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Further Exploring the Brain–Skin Connection: Stress Worsens Dermatitis *via* Substance P-dependent Neurogenic Inflammation in Mice

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A neurogenic component in atopy and allergy is evident and potentially of great pathogenic relevance. Stress was recently shown to activate elements of this component and is vividly discussed as a cause of exacerbation. However, to date, scientific proof of stress-induced neuronal plasticity and neuro-immune interaction in atopy or allergy remains lacking. Here we show early evidence that exposure to sound stress and atopic dermatitis-like allergic dermatitis (AD) equipotently raise the number of cutaneous nerve fibers containing the prototypic stress neuropeptide substance P (SP) in mice. Stress increases AD readout parameters by at least 30% (eosinophil infiltration, vascular cell adhesion molecule-positive blood vessels, epidermal thickness). This dramatic pathologic exacerbation is associated with increased neurogenic inflammation (degranulated mast cells; interstitial neuropeptidergic dense core granules, mast cell apoptosis, endothelial gaping). Key features of AD exacerbation could not be induced in mice lacking the neurokinin-1 SP receptor (NK_1). Interestingly, stress had no significant additional effect on CD4+ cell number, but shifted the cytokine profile toward TH2 in skin. Thus, we conclude that stress primarily exacerbates AD via SP-dependent cutaneous neurogenic inflammation and subsequent local cytokine shifting and should be considered as a therapeutic target, while it offers a convincing pathogenic explanation to affected patients and their frustrated physicians alike.

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INTRODUCTION

Strong psychoemotional stressors have long been expected to exacerbate or even trigger atopic or allergic diseases such as atopic dermatitis (Buske-Kirschbaum and Hellhammer, 2003; Joachim *et al.*, 2004; Peters *et al.*, 2005). The interested reader will find reference to this effect in almost every textbook on dermatology, psychosomatics or psychoneuroimmunology and intriguing hypothesizes based on our knowledge of stress effects on neurogenic inflammation and cytokine production in general have been formulated (c.f. Pallanti *et al.*, 2005; Wright *et al.*, 2005). However, few comprehensive experimental investigations have resulted from this common assumption.

All respective studies on psychopathology in atopic dermatitis so far have been performed in humans. Many authors found associations between personality traits and psychic disturbances such as stress perception, anxiety, or depression and atopic dermatitis severity or even onset (Brown, 1972; Garrie *et al.*, 1974; Gil *et al.*, 1987; Gieler *et al.*, 1990; King and Wilson, 1991; Lammintausta *et al.*, 1991; Scheich *et al.*, 1993; Gupta and Gupta, 1999; Kodama *et al.*, 1999; Kilpelainen *et al.*, 2002; Hashizume *et al.*, 2005). However, an "AD personality" could not be identified, and most of the psychopathological features of atopic dermatitis-like allergic dermatitis (AD) patients were also present in other chronically diseased patients. Also, these studies remained on the level of associating disease severity (SCORAD, itch, IgE, eosinophilia, TH1/TH2 balance) or onset (retrospectively) with psychometric data (life events, anxiety, depression).

Only a small number of investigations focused on immediate stress effects in allergic dermatitis, among them the path-breaking studies by Buske-Kirschbaum and co-workers (Faulstich *et al.*, 1985; Munzel and Schandry, 1990; Arnetz *et al.*, 1991; Buske-Kirschbaum *et al.*, 1998, 2001, 2002a, b; Schmid-Ott *et al.*, 2001), which employed a mainly mental experimental stressor. Such well-conducted studies

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Abbreviations: AD, atopic dermatitis-like allergic dermatitis; NK_1, neurokinin-1 SP receptor; SP, substance P; TH, T-helper cell; TNF-α, tumor necrosis factor α; VCAM, vascular cell adhesion molecule

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exploring stress effects in atopic or allergic disease mostly deal with an abnormal systemic activation of the hypothalamic–pituitary–adrenocortical axis or the sympathetic nervous system (Black, 2002; Buske-Kirschbaum and Hellhammer, 2003). Activation of these systems initiates a systemic response that ultimately leads to a pathogenically relevant shift in the cytokine balance in various inflammatory disease models (Niess *et al*., 2002; Buske-Kirschbaum and Hellhammer, 2003; Dhabhar, 2003; Frieri, 2003). From the psychoneuroimmunological perspective, anxiety and depression can be interpreted as stressors, as was done by Hashizume, but so far only Buske-Kirschbaum and co-workers employed a defined, inducible stress response in correlation with systemic alterations of neuroendocrine and immunological parameters in atopic disease.

Ectodermal organs such as the lung, gut, and the skin however are characterised by their dense innervation and a close neuro-immune crosstalk (Bauer and Razin, 2000; Darsow and Ring, 2001; Black, 2002; Groneberg *et al*., 2004; Renz *et al*., 2004; Wood, 2004), which enables them to respond to systemic stressors by local neuronal plasticity and neuro-immune interaction (Peters *et al*., 2005). Accordingly, more and more reports support a close neuro-immune connection in ectodermal organs such as the skin and the existence of a brain–skin axis has therefore been postulated (Scholzen *et al*., 1998; Slominski and Wortsman, 2000; Arck *et al*., 2001, 2006; Peters *et al*., 2006; Hendrix and Peters, 2007).

We are now aware, that stress exposure can lead to activation at the nerve fiber–mast cell interface, with subsequent neurogenic inflammation at least in mice (Singh *et al*., 1999; Arck *et al*., 2001; Black, 2002; Peters *et al*., 2004, 2005). At the same time a neurogenic component has been identified in the pathogenesis of peripheral inflammatory diseases such as allergic dermatitis (Pincelli *et al*., 1990; Ostlere *et al*., 1995; Huang *et al*., 2003; Mihara *et al*., 2004; Ohmura *et al*., 2004a). These observations led to the frequently drawn conclusion, that stress-induced local neurogenic inflammation plays a role in atopic or allergic disease pathogenesis (Theoharides and Cochrane, 2004). Surprisingly, this conclusion remained a hypothesis to be tested to date.

This hypothesis is especially intriguing in the context of atopic dermatitis, since one of the central cutaneous stress mediators, the neuropeptide substance P (SP), is not only a key player in neurogenic inflammation and itch (Steinhoff *et al*., 2003), but is also a central feature of atopic dermatitis (Mihara *et al*., 2004). SP also affects cellular infiltration by eosinophils (Quinlan *et al*., 1999; Foster and Cunningham, 2003) and cytokine production by T-lymphocytes (Payan *et al*., 1984; Levite, 1998; Kang *et al*., 2000), which are critical features of the exacerbation and chronic course of atopic dermatitis (AD).

To our knowledge, neither a functional *in vivo* animal model nor investigation of local, intracutaneous pathways of stress effects in AD have been introduced to date. Against this background, atopic dermatitis appears an ideal disease model to investigate stress-induced alterations in the interaction between the peptidergic cutaneous innervation and the skin immune system and their effects on the severity of atopic or allergic disease and T-helper cell (TH) cytokine balance. We therefore combined two models long established in dermatitis research (Sawada *et al*., 1997; Cho *et al*., 2001) and stress response research (Arck *et al*., 1995b, 2003), to investigate the effects of noise stress exposure on experimental AD to resolve the following questions:

- Do stress exposure and AD similarly affect neuronal plasticity?
- Does stress exposure change the quality and degree of AD?
- Does stress affect AD via neurogenic inflammation?
- Are the stress-associated effects on AD dependent on SP signalling?
- Does stress exposure alter the cutaneous cytokine balance in AD?

With the data presented here we aim to define the neuro-immune interaction pathways by which stress is linked to the exacerbation of cutaneous inflammation. This will allow us to provide a sound pathogenic framework to explain the contribution of stress to the exacerbation of cutaneous inflammation to affected patients and their frustrated physicians. Based on these findings we lay out a blueprint for the future development of new therapeutic strategies in the management of stress-sensitive recurrent inflammation in the skin (Wright *et al*., 2005).

RESULTS

All C57BL/6 mice sensitised to ovalbumin to establish AD showed the characteristic reddening and scaling of the injected skin site 48 hours after injection as well as increased total and ovalbumin-specific IgE levels (not shown). Upon histological investigation by routine hematoxylin and eosin staining, the dermis and subcutis were thickened and contained an expected cellular infiltrate rich in eosinophils and mononuclear cells (not shown). This infiltrate concentrated around dermal and subcutaneous blood vessels, which appeared dilated and enlarged (not shown). These findings demonstrate effective induction of AD in our model.

SP+ nerve fibers are equally increased in stressed as well as AD skin

By immunofluorescence, a few single SP+ nerve fibers in relative distance to dermal mast cells were present in the interfollicular dermis of untreated control mice as described before (Peters *et al*., 2001) (Figure 1a). After challenge with ovalbumin or stress exposure SP+ nerve fibers became abundant (Figure 1b and c). These nerve fibers also showed close contacts to mast cells, indicating facilitation of neurogenic inflammation.

Stress exposure worsens AD parameters

As compared with control animals and stressed animals, eosinophils (detected by routine Giemsa histopathology) were prominent in AD lesions, and these increased significantly

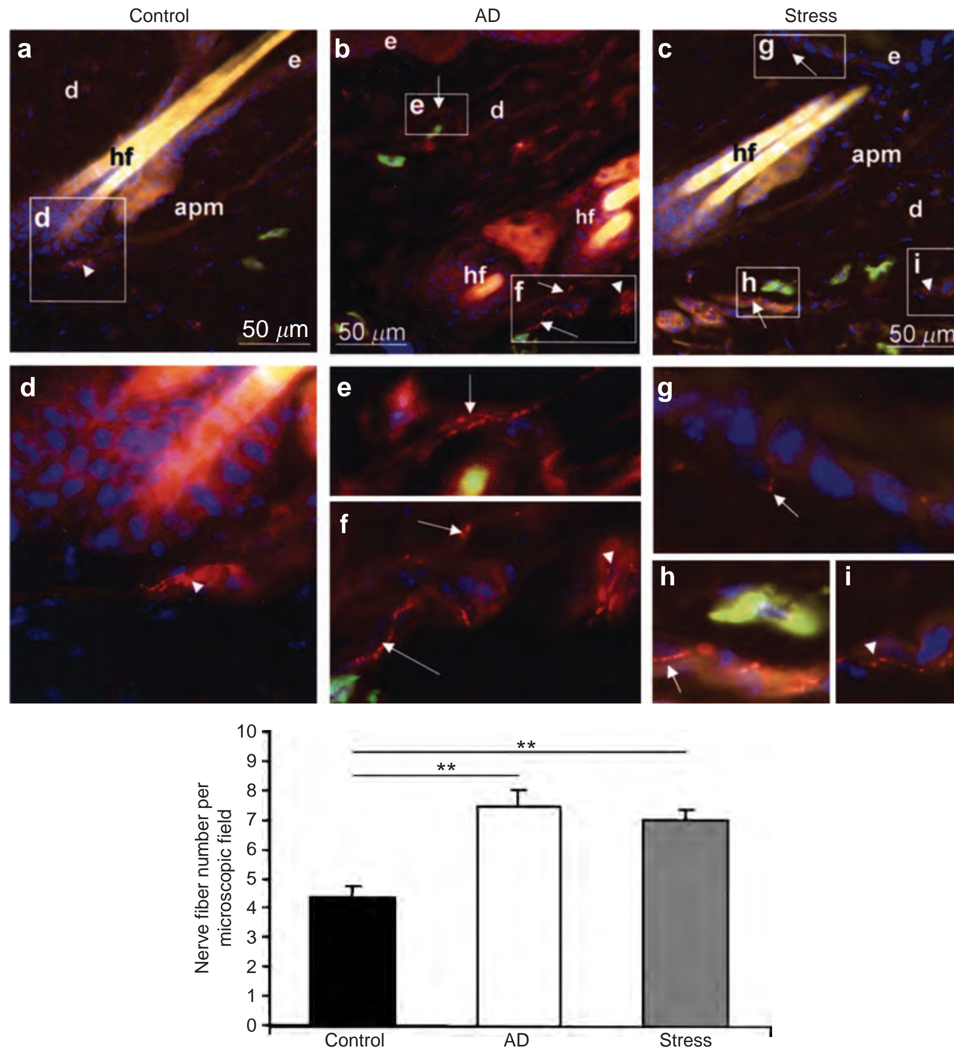

Figure 1. Immunohistochemistry reveals increased nerve fiber density in AD and after stress. Bars represent the mean number of nerve fiber profiles in 10 consecutive microscopic fields per mouse pooled from five different mice per group. *P*-values were determined by Mann–Whitney-*U*; $<0.01=$**. Abbreviations: apm, arrector pili muscle; d, dermis; e, epidermis; hf, hair follicle. White-bordered boxes indicate the location of higher-magnification excerpts labelled with same letter and i as indicated in the boxes. SP+ nerve fibers : red label, mast cells; green label, cell nuclei; blue label, autofluorescence; orange label. (**a, d**) A small SP+ nerve fiber bundle (arrowhead) is visible close to a control animal's hair follicle in telogen. Note the mast cells in the far distance. (**b, e,** and **f**) A mouse sensitised to and challenged with ovalbumin (AD) shows numerous single SP+ nerve fibers (arrows) and a SP+ nerve fiber bundle close by a telogen hair follicle. Note the close proximity to mast cells. Please note, that as compared to (**a**) and (**c**), the hair follicle is cut of at the right, the hair shaft is therefore only partially visible despite identical section orientation and dermal area shown. (**c**, **g**, **h**, and **i**) a stressed mouse shows numerous SP+ nerve fibers and mast cells close by similar to AD.

in number when animals were additionally exposed to noise stress (Figure 2).

Vascular cell adhesion molecule (VCAM)+ blood vessels were rare in control, stressed, and AD mice, and where present, were mostly located in the upper dermis adjacent to the epidermis (Figure 2). Upon stress exposure, numerous VCAM+ blood vessels could be detected in mice with AD throughout the dermis and subcutis (Figure 2). These blood vessels were small to medium size and appeared dilated (Figure 2).

The epidermis overlaying dermal and subcutaneous dermatitis in mice with AD was thickened (Figure 2), with more than two cell layers, as compared with 1–2 cell layers in control animals without AD with or in stressed mice. Thickness of the epidermis was significantly increased if the mice had AD and were additionally exposed to stress, and these mice also displayed pronounced spongiosis and hyperkeratosis (Figure 2).

Stress exposure enhances neurogenic inflammation in AD skin

Mast cells were present in the dermis and subcutis of all mice. Frequently they were located adjacent to the epidermis, close to blood vessels or nerve fiber bundles, and at the dermis–subcutis border. The cells were so densely packed with granules that individual granules could not be distinguished in control mice (not shown) and extracellular

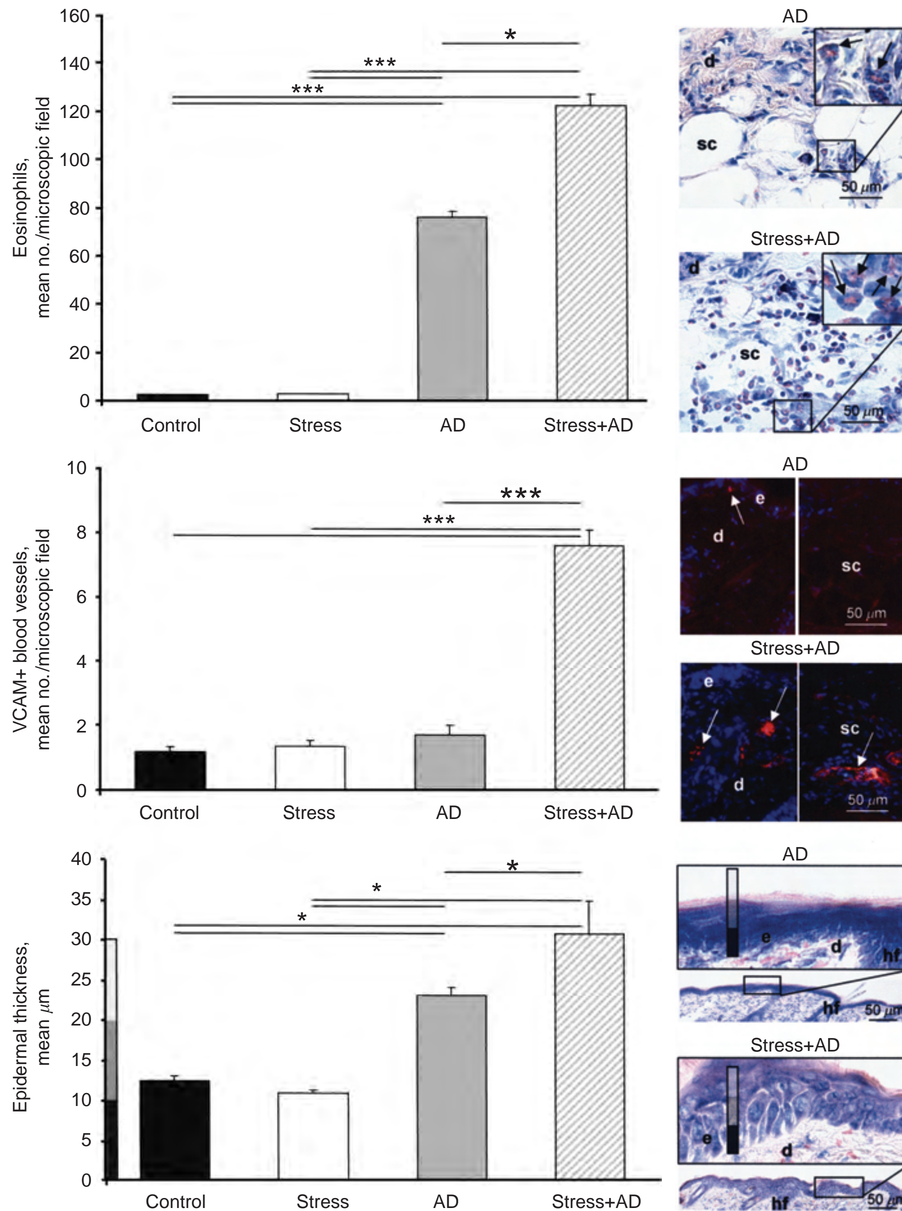

Figure 2. Histomorphometric analysis of allergic dermatitis lesions. Bars represent the mean number of eosinophils or VCAM+ blood vessel profiles or the mean epidermal thickness measured in 10 consecutive microscopic fields per mouse at ×400 magnification. Data were pooled from five different mice per group. *P*-values were determined by Mann–Whitney-*U*; <0.05 = *, <0.001 = ***. Abbreviations: d, dermis; e, epidermis; hf, hair follicle; sc, subcutis. Black-bordered boxes indicate area from which higher-magnification micrographs have been taken. Differences of special interest (between AD and stress+AD groups) are indicated by larger asterisks. Eosinophils: in a hematoxylin and eosin staining, eosinophilic granulocytes (arrows) are highly present in AD skin and their number further and significantly increases upon stress exposure. Eosinophil infiltration was increased 61% over AD in the stress+AD group. VCAM+blood vessels: immunofluorescence labelled some VCAM+ blood vessel profiles (red, arrows) in AD skin and the number dramatically and significantly increases in AD animals exposed to stress. VCAM+ blood vessel count is increased 339% over AD in the stress+AD group. Cell nuclei are counterstained with 4′,6 diamidino-2-phenylindole (blue). Epidermal thickness: in a hematoxylin and eosin staining, epidermal thickness is increased in AD skin showing enlarged cells and more than the normally present 1–2 cell layers. Upon additional stress exposure, epidermal thickness increases significantly. Morphologically, spongiosis, as evidenced by clear spaces between epidermal cells, is present. Epidermal thickness is increased 33% over AD in the stress+AD group. Gray, transparent striped longitudinal bars indicate thickness in micrometers.

granules were only occasionally visible (not shown). In stressed mice and in mice with AD, mast cells appeared less packed and individual granules could be detected within the cytoplasm as well as outside the cell membrane, indicating more frequently occurring degranulation than in control mice (Figure 3). These changes in mast cell morphology were strongly increased in stressed AD mice (Figure 3). Accordingly, the proportion of degranulated mast cells per total number of mast cells was significantly increased compared with AD mice (Figure 3).

Associated with mast cell activation, we observed the deterioration of dermal nerve fibers in stressed AD mice by

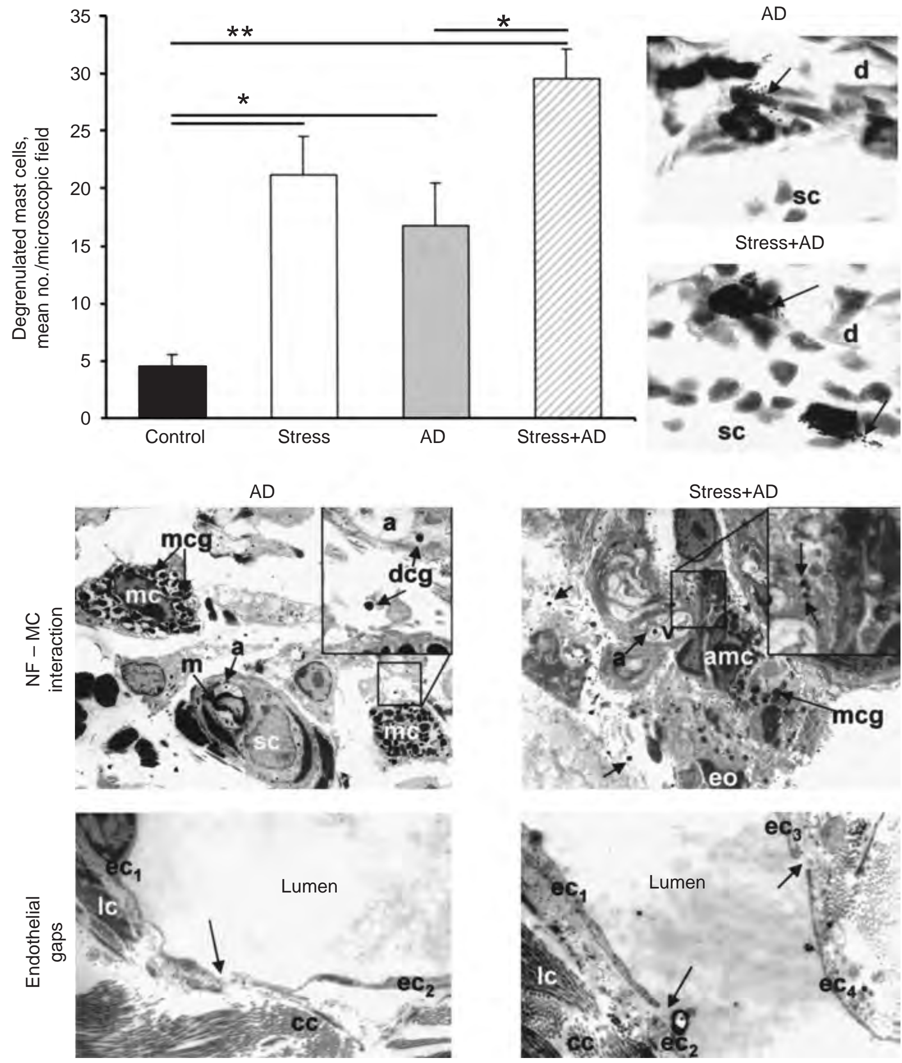

Figure 3. Histochemistry and electron microscopy reveal neurogenic inflammation in AD skin upon stress exposure. Each bar represents the mean percentage of degranulated mast cells as percentage of the total number of mast cells counted in the skin in 10 consecutive microscopic fields/mouse. Data were pooled from five different mice per group. *P*-values were determined by Mann–Whitney-*U*; $^{*}P<0.05$; $^{**}P<0.01$. Abbreviations: a, unmyelinated axon; amc, nucleus of apoptotic mast cell; bve, blood vessel endothelium; cc, circular collagen; dcg, dense core granule; e, epidermis; ec, endothelial cell; eo, nucleus of eosinophil; eog, eosinophil granule; ery, erythrocyte; d, dermis; hf, hair follicle; lc, longitudinal collagen; m, myelinated axon; mc, nucleus of mast cell; mcg, mast cell granule; sc, subcutis; sc, Schwann cell; v, void space. Differences of special interest (between AD and stress + AD groups) are indicated by larger asterisks. Degranulated mast cells: mast cells (lilac cells) are intensely stained by Giemsa staining and show granules outside (arrows) their cytoplasm when degranulated (arrows) in AD skin, and their number significantly increases upon stress exposure. Note the eosinophils close by the degranulated mast cells. NF–MC interaction: several nerve fiber bundles are located close to intact mast cells containing large and round intracellular mast cell granules in the dermis of an AD mouse. Mast cells are densely packed with granules close to unmyelinated nerve fibers and a single dense core granules can be seen in the intercellular space between mast cell and nerve fiber (arrows in insert). In a stressed AD mouse, a deteriorated nerve fiber bundle with disorderly shaped axons and void spaces can be seen containing only few axons with dense core granules. This nerve fiber bundle is located close to a degranulated mast cell with an apoptotic nucleus (amc) and multiple mast cell granules dispersed in the surrounding tissue. Dense core granules in the intercellular space (insert) appear less dense and may shed their content. At the bottom of this image, another cell has degranulated and this cell appears to be an eosinophil as judged by the presence of eosinophil granules. Endothelial gaps: the endothelial cells of a blood vessel form a small gap (arrow) in a blood vessel from an AD mouse. In the skin of a stressed AD mouse, gap formation is prominent in blood vessel endothelia (arrows).

electron microscopy. As a potential sign of massive neuropeptide release, unmyelinated nerve fibers in the dermis lost shape, orderly structure, and granularity (Figure 3). We even observed dense core granules in the intercellular space between nerve axons and mast cells (Figure 3), a rare sight, and these granules were less dense and smaller in stressed AD mice, suggesting release of neuropeptides. In addition, we observed mast cells showing signs of apoptosis and degranulated eosinophils close by the neuropeptide releasing nerve fibers (Figure 3).

Associated with the nerve fiber–mast cell interaction, stressed AD mice also showed prominent separation of endothelial cells (ie, short gaps) lining capillaries (Figure 3). AD mice without stress exposure (Figure 3) and stressed mice

(not shown) showed few gaps, and control mice without any treatment virtually none (not shown).

NK_1−/− mice are resistant to stress-induced worsening of AD
In mice with defective SP receptor neurokinin-1 SP receptor (NK_1)-mediated signalling induced by genetically engineered knockout of the NK_1 gene, AD could effectively be elicited and eosinophil infiltration and epidermal thickening were only slightly but not significantly reduced when compared with littermate wild-type controls (Figure 4). By contrast, mast cell degranulation in AD skin was significantly reduced in NK_1−/− mice compared with age-matched control mice (Figure 4). This observation indicates that neurogenic inflammation plays only a minor role in the initial response to an allergen. However, when NK_1−/− mice were additionally exposed to stress, the stress-induced increase in eosinophil infiltration was significantly reduced as was the increase in epidermal thickening (Figure 4). Correspondingly, the stress induced increase in mast cell degranulation was completely blocked (Figure 4).

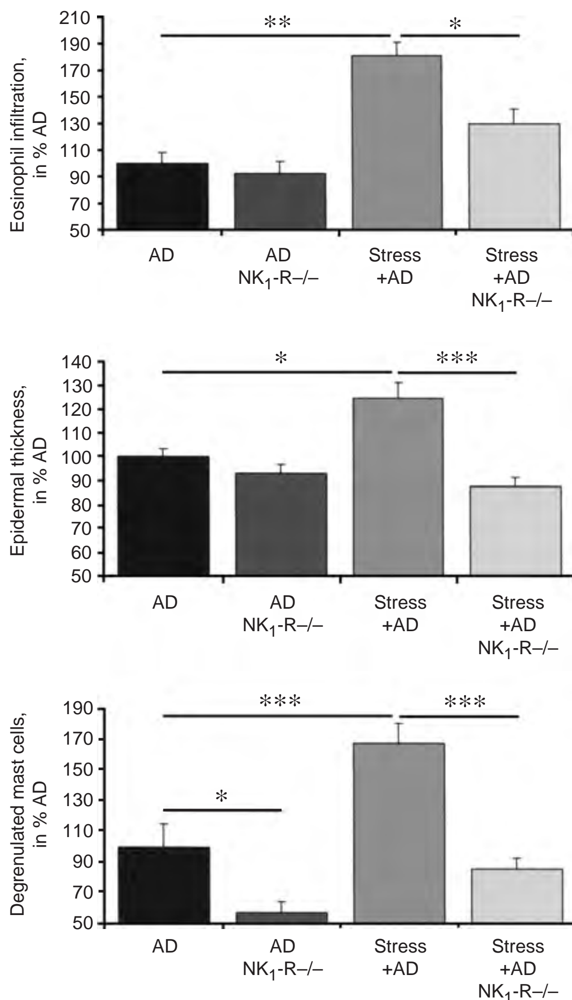

Figure 4. NK1−/− mice are resistant to stress-induced worsening of AD. Each bar represents the mean of eosinophil number, epidermal thickness, or degranulated mast cells expressed percentage of control levels (=100%). Absolute numbers were determined, pooled, and subjected to statistical analysis as described before. *P*-value <0.05=*, <0.01=**, and <0.001=***. Mice genetically engineered to lack the NK_1 receptor for SP do not show worsening of classical AD read-out parameters such as eosinophilic infiltration, epidermal thickening, or mast cell degranulation.

Stress does not affect CD4+ cell numbers, but shifts the cytokine profile toward TH2 in AD mice
Immunohistochemistry revealed a dense infiltrate with CD4+ T cells in mice that were stressed, AD-affected, and in stressed AD-affected mice especially in the interfollicular dermis. However, we could not detect significant differences other than compared with untreated controls (Figure 5). CD4+ cell numbers appeared even less in stressed AD mice compared with controls, but this difference did not reach significance.

Semi-quantitative reverse transcription–PCR performed on skin samples showed significantly increased IL-4, IL-5, and tumor necrosis factor α (TNF-α) levels and mildly increased IFN-γ mRNA levels in AD lesions as compared with controls without AD (Figure 5). This demonstrates the successful induction of an AD-like lesion in our mice, with a prominent expression of cytokines that are traditionally attributed as TH2 (humoral response) but also TH1 (cellular response) cytokines. Stress exposure further increased IL-4 and IL-5 cytokine mRNA expression, while it decreased TNF-α levels 48 hours after termination of stress and induction of AD (Figure 5). These changes resulted in a shift from a more TH1-like cytokine pattern in stressed mice to a more TH2-like cytokine pattern in AD and a prominently TH2 like cytokine pattern in stressed AD mice (Figure 5).

DISCUSSION
With the establishment of a new animal model for the investigation of stress and cutaneous disease, we here demonstrate that the local neuro-immune stress response is a key player in the exacerbation of peripheral inflammatory diseases such as AD. Stress significantly aggravates peripheral inflammation dependent on enhanced local neuro-immune interaction and subsequent neurogenic inflammation in an SP-dependent manner, followed by a cytokine shift. We therefore conclude that exposure to stress has indeed great pathogenic potential in atopic and allergic disease and operates through local neurogenic mechanisms. In detail we found the following answers to the above posed questions:

- Stress exposure is as potent as AD in inducing neuronal plasticity in the skin. This finding is of particular note, as inflammation is currently assumed to be much more potent than the rather mild exposure to a perceived stressor such as noise.
- Stress enhances AD read-out parameters by at least a third above AD levels in a controlled animal experimental setting. The effect can therefore be expected to be of clinical relevance.
- Neurogenic inflammation appears to be a key feature of stress-induced exacerbation of AD. Interference with this

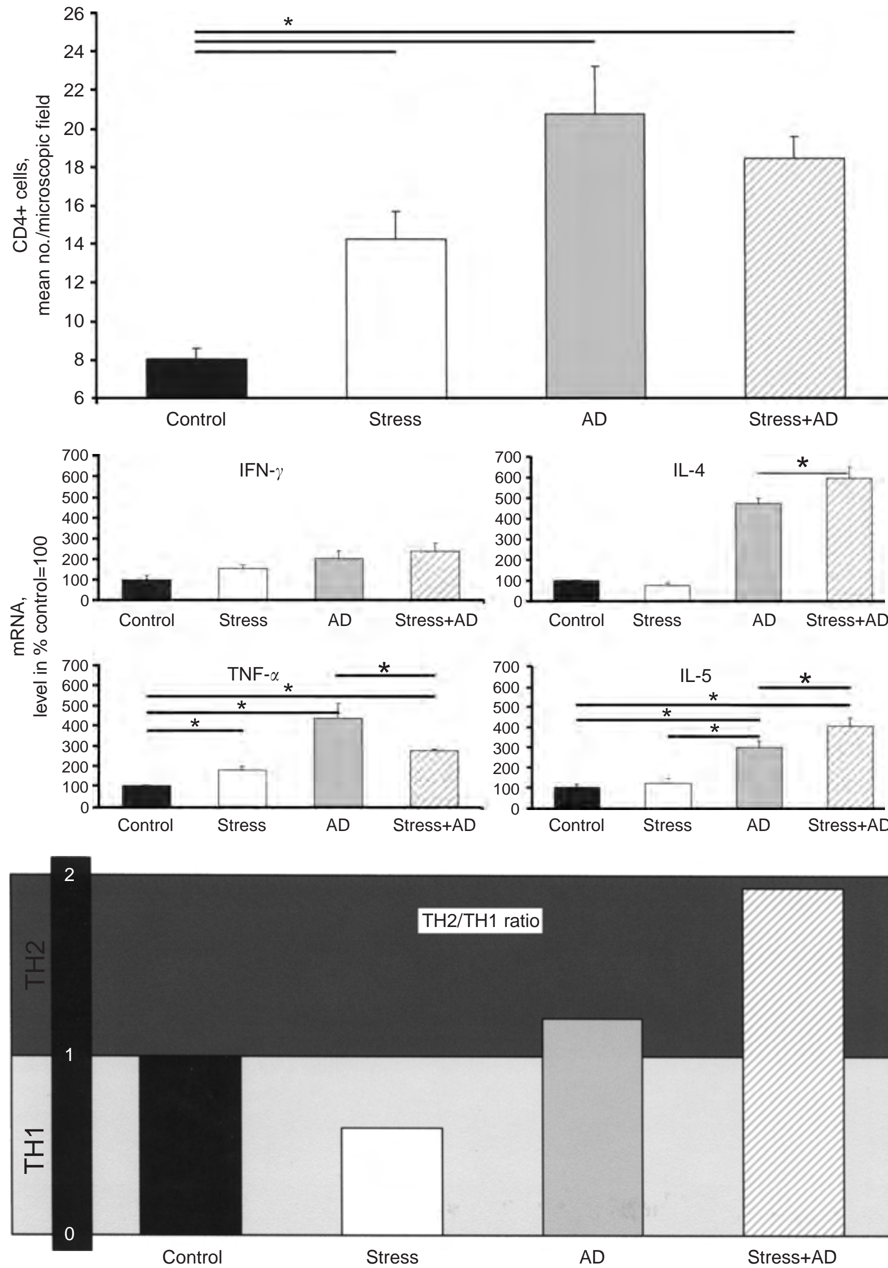

Figure 5. Stress does not increase CD4+ cells in AD skin, but shifts the cytokine balance toward a TH2 pattern. CD4+ cell numbers were determined, pooled, and subjected to statistical analysis as described before. mRNA levels were determined as described in Materials and Methods *P*-values were determined by Mann–Whitney-*U*. *P*-value $<0.05=*$. Differences of special interest (between AD and stress+AD groups) are indicated by larger asterisks. CD4+ cells: stress and AD significantly elevate CD4+ cells numbers above control levels, but stress has no additional effect on AD levels. mRNA: semi-quantitative reverse transcription–PCR reveals no significant changes in IFN-γ levels, decreased levels of TNF-α, and increased IL-4 and IL-5 levels in stressed AD mice in whole skin extracts derived from lesional skin. TH2/TH1 ratio: when IL-4 and IL-5 levels are added and divided by TNF-α and IFN-γ levels, stress is characterised by a ratio characteristic for a TH1 profile, while AD and, even more so, stress+AD skin shows a TH2 profile when compared with control.

feature therefore deserves to be reconsidered as a therapeutic target in stress-induced worsening, for example, by mast cell stabilizers, NK_1 antagonists, interference with neuropeptide processing etc.

- SP receptor NK_1-dependent signalling mediates neurogenic stress effects in skin and can be identified as a key player and potential pharmacological target in stress-induced aggravation of peripheral inflammatory disease.
- Stress further skews the cytokine balance toward a humoral, allergy-relevant pattern. Stress-induced neurogenic inflammation may therefore contribute to establishment as well as chronic course of AD via local alteration of the cytokine milieu.

Histologically, the atopic lesion is characterised by dense infiltrates containing many eosinophils around blood vessels.

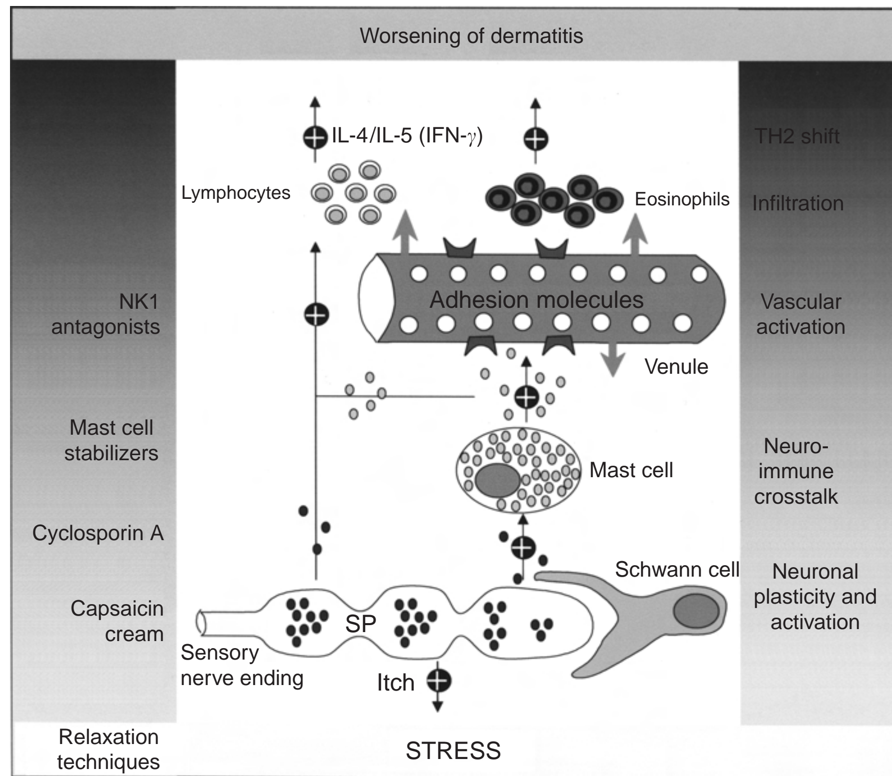

Figure 6. Neuronal plasticity and neurogenic inflammation play a key role in stress-induced worsening of AD. This schematic representation delineates an easy to comprehend and convey scenario of stress-induced worsening of AD. Stress via SP-dependent mechanisms, neuronal plasticity, and neurogenic inflammation elevate the classical features of AD lesions. SP release degranulates mast cells and alters cytokine production of lymphocytes, thereby leading to cellular infiltration and a TH2-dominated cytokine profile, which completes a circulus vitiosus involved in the worsening and chronification of AD lesions. Potential remedies are depicted on the left.

The overlaying epidermis thickens and displays discrete spongiosis and cell adhesion molecule expression on blood vessels increases (Li *et al.*, 2001; Akdis *et al.*, 2002; Ma *et al.*, 2002). All of these features were present in our mouse model and were increased after exposure to stress. This increase was dependent on the NK_1 receptor for SP, demonstrating the clinical relevance of neurogenic inflammation and its key player SP in stress-induced exacerbation of atopic or allergic disease.

Previously we have shown that stress causes an increase in SP+ nerve fibers and in mast cell degranulation and that these changes are induced by nerve fiber growth. This was evidenced by the presence of the nerve-growth marker Gap-43 on cutaneous nerve fibers, a key feature of true neuronal plasticity (Peters *et al.*, 2005). The reported increase is comparable to the increase in SP+ nerve fiber numbers and mast cell degranulation we observe here in AD skin. We conclude that an increase of SP+ nerve fibers in the skin exposed to a danger signal such as noise or inflammation is a prerequisite for an enhanced and deleterious neuro-immune communication upon double challenge (ie, stress and AD). Thus, stress enables peripheral ectodermal organs equipped with nerve fibers and mast cells, such as the skin, to respond faster to an additional stressor by mounting an efficient neurogenic inflammatory response. Normally, this would enhance host defense capacities against, for example, parasites and microbes. However, when a true target is lacking, this would facilitate ongoing and prolonged inflammation in acute and chronic disease (Steinhoff *et al.*, 2003; Peters *et al.*, 2005) and serve as an adjuvant for exacerbated inflammation. We here confirm this hypothesis by the ultrastructural detection of massive mast cell degranulation close to deteriorating nerve fibers in stressed AD skin during the course of stress-induced disease exacerbation (Figure 6).

After consulting the relevant literature on stress and atopic or allergic dermatitis, we find multiple hints at the close potential regulation of cutaneous inflammation by stress-induced neurogenic inflammation, which focus either on stress or atopy.

Stress affects neurogenic inflammation in skin. Upon stress, mast cell degranulation depends on the presence of SP+ nerve fibers (Singh *et al.*, 1999). Stress and SP induce deleterious local neurogenic inflammatory processes in the context of hair growth regulation in the mouse (Arck *et al.*, 2001, 2003; Peters *et al.*, 2004).

Neurogenic inflammation is altered in atopic or allergic dermatitis. Local expression and sensitivity to neuropeptides have been reported (Giannetti and Girolomoni, 1989; Tobin *et al.*, 1992; Ostlere *et al.*, 1995; Sugiura *et al.*, 1997;

Urashima and Mihara, 1998). Mast cell numbers increase and mast cells maintain close contacts to peripheral nerve fibers (Sugiura *et al*., 1992; Toyoda and Morohashi, 1998; Urashima and Mihara, 1998; Bauer and Razin, 2000). In a mouse model of AD, SP+ nerve fibers were found close to putative mast cells, indicating neurogenic inflammation (Huang *et al*., 2003). And last but not the least, increased SP levels and numbers of SP+ mast cells were also detected in the inflamed skin of NC/Nga mice with AD (Katsuno *et al*., 2003; Ohmura *et al*., 2004b). However, to our knowledge this is the first report, which focuses on stress and AD in an animal model, and on the local, cutaneous circuitry in stress aggravation of the disease, providing convincing evidence of a long-suspected interaction, which is clinically relevant at least in mice.

The stress-associated and NK_1-dependent eosinophil influx in AD skin lesions coincided with an increased expression of VCAM in cutaneous blood vessels. This endothelial adhesion molecule facilitates eosinophil extravasation. Intriguingly, VCAM expression on endothelial cells can be upregulated by SP (Quinlan *et al*., 1999), and by various mast cell products. It is thus highly suggestive that increased SP release from nerve fibers and subsequent neurogenic inflammation is responsible for the observed VCAM expression with subsequent eosinophil infiltration. Therefore, stress-enhanced neurogenic inflammation is not only responsible for the classical hallmarks of itch, reddening and swelling, it may also contribute to atopy and allergy-specific infiltration.

It is interesting to note in this context, that the skin has the full capacity to match the central release of stress mediators. Nerve fibers, immunocytes, endothelial cells, keratinocytes, and fibroblasts not only produce and display the corresponding receptors of SP, but also of the classical stress mediators CRH, cortisol, or noradrenaline (McGillis *et al*., 1990; Ansel *et al*., 1993; Staniek *et al*., 1998; Slominski and Wortsman, 2000; Grando *et al*., 2006; Peters *et al*., 2006). Consequently, investigating the role of SP in the context of a cutaneous stress response is merely the beginning of our understanding of the role of stress in cutaneous inflammation.

Moreover, NK_1 is a key player in the central mediation of anxiety (Czeh *et al*., 2006) and relaxation techniques reducing stress and anxiety also reduce disease severity and itch in atopic patients (Kimata, 2003; Hashizume *et al*., 2005). Thus, a behavioral approach for example employing progressive muscle relaxation or enriched environmental housing, as well as a psychopharmacological approach with NK_1 antagonist or i.e. 5-HT(1A) receptor agonists may reduce anxiety on the central level. Thereby, the neuro-immune circuitry may be altered through attenuated central representation of itch and inflammation (Figure 6).

As mentioned above, SP can increase IL-4 and IFN-γ expression in lymphocytes. IL-4 and IL-5—the central cytokines of the so-called TH2 response—contribute to the humoral component of the acute and chronic atopic dermatitis lesion and to increased eosinophilia (IL-4) and a thickened epidermis (IL-5) (Jujo *et al*., 1992; Spergel *et al*., 1999). In chronic AD the central cytokine of the so-called TH1 response—IFN-γ (Werfel and Kapp, 2002) contributes to the cellular component of the disease, for example, with influx of CD4+ T cells.

In our model, stress-induced neurogenic inflammation is associated with a dramatic increase in IL-4 and IL-5 levels, which shifts the cytokine balance in skin to a more prominent TH2 profile associated with eosinophilia and epidermal thickening, but not with an increase in IFN-γ or CD4+ cells. Neurogenic inflammation therefore appears to prominently enhance the humoral more acute aspect of AD lesions, most of which can be induced by SP and blocked by non-functional NK_1 signalling.

It remains to be determined what other players are responsible for the observed stress-induced increase in IL-5 or decrease in TNF-α in AD. Nerve growth factor appears to be a partner mediator to SP in stress-induced neurogenic inflammation and its deleterious effects in skin (Toyoda *et al*., 2002; Kimata, 2003; Peters *et al*., 2004; Tometten *et al*., 2004), and this molecule not only contributes to neurogenic inflammation, but is also a potent inducer of a TH2 cytokine profile, for example in allergic disease (Braun *et al*., 1998; Tokuoka *et al*., 2001; Botchkarev *et al*., 2006).

Taken together, the combined stress–AD model presented here provides good evidence for stress-induced mechanisms of disease exacerbation and offers a new and promising strategy for the dissection of stress-modulated cutaneous inflammatory disease. Therapeutic options suggested by this prominent role of neurogenic inflammation in stress-induced AD worsening which are currently available, include the following: capsaicin crème (Weisshaar *et al*., 1998; Marsella *et al*., 2002; Takano *et al*., 2004), NK_1 antagonists, (Andoh *et al*., 1998; Brain and Cox, 2006; Chahl, 2006; Rost *et al*., 2006; Hill and Oliver, 2007), mast cell stabilisers (Baluk, 1997), relaxation techniques (Haynes *et al*., 1979; Ehlers *et al*., 1995; Kimata, 2003), and cyclosporin A derivatives (Tanaka *et al*., 2007).

The exploitation of our model of stress and AD offers a new and promising target to test pharmacological manipulation and attempt the neutralization/elimination of stress-mediated effects to benefit patients suffering from highly irritating allergic and atopic inflammatory diseases. Future studies will follow up on these options and explore additional stress mediator effects and their impact on disease development, as well as alternative stressors that allow us to differentiate between acute and chronic stress effects.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice (Charles River, Sulzfeld, Germany) were randomly distributed into experimental groups of 10 mice per community cage and left undisturbed for 1 week to adjust to their new environment. Breeding pairs of C57BL/6 NK_1-R−/− mice were obtained from the University College London, UK, and a respective mouse colony was initiated. All mice were kept in the animal facility at the Charité, Virchow Hospital, University Medicine Berlin, Germany under pathogen free conditions in a barrier facility with a 12 hours light/dark cycle. Six- to 8-week-old mice in the telogen stage of the hair cycle were used for subsequent experiments, since

cutaneous innervation and immune response depend on hair cycle stage (Paus *et al.*, 1997, 1998; Peters *et al.*, 2005). Animal care and experimental procedures were followed according to institutional guidelines and conformed to requirements of state authority for animal research conduct (LaGetSi, Berlin, Germany).

The AD–stress model

Mice were sensitised to ovalbumin (20 μg, Grade VI; Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) diluted in 100 μl sterile isotonic phosphate-buffered saline containing 2.25 mg. Aluminium hydroxide ($AL(OH)_3$ AlumImuject; Pierce, Rockford, IL) (Sawada *et al.*, 1997; Cho *et al.*, 2001). AD was induced by intradermal injection of ovalbumin (50 μg, Grade V; Sigma-Aldrich Chemie GmbH). This protocol rather than epicutaneous sensitization was applied since it allows exact timing of the onset of AD, which is a prerequisite for the combination of this protocol with the stress protocol. In the stress protocol, mice were exposed to a sound stress (300 Hz tone emitted at irregular intervals four times per minute) (rodent repellent device; Conrad Electronics, Nuremberg, Germany) for a single 24-hour period (Arck *et al.*, 1995a, b; Peters *et al.*, 2004). Stress exposure immediately preceded cutaneous ovalbumin challenge. Mice that were neither stressed nor challenged, stressed mice and AD mice without stress exposure served as controls. Mice were killed 48 hours after challenge or after termination of stress exposure, followed by processing of the skin as indicated below.

Tissue collection

For immunohistochemistry, mice were perfusion-fixed using a mixture of paraformaldehyde and picric acid (Botchkarev *et al.*, 1997; Peters *et al.*, 2002a). All mice included into the study showed hair follicles in the resting stage of the hair cycle (telogen) in their back skin. This is important since during the growth phase of the hair cycle (anagen) the skin thickness increases up to two-fold and the entire architecture of the skin including innervation, blood vessels, and immunocytes changes dramatically (Hoffman *et al.*, 1996; Botchkarev *et al.*, 1997; Paus *et al.*, 1999a; Mecklenburg *et al.*, 2000; Peters *et al.*, 2001). For real-time reverse transcription–PCR, mice were killed by cervical dislocation and skin was immediately snap frozen.

Routine histochemistry

Epidermal thickness and the presence of eosinophils and mast cells in murine back skin were determined on 10-μm thick sections processed for Giemsa or hematoxylin and eosin staining (Merck, Darmstadt, Germany) (Paus *et al.*, 1999b) using a digital image analysis system (AxioVision; Zeiss, Göttingen, Germany). Cells were classified as eosinophils when they showed the classical eosinophilic granular cytoplasm and the nuclei had ring-like morphology. Mast cells were classified as 'degranulated' when eight or more granules could be found outside the cell membrane. CD4+ T cells (BD Biosciences, Plymouth, UK, dilution 1:100) were detected using the ABC staining method (Paus *et al.*, 1998).

Immunofluorescence

SP+ nerve fibers and VCAM+ blood vessels were determined in 14-μm thick sections. Primary antibody binding (SP antiserum, monoclonal; Chemicon, Temecula, CA, 1:100, or 1:500; VCAM antiserum, monoclonal; BD Biosciences, 1:500) was either detected by a rhodamine-labelled secondary antibody (Dianova, Hamburg, Germany, dilution, 1:200) (Peters *et al.*, 2001) or by tyramide amplification (Renaissance TSAFM-Direct [Red]; NENTM Life Science Products, Boston, MA) (Peters *et al.*, 2002b). Nuclei were counterstained with 4′,6 diamidino-2-phenylindole (Mecklenburg *et al.*, 2000) and mast cells with fluorescein-labelled streptavidin (Botchkarev *et al.*, 1997). The use of antibody abbreviations in expressions like ''SP-immunoreactive'' or ''SP+'' implies labelling with the antibody to that antigen in recognition that an antibody could possibly be cross-reacting with some other antigen.

Qualitative histomorphometry

Epidermal thickness and number of eosinophils, CD4+ cells, VCAM+ blood vessels, mast cells, and immunoreactive nerve fibers were determined in the center of the AD lesion in defined areas of the skin such as the epidermis, hair follicle epithelium, dermis, and subcutis (Peters *et al.*, 2002a, b). For each experimental group, at least 10 microscopic fields per mouse of five different mice per group were studied at a magnification of $\times 400$ (ie, more than 100 microscopic fields were studied for each parameter and experimental group). Representative staining patterns were photo-documented using a digital imaging device (Visitron Systems, Puchheim, Germany).

Electron microscopy

Skin sections (1×1 mm) were fixed in sodium cacodylate-buffered 2.5%, glutaraldehyde and 4% paraformaldehyde, post-fixed in osmium tetroxide and embedded in araldite resin. Ultrathin sections were stained with uranyl acetate and lead citrate. All cutaneous compartments mentioned above were screened for the presence of nerve fibers with or without contact to mast cells, mast cell degranulation, eosinophils, and gap formation between blood vessel endothelial cells.

RNA isolation, DNAse treatment, reverse transcription–PCR, and real-time PCR

About 100 mg skin tissue /mouse were treated with 1 ml Trizol (Invitrogen, Karlsruhe, Germany) and RNA was then extracted. The total RNA was then treated with DNAse I 1 U/1 μg-RNA (Invitrogen), followed by inactivation with EDTA (Invitrogen). There was no detection of genomic DNA via PCR. First-strain cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen).

Real-time PCR was employed to obtain quantitative data on differences between IL-4, IL-5, TNF-α and IFN-γ mRNA expression between AD mice with and without stress exposure. 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) and a fluorogenic probe for the housekeeping gene hypoxanthine phosphoribosyl transferase was used to normalize our samples in real-time PCR.

The real-time PCR reactions were normalised to hypoxanthine phosphoribosyl transferase by calculating the difference between the C_T for hypoxanthine phosphoribosyl transferase and the C_T for the respective transcript as $\Delta C_T = C_{T\ HPRT} - C_{T\ transcript} - C_T$. Amount mRNA was calculated $1/2^{\Delta C_T}$ and expressed as difference to control, when control equals 100.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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