

Mast cell-driven skin inflammation is impaired in the absence of sensory nerves

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Background: Mast cells (MCs) and nerves can induce cutaneous inflammatory responses, both independently and by interacting with each other. However, little is known about the role of skin nerves and neuropeptides in the regulation of MC-mediated skin inflammation, and the contribution of MCs in neurogenic inflammation is still controversial.

Objective: The aim of this study was to investigate the effects of cutaneous sensory nerves on MC-driven inflammatory responses.

Methods: Passive cutaneous anaphylaxis, a model for type I allergic skin responses, was studied in the presence or absence of sensory nerves by using a murine model of selective cutaneous denervation.

Results: Passive cutaneous anaphylaxis was significantly impaired in the absence of sensory nerves. This effect was not a result of an alteration of mast cell numbers in denervated skin. Moreover, IgE-mediated activation of mast cells was markedly decreased in denervated compared with normal skin. Notably, pretreatment of mice with selective antagonists of the neuropeptides substance P and/or calcitonin gene-related peptide also resulted in decreased inflammatory responses after MC activation.

Conclusion: These data suggest that sensory skin nerves augment MC-driven inflammatory responses by releasing neuropeptides that increase MC degranulation.

(J Allergy Clin Immunol 2007;119:1101-1109.)

Key words: Mast cells, sensory nerves, neuropeptides, allergic skin inflammation, urticaria

Mast cells (MCs) are well accepted for their pivotal role in the induction of allergic reactions¹ and their sentinel functions in host-protecting innate immunity and tissue remodeling.²⁻⁷ In

Abbreviations used

CGRP: Calcitonin gene-related peptide
HSA: Human serum albumin
MC: Mast cell
MF: Microscopic field
NGF: Nerve growth factor
PCA: Passive cutaneous anaphylaxis
PIPES: Piperazine-N,N'-bis(2-ethanesulfonic acid)
SN: Sensory nerve
SP: Substance P

recent years, MCs have also been linked to the modulation of adaptive immune responses⁸⁻¹²; however, as of yet, they remain widely underappreciated in the field of neuroimmunology.¹³⁻¹⁶ Many independent lines of evidence indicate the existence of an intensive bidirectional crosstalk between MCs and sensory nerves (SNs) and suggest that MCs and SNs may be viewed as a functional unit.^{7,16}

1. Anatomically, MCs and SNs are closely related within various tissues. In the skin, MCs are frequently colocalized with substance P (SP) and calcitonin gene-related peptide (CGRP)-expressing nerve fibers¹⁷ and peptidergic nerve fibers have been reported to communicate with MCs along their entire route.¹⁸ Moreover, MCs have been shown to be directly innervated by SNs^{17,19} and can even be found within SNs.²⁰
2. Mast cells and SNs share a variety of activating signals, and both MCs and SNs respond by degranulation—that is, the exteriorization of granules loaded with preformed mediators, many of which are produced by either cell type (eg, nerve growth factor [NGF], neuropeptides, and endothelin 1).
3. Both MCs and SNs are long-lived cells that undergo repetitive cycles of degranulation and regranulation. Moreover, MCs can be potentially activated by SN products such as SP^{21,22} and endothelin 1.²³⁻²⁵ Neurogenic inflammatory responses are mediated by the release of neuropeptides from SNs and reportedly are accompanied by pronounced MC degranulation.^{21,22}
4. Conversely, many MC products, including histamine, serotonin, and tryptase, have been shown to activate SNs and therefore are thought to contribute to neurogenic inflammation.^{26,27}
5. In addition, MC proteases have been described to both activate and degrade nerve products by enzymatic cleavage,²⁸⁻³⁰ thus inducing as well as potentially limiting the effects of neurogenic signals.^{7,15,31}

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Supported in part by grants from the Deutsche Forschungsgemeinschaft (SPP1110, B10SFB548 to M. Maurer, B11SFB507 to S.H., PE 890/4-1 to E.M.J.P.), Dr Med Otto Eppenhauer (MD) und Lieselotte Gutzeit Stiftung, ECARF (to M. Maurer), and the Global Allergy and Asthma European Network.

Disclosure of potential conflict of interest: E. M. J. Peters has received grant support from the German Research Foundation. The rest of the authors have declared that they have no conflict of interest.

Received for publication September 4, 2007; revised November 13, 2007; accepted for publication November 15, 2007.

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0091-6749/\$34.00

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doi:10.1016/j.jaci.2007.11.013

6. Sensory nerve products such as NGF and neurotrophins (neurotrophin 3, neurotrophin 4, brain-derived neurotrophic factor) have been implicated in the modulation of MC proliferation, migration, differentiation, mediator production, and priming to other stimulatory signals.³¹⁻³³ Similarly, MC products such as NGF and TNF are thought to regulate SN development, degeneration, and regeneration.³⁴⁻³⁶
7. Finally, MCs and SNs have been suggested to co-orchestrate a variety of physiological and pathological processes such as hair follicle cycling,³⁷ wound healing,³⁸ responses to stress,³⁹⁻⁴¹ and the pathogenesis of inflammatory and autoimmune diseases.^{7,16,42}

This overwhelming body of circumstantial evidence suggests that the crosstalk between MCs and SNs could be of crucial importance in neuroimmunologic pathways.^{14,43} However, as yet, the functional significance of this interaction remains widely unresolved. Therefore, we investigated the mechanisms and relevance of MC/SN crosstalk in MC-driven inflammatory skin responses.

METHODS

Animals and treatment

C57BL/6 mice as well as genetically MC-deficient WBB6F₁ *Kit^W/Kit^{W-v}* (*Kit^W/Kit^{W-v}*) mice and their congenic normal WBB6F₁ *Kit⁺/Kit⁺* littermates were bred under SPF (specific pathogen-free) conditions at the animal facility of Johannes Gutenberg University, Mainz, and the Charité-Universitätsmedizin Berlin. *Kit^W/Kit^{W-v}* mice express <1% of the numbers of dermal MCs present in the skin of *Kit⁺/Kit⁺* mice.^{44,45} Mice were kept in community cages (5-10 mice per cage) at light periods of 12 hours and were fed water and mouse chow (ssniff R/M-H, 10 mm; Sniff, Soest, Germany) *ad libitum*. For experiments with neuropeptide antagonists, mice were injected with specific peptide receptor antagonists to either SP ([D-Arg¹, D-Phe⁵, D-Trp^{7,9}, Leu¹¹]-SP 40 ng in 100 μ L, intraperitoneally [i.p.]; Sigma, Steinheim, Germany) or CGRP ([CGRP Fragment 8-37] 50 ng in 100 μ L, i.p.; Sigma), or with vehicle (NaCl, 100 μ L, i.p.) 15 minutes before challenge with dinitrophenol-human serum albumin (DNP-HSA). All animal care and experimentation were conducted in accordance with current federal, state, and institutional guidelines.

Unilateral surgical skin denervation of back and ear skin

For denervation of back skin areas, a previously described denervation technique was performed.⁴⁶ Briefly, mice were anesthetized, and an incision 2.5 to 3 cm at the dorsal midline of the back skin was made. The dorsal cutaneous nerves (T3-12) were exposed under a dissection microscope at both sides. On the right back side, nerves were dissected close to their anatomical entry into the skin. The left back side was kept as a sham-denervated control. The skin was closed by using steel wound clips. The immediate success of denervation was verified by testing the appropriate skin region for pinch sensitivity. The denervation of ear skin was adopted from the denervation technique described.⁴⁶ Mice were anesthetized, and an incision 0.5 to 1 cm at the dorsal base of both ears was performed. The upper and lower main trunks of the ear innervating nerves were exposed under a dissection microscope, and a 1-mm piece of each trunk was removed. For appropriate controls, nerve trunks were exposed but not dissected. Skin was closed by 2 stitches and fibrin glue.

Passive cutaneous and systemic anaphylaxis

Mice were sensitized by either intracutaneous (100 ng in 50 μ L Hanks Minimum Essential Medium (HMEM) 1,4-piperazine-diethanesulfonic acid [PIPES]) or intravenous (20 μ g in 100 μ L HMEM-PIPES) injection of specific

IgE antialbumin-dinitrophenol (dinitrophenol-HSA) antibody, or they were injected with vehicle. In back skin, IgE antidinitrophenol and vehicle were injected into both denervated and sham-denervated skin areas. Twenty-four hours later, mice were systemically challenged by intravenous injection of the corresponding antigen dinitrophenol-HSA (200 μ g in 100 μ L). The extent of allergic inflammation in back skin was assessed by measuring Evans blue extravasation (Sigma) as described previously.⁴⁶ Briefly, mice were injected intravenously with 1% Evans blue (200 μ L in PBS) 30 minutes before antigen challenge. Skin was harvested after 1 hour and incubated in 2 mL 99% N,N-dimethyl formamide (37°C overnight). Evans blue levels were measured photometrically at 650 nm and calculated as Evans blue per gram tissue using a standard curve and the weight of the individual skin samples. For the induction of passive cutaneous anaphylaxis in ear skin, mice were sensitized with IgE antidinitrophenol (100 ng in 50 μ L, intracutaneously, left ear) or injected with vehicle (50 μ L HMEM-PIPES, right ear) and subsequently challenged with dinitrophenol-HSA (200 μ g in 100 μ L, intravenously) 24 hours later. The extent of inflammation was assessed by measuring increases in ear thickness using a caliper. Systemic anaphylaxis was challenged by intravenous injection of 200 μ g dinitrophenol-HSA in 100 μ L HMEM-PIPES 24 hours after sensitization.

Induction of cutaneous neurogenic inflammation

Mouse ears were treated topically with either capsaicin 0.1% or vehicle (50 μ L; ethanol [20 μ L], acetone [20 μ L], and N,N-dimethylacetamide [10 μ L]). The extent of inflammation was assessed for 6 hours thereafter by measuring increases in ear thickness using a caliper.

Analysis of MC degranulation *in vivo*

Skin was harvested, fixated in Karnovsky solution for 2 hours, placed in 0.1 mol/L cacodylate buffer for 24 hours, and embedded in epon. Sections 1 of μ m were stained with alkaline Giemsa (Sigma), and MC degranulation was assessed histomorphometrically as described previously.^{23,47,48} Briefly, MCs were classified at 400 $\times$ magnification as extensively degranulated (>50% of cytoplasmic granules exhibiting staining alterations, fusion and/or exteriorization), moderately degranulated (10% to 50% of granules affected), or not degranulated (<10% granules affected) and expressed as a percentage of all MCs.

Immunohistochemistry and planimetric histomorphometry

Mice were perfusion-fixed by intracardial injection of Lana fixative (4% paraformaldehyde, 14% saturated picric acid, 160 mmol/L sodium phosphate) 10 days after surgical denervation. Denervated and sham-denervated back and ear skin was harvested, cryopreserved, and stained to detect neuronal marker proteins according to previously described protocols.^{17,42,49-51} Briefly, cryosections (15 μ m) were incubated overnight at room temperature with primary rabbit antimouse antibodies to SP (Chemicon, Temecula, Calif), CGRP (Amersham, Buckinghamshire, United Kingdom), and protein gene product 9.5 (Paesel and Lorei, Frankfurt, Germany) followed by incubation with tetramethylrhodamine-isothiocyanate conjugated F(ab)2 fragments of goat anti-rabbit (Jackson ImmunoResearch, West Grove, Pa) for 1 hour at 37°C. Sections were analyzed histomorphometrically as described previously.⁵⁰⁻⁵² To assess MC numbers in denervated, sham-denervated, and untreated skin areas, skin was harvested 10 days after surgical denervation, fixated in 4% buffered formalin, and stained with Giemsa. The number of cutaneous and subcutaneous MCs was assessed in 50 microscopic fields (MFs) per section at a 400 $\times$ magnification and expressed as MCs per MF.⁵³

Data presentation and statistics

All data presented are derived from 3 independent sets of experiments, and they are expressed as means $\pm$ SEMs unless indicated otherwise. The results of histomorphometrical analyses (quantification of MC degranulation) were compared by χ^2 test. All other data were tested for statistical significance using the unpaired 2-tailed Student *t* test for single point analysis.

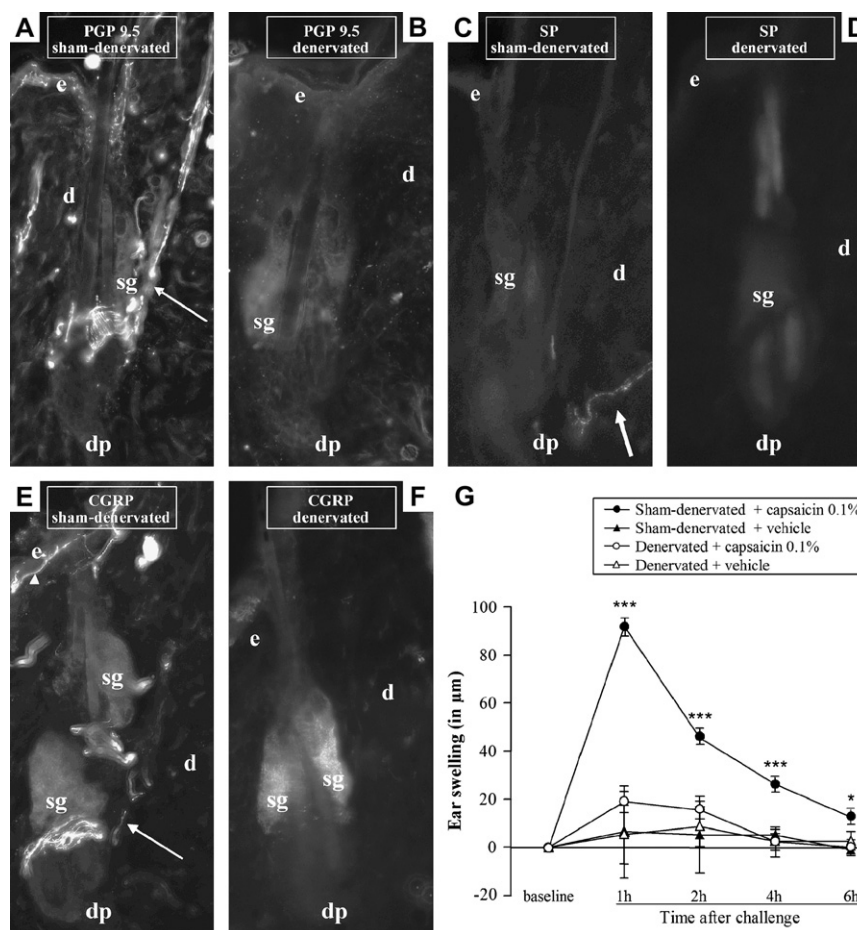


FIG 1. Unilateral surgical skin denervation results in loss of functional skin nerves. SN markers (**A and B**, PGP 9.5; **C and D**, SP; **E and F**, CGRP) are virtually undetectable 10 days after denervation (**B, D, F**) compared with sham-denervation (arrows in **A, C, E**). **G**, Denervated ears fail to develop inflammatory reaction in response to capsaicin. *d*, Dermis; *dp*, dermal papilla; *e*, epidermis; *sg*, sebaceous gland. * $P < .05$; *** $P < .005$.

RESULTS

Unilateral surgical skin denervation results in the absence of cutaneous innervation

Unilateral surgical skin denervation of murine back and ear skin resulted in a complete loss of sensory nerves in the corresponding skin areas as assessed by immunohistochemical detection of several SN marker proteins—that is, PGP 9.5, CGRP, and SP—10 days after surgical denervation (Fig 1 and data not shown). In contrast, PGP 9.5⁺, CGRP⁺, and SP⁺ nerve fibers were present at physiological sites and numbers at the contralateral, sham-denervated skin areas (Fig 1). To test whether unilateral surgical skin denervation also results in a loss of nerve function, we treated denervated and control skin sites with capsaicin, which induces strong cutaneous neurogenic inflammation in normal skin. Notably, skin responses to capsaicin were dramatically diminished—that is, reduced to levels in vehicle treated sites—in denervated compared with innervated control skin (Fig 1).

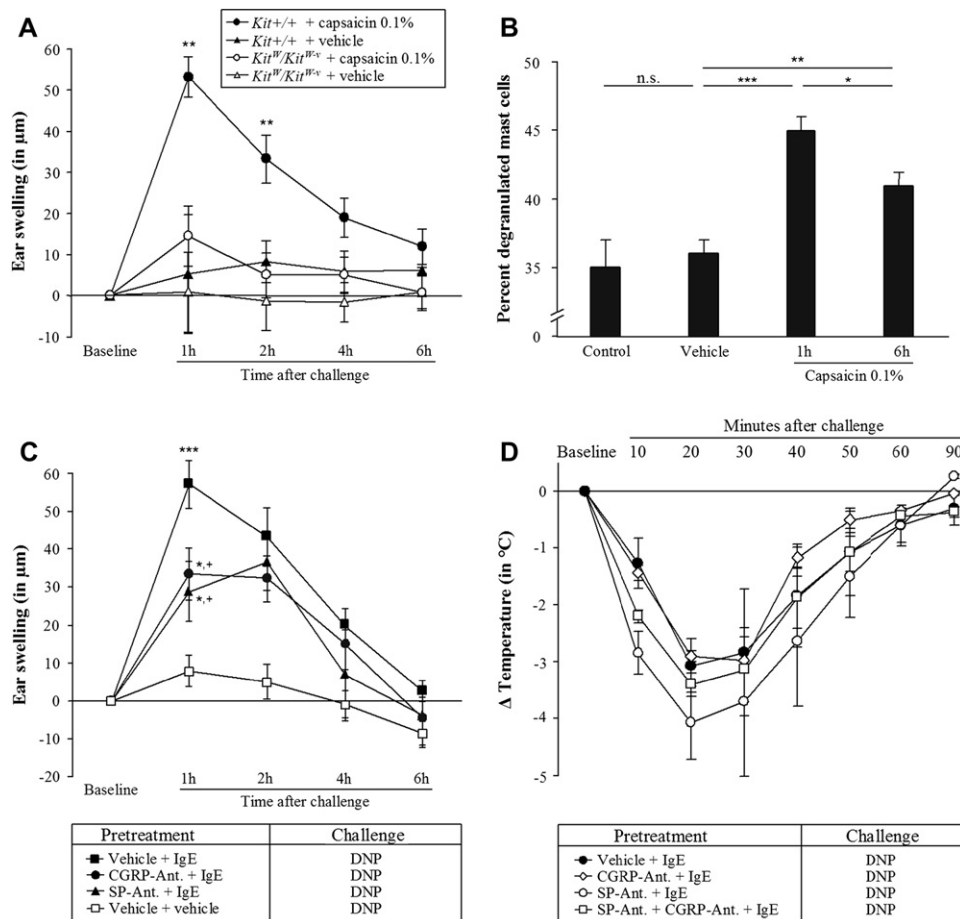
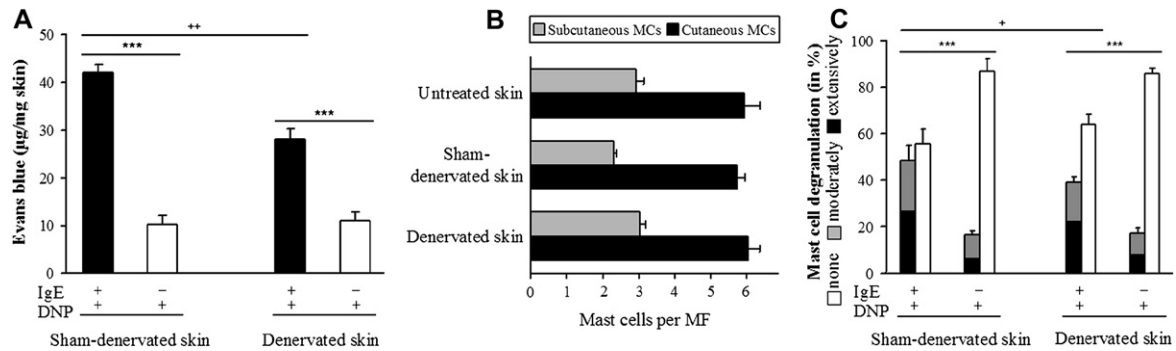
MC-driven cutaneous inflammation is impaired in the absence of sensory nerves

To evaluate the effect of SNs on MC-dependent inflammatory responses, we assessed type I allergic responses induced by

passive cutaneous anaphylaxis (PCA) in denervated and sham-denervated skin. Interestingly, skin inflammation was significantly decreased (by 26.2%; $P < .01$) in denervated compared with sham-denervated skin (Fig 2). No differences in the numbers of cutaneous or subcutaneous MCs were detected among denervated, sham-denervated, or untreated skin areas as assessed by planimetric histomorphometry. Notably, PCA induction resulted in markedly reduced MC degranulation in the absence of SNs (Fig 2), suggesting that nerve products such as neuropeptides contribute to the degranulation response of MCs after allergic activation.

Substance P and CGRP are required for normal MC-driven skin inflammation

To characterize better the mechanisms involved in the promotion of MC-mediated cutaneous inflammation by SNs, we assessed and compared capsaicin-induced skin neurogenic inflammation, which is mediated by the release of neuropeptides such as SP and CGRP from SNs, in the absence and in the presence of MCs. As expected, the application of capsaicin to the ear skin of normal *Kir*^{+/+} mice resulted in pronounced inflammatory responses—that is, ear thickening—over a period of 6 hours.



In contrast, capsaisin-induced skin inflammation was dramatically reduced in MC-deficient *Kit^W/Kit^{W-v}* mice (Fig 3, A). Significant MC degranulation was detected at capsaisin treatment sites compared with untreated or vehicle-treated control skin (Fig 3,

B), indicating that SNs may promote skin inflammation by releasing neuropeptides that function as MC secretagogues. To assess the effects of neuropeptides released from SNs on type I allergic skin inflammation, we investigated PCA reactions in mice that

had been treated with selective antagonists to SP ([D-Arg¹, D-Phe⁵, D-Trp^{7,9}, Leu¹¹]-SP), CGRP (CGRP fragment 8-37), or vehicle. Interestingly, inflammatory responses after PCA were decreased by about 30% in both (D-Arg¹, D-Phe⁵, D-Trp^{7,9}, Leu¹¹)-SP and CGRP fragment 8-37-treated mice compared with vehicle-treated control animals (Fig 3, C). Mice treated with both (D-Arg¹, D-Phe⁵, D-Trp^{7,9}, Leu¹¹)-SP and CGRP fragment 8-37 exhibited similar, but not greater, reduction of inflammation (data not shown). Most interestingly, the induction of passive systemic anaphylaxis was not impaired by the antagonism of these neuropeptides (Fig 3, D).

DISCUSSION

Several lines of independent evidence suggest a functional interaction between the immune and nervous system, which contributes to the orchestration of a variety of physiological and pathological situations. Such effects are thought to be mediated by various cell types involved in innate and adoptive immune responses that can respond to neurogenic signals. MCs are the key effector cells in type I allergic inflammation and are potent inducers of tissue inflammatory responses in innate and acquired immune responses.⁵⁴ However, detailed information on the functional relevance of neurogenic signals in MC-mediated inflammatory responses as well as on the bidirectional interaction of MCs and SNs in general is very limited. Therefore, the aim of this study was to investigate the effect of MC/SN crosstalk in MC-driven inflammatory responses to understand better how SNs control MC functions and how MCs might make use of SNs in inducing inflammation.

The model of unilateral surgical skin denervation leads to complete degeneration of cutaneous nerve fibers at treatment sites as reported previously.⁴⁹ It has recently been discussed whether the unilateral injury of nerve fibers results in bilateral loss of distal innervation even without any known anatomical connection between the neurons that innervate corresponding body sites.^{54,55} The degeneration of contralateral innervation is thought to be a creeping process induced by the loss of signal transmission from corresponding denervated areas. It has been reported that ipsilateral denervation leads to the loss of about 50% of contralateral neurites 3 weeks after surgery, with claims that corresponding contralateral areas are inappropriate controls for long-term investigations.^{55,56} According to the time points of our investigations, we still found a normal distribution of nerve fibers in corresponding sham-denervated skin areas 10 days after surgical denervation. Therefore, these skin areas were considered appropriate controls for the sets of experiments presented in this study.

The impaired inflammatory responses after induction of PCA in mice that had undergone unilateral surgical skin denervation of SNs demonstrate that neurogenic signals contribute to type I allergic reactions in mouse skin. The inflammatory response to PCA critically depends on the presence of cutaneous MCs and has been shown to be virtually absent in MC-deficient *Kit^W/Kit^{W-v}* mice.⁵⁷ Selective IgE antibodies bound to the high-affinity FcεRI on MCs recognize specific antigens, which subsequently result in MC degranulation and inflammatory response as assessed by Evans blue extravasation in the current study. Most interestingly, IgE-mediated activation of MCs was found to be markedly reduced in the absence of SNs, whereas no differences were found in MC numbers or distribution comparing denervated, sham-denervated, and untreated skin areas. These results indicate that

signals derived from SNs contribute to the extent of type I allergic skin responses and suggest that SNs augment MC-driven inflammatory responses, at least in part, by enhancing IgE-mediated MC degranulation.

Mast cells and SNs have been shown to produce and express several mediators and receptors, which allow for an intensive bidirectional interaction.^{16,40,58} For example, it has been reported that MC-derived mediators—that is, histamine—are able to activate and deplete SNs via histamine receptors expressed on SNs.⁵⁹ Recently, novel proteinase-activated receptors expressed on SNs have been described, which can be targeted by MC-derived proteases and thus contribute to neurogenic inflammation.²⁷ Whether such signals are involved in the transmission of SN-mediated effects on type I allergic responses is as yet ill defined. Therefore, the effect of SN-mediated responses on MC function was investigated by the induction of capsaicin-induced neurogenic inflammation. Neurogenic inflammation is conducted by the release of SN products—that is, neuropeptides—from SNs, leading to inflammatory responses. Most interestingly, capsaicin treatment failed to induce cutaneous inflammation in the absence of MCs, whereas the application of capsaicin was accompanied by pronounced MC activation in wild-type mice. We demonstrated that the treatment of mice with selective receptor antagonists of the neuropeptides SP or CGRP before the elicitation of MC-driven type I allergic responses results in remarkable reduction of inflammation. This effect is likely to be mediated, at least in part, by a direct effect of these neuropeptides on MCs, for example by increasing MC releasability or enhancing IgE-mediated MC degranulation.

In fact, SP and CGRP have been reported also to mediate vasoactive effects during cutaneous inflammatory responses¹⁴ by targeting their receptors expressed on endothelial cells. Therefore, neuropeptides released from SNs during IgE-mediated MC-driven responses may also augment vasodilatation and extravasation by additive effects on endothelial cells. In addition, it has been previously reported that vasoactive responses in neurogenic inflammation are controlled by neuropeptides released from both SNs and MCs.⁶⁰ This is in line with our results showing that cutaneous neurogenic inflammation is dramatically impaired in the absence of MCs. However, the treatment of mice with antagonists to SP or CGRP had no effect on the extent of inflammatory responses induced by passive systemic anaphylaxis. This demonstrates that the effect of neuropeptides in controlling inflammation is presumably a compartment specific mechanism.

In summary, these results support and extend the view of neurogenic signals involved in MC-driven inflammatory responses and suggest that the impairment of type I allergic skin responses in the absence of SNs is, at least in part, a result of the release of neuropeptides—that is, SP and CGRP—from SNs during IgE-mediated inflammatory responses. Therefore, this study demonstrates for the first time that the bidirectional crosstalk between MCs and SNs is of relevance for the induction and mediation of MC-driven type I allergic skin inflammation.

The interaction of nerve-derived signals and immune cells has been previously reported to be involved in the pathogenesis of several inflammatory and allergic diseases, such as psoriasis, prurigo nodularis, urticaria, contact hypersensitivity, atopic dermatitis, and allergic asthma.⁶¹⁻⁶⁷ More specifically, the activation of neuropeptide-releasing nerve fibers by MCs and vice versa has been implicated in aggravating cutaneous pruritogenic inflammation as well as bronchial hyperreactivity, hypersecretion, eosinophilia, and tissue remodeling.⁶⁸⁻⁷⁴

Further detailed characterization of the complex interactions between SNs and MCs will increase understanding of the pathology of several inflammatory and allergic diseases that involve both MC-driven and SN-mediated signals. Targeting the interactions between SNs and MCs ideally will lead to the development of novel therapeutic strategies.

We thank S. Dinges and S. Lescau for excellent technical support and J. Urcioli for proofreading the manuscript.

Clinical implications: Interactions between cutaneous SNs and MCs are promising targets of novel treatment approaches for allergic skin conditions such as atopic dermatitis or urticaria.

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