

Nerve Growth Factor Partially Recovers Inflamed Skin from Stress-Induced Worsening in Allergic Inflammation

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Neuroimmune dysregulation characterizes atopic disease, but its nature and clinical impact remain ill-defined. Induced by stress, the neurotrophin nerve growth factor (NGF) may worsen cutaneous inflammation. We therefore studied the role of NGF in the cutaneous stress response in a mouse model for atopic dermatitis-like allergic dermatitis (AID). Combining several methods, we found that stress increased cutaneous but not serum or hypothalamic NGF in telogen mice. Microarray analysis showed increased mRNAs of inflammatory and growth factors associated with NGF in the skin. In stress-worsened AID, NGF-neutralizing antibodies markedly reduced epidermal thickening together with NGF, neurotrophin receptor (tyrosine kinase A and p75 neurotrophin receptor), and transforming growth factor- β expression by keratinocytes but did not alter transepidermal water loss. Moreover, NGF expression by mast cells was reduced; this corresponded to reduced cutaneous tumor necrosis factor- α (TNF- α) mRNA levels but not to changes in mast cell degranulation or in the T helper type 1 (Th1)/Th2 cytokine balance. Also, eosinophils expressed TNF receptor type 2, and we observed reduced eosinophil infiltration after treatment with NGF-neutralizing antibodies. We thus conclude that NGF acts as a local stress mediator in perceived stress and allergy and that increased NGF message contributes to worsening of cutaneous inflammation mainly by enhancing epidermal hyperplasia, pro-allergic cytokine induction, and allergy-characteristic cellular infiltration.

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INTRODUCTION

The association between stress and the worsening of atopic disease is widely accepted and generally attributed to neuroimmune dysregulation. Two parallel observations contributed to the generation of this conviction (c.f. Theoharides and Kalogeromitros, 2006): (i) allergic inflammation is characterized by mast cell degranulation and altered neuropeptide

levels (Sugiura *et al.*, 1992; Black, 2002; Huang *et al.*, 2003; Peters *et al.*, 2006a; Cevikbas *et al.*, 2007) and (ii) stress is able to degranulate mast cells in a neuropeptide-dependent manner even when generated by nonphysical, noninflammatory, perceived stressors such as noise or restraint (Singh *et al.*, 1999; Peters *et al.*, 2005, 2006a; Arndt *et al.*, 2008). From these observations, it was concluded that stress leads to neurogenic inflammation, which in turn can worsen allergic inflammation, albeit scientific proof of this hypothesis was unavailable (Peters *et al.*, 2006a; Arndt *et al.*, 2008).

Recently, we confirmed that acutely administered stress employs neurogenic inflammation to worsen allergic inflammation *in loco* in a mouse model of atopic dermatitis-like allergic dermatitis (AID; Pavlovic *et al.*, 2008). Neurogenic inflammation thereby initiates classic features of atopic dermatitis development and chronification, such as epidermal thickening, infiltration by eosinophils, and altered cytokine balance, and it depends on substance P (SP) signaling (Pavlovic *et al.*, 2008), a neuropeptide mediator of the cutaneous stress response.

Neurotrophin nerve growth factor (NGF) contributes to cutaneous innervation development and turnover (Tominaga *et al.*, 2009) and maintains SP+ nerve fibers in the skin (Botchkarev *et al.*, 2004; Schulte-Herbruggen *et al.*, 2007). It is also involved in stress-induced cutaneous hyperinnervation

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Abbreviations: AID, atopic dermatitis-like allergic dermatitis; HPRT, hypoxanthine phosphoribosyl transferase; NGF, nerve growth factor; p75NTR, p75 neurotrophin receptor; SP, substance P; TEWL, transepidermal water loss; TGF- β , transforming growth factor- β ; Th cell, T helper cell; TNF- α , tumor necrosis factor- α ; TNFR1, tumor necrosis factor receptor type 1; TrkA, tyrosine kinase A

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(Peters *et al.*, 2007) and induces keratinocyte proliferation (Botchkarev *et al.*, 2004), mast cell degranulation (Tal and Liberman, 1997), and alterations of the cytokine balance

that promote allergic inflammation (Bonini *et al.*, 2003). Taken together, these findings show that NGF is potentially a key molecule that affects all hallmarks of allergic inflammation and cutaneous stress responsiveness upstream of SP (Botchkarev *et al.*, 2006; Peters *et al.*, 2007).

In this study, we therefore characterized NGF function in the cutaneous stress response during allergic inflammation. We determined the impact and mechanisms by which NGF alters stress-induced exacerbation of AID in the established murine AID-stress model (Pavlovic *et al.*, 2008). With this approach, we intend to clarify the potential and predominant mechanism by which NGF acts as a stress mediator in allergic inflammation, with the aim of identifying key features of disease pathology that can be improved by NGF neutralization.

RESULTS

AID and noise stress increase NGF expression in murine telogen skin

We first sought to determine the impact of noise stress on local and systemic NGF production in telogen mice. We found that NGF mRNA expression in the hypothalamus (Figure 1a) and NGF serum levels (Figure 1b) were unaltered 48 hours after termination of stress exposure. By contrast, stress increased cutaneous NGF transcription and immunoreactivity especially in epithelial structures and mast cells in the skin of stressed mice as compared with unaffected, unstressed controls (Figure 1c).

Microarray analysis of stressed skin reveals upregulation of NGF-associated growth factor and inflammatory signaling

To gain a general idea of the inflammation-modulating effects of NGF under stress, we employed the Affymetrix Mouse Genome 430A 2.0 Array (Santa Clara, CA). We analyzed 22,600 probe sets representing 14,000 genes. Of these genes, the expression of 272 was upregulated and that of 416 downregulated after stress exposure. A fold change >2 was considered significant. Among these regulated genes, Kyoto Encyclopedia of Genes and Genomes analysis revealed pathways involved in processes such as neuronal maintenance, focal adhesion, and inflammation, all of which may be affected by altered NGF signaling. We selected 76 genes for focused analysis, including interacting growth factors as well as cytokines and adhesion molecules reported to be modulated by NGF. None was downregulated whereas gene expression of transforming growth factor- β (TGF- β) 2, tumor necrosis factor receptor type 1B (TNFR1B encoding TNFR1), and vascular endothelial growth factor A and



Figure 1. Nerve growth factor (NGF) is increased in skin but not in serum or hypothalamus. (a) In the hypothalamus, the key brain area mediating stress, stress did not alter NGF mRNA levels. (b) In the serum, NGF protein level was unaltered by stress. (c) In the skin, stress exposure leads to a strong albeit not significant increase in NGF mRNA. Immunohistochemistry (IHC) confirms the mRNA data on protein level and demonstrates a significant increase in epidermal NGF expression. Representative photomicrographs of epidermal and mast cell NGF expression (red) are given below the corresponding mRNA levels. Fluorescein isothiocyanate counterstaining reveals identity of mast cells. Arrows point at NGF+ mast cell in stressed dermis. d, dermis; e, epidermis; hfe, hair follicle epithelium; IR, immunoreactivity. Cell nuclei are counterstained with 4',6-diamidino-2-phenylindol (blue). **P-values <0.01 . Bar = 50 μ m.

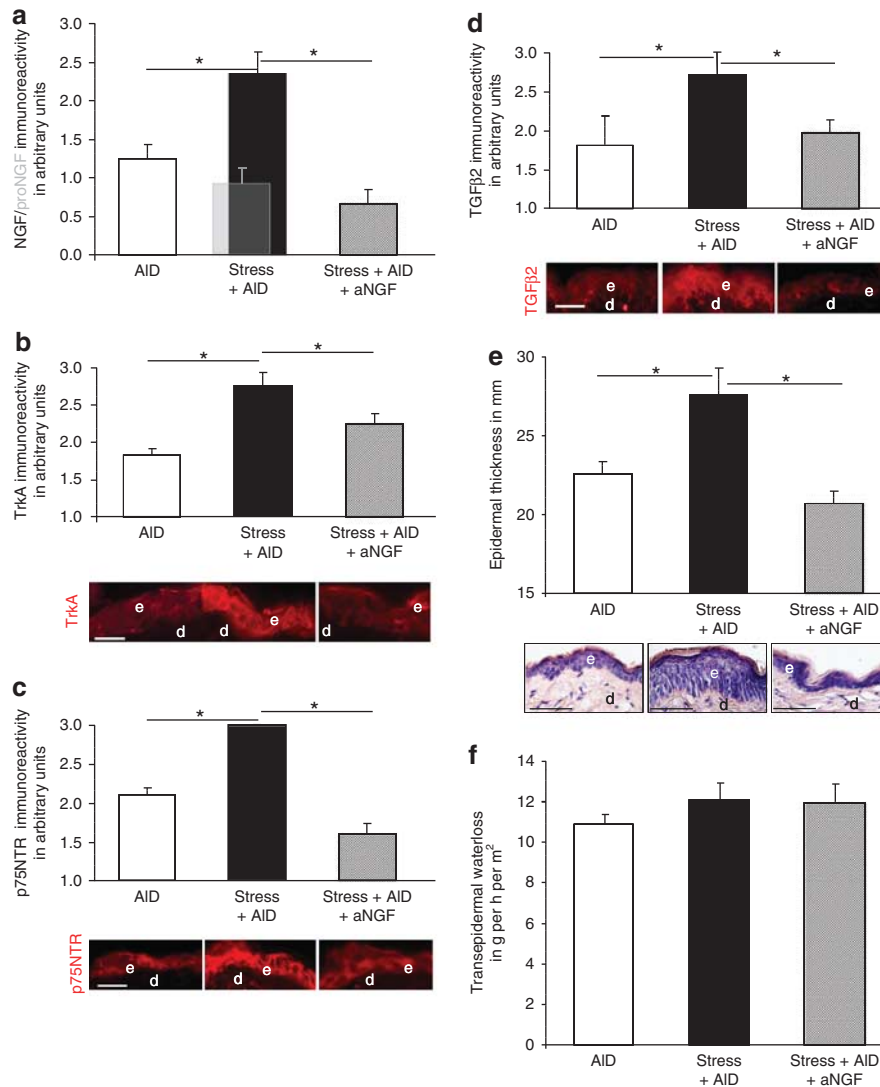


Figure 2. NGF modulates growth factor expression and epidermal hyperplasia but not transepidermal water loss (TEWL). AID skin shows prominent epidermal immunoreactivity for (a) nerve growth factor (NGF), (b) tyrosine kinase A (TrkA), (c) p75 neurotrophin receptor (p75NTR), and (d) transforming growth factor-β (TGF-β) 2. After stress, increased expression of NGF (a–d) and proNGF (gray transparent bar in a) is reverted after treatment with anti-NGF (a–d). (e) AID epidermis is multilayered and thickened to more than 20 μm by stress. Stress exposure markedly and significantly increased epidermal thickness and produced prominent spongiosis, which could be reverted with anti-NGF. (f) Stress did not induce a significant increase in AID-enhanced TEWL nor did the treatment with NGF-neutralizing antibodies improve barrier function. Representative photomicrographs are shown below the respective statistical analysis. aNGF, anti-NGF neutralizing antibody; d, dermis; e, epidermis. **P* < 0.05. Bar = 50 μm.

C were significantly upregulated in the skin of stressed mice (Supplementary Table S1 online).

Anti-NGF treatment reduces epidermal NGF, NGF receptor, and TGF-β2 expression and epidermal thickening, but not transepidermal water loss

We next assessed epithelial NGF and NGF receptor expression as well as that of the epithelial growth factors highlighted by the above microarray analysis. By quantitative immunomorphometry, NGF was significantly increased in the epidermis of stressed AID over AID mice (Figure 2a). Also, proNGF—the biologically active precursor molecule of NGF—was detectable only in stressed AID skin (Figure 2a).

Expression of tyrosine kinase A (TrkA)—the high-affinity receptor for NGF with high epidermal proliferative potential—was strongest in epidermal keratinocytes. In control epidermis, TrkA immunoreactivity highlighted exclusively the basal layer (Peters *et al.*, 2006b), whereas in the stressed AID skin, the staining spread over all epidermal layers (Figure 2b). Expression of p75 neurotrophin receptor (p75NTR)—the low-affinity NGF receptor and enhancing co-receptor of TrkA—(Figure 2c) as well as that of TGF-β2 (Figure 2d) followed the pattern of NGF with the highest staining intensity in the epidermis of stressed AID mice. Semiquantitative histomorphometric analysis of expression intensity revealed an additive effect of stress on AID expression patterns. Treatment

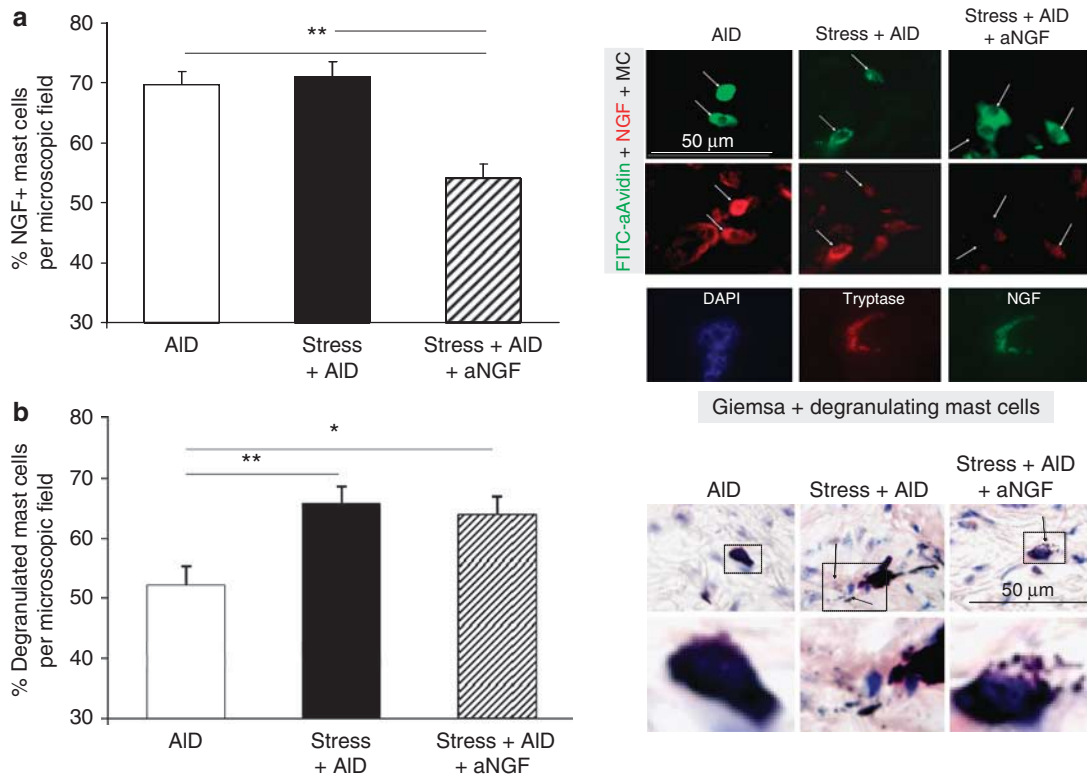


Figure 3. Anti-nerve growth factor (NGF) downregulates NGF immunoreactivity in mast cells but does not affect mast cell degranulation. (a) Treatment with NGF-neutralizing antibodies markedly reduced the NGF immunoreactivity (red label) in fluorescein isothiocyanate-avidin + (green label) mast cells (MC; arrows) as well as the percentage of NGF-immunoreactive mast cells. NGF+ mast cells were also identified by tryptase staining. (b) Stress increased the percentage of degranulated mast cells in the dermis and subcutis of mice with AID, but treatment with anti-NGF did not bring down mast cell degranulation. Giemsa staining reveals multiple extracellular mast cell granules in the back skin of both stressed AID mice and anti-NGF-treated stressed AID mice (arrows, boxes indicate magnifications as shown below). aNGF, anti-NGF neutralizing antibody. * $P < 0.05$; ** $P < 0.01$. Bar = 50 μ m.

with NGF-neutralizing antibody was sufficient to reduce NGF, proNGF, TrkA, and p75NTR as well as TGF- β 2 immunoreactivity to AID levels without stress exposure (Figure 2a-d).

We had previously shown that epidermal thickening is a useful readout parameter for assessing dermatitis severity after stress exposure in AID mice (Pavlovic *et al.*, 2008). In the present study we confirmed a significant increase (by more than 30%) in epidermal thickness after stress exposure (Pavlovic *et al.*, 2008). Furthermore, we observed that treatment with NGF-neutralizing antibodies reduced epidermal thickness to AID levels without stress exposure (Figure 2e). Altered epidermal thickness is frequently associated with a disruption of epidermal barrier function, which is also a characteristic feature and indicator of disease severity in cutaneous allergic inflammation (Takahashi *et al.*, 2005; Gupta *et al.*, 2008). We therefore assessed baseline transepidermal water loss (TEWL) as a measure of epidermal barrier disruption. We were able to detect increased TEWL in stressed mice and most strikingly in AID mice as compared with controls (control: 4.76 g per hour per m^2 , stress: 6.28 g per hour per m^2 , AID: 10.91 g per hour per m^2). However, barrier function was not significantly further disturbed by stress in AID mice, nor was it affected by treatment of stressed AID mice with NGF-neutralizing antibodies (Figure 2f).

Mast cell expression of NGF but not mast cell degranulation is reduced by treatment with anti-NGF

To assess the impact of NGF on relevant innate immune responses in allergy, we focused on mast cells and found that absolute mast cell numbers are unaffected by our experimental protocol. Compared with untreated controls, we observed a significant increase in the NGF immunoreactivity of dermal and subcutaneous mast cells as well as in the percentage of NGF+ mast cells in both AID and stressed AID mice (not shown). When AID mice were compared with stressed AID mice, stress did not further enhance NGF expression by mast cells (Figure 3a). By contrast, mast cell degranulation, as a sign of enhanced neurogenic inflammation, was prominently increased by stress in AID mice (Figure 3b; Pavlovic *et al.*, 2008). Anti-NGF treatment significantly reduced the number of NGF+ mast cells in stressed AID mice (Figure 3a), but did not reduce the percentage of degranulated mast cells in AID skin after stress exposure (Figure 3b), which shows that altered NGF expression is not associated with altered mast cell numbers or mast cell degranulation.

TNF- α but not T helper 1/2 cytokine production is altered by anti-NGF

Using quantitative real-time PCR, we determined the mRNA levels of the classic T helper type 1 (Th1) cytokine IFN- γ or the classic Th2 cytokines IL-4 and IL-5 to assess cytokine

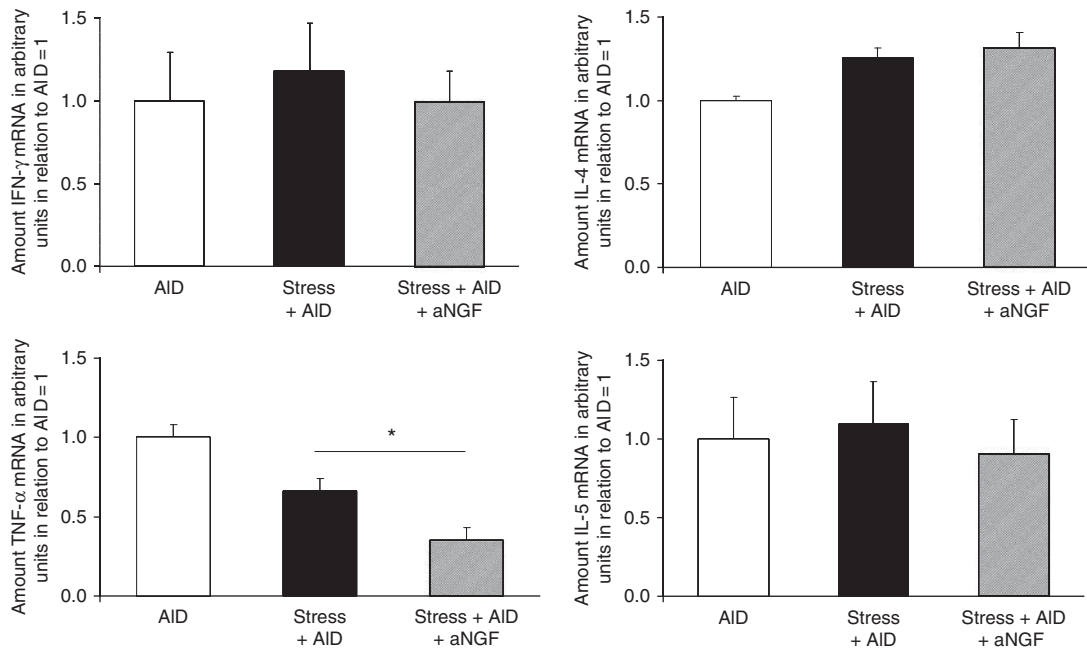


Figure 4. Anti-nerve growth factor (NGF) decreases tumor necrosis factor (TNF)- α mRNA levels in AID skin. Values are expressed in relation to AID levels when AID equals 1. Treatment by NGF-neutralizing antibodies did not affect mRNA levels of IFN- γ , IL-4, or IL-5 when compared with stressed AID full-thickness skin extracts. By contrast, anti-NGF treatment significantly reduced TNF- α levels. * $P < 0.05$. aNGF, anti-NGF neutralizing antibody.

balancing. These parameters did not show significant differences between stressed AID mice and anti-NGF-treated stressed AID mice (Figure 4). A quotient build from Th1/Th2 cytokines using the formula $\text{IFN-}\gamma/(\text{IL-4} + \text{IL-5})/2$ did not reveal a significant shift in the cytokine balance ($\text{AID} = 1$, $\text{stress} + \text{AID} = 1$, $\text{stress} + \text{AID} + \text{anti-NGF} = 0.9$). By contrast, the mainly mast cell-derived pro-inflammatory cytokine TNF- α was markedly and significantly reduced by anti-NGF treatment (Figure 4).

Eosinophils are reduced by anti-NGF

Finally, we analyzed skin infiltration by eosinophils, a crucial hallmark of cutaneous allergic inflammation (Pavlovic *et al.*, 2008). Immunohistochemistry revealed that skin-infiltrating eosinophils stained weakly positive for p75NTR (not shown) and strongly positive for TNF receptor type 2 (TNFR2) in all experimental groups, with the strongest expression in AID mice (Figure 5a). Again, treatment with NGF-neutralizing antibodies significantly reduced cutaneous eosinophil infiltration in the dermis and subcutis of stressed AID mice to levels measured in AID without stress exposure (Figure 5b).

DISCUSSION

Using our mouse model of stress and AID with analogy to human extrinsic atopic or allergic dermatitis, we obtained conclusive evidence of a role for NGF in stress-induced worsening of allergic inflammation. Specifically, we found that NGF is involved in the enhancement of epidermal hyperplasia, TNF- α expression, and eosinophilia under stress but does not contribute to epidermal barrier dysfunction, mast cell degranulation, or alteration of the Th1/Th2 cytokine balance. It accounts for a stress-induced worsening of disease

parameters by ~30–50%. Furthermore, our data document a therapeutic effect of anti-NGF treatment on stress-induced exacerbation of peripheral inflammatory events.

Supporting a role for NGF in allergy and stress, increased NGF levels were reported in atopic individuals and NC/Nga mice, a mutant developing spontaneous AID-like lesions (Toyoda *et al.*, 2002; Takano *et al.*, 2007), as well as in stressed healthy individuals (Aloe *et al.*, 1994; Alleva *et al.*, 1996), whereas relaxing activities led to a reduction in both disease activity and NGF levels (Kimata, 2004; Takano *et al.*, 2005; Tominaga *et al.*, 2009). In the NC/Nga mouse, topical treatment with TrkA antagonists downregulated epidermal TrkA as well as NGF, and anti-NGF treatment reduced epidermal thickening (Takano *et al.*, 2005). In this study, we observed an additive effect of AID and stress on NGF expression, which can be specifically downregulated by anti-NGF treatment together with stress enhancement of epidermal hyperplasia. This observation indicates the existence of a positive-feedback loop in stress-induced epidermal NGF signaling, which can be specifically blocked by anti-NGF treatment (Spillantini *et al.*, 1989).

As players in the innate immune response, mast cells have long been shown to synthesize, store, and release NGF, particularly in the context of inflammation (Leon *et al.*, 1994; Groneberg *et al.*, 2005). In our experiments, mast cells express NGF and p75NTR and downregulate NGF expression after anti-NGF treatment. Intriguingly, this corresponds to cutaneous TNF- α levels but not mast cell degranulation (Black, 2002; Steinhoff *et al.*, 2003; Pavlovic *et al.*, 2008). This observation is in good agreement with a recent report indicating that physiological concentrations of NGF do not directly induce mast cell degranulation (Kulka *et al.*, 2008).

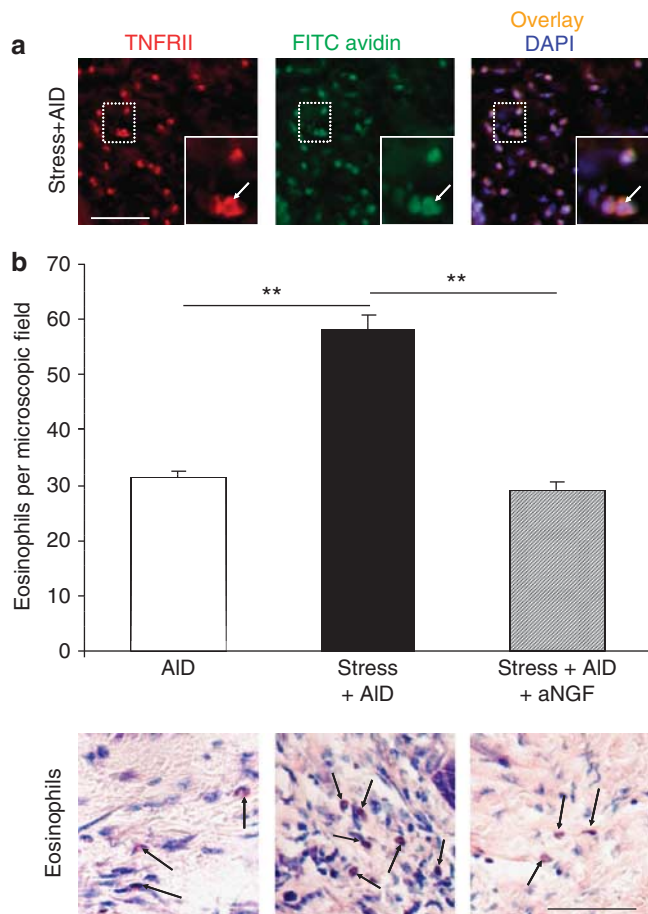


Figure 5. Anti-nerve growth factor (NGF) effectively reduces the number of eosinophil granulocytes in stressed AID skin. (a) Eosinophils were strongly immunoreactive for tumor necrosis factor receptor type 2 (TNFR1I; arrow in insert, indicated by dotted lines) in all groups. A representative photomicrograph of the dermis of a stressed AID mouse is shown. (b) Stress increased the number of eosinophil granulocytes infiltrating the skin of mice with AID as expected and reported earlier (Pavlovic *et al.*, 2008). Treatment with anti-NGF reduced eosinophil granulocyte levels to the AID level without stress exposure. The photomicrographs are selected representative views of eosinophil granulocyte infiltration at the dermis subcutis border as detected by Giemsa staining (arrows). ** $P < 0.01$. aNGF, anti-NGF neutralizing antibody. Bar = 50 μ m.

Previously reported biological effects of NGF on mast cell degranulation in the skin may thus reflect NGF-induced relative peptidergic hyperinnervation (Davies *et al.*, 1987; Kinkelin *et al.*, 2000; Peters *et al.*, 2004).

Finally, several *in vitro* reports indicate that NGF may promote a bias toward the production of Th2 cytokines (Braun *et al.*, 1998; Tokuoka *et al.*, 2001), which are prominently involved in atopy as well as in chronic stress responses (Pavlovic *et al.*, 2008). Intriguingly, our data do not support this hypothesis because we did not see any substantial alteration of Th1 or Th2 cytokines after treatment with anti-NGF. This unexpected finding underscores the necessity of analyzing biological function in the context of living organisms, so that all potentially counterregulating pathways that determine clinical outcome are present.

With respect to the skin infiltration by eosinophils, which express p75NTR and TrkA receptor (Nassenstein *et al.*, 2003; Raap *et al.*, 2005; Hahn *et al.*, 2006), we observed p75NTR receptor expression on eosinophils and decreased NGF levels corresponding to the significant downregulation of cutaneous eosinophil granulocyte infiltration by anti-NGF treatment of stressed AID mice. These results provide first evidence for a functional role for NGF in eosinophil granulocyte infiltration of the allergically inflamed skin in response to stress.

Finally, in healthy skin, TNF- α is exclusively located on mast cells (Ackermann and Harvima, 1998), and the number of TNF- α + mast cells is increased in lesional human AID skin (Ackermann and Harvima, 1998). TNF- α participates in eosinophilic infiltration of the skin (Thomas, 2001) and may further enhance cutaneous NGF expression (Hattori *et al.*, 1993). In our experiments, TNFR1I was detected on eosinophils, and after anti-NGF treatment, TNF- α was markedly and significantly reduced together with NGF in stressed AID skin, indicating a close association between both their expression patterns and their capacity to promote eosinophil survival.

These findings indicate that targeting NGF signaling can be an important therapeutic tool to prevent lichenification and chronic allergic inflammation in the skin, especially in response to stress. Figure 6 illustrates selected NGF effects directly exerted on epidermal hyperplasia and eosinophil granulocyte infiltration, mast cell NGF and TNF- α signaling, and NGF control of neuronal plasticity, leaving mast cell degranulation and cytokine balance out of the cycle. This insight into NGF function in allergic inflammation and the cutaneous stress response explains why anti-NGF treatment does not recover all atopic symptoms (Takano *et al.*, 2005). Thus, future treatment strategies aiming at neurogenic inflammation should consider a combination of pharmaceuticals that affect different aspects of the neuroimmune cross-talk, such as NGF and SP.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice (Charles River; Sulzfeld, Germany) were randomly distributed into five experimental groups (control, stress, AID, stress + AID, anti-NGF + stress + AID) and left for 1 week to adjust to their new environment at the animal facility, University Medicine Charité, Virchow Campus, Berlin, Germany (Paus *et al.*, 1997, 1998; Peters *et al.*, 2005; Pavlovic *et al.*, 2008). Animal care and experimental procedures were approved by the institutional review board and conformed to the requirements of the state authority for animal research (LaGetSi, Berlin, Germany).

The AID-Stress Model

As described previously (Sawada *et al.*, 1997; Cho *et al.*, 2001; Pavlovic *et al.*, 2008), mice were sensitized to ovalbumin (20 μ g, grade VI, Sigma-Aldrich Chemie, Schnelldorf, Germany) on days 7 and 28 of the experimental protocol. AID was induced by intradermal injection of ovalbumin (50 μ g, grade V, Sigma-Aldrich Chemie) on day 35. In the stress protocol, mice were exposed to noise (300 Hz tone emitted at irregular intervals four times per minute; rodent repellent device, Conrad Electronics, Hirschbach, Germany) for a single 24-hour period (Peters *et al.*, 2004, 2005;

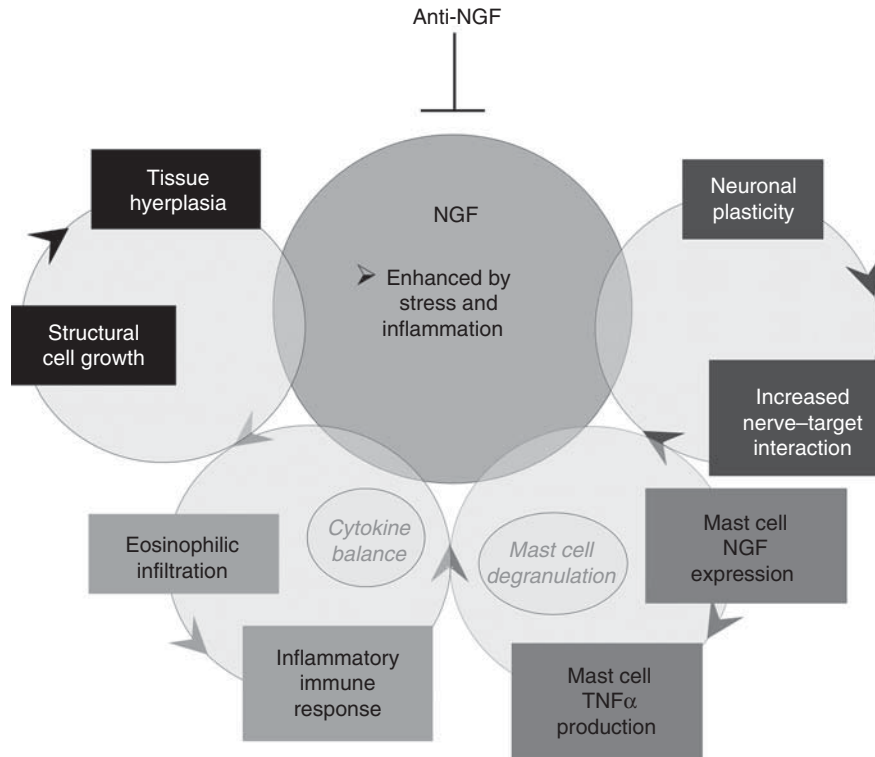


Figure 6. Hypothetical scenario of nerve growth factor (NGF) signaling in stressed allergic skin. NGF shown in the center of its biological functions in skin, all of which are affected in stressed skin and in atopic dermatitis-like allergic dermatitis (AID). By blocking NGF signaling, anti-NGF can effectively block all affected NGF interactions in stressed AID skin.

Pavlovic *et al.*, 2008). Stress exposure immediately preceded cutaneous ovalbumin challenge. Mice were killed 48 hours after challenge or after termination of stress exposure.

Application of anti-NGF

Two hundred microliters of polyclonal rabbit anti-mouse NGF antibody (Sigma-Aldrich, Munich, Germany) was injected intraperitoneally twice in stressed AID mice, at a dilution of 1:1,500 in phosphate-buffered saline. The injections were administered just before and immediately after stress exposure. The second injection preceded AID induction by 30 minutes. According to the manufacturer, a 1:4,000 dilution of this antibody blocks bioactivity of 5 ng ml⁻¹ NGF. The dosage of anti-NGF at 1:1,500 was based on previously published data (Peters *et al.*, 2004). We employed this NGF-blocking approach rather than NGF-receptor blockade because the complex interplay between NGF and its receptors TrkA and p75NTR requires blockade of the ligand in an *in vivo* setting to target biologically relevant interaction. Likewise, a transgenic approach to targeting the role of NGF can focus only on individual receptors owing to the lethality of the NGF knockout mutation (Conover and Yancopoulos, 1997).

Tissue collection

For immunohistochemistry, mice were perfusion-fixed using a mixture of paraformaldehyde and picric acid (Botchkarev *et al.*, 1997; Peters *et al.*, 2002). For purposes of microarray and quantitative real-time PCR, mice were killed by cervical dislocation and the skin was immediately snap-frozen.

RNA isolation, DNase treatment, reverse transcription, and real-time PCR

About 100 mg of skin tissue from each of three mice per group was treated with 1 ml Trizol (Invitrogen, Karlsruhe, Germany). The total RNA was extracted and treated with 1 U DNase I per microgram of RNA (Invitrogen), followed by inactivation with EDTA (Invitrogen). There was no detection of genomic DNA via PCR. First-strand cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was employed to obtain quantitative data on differences in NGF, pre-protachikinin 1 (precursor mRNA encoding SP), IL-4, IL-5, TNF- α , and IFN- γ mRNA expression between experimental groups. This assay exploits the 5'-nuclease activity of AmpliTaq Platin (Invitrogen) DNA polymerase to cleave a fluorogenic probe designed for the above transcripts (TipMolBiol, Berlin, Germany). The quantitative real-time PCR reactions were normalized to hypoxanthine phosphoribosyl transferase (HPRT) by calculating the difference between the CT for HPRT and the CT for the respective transcript as $\Delta CT = CT_{HPRT} - CT_{transcript}$. The amount of mRNA was calculated as $1/2^{\Delta CT}$ and expressed as the difference from control or the CT detected in AID, set as 1. Each sample was analyzed in duplicate.

Microarray analysis

A total of three mice were pooled for the untreated control and the stress groups. In all, 90 ng of total RNA of each skin sample was applied for two rounds of RNA amplification with RiboAmp RNA Amplification Kit (Arcturus, Sunnyvale, CA) and antisenseRNA was biotin labeled with a BioArray HighYield RNA Transcript Labeling Kit

(ENZO Diagnostics, Farmingdale, NY). A total of 10 µg of antisense RNA was hybridized to Gene Chip Mouse Genome 430A 2.0 (Affymetrix; inquires for ~14,000 murine genes) arrays using standard Affymetrix protocols. Raw data were log2 transformed and quintile normalized, and expression levels were calculated using Affymetrix Microarray Suite (MAS) 5.0 and Operating Software GCOS 1.4 (Affymetrix, Santa Clara, CA). Genes were annotated employing the NetAffX analysis center (<http://www.affymetrix.com/analysis/index.affx>). Pathway and gene ontology analysis was done using Database for Annotation, Visualization and Integrated Discovery 2007 (Dennis *et al.*, 2003; Hosack *et al.*, 2003).

ELISA

Serum NGF was measured with a ChemiKine murine NGF ELISA kit (Chemicon, Temecula, CA; >10 pg ml⁻¹) according to the manufacturer's instructions. Briefly, serum was collected from five or six animals per group. A total of 100 µl standard or serum per well was incubated overnight at 4 °C and labeled by peroxidase-anti-NGF-antibodies for 2 hours at room temperature, followed by 3,3',5,5'-tetramethylbenzidine substrate addition. The reaction was stopped after 15 minutes by adding HCl, and the plate was immediately read with the help of a standard ELISA reader (BMG Labtech, Jena, Germany) at 450 nm. Data were evaluated using the Optima Program with the Optima Microplate Reader (BMG Labtech).

Routine histochemistry

Epidermal thickness and the presence of eosinophils and mast cell degranulation in back skin from five or six mice per group were determined on 10-µm thick sections processed for Giemsa staining (Merck, Darmstadt, Germany) in a standard method according to previously published protocols (Botchkarev *et al.*, 1997; Peters *et al.*, 2005; Pavlovic *et al.*, 2008). Cells were classified as eosinophils when they showed the classic eosinophil granulocyte cytoplasm and ring-like nuclei. Mast cells were classified as "degranulated" when eight or more granules could be found outside the cell membrane.

Immunofluorescence

NGF, TrkA, p75NTR, TGF-β2, and TNFR1 immunoreactivity was determined in 10-µm thick sections from five or six mice per group. Primary antibody binding (NGF antiserum, polyclonal, 1:100; TrkA antiserum, polyclonal, 1:100; TGF-β2 antiserum, polyclonal, 1:50, all acquired from Santa Cruz, Heidelberg, Germany; p75NTR antiserum, polyclonal, 1:100, acquired from Chemicon; TNFR1 antiserum, polyclonal, 1:50, acquired from Promo Kine, Heidelberg, Germany) was detected either by a rhodamine-labeled secondary antibody (Dianova, Hamburg, Germany, dilution 1:200) or by tyramide amplification (Renaissance TSA-FM-Direct (Red); NENTM Life Science Products, Boston, MA; Pavlovic *et al.*, 2008). NGF was also counterstained with tryptase to verify the immunoreactivity of mast cells (MCP6 antiserum, polyclonal, 1:100, R&D Systems, Wiesbaden, Germany). Nuclei were counterstained with 4',6-diamidino-2-phenylindol and mast cells with fluorescein-labeled streptavidin.

Qualitative histomorphometry

Epidermal thickness and immunoreactivity to the above listed antibodies and number of eosinophils, degranulated mast cells, and immunoreactive mast cells, as well as immunoreactivity of eosinophils, were determined in the center of the AID lesion

(Pavlovic *et al.*, 2008). For each experimental group, at least 10 microscopic fields per mouse of five or six mice per group were studied at a magnification of ×400 (i.e., more than 50 microscopic fields were studied for each parameter and experimental group). Representative staining patterns were photodocumented using a digital imaging device (Visitron Systems, Puchheim, Germany).

TEWL

To prepare the test area, the flanks of the five mice per group were shaved, avoiding any damage to the skin. Two days later, TEWL was evaluated in the shaved areas using a C&K Tewameter TC350 (Courage + Khazaka, Cologne, Germany). The mean TEWL values were internally recalculated every 2 seconds by the Tewameter, and values were recorded after an overall measurement time of 1 minute. Measurements were made under standardized conditions (temperature 22–24 °C, constant air humidity (40–50%), minimal air movement, no direct exposure to light).

Statistical analysis

Means were calculated and significant differences were determined with the Mann–Whitney *U*-test for unpaired samples. Significance was assumed if **P*<0.05 or ***P*<0.01.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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