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Substance P Is a Key Mediator of Stress-Induced Protection from Allergic Sensitization via Modified Antigen Presentation

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Interaction between the nervous and immune systems greatly contributes to inflammatory disease. In organs at the interface between our body and the environment, the sensory neuropeptide substance P (SP) is one key mediator of an acute local stress response through neurogenic inflammation but may also alter cytokine balance and dendritic cell (DC) function. Using a combined murine allergic inflammation/noise stress model with C57BL/6 mice, we show in this paper that SP—released during repeated stress exposure—has the capacity to markedly attenuate inflammation. In particular, repeated stress exposure prior to allergen sensitization increases DC-nerve fiber contacts, enhances DC migration and maturation, alters cytokine balance, and increases levels of IL-2 and T regulatory cell numbers in local lymph nodes and inflamed tissue in a neurokinin 1-SP-receptor (neurokinin-1 receptor)-dependent manner. Concordantly, allergic inflammation is significantly reduced after repeated stress exposure. We conclude that SP/repeated stress prior to immune activation acts protolerogenically and thereby beneficially in inflammation. *The Journal of Immunology*, 2011, 186: 000–000.

Organs at the interface between our body and the environment frequently encounter and respond to a wide variety of environmental challenges that can also be defined as stressors. A stress response can be provoked by stimuli ranging from physical (heat, cold, etc.) to inflammatory (microbial, allergen, etc.) and to psychosocial (noise, restraint, aggression, etc.) stressors, which all result in a similar arousal of the hypothalamus–pituitary–adrenal-axis (HPA) and the sympathetic axis (SA) and generally promote a proallergenic Th2 bias of the immune response.

It has lately become accepted that in addition to response elements for the HPA and SA, organs such as the skin, the lung, or the gut possess local neuroimmune stress-response elements to meet these challenges on-site. One central player along this third stress axis, which involves neuropeptides and neurotrophins (NNA), is the sensory neuropeptide substance P (SP). It activates the neurokinin 1 receptor (NK-1 R) and is generally described to act locally in a proinflammatory way through close contacts between SP containing peptidergic nerve fibers with mast cells and subsequent neurogenic inflammation (1).

Organs at the interface with the environment frequently develop allergic disease. In allergic disorders—especially in people with an atopic predisposition—the net effect of the stress response was generally reported to contribute to acute exacerbation and even onset of disease (2–7). The key mechanisms involved are altered immune competence and neuronal plasticity after stress experience as a result of an HPA- and SA-generated Th2 bias on the one hand and local proinflammatory NNA activation on the other hand (8–10).

Intriguingly, however, in the course of atopic disease, clinical and experimental observations also demonstrate the occurrence of improved inflammation after stress (11). In fact, several findings indicate a balancing role for nerve-immune cell interaction under defined conditions (2–4, 6, 12). For example, stress during allergen sensitization can block contact hypersensitivity (12), and the following observations suggest that stress-induced suppression of inflammation may involve the NNA: SP is released by the repeated application of either capsaicin (13), UV light (14–16), physical exercise (17–19), or calcineurin inhibitors (20), all of which can be considered as repeated mild stress exposures and generally result in improved allergic inflammation in atopics.

However, the involvement of the NNA in stress-induced inflammation control has never been investigated. One prerequisite for a potentially protective role of nerve-immune cell interaction is the close contacts between dendritic cells (DC) such as Langerhans cells (LC) and peptidergic nerve fibers (21, 22). DC present Ag as their key function connecting innate and adaptive immune response and are therefore key players in allergy development (23, 24). Through these contacts SP can induce a Th1 shift (21, 25–27) and counterbalance the predominating Th2 cytokine release and humoral immune responses in allergy. Also, in addition to SP, calcitonin gene-related peptide (CGRP) is released, which suppresses Ag presentation (22, 28). SP and CGRP release following stress may thus exert anti-inflammatory properties, for example, in allergy, with the mechanisms involved still unknown.

These observations trigger the questions: can specific stress paradigms improve allergic inflammation rather than enhance it, and which role do neuropeptides play in this scenario? We hypothesize that a stress paradigm, which involves repeated, mild or combined exposure to various triggers of neuroimmune activation,

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Abbreviations used in this article: AID, atopic dermatitis-like allergic dermatitis; CBA, cytometric bead array; CGRP, calcitonin gene-related peptide; DC, dendritic cell; HPA, hypothalamus–pituitary-axis; LC, Langerhans cell; MHCII, MHC class II; NK-1 R, neurokinin-1 receptor; NK-1 Ra, neurokinin-1 receptor antagonist; NNA, neuropeptide and neurotrophin; PGP 9.5, protein gene product 9.5; SA, sympathetic axis; SP, substance P; Treg, T regulatory cell.

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may alter the function of the nerve–DC interface and thereby lead to tolerance induction, especially during allergen sensitization. To test this hypothesis, we modified our previously published mouse model of stress-exacerbated atopic dermatitis-like allergic inflammation (AID). We introduced repeated stress exposure prior to allergen sensitization and analyzed neuroimmune interaction and DC behavior, as well as the course of allergic inflammation. By using this paradigm, we found new proof of an anti-inflammatory function of the nerve–immune cell interface centering on SP that can be triggered by stress.

Materials and Methods

Mice

Female C57BL/6 mice were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany) and maintained in the animal facility at the Charité, Virchow Hospital, University Medicine Berlin (Berlin, Germany), under pathogen-free conditions in a barrier facility with a 12-h light/dark cycle. Animal care and experimental procedures were followed according to the requirements of the state authority for animal research conduct (LaGeSo, Berlin, Germany). Six- to 8-wk-old mice with skin in the telogen stage of the hair cycle were randomized into experimental groups and left for 1 wk to adjust to their new environment (29).

The AID-stress model

The mice were sensitized by a s.c. injection of chicken OVA (20 µg, grade VI; Sigma-Aldrich Chemie, Schnellendorf, Germany) diluted in 100 µl sterile isotonic PBS containing 2.25 mg aluminum hydroxide (Al(OH)₃, AlumI-muject; Pierce, Rockford, IL) into the abdominal skin above the left leg (30, 31) on days 0 and 14. To trace DC migration into the lymph nodes, fluorescein-conjugated OVA (Invitrogen/Molecular Probes, Eugene, OR) was injected in a pilot experiment. One week after the second sensitization, the mice were challenged by an intradermal injection of OVA (50 µg; grade V) into the skin of the lower back as described previously (32–34).

In the stress protocol, mice were exposed to an inescapable sound stress (i.e., noise [at the frequency of 300 Hz and sound pressure level 75–80 dB]) emitted at irregular intervals four times per minute by a rodent repellent device (Conrad Electronic) placed in the mouse cage for the duration of 24 h (32–34). In the combined AID-stress model, stress was applied twice, 24 h prior to each sensitization. Effective induction of a stress response was confirmed by the altered behavior of the mice during stress exposure (e.g., restless movement around the cage) and determination of mast cell degranulation in the skin of the mice 24 h after stress exposure as investigated previously (34, 35). Mice that were neither stressed nor challenged served as controls. In addition, one group of stressed AID-induced mice was injected i.p. with the highly specific NK-1 R antagonist (NK-1 Ra) [D-Arg1, D-Phe5, D-Trp7,9, Leu11]-SP (Sigma-Aldrich Chemie) prior to and after each stress exposure. The mice were sacrificed and processed either immediately or 48 h after the challenge.

Histochemistry

Epidermal thickness and eosinophils infiltrating AID skin were detected by Giemsa staining (Merck, Darmstadt, Germany) (35) on 10-µm cryosections. MHC class II (MHCII) (BMA Biomedicals, Augst, Switzerland) staining was performed on 10-µm cryosections at a 1:100 dilution for 1 h, followed by incubation with biotinylated goat-anti-rat secondary Ab (1:200, 30 min; Dianova, Hamburg, Germany) and avidin–biotin complex labeled with alkaline phosphatase.

For immunofluorescence, the mice were perfusion fixed using a mixture of paraformaldehyde and picric acid (29). For double labeling of Langerin (CD207)-positive LC and protein gene product 9.5 positive nerve fibers, 14-µm-thick cryosections were used. Primary Ab binding (Langerin-antiserum, polyclonal, 1:5000; Santa Cruz Biotechnology, Heidelberg, Germany; protein gene product (PGP)-antiserum, polyclonal, 1:400; Biotrend, Cologne, Germany) was detected either by tyramide amplification (Renaissance TSAFM-Direct [Red]; NEN Life Science Products, Boston, MA) (29) or by FITC-labeled secondary Ab (dilution 1:200; Dianova) (29). Nuclei were counterstained with DAPI (29). Tyramide amplification was also applied to 10-µm-thick cryosections to detect Foxp3-immunoreactive T regulatory cells (Treg) in skin. All sections were counterstained with DAPI (Boehringer Mannheim, Mannheim, Germany) for identification of cell nuclei. All immunohistochemical staining steps were performed in light-protected humidity chambers and interspersed by washing steps in TBS.

Histomorphometry

Immunoreactivity patterns were visualized with a Leica laser-scanning confocal microscope (Leica, Wetzlar, Germany). The number of contacts between LC and nerve fibers or of immunoreactive cell populations was determined by standard histomorphometry as published previously (29). Briefly, numbers were counted per microscopic field at $\times 400$ magnification in at least 10 consecutive microscopic fields per experimental mouse and in at least five mice per experimental group by two blinded independent researchers. The data were then pooled and expressed as means per group $\pm$ SEM.

Epidermal sheet culture and evaluation of LC migration

Epidermal sheet culture was conducted based on established methods (36). Briefly, four biopsies per mouse (six mice per group, control, stress, AID, and stress plus AID) were taken from telogen back by an 8-mm punch shortly after inducing AID in the presence of 200 µg/ml gentamycin. After removing the subcutis, to split the epidermis from the dermis, the skin explants were incubated in dispase I (final concentration, 1.2 U/ml; Roche, Mannheim, Germany) dissolved in HBSS without Ca²⁺ and Mg²⁺ at 4°C overnight. The epidermis was detached with fine forceps and cultured separately (one sheet per well) for 72 h at 37°C in 24-well plates, each well containing 1.5 ml culture medium consisting of RPMI 1640, 10% FCS, 50 µg/ml glutamine, and 1% streptomycin/penicillin.

Cells that had emigrated from the epidermis into the culture medium were harvested. DC could be readily and unequivocally identified by their hairy and veiled appearance. They were counted using $\times 40$ objective lenses and a calibrated grid under the hemocytometer. Dead cells were excluded by trypan blue staining. Additionally, the phenotype of emigrated DC was confirmed by staining cytocentrifuge smears with MHCII mAb (as described above). The percentage of emigrated epidermal DC (LC) out of all emigrated cells was counted and expressed as mean $\pm$ SEM. To analyze migration of LC upon neuropeptide treatment, dispase-procured epidermal sheets were cultured in the presence of SP (1 µM), CGRP (1 µM) (both purchased from Sigma-Aldrich), or medium only.

FACS standard methodology

Cells were isolated from skin-draining lymph nodes, and flow cytometry was performed following established protocols (37). In brief, cells were washed and resuspended at a final cell concentration of 2×10^7 /ml. Cells were then incubated for 30 min at 4°C with previously optimized amounts of one or more of the following conjugated murine mAbs: FITC-labeled mAb against CD86 and CD4, DC-labeled mAb against CD11c and CD25, and PE-labeled anti-CD80, –VLA-4, and –LFA-1 (all purchased from BD Biosciences, San Diego, CA). Controls were stained with the corresponding isotype-matched mAb. Acquisition was performed using the FACSCalibur system (BD Biosciences). Data were analyzed using CellQuest software. Instrument compensation was set in each experiment using single-color-stained samples. Results were expressed as the percentage of cells positive for the surface marker evaluated.

DC–T cell coculture

For DC–T cell coculture, DC were purified from skin-draining lymph nodes (axillar, inguinal, and sciatic) from AID-induced, stressed, and AID plus stress C57BL/6 mice. Cell suspensions were enriched by positive selection using anti-CD11c⁺ immunomagnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). To obtain CD4⁺ T cells, spleens from 6-wk-old BALB/c were harvested, and the splenocytes were enriched as described by the manufacturer (Miltenyi Biotec). The resulting DC and T cells were routinely >85 – 90% positive for CD11c and CD4, respectively, as determined by flow cytometry. CD11c⁺ cells (1.6×10^4 /well) were seeded as stimulators with CD4⁺ cells (10^5 /well) as responders in 24-well plates (38). Cell cultures were incubated at 37°C, 5% CO₂ for 72 h, in a final volume of 400 µl/well complete RPMI 1640 medium, containing 10% heat-inactivated (56°C, 30 min) FCS (Life Technologies, Rockville, MD), 1% penicillin/streptomycin, and 2 mM glutamine.

Cytometric bead array

Cytokines were analyzed in cell culture supernatants using cytometric bead array (CBA). Briefly, upon 72 h of DC–T cell coculturing, supernatants were harvested and stored at 70°C until cytokine determination. IL-4, IL-5, TNF-α, IFN-γ, and IL-2 were detected simultaneously using the mouse Th1/Th2 cytokine CBA kit (BD Pharmingen, San Diego, CA) following the manufacturer's instructions. In short, 50 µl of each sample was mixed with 50 µl of mixed capture beads and 50 µl of the mouse inflammation PE detection reagent. The samples were incubated at room temperature for 2 h in the dark.

After incubation with the PE detection reagent, samples were washed and resuspended in buffer before acquisition on a FACSCalibur cytometer (BD Biosciences). Data were analyzed using CBA software (BD Biosciences). Standard curves were generated for each cytokine using the mixed cytokine standard provided by the kit. The concentration of each cytokine in cell supernatants was determined by interpolation to the corresponding standard curve. Absolute cytokine levels ranged from 1.25 to 19.31 pg/ml. Changes in cytokine levels were expressed as percent change over control harvested on the same day. To then summarize the data in a Th2/Th1 ratio to delineate changes in pro- versus anti-inflammatory conditions in response to inflammatory stimulus and/or stress, we followed established protocols (4, 37, 39, 40).

Statistical analysis

Means were calculated and significant differences determined by Mann-Whitney *U* test for unpaired samples. Significance was assumed if $*p < 0.05$ or $**p < 0.01$.

Results

Interaction between LC and epidermal nerve fibers is increased under stress

Intimate contacts between peptidergic nerve fibers and DC may be responsible for altered DC activation after stress. We therefore foremost investigated whether exposure to stress by inducing skin neuronal plasticity can alter interaction between DC and nerve endings. We assessed the number of contacts in the epidermis, as the first site of allergen encounter, by double immunohistochemical staining for LC marker Langerin and nerve fiber marker PGP 9.5. Exposure to 24 h of noise stress results in a significant and robust increase in the number of contacts between LC and peripheral nerves (stressed mice 5.9 ± 0.88 versus nonstressed mice 3.1 ± 0.53) (Fig. 1).

Stress induces SP-dependent migration of DC out of the skin

To further explore by which mechanisms stress and its mediator SP might interfere with inflammation, we investigated the fate of APC. We first analyzed whether repeated stress exposure during

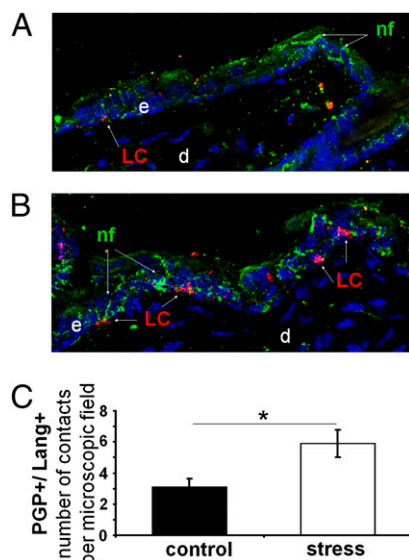


FIGURE 1. Stress increases the number of LC-nerve fiber contacts. Double staining of Langerin (red) and PGP 9.5 (green) in the epidermis of control (nontreated) (A) and stressed mice (B), as assessed by confocal microscopy (original magnification $\times 400$). C, Number of contacts in stressed and control group as evaluated by histomorphometry (29) in 10 consecutive microscopic fields per mouse using conventional fluorescence microscopy. Data were pooled from five different mice per group and are expressed as mean number of contacts per microscopic field $\pm$ SEM; $*p \leq 0.05$. e, epidermis; d, dermis; nf, nerve fiber.

sensitization changes the number of MHCII-immunoreactive cells both in the epidermis (all LC) (Fig. 2A, 2B), as well as in the dermis (mostly dermal DC) (Fig. 2A, 2B) *in vivo*. Compared with control (nontreated) mice, repeated stress exposure during sensitization alone did not induce a significant change in the number of LC (Fig. 2B). However, the number of dermal DC increased significantly, as previously reported, under the influence of acute stressors (39). Also, induction of AID was accompanied by a vast increase in the number of MHCII-immunoreactive DC both in the epidermis and the dermis (Fig. 2A, 2B) (41). Repeated stress exposure during sensitization, however, significantly reduced the number of MHCII-immunoreactive cells (both in the epidermis and the dermis) compared with the nonstressed AID-mice (Fig. 2A, 2B).

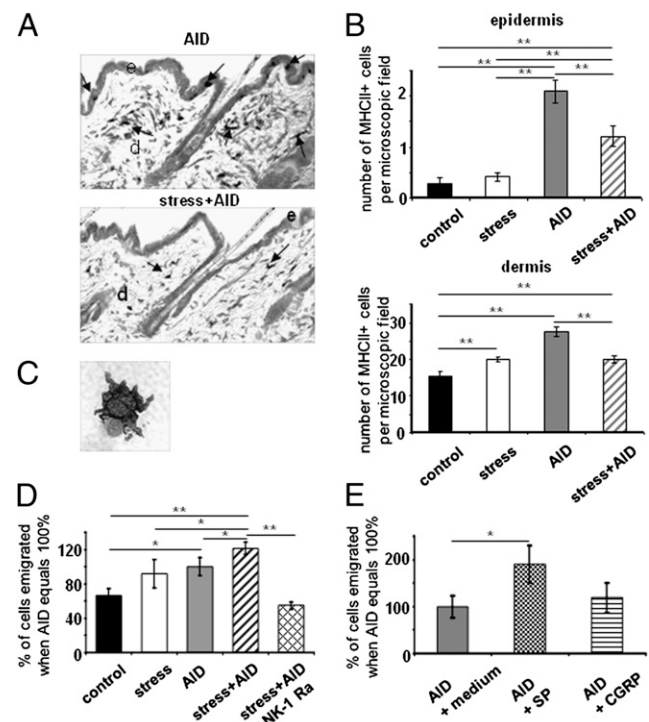


FIGURE 2. Stress enhances DC migration out of allergic skin via SP-dependent mechanisms. A, Representative photomicrographs show MHCII-immunoreactive cells (black arrows) in skin samples obtained from AID (A, upper panel) and stressed AID (A, lower panel) mice (original magnification $\times 400$). B, Stress reduces the number of both epidermal and dermal DC in AID skin 48 h after allergy induction. Bars represent mean $\pm$ SEM number of MHCII-immunoreactive cells in the epidermis (upper panel) and the dermis (lower panel) counted in 10 consecutive microscopic fields/mouse at $\times 400$ magnification. C, The phenotype of emigrated DC, as confirmed by staining of cytocentrifuge smears with MHCII mAb, in this paper are shown as a typical conjugate of an MHCII-immunoreactive cell and a lymphocyte in magnification. D, Percentage of LC out of all cells emigrated over 72 h out of epidermal sheets that had been isolated shortly after allergic challenge (for details, see *Materials and Methods*). Results of two experiments, four explants per mouse, and six mice per group were pooled, and all groups were expressed as relative to the values obtained in AID group. Bars represent mean $\pm$ SEM. $*p \leq 0.05$; $**p \leq 0.01$, as determined by nonparametric Mann-Whitney *U* test. E, To find out whether the neuropeptides SP or CGRP are accountable for these findings in D, epidermal sheets isolated from skin biopsies of AID mice were incubated with SP, CGRP (both at concentrations of $1 \mu\text{M}$), or only with culture medium for 72 h. The number of LC was expressed as a percentage of all emigrated cells and as relative to medium-only treated group. Data pooled from two experiments and five different mice per group are presented as mean $\pm$ SEM. $*p \leq 0.05$; $**p \leq 0.01$, as evaluated by nonparametric Mann-Whitney *U* test. e, epidermis; d, dermis.

Bearing in mind that 48 h is the expected time required for most "activated" DC to migrate to the local lymph nodes (42), we cultured epidermal sheets isolated from skin biopsies immediately after the challenge and determined LC emigration. We confirmed the phenotype of the emigrated LC by staining cytocentrifuge smears with MHCII mAb (Fig. 2C). We found that AID enhanced the migration of LC into the cell culture medium compared with control mice. Interestingly, a significant further increase could be observed in stressed AID skin (Fig. 2D).

To find out whether SP may be involved in the enhancing effect of repeated stress exposure during sensitization on LC migratory activity, we treated stressed AID mice with NK-1 Ra. Strikingly, treatment with NK-1 Ra abolished the effect of repeated stress exposure during sensitization on LC migration (Fig. 2D), shifting the response down to control levels in the stressed AID mice treated with NK-1 Ra.

To further confirm the role of SP as the neuropeptide stress mediator responsible for LC migration out of stressed AID skin, we treated epidermal sheets isolated from AID skin with SP or the LC-modulating neuropeptide CGRP. In this experiment, the number of emigrated LC was significantly increased by SP in comparison with nontreated explants or after treatment with CGRP (Fig. 2E). Thus, SP was capable of inducing the observed *in vivo* response *in vitro*, supporting the conclusion derived from the NK-1 Ra treatment experiment that SP is responsible for the stress effects observed in AID skin.

Stress promotes DC maturation in skin-draining lymph nodes

The immune function of DC such as skin derived LC is largely attributed to their ability to migrate to the local lymph nodes, which is accompanied by morphological changes and includes the coordinate activation of adhesion molecules, such as α_4 integrin (VLA-4) (43), LFA-1, and ICAM-1 (39, 44, 45). As we observed an increased rate of LC migrating out of the epidermis in stressed AID skin, we wished to determine whether repeated stress exposure during sensitization influences the number and state of maturation (46, 47) of DC in local skin-draining lymph nodes.

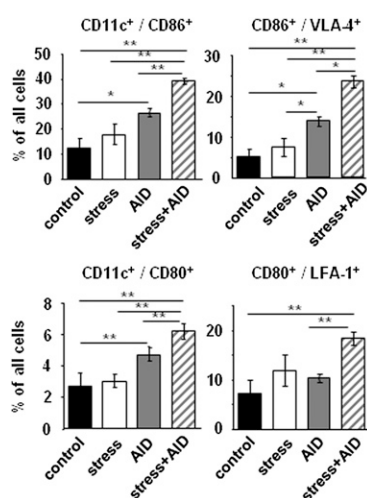


FIGURE 3. Stress increases the proportions of lymph node CD11c⁺ DC expressing markers of DC maturation and Th1-distinctive adhesion molecules. DC and DC phenotypes were characterized by flow cytometry by labeling cells isolated from skin-draining lymph nodes 48 h after allergic induction. Each column shows the mean of percentage of double-positive cells for given markers out of all measured cells (10,000 cells assessed). Results are representative of two separate experiments consisting of five individually analyzed mice per group. Significances are assumed if **p* ≤ 0.05 or ***p* ≤ 0.01.

At first, to prove that cutaneous DC actually reach the lymph nodes in our experimental setting, and as a prerequisite of further experiments, we used FITC-conjugated OVA for AID induction. By FACS analysis, we were able to detect sizeable numbers of FITC-positive cells in all tested skin-draining lymph nodes in all experimental settings (data not shown).

Final experiments were carried out with unlabeled OVA to avoid unwanted FITC effects. By measuring the expression of general DC marker CD11c, we determined the proportions of DC in skin-draining lymph nodes in parallel with the level of expressed surface markers of maturation. We found that repeated stress exposure during sensitization resulted in a significant increase in the number of dendritic CD11c⁺ cells expressing the costimulatory molecules CD80 and CD86 (B7-1/-2) in skin-draining lymph nodes of AID mice compared with nonstressed AID mice (Fig. 3). We further investigated coexpression of diverse adhesion molecules on the surfaces of CD11c⁺ cells expressing costimulatory molecules and found that CD80⁺ cells from stressed AID mice coexpressed enhanced levels of LFA-1 (ICAM-1 ligand), whereas the expression of adhesion molecule VLA-4 (VCAM-1 ligand) was significantly

A CD11c⁺ dendritic cell- CD4⁺ T cell co-culture

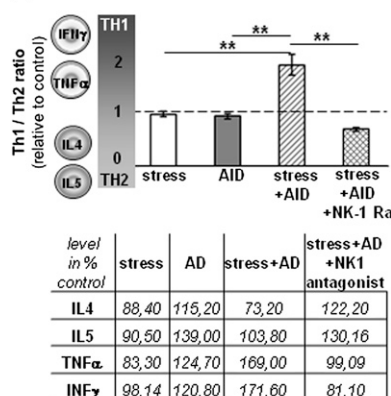


FIGURE 4. Stress shifts cytokine production in DC-T cell cocultures from allergic mice toward Th1 and Treg patterns. DC and T cells were isolated from C57BL/6 or BALB/c mice and enriched. A, Bars represent ratio of Th1/Th2 cytokines as measured by CBA in cell cocultures of CD11c⁺ DC isolated from given experimental groups and allogeneic CD4⁺ T cells after 72 h of incubation. Assays were run in a series of experiments. Absolute individual cytokine values varied from 5.35 to 13.08 pg/ml. To determine and compare the Th1 to Th2 ratios, the level of each cytokine (TNF-α, IFN-γ, IL-5, and IL-4) per treatment group was calculated as a percentage of the level of the same cytokine released from control samples (coculture of T cells with DC from control, nontreated animals) harvested on the same day. Means pooled from five different mice per group are given in the table below the graph. To calculate Th1 to Th2 ratios, percentages of each Th1 cytokine were divided by the percentage of each Th2 cytokine (IFN-γ/IL-4, IFN-γ/IL-5, TNF-α/IL-5, and TNF-α/IL-4) per mouse and treatment group, and a mean was calculated per mouse. Data displayed in the graph are pooled from five different mice per group, and the SEM is given. B, Concentration of IL-2 measured in DC-T cell cocultures originating from differently treated groups and expressed as relative to IL-2 levels measured in control (nontreated) group. Data are shown as mean ± SEM (*n* = 5). **p* ≤ 0.05; ***p* ≤ 0.01.

increased in the CD86⁺ subpopulation (Fig. 3). However, not all of the DC identified by CD11c and CD80 or CD86 coexpressed the adhesion molecule ligands.

Stress induces an elevated release of Th1 cytokines and IL-2 in DC–T cell coculture in an SP-dependent manner

Depending on the extracellular costimulatory molecule milieu, DC may drive Th naive cell differentiation in various directions (47, 48). To analyze the effect of stress on the DC–T cell interface and subsequent cytokine balance, we cocultured CD11c⁺ DC isolated from differently treated mice, with allogeneic CD4⁺ T lymphocytes and measured the subsequent release of Th1 (IFN- γ and TNF- α) and Th2 (IL-5 and IL-4) cytokines. To this effect, DCs primed in skin under the respective *in vivo* conditions were isolated from the skin-draining lymph nodes, which is where Ag presentation of skin-derived Ags and subsequent induction of proallergic immune responses take place (11–13). Repeated stress exposure during sensitization significantly skewed the cytokine balance induced by CD11c⁺ DC from AID mice toward a Th1

response, when compared with all other groups (Fig. 4A). In addition, we were able to demonstrate that SP is a relevant neuropeptide stress mediator in this context, as this effect was abolished in animals that additionally received NK-1 Ra (Fig. 4A). Specifically, stress reduced the levels of the Th2 cytokines IL-4 and IL-5 in AID, which was restored by SP blockade, and stress further enhanced the levels of the Th1 cytokines TNF- α and IFN- γ , which was completely blocked by SP blockade to levels even below AID. Thus, SP appears to directly block the production of Th2 cytokines in AID (18, 19), whereas it is directly responsible for the rise in Th1 cytokines both in AID and stressed AID (14–17).

Strikingly, we also measured a significant increase in the level of a cytokine relevant for CD25⁺ Treg survival and proliferation, IL-2 (49), in cocultures of CD11c⁺ DC and allogeneic T cells from stress plus AID mice. Again, the effect of repeated stress exposure during sensitization was absent in animals in which NK-1 Ra was applied, demonstrating the effect to be mediated by SP (Fig. 4B). Regulatory properties of DC have been described before to associate with maturation and production of IL-2, IFN- γ , and TGF- β (50). Interestingly, parallel to the increased production of IL-2 and IFN- γ in AID after stress, we had also previously found a significant 2-fold increase in the expression of TGF- β 2 by MicroArray gene chip technology in the skin of stressed versus control mice (51). Stress is therefore highly capable of mediating a Treg-promoting cytokine profile.

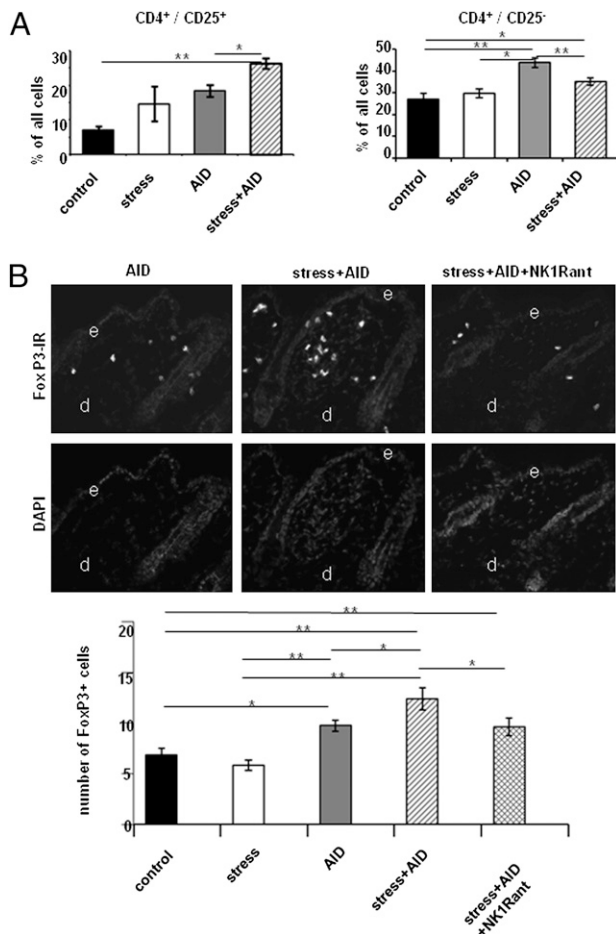


FIGURE 5. Stress increases the number of Treg in the skin-draining lymph nodes and skin of AID mice. *A*, Bars represent percentage of IL-2-responsive CD4⁺CD25⁺ and CD4⁺CD25⁻ cells in skin-draining lymph nodes as measured by FACS. Data are presented as mean $\pm$ SEM ($n = 10$). *B*, In the *upper panel*, Foxp3-immunoreactive dominant tolerance mediating cells in the dermis of stressed, nonstressed, and AID mice that received both stress and NK-1 Ra are shown. Corresponding DAPI⁺ cell nuclei are shown in the *lower panel*. Bars represent the average number of Foxp3-immunoreactive cells as evaluated in dermis of treated animals in 10 consecutive microscopic fields/mouse and five mice per group, using conventional fluorescence microscopy (original magnification $\times 400$). * $p \leq 0.05$; ** $p \leq 0.01$. Abbreviations: d, dermis; e, epidermis.

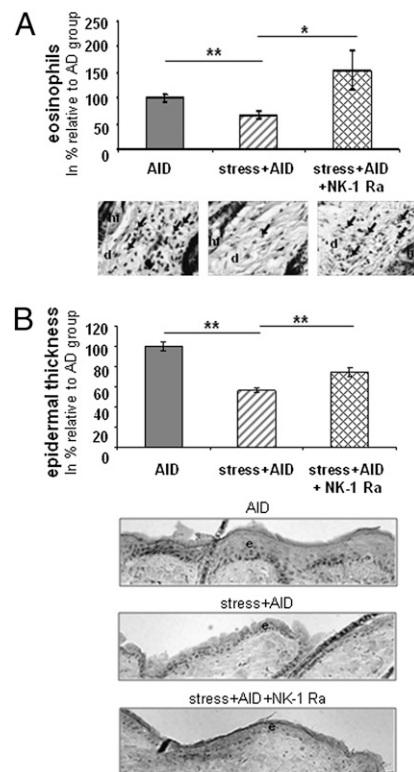


FIGURE 6. Stress exposure prior to allergen sensitization reduces allergic skin inflammation in an SP-dependent manner. *A*, Eosinophil infiltration as visualized by Giemsa staining was evaluated per microscopic field. Eosinophilic granulocytes (arrows) are highly present in AID skin, and their number significantly decreases upon stress exposure (stress + AID). Application of NK-1 Ra abolishes the effect of stress (stress + AID + NK-1 Ra). Number of eosinophils in dermis as mean $\pm$ SEM out of two independent experiments ($n = 10$) is expressed as percent relative to AID group (*B*) epidermal thickness in micrometers was evaluated in 10 consecutive microscopic fields per mouse (five mice per group) in two independent experiments. Columns represent mean $\pm$ SEM ($n = 10$) expressed relative to AID group. * $p \leq 0.05$; ** $p \leq 0.01$. d, dermis; e, epidermis; hf, hair follicle.

Additionally, we asked whether repeated stress exposure during sensitization changes DC potency to induce proliferation of naive allogeneic CD4⁺ helper cells. Using CFSE prelabeling, we measured no significant changes in DC stimulatory capacity between the AID and stress plus AID groups. However, stress plus AID treated with NK-1 Ra prior to and after each exposure to stress showed a significant drop in the number of responding CD4⁺ lymphocytes (data not shown), indicating a basic requirement of SP for T cell proliferation.

Stress promotes induction and skin redistribution of Treg

To test whether stress-induced changes in the activation and maturation of DC and increased IL-2 production might be accompanied by alterations in the distribution of different T lymphocyte subpopulations, we subjected cells isolated from skin-draining lymph nodes to FACS analysis. Remarkably, analysis revealed no changes in the total percentage of CD4⁺ Th cells, but a significant rise in the percentage of CD4⁺ cells expressing CD25⁺, the IL-2R α -chain. These cells are capable of suppressing immune responses in allergy. This corresponded with a pronounced decline in CD4⁺CD25⁻ T cells (Fig. 5A). Hence, stress altered the proportions of CD4⁺CD25⁺ and CD4⁺CD25⁻ cells but not their total number. This result corresponds well to the proliferation assay reported above, where we could not detect enhanced T cell proliferation induced by DC derived from stressed AID skin.

We further questioned whether the increased number of CD4⁺CD25⁺ Treg in skin-draining lymph nodes results in an increased frequency of dominant tolerance inducing Treg in the skin (52). We therefore evaluated the number of Foxp3-immunoreactive cells, which account for the suppressive Treg phenotype in peripheral tissues and decidedly lack effector T cell properties (Fig. 5B) and found that repeated stress exposure during sensitization itself did not induce any changes in cutaneous Foxp3-immunoreactive Treg numbers, whereas AID resulted in a significantly increased number of Foxp3-immunoreactive cells in the skin compared with controls. Moreover, in stress plus AID mice, the increase in the number of Foxp3-immunoreactive cells was significantly enhanced compared with AID mice (Fig. 5B). Again, supporting the role of SP, NK-1 Ra abolished the additional effect of repeated stress exposure during sensitization (Fig. 5B).

Stress prior to allergen sensitization reduces cutaneous allergic inflammation

To find out what the relevance of a stress-modified nerve fiber–DC interaction during sensitization would be, we determined the level of cutaneous inflammation by standard AID-readout parameters such as cellular infiltration and level of epidermal thickening. When compared with control, AID animals (data not shown) showed massive eosinophil infiltration. Surprisingly, the number of eosinophils decreased greatly in animals exposed to repeated stress during sensitization (Fig. 6A). Additionally, in contrast to the epidermis overlaying dermatitis in control and only stressed animals (data not shown), the epidermis of mice with AID was markedly thickened with more than two cell layers (Fig. 6B). Stress exposure at the time of Ag presentation resulted in a large reduction of the AID-induced epidermal thickening to almost control levels at 48 h after AID induction (Fig. 6B). Again, SP signaling was identified as a key signaling mediator in the observed stress-induced ameliorating effect on ongoing inflammation, because treatment with NK-1 Ra significantly counteracted the stress effect, both on eosinophil infiltration and epidermal thickening and returned them to unstressed levels (Fig. 6A, 6B).

Discussion

Neuroimmune interaction connects two key systems, the nervous system and the immune system, which allow the body to adapt to a wide variety of environmental challenges. At the interface between body and environment, the immune system alerts the nervous system to immunological provocations such as microbes, whereas the nervous system alerts the immune system to environmental challenges such as the possibility of injury. This enables the body to be prepared to meet and rapidly respond to stressors in the attempt to maintain a healthy homeostasis.

Dysfunctional activation, however, may be involved in the promotion of inflammatory diseases, especially in the context of allergy and stress-induced exacerbation of disease, as has been shown for atopic dermatitis, bronchial asthma, or colitis (3, 4, 34, 53). These diseases are characterized by a prominent production of Th2 cytokines and local neurogenic inflammation. Stress-induced activation of the HPA and SA supports the Th2 imbalance, whereas activation

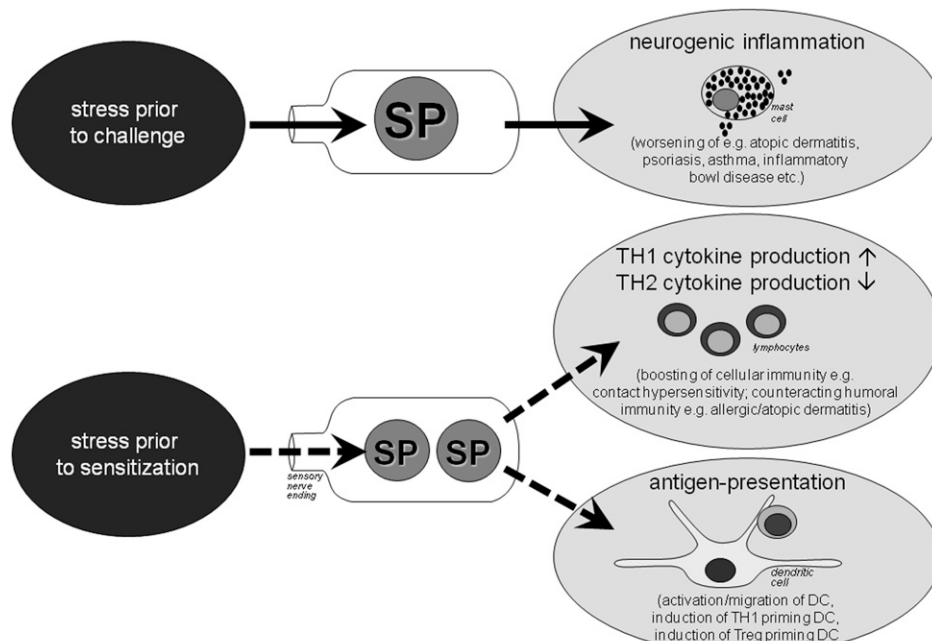


FIGURE 7. Schematic representation of SP immune regulation in peripheral organs at self-environment interfaces. Note the differential effects of various stress- and SP-release qualities. Thick arrow indicates singular release boost, and dashed arrow indicates repeated dosed release.

of the NNA supports neurogenic inflammation. Stress as a trigger and enhancer of Th2-driven inflammation, which also involves neurogenic inflammation, is therefore widely accepted.

One key mediator in this scenario is SP. Close proximity of SP-immunoreactive nerve fibers and cells of the immune system that express the NK-1 R enables interaction. Besides mast cell degranulation, the key feature of neurogenic inflammation, this interaction may also involve DC and enhanced production of proinflammatory cytokines such as IFN- γ . In the context of allergy, however, this may also promote an antiallergic Th1 bias.

Using the skin as a model organ for studying neuroimmune interactions at the interface between body and environment, we show in this article, to our knowledge, for the first time, that stress in fact increases the interaction between peripheral peptidergic nerve fibers and DC and that stress-induced plasticity of the nerve–DC interface during allergen sensitization can result in a reduced allergic response to challenge. A defined stress paradigm using repeated stress exposure during allergen sensitization thereby effectively enhances DC migration and upregulation of costimulatory molecule expression (47) in a neurokinin 1-SP-receptor (NK-1 R)-dependent fashion. To our knowledge, this is the first report of SP-dependent Treg responses after stress. Our experiments show that repeated stress exposure during sensitization as well as SP increase IL-2 production, which closely correlates with Treg homeostasis and anti-inflammatory function in interaction with CD25 and TGF- β (49, 50, 54, 55). Consequently, we were able to demonstrate skin infiltration by Foxp3-immunoreactive anti-inflammatory Treg, in association with reduced cutaneous inflammation in AID skin after repeated stress exposure during sensitization. Certainly, the mechanisms underlying migration of Treg into the skin and precise Treg subtypes involved in suppression of the effector phase (56) remain to be defined. However, that the sensory neuropeptide SP occupies a central position in the modulation of DC function and their ability to determine Treg responses is a surprising new finding.

As described above, to date, SP is exclusively discussed as a proinflammatory neuropeptide. A recent report (57) states a constitutive NK-1 R expression by LC and dermal DC in skin (26). In these experiments, SP activation of DC during the effector phase of a Th1-driven inflammation triggered a massive and rapid LC mobilization out of the epidermis and enhanced inflammation. An inflammation-promoting effect of SP signaling was also demonstrated in other Th1 and neurogenic inflammation-dominated neuroimmune constellations, such as during the elicitation of contact hypersensitivity (7, 58, 59), during provocation of AID (32–34), in hair loss (60), and in healthy murine skin and lymphocytes (34, 39, 40). This effect appears to be further facilitated by stress- and nerve growth factor-induced plasticity of the peripheral nervous system and malfunctioning HPA responsiveness (32, 34, 39, 61, 62). The DC-activating capacities of SP are therefore suited to boost innate and cellular immunity.

We show in this study that SP-induced DC activation may also be suited to attenuate humoral immunity in a disease characterized by a Th2 bias (Fig. 7). In this context, stress exposure, which coincides with allergen sensitization, serves to reduce the inflammatory response in Th2-driven inflammation (63–65). In addition, the anti-allergic enhancing effect of SP on Th1 cytokine production and the suppressive effect of SP on Th2 cytokine production is complemented by the expansion of Treg activity, which restricts the Th effector phase responsible for allergic inflammation (66, 67). These observations explain previously puzzling observations in allergic disease such as increased SP levels associated with successful antihistamine treatment for atopic disease (68) or in atopic children with low allergic sensitization (69).

Taken together, our results contribute to the understanding of proinflammatory effects of SP versus anti-inflammatory effects and entail reconsideration of the familiar concept of stress as primarily an inductor and enhancer of allergy. It surely depends on the time point, frequency, and dosage of exposure to a variety of challenges and neuronal stress mediators (53, 70, 71) whether we observe: 1) enhanced neurogenic inflammation that worsens inflammation, for example, after singular and immediate stress exposures prior to inflammatory challenges (34); 2) allergy-facilitating Th2-dominated cytokine production under the regimen of chronic HPA activation (8), which is counteracted by SP-induced Th1 induction; or finally 3) tolerance induction by repeated stress exposure coinciding with allergen sensitization (Fig. 7). We may even expect inhibited proinflammatory Th17 responses using the experimental protocol described in this paper—a promising future research option (52, 72).

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Disclosures

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