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Neuronal branching of sensory neurons is associated with BDNF-positive eosinophils in atopic dermatitis

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Abstract

Background: Pruritus is a major symptom of atopic dermatitis (AD) and is transmitted by a subpopulation of non-myelinated C-type free nerve endings in the epidermis and upper dermis. Stimulation of these nerve terminals is affected by histamine, neurotrophins and physical factors. Eosinophils of patients with AD are a source of neurotrophins, including brain-derived neurotrophic factor (BDNF), levels of which correlate with disease severity.

Objective: The purpose of this study was to determine the anatomical localization of eosinophils in the skin of patients with AD with regard to peripheral nerves and to investigate whether eosinophils induce sprouting and neurite outgrowth in murine sensory neurons.

Methods: Cryosections of skin derived from AD and control (NA) patients were subjected to immunofluorescence analysis with markers for eosinophils, BDNF and neuronal cells. Stimulated eosinophil supernatants were used for the treatment of cultured peripheral mouse dorsal root ganglia (DRG) neurons followed by morphometric analysis.

Results: Dermal axon density and the proximity of eosinophils to nerve fibres were significantly higher in AD patients vs NA. Both neuronal projections and eosinophils expressed BDNF. Furthermore, activated eosinophil supernatants induced BDNF-dependent mouse DRG neuron branching.

Conclusions and Clinical Relevance: Our results indicate that BDNF-positive eosinophils are also localized in close proximity with nerve fibres in AD, suggesting a functional relationship between BDNF-expressing eosinophils and neuronal projections. These observations suggest that eosinophils may have considerable impact on pruritus by supporting sensory nerve branching.

KEYWORDS

atopic dermatitis, BDNF, DRG neurons, eosinophils

Daria Guseva and Urda Rüdrich equal contributions to this work.

1 | INTRODUCTION

Pruritus is a common feature of atopic dermatitis (AD), which negatively influences patient quality of life.[1] Itch often begins before any lesions appear and is transmitted by a subpopulation of non-myelinated C-type free nerve endings in the epidermis and upper dermis.[2] Stimulation of these nerve terminals can be affected by various chemical and physical factors but also through mediators such as histamine or neurotrophins.[2] In patients with AD, neurotrophin levels are increased in plasma, serum[3-5] and within the skin.[6] More importantly, serum levels of the neurotrophin brain-derived neurotrophic factor (BDNF) and the neurokinin substance P (SP) correlate with disease severity in AD patients.[4,5,7] In addition, BDNF levels in AD patients correlate with eosinophilic cationic protein (ECP).[8] ECP itself represents a sensitive readout for disease activity in AD and is released by eosinophils, and eosinophilia is a characteristic feature of AD where the number of eosinophils is increased in both the peripheral blood and skin. Interestingly, eosinophils not only secrete various chemokines and cytokines, but also neurotrophins, including BDNF. Because eosinophils express different receptors of the neurotrophin family such as pan-neurotrophin receptor ($p75^{NTR}$) and tyrosine kinase (trk)B,[9] eosinophils can also be functionally activated through neurotrophins. Furthermore, eosinophils express neurokinin (NK) receptors, including the NK2R,[10] which renders them sensitive to neurokinins such as SP. The accumulation of degranulated eosinophils near SP-positive nerve fibres in an AD-stress mouse model[11] suggests a direct communication of eosinophils with nerves. Indeed, SP leads to an activation of human peripheral blood eosinophils.[10] Additionally, activation of eosinophils leads to the release of BDNF and IL-31.[7,12] In rats, BDNF was shown to induce the sprouting of DRG neurons,[13] where touch-sensitive neurons are known to express trk receptors, particularly the trkB receptor for BDNF.[14] Additionally, Foster et al[15] reported that lesional skin of humans with AD as well as that from a transgenic mouse model of AD has more peripheral nerves than non-lesional skin and that the levels of eosinophil granule proteins are increased near nerve terminals. Moreover, the authors demonstrated that eosinophil culture supernatant induces the branching of DRG neurons, which is not dependent on nerve growth factor (NGF).[15]

Previously we have shown that eosinophils from AD patients release high amounts of BDNF, which can be further increased by stimulation with platelet-activating factor (PAF).[7] In this study, we observed that many eosinophils are co-localized with nerve fibres in skin samples from AD patients. In addition, we found morphogenic effects of PAF-stimulated eosinophils derived from AD patients on DRG neurons, which was not the case in control patients.

2 | METHODS

2.1 | Patient materials

Skin samples (3 patients with AD; mean age 32 ± 4 years; 3 woman; SCORAD score 23.5 ± 7.2 and 4 control patients, mean age 31 ± 3 years; female:male 3:1) were obtained from patients with established AD as well as control patients (NA) from the volar forearm at the Department of Dermatology and Allergy of Hannover Medical School. Procedures were approved by the Ethics Committee of Hannover Medical School (ethics approval number 5808). Additional skin samples, including bullous pemphigoid (BP) patients, were obtained from the Universities of Oldenburg and Düsseldorf and described in the Figure S2. Here, BP (perilesional skin) and NA skin was obtained from the University of Oldenburg and AD skin from the University of Düsseldorf, following approval by the local Ethics Committees (reference numbers 2017-068, 2017-106 and 6132R [PruSearch], respectively).

The samples were fixed overnight at 4°C in 4% paraformaldehyde (PFA) in 0.1 mol/L sodium cacodylate buffer, pH 7.3. The tissue was then immersed in a 15% sucrose solution followed by a 30% sucrose solution in 0.1 mol/L cacodylate buffer, pH 7.3, for 1 day each, at 4°C. Afterwards, the tissue was immersed in TissueTek® (Sakura Finetek Europe) in plastic Biopsy Cryomold® (Science Services) and frozen on dry ice. For sectioning, samples were attached to a cryostat specimen holder using TissueTek®. Serial transverse sections of 12 μm thickness were cut on a cryostat (Thermo Scientific, Microm HM 560 M) and picked up on SuperFrost® Plus glass slides (Roth). The sections were air-dried at RT and used for immunohistochemistry. One sample per patient was fixed in neutral buffered 4% PFA, processed routinely, embedded in paraffin, sectioned at 5-6 μm and stained with haematoxylin and eosin (H&E).

2.2 | Immunohistochemistry

Cryoslices were briefly washed with PBS, and antigen retrieval was performed by immersion in 0.01 mol/L sodium citrate solution, pH 9.0, heated to 80°C in a water bath for 30 minutes. After short washing in PBS, blocking of non-specific binding sites was performed using PBS containing 0.2% Triton X-100 (Sigma Aldrich Chemicals), 0.02% sodium azide (Merck) and 5% normal donkey serum for 1 hours at RT. Incubation with primary antibodies against BDNF (sheep, 1:1000, Millipore, AB3004186), human ECP (EG1, mouse, 1:100, Pharmacia & Upjohn), MBP (PRG2, rabbit, conjugated to FITC, 1:100, Biorbyt, 222526; used in Figure S2) and βIII-Tubulin (rabbit, 1:1000, Synaptic System, 302302), diluted in PBS containing 0.5% lambda-carrageenan (Sigma Aldrich Chemicals) and 0.02% sodium azide, was carried out at 4°C for 3 days. After washing in PBS, appropriate DyLight-conjugated secondary antibodies (Jackson ImmunoResearch Europe) diluted 1:200 in PBS-carrageenan solution were applied for 2 hours at RT. After subsequent washing in PBS, cell nuclei were stained for 10 minutes at RT with bisbenzimide solution (Hoechst 33258 dye, 5 μg/mL in PBS, Sigma Aldrich Chemicals). Specificity of staining was controlled by omitting the primary antibody (these controls were all negative). For the anti-BDNF antibody, as a control, we utilized the BDNF blocking peptide (50 ng/μL, R&D Systems, 248-BD), which was pre-incubated with BDNF blocking peptide for 1 hour at RT. These controls revealed no

positive reaction (Figure S1A). Finally, the sections were mounted in anti-quenching medium (Fluoromount G; Southern Biotechnology Associates) and, unless stated otherwise, analysed using a confocal microscope (Zeiss LSM780) with a 60× oil-immersion objective.

2.3 | Isolation of human peripheral blood eosinophils

Peripheral venous blood was obtained from patients with AD and control patients who had not received any immunosuppressive treatment within the last 4 weeks (approved by the ethics committee of the Hannover Medical School, ethics number 5808). Human eosinophil granulocytes were separated by Ficoll-density centrifugation. After removing the interphase and the supernatant, the erythrocytes in the pellet were lysed with lysis buffer (155 mmol/L NH_4Cl; 10 mmol/L $KHCO_3$; 0.1 mmol/L EDTA). Eosinophils were further isolated with subsequent negative immunomagnetic selection using CD16 microbeads (Miltenyi Biotech®) as previously described.[7] The purity of eosinophils was 99.5% as determined by microscopic examination with Kimura staining[16] and FACS analysis (CD49d/CD16, Beckmann Coulter). Viability was 99% as assessed by trypan blue exclusion.

2.4 | Culture of isolated eosinophils

Eosinophils were cultivated in RPMI 1640 medium with 10% heat-inactivated fetal calf serum (FCS) including 2 mmol/L L-glutamine, 10 000 U/mL penicillin and 10 mg/mL streptomycin (all Seromed, Biochrom AG) at 37°C and 5% CO_2. To stimulate BDNF release, eosinophils were activated with 0.1 µmol/L PAF for 48 hours and centrifuged. Supernatants were harvested and stored at −80°C until further analysis. BDNF in the supernatant was measured using a commercial ELISA (R&D Systems) according to the manufacturer's recommendations. The specificity of the assay was confirmed by the manufacturer, and the detection limit was 39 pg/mL.

2.5 | Isolation and culture of DRG neurons

DRGs were isolated from 21-day-old wild-type C57BL/6J mice, washed in ice-cooled F-12 medium (PAA Laboratories, Gölbe) and incubated with 1 mL of 0.25% trypsin (Sigma Aldrich Chemicals) and 0.03% collagenase (Sigma Aldrich Chemicals) in Dulbecco's modified Eagle medium (DMEM, PAA) for 1 hour at 37°C. Enzymatic digestion was terminated by rinsing twice with F-12 medium. The tissue samples were then transferred into complete (DMEM/Ham's F-12) medium containing 0.01% DNase I (Sigma Aldrich Chemicals) and mechanically dissociated by gentle trituration with a fire-polished Pasteur pipette. The complete medium (DRG medium) consisted of equal parts of DMEM and F-12 media, and was supplemented with 100 IU/mL penicillin/streptomycin, 2 mmol/L L-glutamine, 100 mmol/L sodium pyruvate (all from PAA), 0.05 mg/mL insulin, 0.05 mg/mL transferrin, 15 nmol/L triiodo-thyronine, 5 nmol/L hydrocortisone, 50 nmol/L putrescine, 10 nmol/L progesterone, 15 nmol/L sodium selenite, 0.1 mg/mL bovine serum albumin (all from Sigma Aldrich Chemicals) and 5% horse serum (Invitrogen). After adding of DMEM/F-12, the cell suspension was centrifuged at 500 *g* for 5 minutes at 4°C. The supernatant was discarded and the cells were resuspended in 1 mL fresh pre-warmed (37°C) DRG medium, counted and plated at a density of 5×10^3 to 5×10^4 cells/mL (500 µL/well) on 0.01% poly-L-lysine-coated coverslips. At least three independent cell cultures were analysed.

2.6 | Treatment of DRG neurons

DRG neurons were cultivated (a) in the supernatant from AD eosinophils and eosinophils of control patients that were either unstimulated or (b) stimulated with PAF (0.1 µmol/L), (c) in DRG neuron medium (described above), or in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, 10 000 U/mL penicillin and 10 mg/mL streptomycin with (d) and without 50 ng/mL BDNF (e) as a positive control. As additional controls, the cultures treated with PAF (f), anti-BDNF antibody alone (g), supernatant from AD eosinophils stimulated with PAF and pre-incubated with anti-BDNF antibody (h) were analysed.

2.7 | Immunocytochemistry

Forty-eight hours after plating, cultures of DRG neurons were briefly washed with PBS and fixed in 4% PFA for 15 minutes at RT. After washing twice in PBS, cells were incubated with 0.5 mol/L glycine in H_2O for 15 minutes to remove free PFA from the cell surface, washed with PBS and incubated with PBS containing 0.2% Triton X-100 (Sigma Aldrich Chemicals), 0.02% sodium azide (Merck) and 5% normal donkey serum for 30 minutes at RT to block non-specific binding sites. Incubation with primary antibody against βIII-Tubulin (rabbit polyclonal, 1:2000, Covance, PRB-435P-100) and Fluorescein labelled Griffonia Simplicifolia Lectin I (GSL I) isolectin B4 (IB4, 1:200, Vector Laboratories, FL-1201) diluted in PBS containing 0.5% lambda-carrageenan (Sigma Aldrich Chemicals) and 0.02% sodium azide was carried out at 4°C overnight. After washing in PBS, appropriate donkey anti-rabbit Alexa Fluor® 594-conjugated secondary antibody (Jackson ImmunoResearch Europe) diluted 1:200 in PBS-carrageenan solution was applied for 1 hour at RT. After a subsequent washing in PBS, coverslips were mounted in anti-quenching medium. In our experiments, we selected a population of medium to large myelinated neurons, which are known to have high-affinity receptors to BDNF—trkB.[14] To exclude small diameter neurons from the analysis, which do not express BDNF receptors,[17] we measured the neurite outgrowth of IB4-negative neurons (Figure S1B), a population of peptidergic neurons which are substantially expressed (*ca* 50%) in DRG in vivo. Ten neurons per each condition were measured.

2.8 | Image analysis and quantification

To assess the changes in density of axons in the upper dermis of AD patients, cryosections were subjected to confocal microscopy after immunofluorescent analysis with antibody against βIII-Tubulin (Zeiss LSM 780, 20× objective was used) and βIII-Tubulin-positive projections quantified using MATLAB®. During analysis, images were converted to binary system and the area covered in white was calculated as a measurement for axon density (Figure 1C). The number of axon profiles was determined using image analysis of multiple fields from non-adjacent sections. Although some labelled profiles may represent multiple axons, the method was applied uniformly to all tissue examined, allowing comparison among the different conditions employed. ImageJ was used to assess the proximity of eosinophils to dermal nerve fibres. In this analysis, we quantified the number of eosinophils ($EG1^+$-cells) per mm^2 of nerve fibre area.

Neuron images were obtained by confocal microscopy (Olympus FV1000) at 2048 × 2048 pixel resolution for morphological analysis (20× and 40× objectives were used). The confocal settings were equal for all of the scans. Morphometric analysis and quantification were performed with Motic Images Plus 2.0 and ImageJ software with the Sholl plug-in[18] for measurements of total neurite length and Sholl analysis, respectively. At least 10 neurons per experiment were measured. For analysis of the association between nerve fibres with BDNF-positive eosinophils, we utilized ImageJ and quantified the number of eosinophils per mm^2 of the nerve fibre area.

To estimate the co-localization of βIII-tubulin and BDNF in skin sections of AD and NA patients, microscopic images (n = 3) were obtained by confocal microscopy at ×40 magnification. Whole images were used for the analysis of Pearson's correlation coefficient by using of Fiji software (http://imagej.nih.gov/ij/) with plug-in Coloc 2.

2.9 | Statistical analysis

All data are presented as means ± SEM. Statistical analysis was performed with GraphPad Prism 5. Normality test was performed with D'Agostino & Pearson Omnibus normality test. Since the data showed no Gaussian distribution, analyses were performed using the Kruskal-Wallis test followed by the Dunn's post-test.

3 | RESULTS

3.1 | The density of neuronal projections in the skin of patients with AD is enhanced and is surrounded by BDNF-releasing eosinophils

Morphological analysis of the skin and cutaneous nerves performed by light microscopy after H&E staining showed an increased number of axons in the upper dermis of patients with AD in comparison with controls (NA; Figure 1A). In order to obtain more detailed information regarding the changes in dermal axon density in patients with AD, we performed a quantification analysis of βIII-Tubulin–positive dermal nerves using MATLAB® Software. Immunofluorescence analysis revealed a significantly increased area covered by dermal axons in the skin of patients with AD compared to controls (Figure 1B and C).

To investigate the possible interplay between eosinophils and nerve fibres in patients with AD, we performed immunofluorescence analysis of skin biopsies by co-staining with antibodies against EG1 (marker for eosinophils), βIII-Tubulin (neuronal marker) and BDNF (Figure 1B). Fluorescent confocal microscopy analysis revealed high expression levels of BDNF in eosinophils. Moreover, we observed EG1-positive eosinophils expressing BDNF localized in close proximity with βIII-Tubulin–positive nerve fibres (Figure 1B). The specificity of the BDNF antibody was verified by assessing the effects of a corresponding blocking peptide (Figure S1A). Quantitative analysis revealed a significant increase in eosinophils associated with dermal nerve fibres in AD patients compared with controls (Figure 1D). This suggests a functional relationship between BDNF-expressing eosinophils and neuronal projections. We also detected a high amount of BDNF in nerve fibres (βIII-Tubulin–positive fibres and BDNF-positive fibres) and quantification analysis of the Pearson's correlation coefficient between BDNF immunofluorescence and βIII-Tubulin immunofluorescence revealed a significantly higher high level of co-localization in samples of AD patients compared with control subjects (Figure 1E). This finding is in line with earlier studies showing BDNF expression in nerve fibres in mouse skin[19] and an increase in BNDF levels during allergic inflammation in neurons, structural cells and invading immune cells.[20]

3.2 | Factors released by stimulated eosinophils induce neurite outgrowth and branching of DRG neurons

It has previously been shown that lesional skin of AD patients contains increased nerve fibres.[21] In addition, murine eosinophils have been reported to induce branching of DRG neurons.[15] Therefore, we investigated whether human eosinophils are able to stimulate morphological changes in neurons. To this end, peripheral blood eosinophils, isolated from healthy control patients (NA) and patients with AD, were incubated with PAF, which we previously reported to stimulate BDNF release from AD eosinophils.[7] The supernatants were then added to the cell culture of DRG neurons. After treatment, DRG neurons were fixed and subjected to immunocytochemistry with antibodies against neuronal βIII-Tubulin to visualize the neurites of DRG neurons (Figure 2A). Quantitative analysis of different preparations revealed that the treatment of DRG neurons with culture medium from non-stimulated eosinophils as well as with PAF alone had no effect on neurite outgrowth (Figure 2A and B). In contrast, treatment of neurons with the supernatant from PAF-stimulated AD eosinophils led to a significant increase in neurite outgrowth of DRG neurons (Figure 2A and B). Supernatants of unstimulated and PAF-stimulated eosinophils of control subjects had no effect on neurite outgrowth (Figure S1C). Interestingly, pretreatment of DRG neurons with blocking anti-BDNF antibody followed

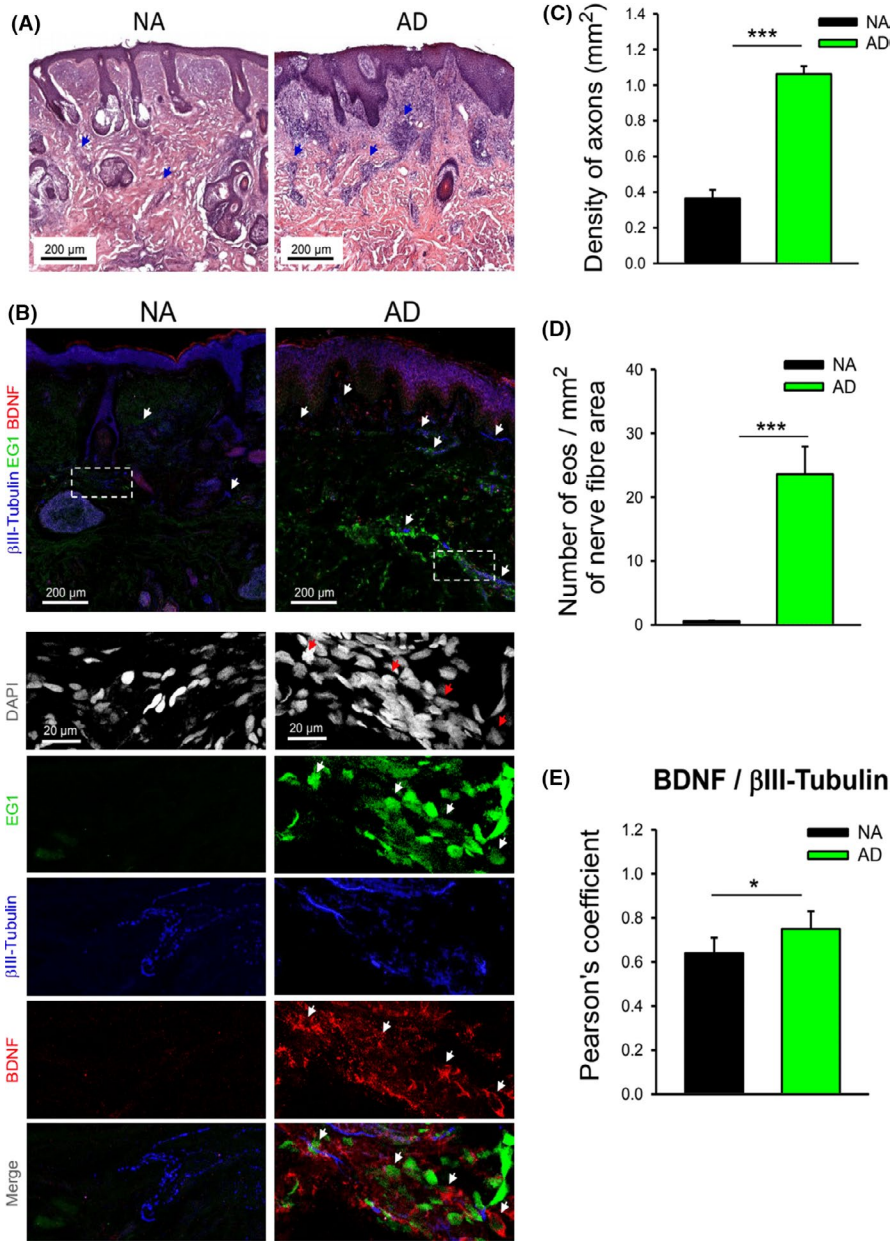
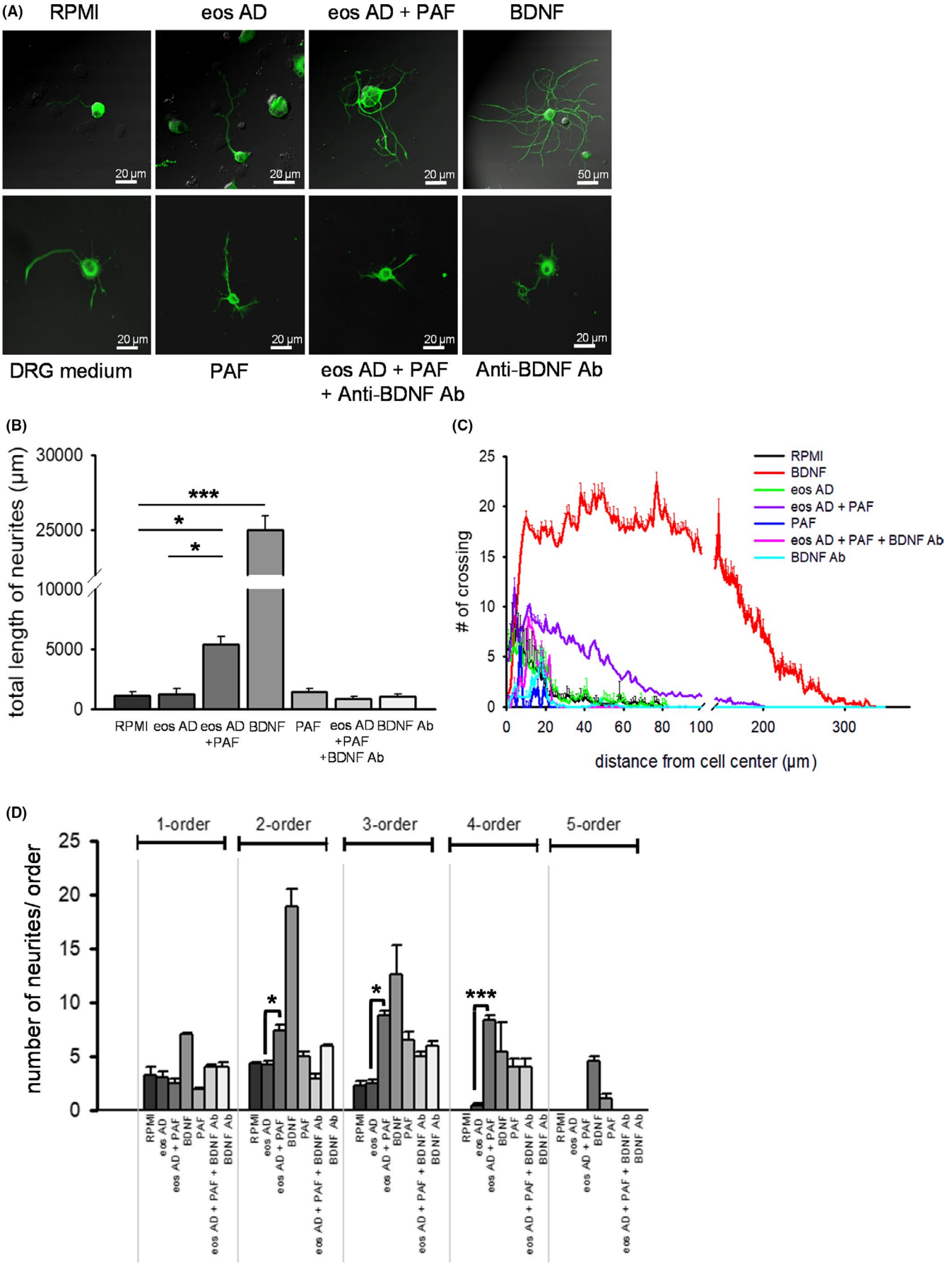


FIGURE 1 Dermal nerve fibres and eosinophils co-localize in the upper dermis of AD patients. A, Representative light micrographs of H&E-stained skin tissue sections from AD and control (NA) patients (blue arrows indicate dermal nerve fibres). B, Representative immunofluorescence images of the skin from AD and NA patients. Eosinophils and nerve fibres of the AD patient both express BDNF (right panel, $EG1^+BDNF^+$-cells and $\beta III\text{-}Tubulin^+BDNF^+$-fibres, indicated by white arrows). Lower panels—higher magnification of corresponding region shown in the upper panel. In AD patients, $EG1^+BDNF^+$-eosinophils are closely associated with nerve fibres indicated by white arrows. EG1—marker for eosinophils (green), βIII-tubulin—neuronal marker (blue), BDNF (red). C, Morphological analysis of the density of axons in skin of patients with AD vs controls (NA) (***$P < .001$). D, Quantitative analysis of the number of eosinophils per mm^2 of nerve fibre area (***$P < .001$). E, Pearson's correlation coefficient between the expression of BDNF and βIII-tubulin (*$P < .05$)

by stimulation with eosinophil-conditioned medium blocked morphological changes in DRG neurons. These data suggest that the main morphogenic substances within supernatants from PAF-stimulated eosinophils isolated from patients with AD is BDNF. As a positive control, we used BDNF-stimulated neurons, which displayed an approximately ten-fold increase in DRG neurite total length (Figure 2A and B). This effect was BDNF-specific, because it was completely blocked by pretreatment with blocking anti-BDNF antibody. Overall, neurite length can be increased by several factors including the number of branches. To distinguish between these possibilities, we performed a Sholl analysis on the same cells. This experiment demonstrated that the axonal arborization, in particular at distances between 10 and 60 µm from the cell body, was significantly increased in DRG neurons treated with the supernatants of PAF-stimulated AD eosinophils (Figure 2C). This effect was more pronounced after BNDF treatment, where the number of crossings was significantly increased even at a distance of 200 µm from the cell somata. Finally, we performed a detailed analysis of the branching by calculating the number of branches of the 1st-6th order. As shown in Figure 2D, treatment of neurons with supernatants of PAF-stimulated AD eosinophils induced a symmetric distribution with the maximal amount of branches of 2nd, 3rd and 4th orders. It is noteworthy that treatment with BNDF resulted in a maximal increase of branches of the 2nd order (Figure 2D). These results demonstrate that, in addition to BNDF, stimulated AD eosinophils might release other factors which modulate axonal branching in DRG neurons.

4 | DISCUSSION

Patients with AD often suffer from strong pruritus, which has a substantial impact on the quality of life. It has been hypothesized that pruritus can be mediated by an increased number of nerves in the skin of


(A)
RPMI
eos AD
eos AD + PAF
BDNF
20 μm
20 μm
20 μm
50 μm
20 μm
20 μm
20 μm
20 μm
DRG medium
PAF
eos AD + PAF + Anti-BDNF Ab
Anti-BDNF Ab
(B)
total length of neurites (μm)
30000
25000
10000
5000
0
***
*
*
RPMI
eos AD
eos AD +PAF
BDNF
PAF
eos AD +PAF +BDNF Ab
BDNF Ab
(C)
# of crossing
25
20
15
10
5
0
0
20
40
60
80
100
200
300
distance from cell center (μm)
RPMI
BDNF
eos AD
eos AD + PAF
PAF
eos AD + PAF + BDNF Ab
BDNF Ab
(D)
number of neurites/ order
25
20
15
10
5
0
1-order
2-order
3-order
4-order
5-order
*
*
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RPMI
eos AD
eos AD + PAF
BDNF
PAF
eos AD + PAF + BDNF Ab
BDNF Ab

FIGURE 2 DRG neurons cultured with medium from PAF-stimulated AD eosinophils display enhanced neuronal outgrowth and branching. A, representative images of βIII-Tubulin–positive DRG neurons: RPMI (control, eosinophil culture medium), eos AD (unstimulated eosinophils of AD patients), eos AD + PAF (supernatant of AD eosinophils stimulated with PAF), BDNF (treatment of the DRG neuronal culture with BDNF), DRG medium (as described above), PAF (PAF applied in RPMI), eos AD + PAF + anti-BDNF Ab (supernatants from AD eosinophils stimulated with PAF, which were pre-incubated with blocking anti-BDNF antibody), anti-BDNF Ab (anti-BDNF antibody applied in RPMI). B, Total length of neurites per neuron (*P < .05; ***P < .001). C, Neuronal branching evaluated by Sholl analysis. D, Number of neurites per certain order of the DRG neuron (*P < .05; ***P < .001)

affected individuals. However, this topic is controversial since recent data demonstrated a lower density of nerve fibres in patients with AD than in healthy subjects.[22] In earlier investigations, an increase of nerve fibre density was presented in the epidermis of AD patients.[23,24]

In the present study, we demonstrated that eosinophils in the skin of AD patients express BDNF and are largely located in close proximity to peripheral nerves. Further, we could show that PAF-stimulated human eosinophils from AD patients strongly induce axonal outgrowth and neurite's branching of DRG neurons, which was not seen using eosinophils of control patients. In contrast to the results obtained by Foster et al,[15] no increased branching was observed in DRG neurons cultivated with the supernatant of unstimulated human AD eosinophils. Previous studies of our group have shown that human AD eosinophils release higher amounts of BDNF than eosinophils of healthy subjects.[7] The amount of BDNF released could be further increased by stimulating eosinophils with PAF, which is secreted by various cells such as keratinocytes,[25] mast cells[26] or fibroblasts.[27] Our study revealed that stimulation of AD eosinophils with PAF induces the release of BNDF at a concentration required for the induction of morphological changes in DRG neurons (Figure 2). In addition, Noga and colleagues showed that eosinophils derived from patients with AD express increased BDNF mRNA.[28] These data suggest that BDNF could be an important factor in the initiation of morphological changes in neurons of patients with AD. However, based on our results shown in Figure 2, we cannot exclude that other specific factors released by eosinophils such as eosinophil peroxidase (EPO), major basic protein (MBP)[29] or NGF[30] could also influence neuronal morphology.[31] In addition, the pruritogenic cytokine IL-31, which is produced and released by eosinophils,[12] can also promote the outgrowth of sensory nerves.[32] In this regard, NGF is not directly involved in neuronal outgrowth since application of neutralizing NGF antibody did not prevent the increase of neurite branching in co-cultures of DRG neurons and eosinophils.[15] Thus, cationic proteins from eosinophils may contribute to hyperresponsiveness of sensory neurons as also observed in airway inflammation.[33]

Our results clearly suggest that the majority of BDNF-expressing cells in close proximity to nerve fibres in AD skin are eosinophils rather than other reported potential sources such as Th2 cells[34] as well as megakaryocytes and platelets.[35] It remains to be seen whether the concept of sensory neurite outgrowth is regulated by eosinophils in other pruritic diseases. We have previously shown that eosinophils play a major role in the autoimmune blistering disease BP.[36,37] Interestingly, our preliminary immunofluorescence microscopy data, based on perilesional skin sections from BP patients, show that the same proof of concept applies in BP too. Here, we observed a striking accumulation of eosinophils that expressed BDNF in the vicinity of peripheral nerves in the upper dermis of BP skin, as well as verifying our previous findings in AD skin (results shown in Figure S2A). Furthermore, we could again show that skin eosinophils are BDNF-positive in AD in comparison with NA (Figure S2B), which supports our previous finding that eosinophils are a major source of BDNF in AD compared with NA in the peripheral blood.[7]

Taken together, our results indicate that eosinophils from AD patients activated with PAF, a known factor to stimulate the release of BDNF, induce neurite outgrowth and stimulate axonal branching of DRG neurons. This finding suggests that BDNF is one of the neurite outgrowth stimulating factors, which may induce the sensation of pruritus *via* stimulation of skin nerve branching. However, other factors such as IL-31 may also be involved in these neuronal morphological changes, which will need to be addressed in future studies.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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