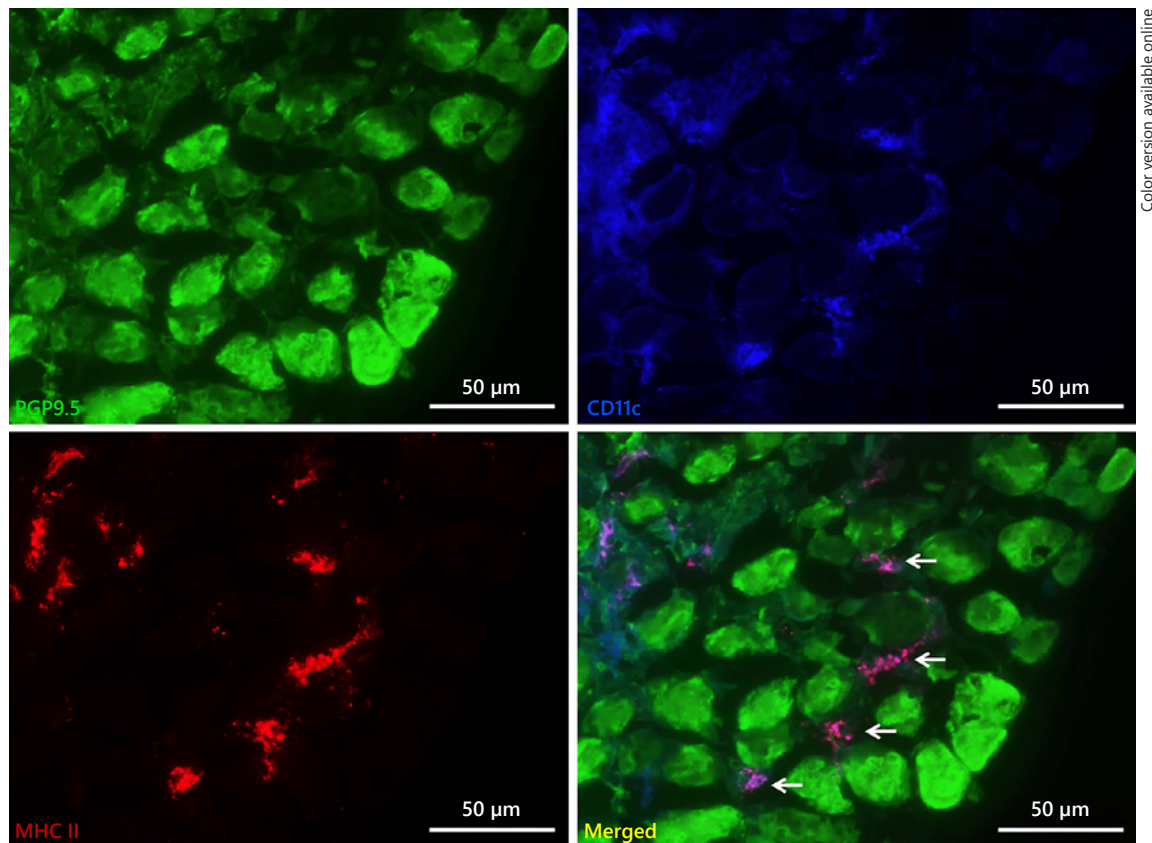


Fig. 5. Immunohistochemical characterization of DCs in JNC: exemplary immunohistochemical staining of JNC to define the immigrated DCs as MHCII⁺CD11c⁺ cells (arrows). Images were acquired using epifluorescence microscopy (Axio Imager.M2, Zeiss, Germany) and AxioVision software. $\times 400$.

We recently described the anti-inflammatory activity of chalcone 4 in a murine model of allergic airway hyper eosinophilia [6, 14, 15]. The present study confirmed the anti-inflammatory effect of chalcone 4 treatment in asthma, with decreased numbers of inflammatory cells in BALF. In addition, this anti-inflammatory effect was associated with decreased CD11c⁺ DC infiltration into BALF, as well as with decreased MHC II⁺ DC numbers in airway sensory JNC.

The CXCR4/CXCL12 axis is essential for the migration of DCs to their effector spot for antigen presentation [24, 25]. The present immunofluorescence results indicate that CXCR4 was not expressed at the surface of DCs located at the vicinity of nerve cell bodies within the JNC. This would suggest a downregulation of CXCR4 expression on DCs located in the JNC close to nerve cell bodies. The chalcone 4-induced effect would then be linked to inhibition of the CXCL12-induced DC migration to the JNC before differentiating into CD11c⁺ DCs and nerve

cell body localization [26–28]. In addition, we may also hypothesize that the effect of chalcone 4 – and subsequently of CXCL12 – is indirect, which would altogether indicate that the leverage point of chalcone 4 on the migration of DCs is upstream in the trafficking process. This trafficking process is still poorly understood, and new studies could provide additional information on the mechanism of CXCL12-induced DC migration into the JNC.

Airway vagal sensory neurons play a pivotal role in the neurogenic inflammation of the airways [11] via neuronal reflex and by triggering the release of a variety of neuropeptides [29]. We showed here that the increased DC/neuron ratio in the OVA compared to the saline groups was identical in this OVA model as in the house dust mite model previously reported [11]. Furthermore, chalcone 4 decreased this DC/neuron ratio in the OVA group. However, the DC/neuron ratios in JNC were almost identical in the saline-challenged groups treated either with chal-

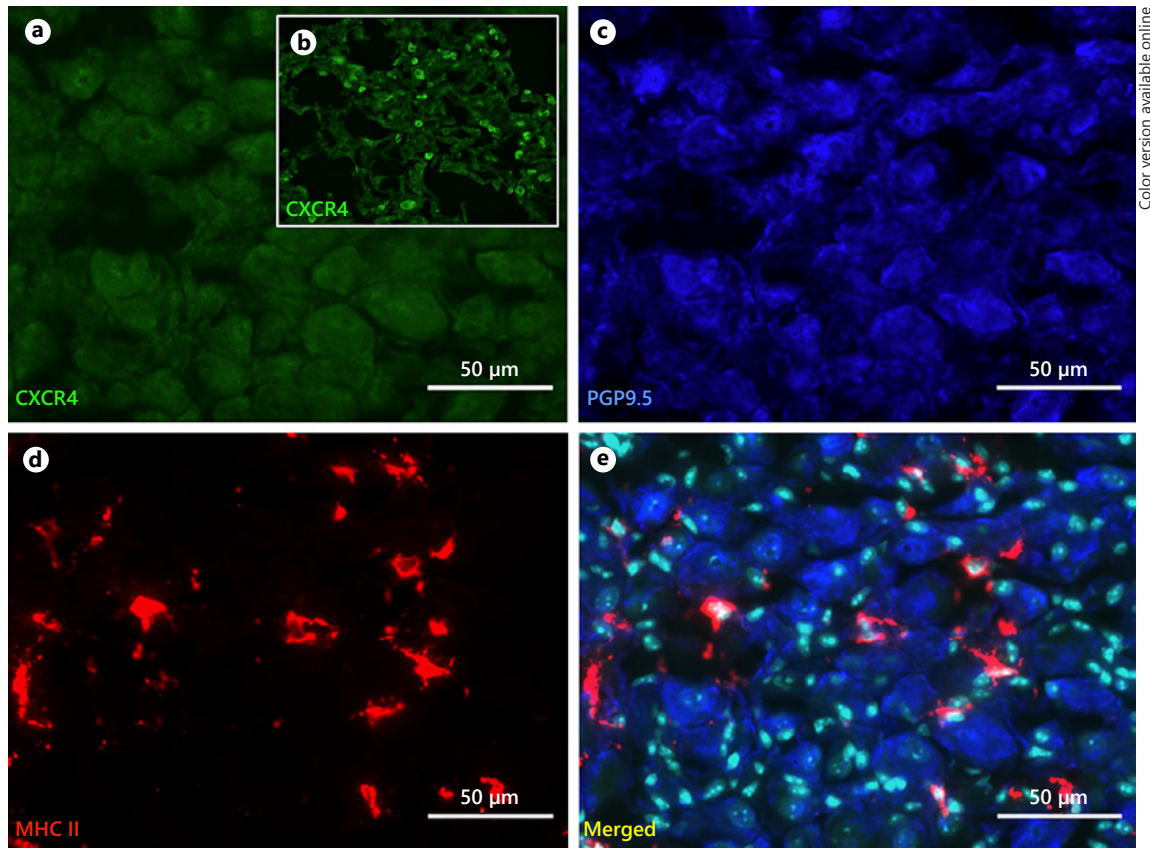


Fig. 6. Immunohistochemical characterization of dendritic cells (DCs) in the jugular-nodose ganglion complex (JNC): exemplary immunohistochemical staining of JNC to analyze the expression of CXCR4 on DCs in the different groups: CDx (a); chalcone 4 (c); OVA (d); and OVA + chalcone 4 (e). $\times 400$. b Positive control of the CXCR4 antibody in lung tissue. $\times 200$. Images were acquired using epifluorescence microscopy (Axio Imager M2; Zeiss) and AxioVision.

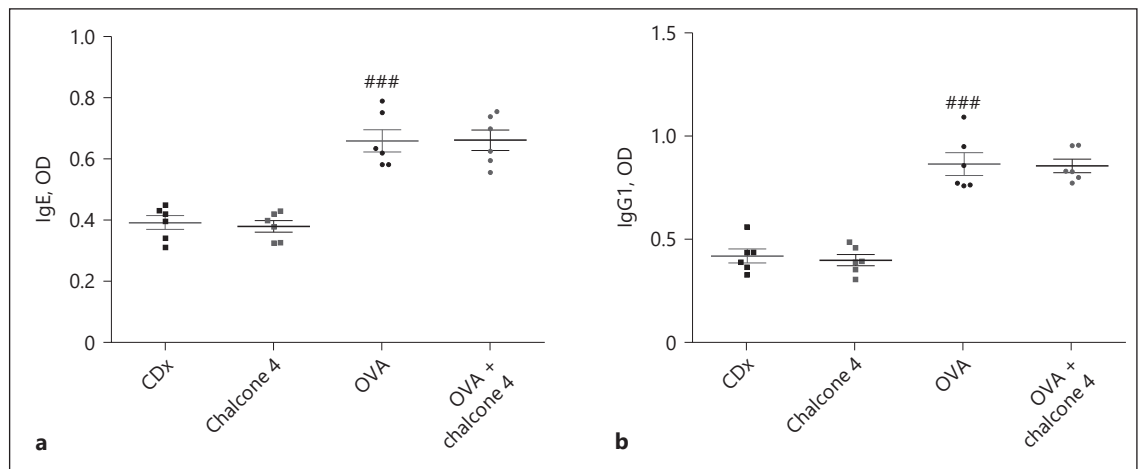


Fig. 7. Chalcone 4 did not affect the ovalbumin (OVA)-specific IgE (a) and IgG1 (b) levels in plasma. Plasma samples were collected 24 h after the final OVA challenge. Serum levels of OVA-specific IgE and IgG1 were measured by ELISA. One-way ANOVA with a Bonferroni multicomparison post hoc test was conducted using Graph-Pad Prism5 software. ### $p \leq 0.001$, OVA + chalcone 4 vs. CDx.

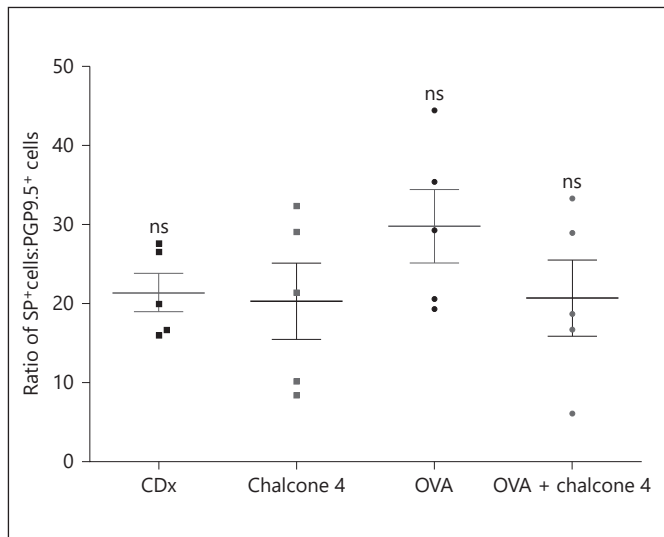


Fig. 8. Chalcone 4-dependent substance P (SP) expression in the jugular-nodose ganglion complex (JNC). Effects of chalcone 4 (gray) treatment in an OVA (circles) mouse model of allergic asthma on calcitonin gene-related peptide cell body expression in JNC. There were no significant differences among the different groups.

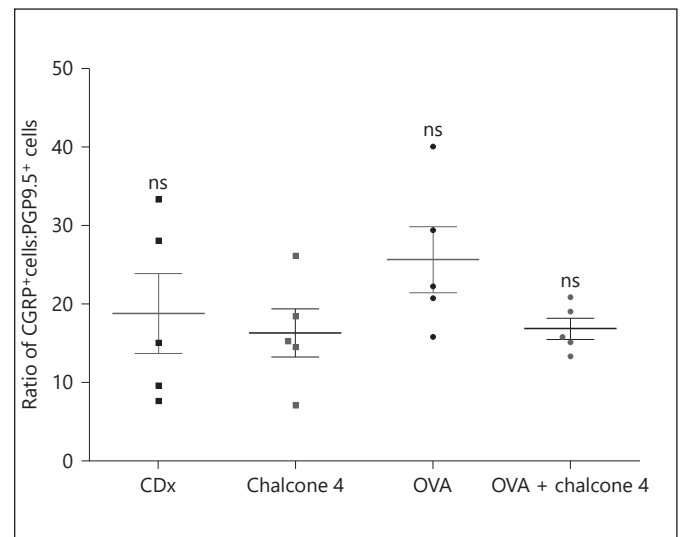


Fig. 9. Chalcone 4-dependent calcitonin gene-related peptide (CGRP) expression in the jugular nodose-ganglion complex (JNC). Effects of chalcone 4 (gray) treatment in an ovalbumin (OVA)-challenged (circles) mouse model of allergic asthma on CGRP cell body expression in JNC. There were no significant differences among the different groups.

cone 4 or solvent, indicating that chalcone 4 did not affect the steady-state level of ganglionic DCs. This strengthens the evidence of an attenuated migratory behavior of DCs caused by the inhibition of CXCL12 binding to the CXCR4 receptor by chalcone 4.

Concerning the CGRP⁺ cell bodies in the JNC, we observed a high baseline level of the CGRP⁺/PGP9.5 ratio in the control saline-challenged animals (18.8%), whereas the baseline ratio was only 7.6% in a previous study [9]. This may be because in this study, the mice were all sensitized, but only a subgroup was challenged with allergen (OVA group), whereas the other subgroup was challenged with saline (controls). After OVA challenge, there was a trend towards an increase in SP and CGRP nerve cell bodies, but the difference was not significant in our acute model of OVA-challenged mice. This was in contrast to a previous report by Le et al. [11] in a house dust mite model where a significant increase in CGRP cell bodies was detected. On the other hand, this may be related to the acute (4 days in this study) versus chronic and long-term (47 days in the study by Le et al. [11]) allergen exposure, which left time for increased numbers of SP/ CGRP nerve cell bodies in the JNC. Additionally, we showed here that the neutraligand chalcone 4 stabilized the sensory nerve cell body ratio CGRP/PGP as well as

SP/PGP to their respective baseline levels. This effect of CXCL12 neutralization on nerve cell bodies will be interesting to investigate in more detail to elucidate the mechanism of the increased number of sensory nerve cell bodies over allergen exposure.

In conclusion, our results indicate that the CXCL12 neutraligand chalcone 4, a potent inhibitor of the CXCL12-CXCR4 axis, prevented DC migration to the airways and airway JNC during allergic airway inflammation. The discovery of the CXCR4-CXCL12 pathway and its role in the pathophysiological mechanism of asthma thereby represents a promising future target for asthma therapy.

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Disclosure Statement

All authors confirm that they have no conflict of interest.

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Author Contributions

S.H. processed the JNC from removal to histological staining, analyzed the data, and wrote the manuscript together with Q.T.D., F.D., N.F., D.D.L., M.S., and R.B.; F.D. performed the in vivo study including the treatment of mice, IgE/IgG1 ELISA, and BALF analysis; N.F. and Q.T.D. supervised the study.

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