



Pituitary adenylate cyclase-activating polypeptide promotes cutaneous dendritic cell functions in contact hypersensitivity

Yasuo Yamamoto, MS,^{a,b} Atsushi Otsuka, MD, PhD,^{a,c} Yoshihiro Ishida, MD, PhD,^a Lai San Wong, MD,^{a,d} Judith A. Seidel, PhD,^a Yumi Nonomura, MD, PhD,^a Chisa Nakashima, MD, PhD,^a Saeko Nakajima, MD, PhD,^a Akihiko Kitoh, MD, PhD,^a Takashi Nomura, MD, PhD,^a Teruki Dainichi, MD, PhD,^a Tetsuya Honda, MD, PhD,^a Wataru Amano, DVM, PhD,^{a,b} Noriko Konishi, PhD,^b Mikio Hayashi, PhD,^b Mutsuyoshi Matsushita, PhD,^b and Kenji Kabashima, MD, PhD^{a,e} *Kyoto and Takatsuki, Japan; Kaohsiung, Taiwan; and Biopolis, Singapore*

Background: Sensory nerves regulate cutaneous local inflammation indirectly through induction of pruritus and directly by acting on local immune cells. The underlying mechanisms for how sensory nerves influence cutaneous acquired immune responses remain to be clarified.

Objective: This study aimed to explore the effect of peripheral nerves on cutaneous immune cells in cutaneous acquired immune responses.

Methods: We analyzed contact hypersensitivity (CHS) responses as a murine model of delayed-type hypersensitivity in absence or presence of resiniferatoxin-induced sensory nerve denervation. We conducted ear thickness measurements, flow cytometric analyses, and mRNA expression analyses in CHS.

Results: CHS responses were attenuated in mice that were denervated during the sensitization phase of CHS. By screening neuropeptides, we found that pituitary adenylate cyclase-activating polypeptide (PACAP) mRNA expression was decreased in the dorsal root ganglia after denervation.

Administration of PACAP restored attenuated CHS response in resiniferatoxin-treated mice, and pharmacological inhibition of PACAP suppressed CHS. Flow cytometric analysis of skin-draining lymph nodes showed that cutaneous dendritic cell migration and maturation were reduced in both denervated mice and PACAP antagonist-treated mice. The expression of chemokine receptors CCR7 and CXCR4 of dendritic cells was enhanced by addition of PACAP *in vitro*.

Conclusion: These findings indicate that a neuropeptide PACAP promotes the development of CHS responses by inducing cutaneous dendritic cell functions during the sensitization phase. (*J Allergy Clin Immunol* 2021;148:858-66.)

Key words: PACAP, denervation, dendritic cells, contact hypersensitivity, resiniferatoxin, neuropeptides, peripheral sensory nerves

Contact hypersensitivity (CHS) has been widely used to study cutaneous acquired immune responses as a mouse model of human allergic contact dermatitis. CHS is mediated by antigen-specific T cells and consists of sensitization and elicitation phases.¹⁻³ During the sensitization phase of CHS, a hapten is acquired by cutaneous dendritic cells (DCs), which then migrate to skin-draining lymph nodes (LNs) to present the antigen to naive T cells.^{4,5} In the subsequent elicitation phase, reexposure to the cognate hapten results in the recruitment of antigen-specific T cells and other non-antigen-specific leukocytes, resulting in local inflammation.³

The skin is interwoven with a meshwork of peripheral nerves, and skin sensations such as pain and pruritus are transmitted by nociceptive sensory nerves. Single-cell RNA sequencing analysis decomposed dorsal root ganglion (DRG) neurons into multiple subtypes,⁶ including nonpeptidergic and peptidergic nociceptor clusters, which express nociceptors such as the transient receptor

From ^athe Department of Dermatology, and ^cthe Translational Research Department for Skin and Brain Diseases, Kyoto University Graduate School of Medicine; ^bthe Central Pharmaceutical Research Institute, Japan Tobacco, Takatsuki; ^dthe Department of Dermatology, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine; and ^ethe Singapore Immunology Network and Skin Research Institute of Singapore, Technology and Research, Biopolis.

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare of Japan (to K.K.) and the Platform Project for Supporting Drug Discovery and Life Science Research (Platform for Drug Discovery, Informatics, and Structural Life Science) from the Japan Agency for Medical Research and Development (AMED) under grant JP19gm1210006h0001 (to A.O. and K.K.), and a research grant from Japan Tobacco Inc (to K.K.).

Disclosure of potential conflict of interest: Y. Yamamoto, W. Amano, N. Konishi, M. Hayashi, and M. Matsushita are employed by Japan Tobacco. A. Otsuka has received honoraria from Ono Pharmaceutical; and a research grant from Eisai. Y. Nonomura has received honoraria from Novartis. S. Nakajima has received a research grant from Toyota. A. Kitoh has received honoraria from Novartis, Sanofi, Chugai Pharmaceutical, Kyowa Hakko Kirin, and Tanabe Mitsubishi. T. Dainichi has received a research grant from Rohto Pharmaceutical. T. Honda has received honoraria from Eli Lilly

Japan, Janssen Pharmaceutical, Taiho Pharmaceutical, and Celgene; and research grants from Eisai, Novartis, Kyowa Hakko Kirin, Maruho, AbbVie, Sanofi, Torii Pharmaceutical, and Tanabe Mitsubishi. K. Kabashima has received research consulting fees or advisory board honoraria from Chugai Pharmaceutical, Japan Tobacco, Maruho, and Pola Pharma; and research grants from AbbVie, Eli Lilly Japan, Japan Tobacco, Kyorin Pharmaceutical, Kyowa Hakko Kirin, LEO Pharma, Ono Pharmaceutical, P&G Japan, Pola Pharma, Sanofi, and Tanabe Mitsubishi. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication September 5, 2020; revised January 17, 2021; accepted for publication February 5, 2021.

Available online February 18, 2021.

Corresponding authors: Atsushi Otsuka, MD, PhD, or Kenji Kabashima, MD, PhD, Department of Dermatology, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawara-cho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: otsukamn@kuhp.kyoto-u.ac.jp. Or: kaba@kuhp.kyoto-u.ac.jp.

The CrossMark symbol notifies online readers when updates have been made to the article such as errata or minor corrections

0091-6749/\$36.00

© 2021 American Academy of Allergy, Asthma & Immunology

<https://doi.org/10.1016/j.jaci.2021.02.005>

Abbreviations used

BMDC:	Bone marrow-derived dendritic cell
CGRP:	Calcitonin gene-related peptide
CHS:	Contact hypersensitivity
DC:	Dendritic cell
DNFB:	2,4-Dinitrofluorobenzene
DT:	Diphtheria toxin
DRG:	Dorsal root ganglion
FITC:	Fluorescein isothiocyanate
KikGR:	Kikume Green-Red
LN:	Lymph node
PACAP:	Pituitary adenylate cyclase-activating polypeptide
RTX:	Resiniferatoxin
T _C 1:	T cytotoxic 1 cell
TCR:	T-cell receptor
TRPA1:	Transient receptor potential ankyrin 1
TRPV1:	Transient receptor potential vanilloid 1

potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1).⁶ Several recent studies have reported that nociceptive sensory nerves also play an essential role in cutaneous immunity.⁷⁻⁹ Riol-Blanco et al⁸ demonstrated that DCs reside in close proximity to peripheral nerves in the skin, and that TRPV1⁺ nociceptive sensory nerves interact with DCs to regulate the iIL-23/IL-17 pathway in a psoriasis-like dermatitis model.⁸ Consistently, denervation is found to reduce disease severity in patients with psoriasis.¹⁰

Several neuropeptides are released from TRPV1⁺ neurons and DRG neurons^{11,12} and influence skin immune cell functions and vascular permeability.¹³ A neuropeptide calcitonin gene-related peptide (CGRP) is known to act upstream of dermal DCs to drive IL-23 expression during *Candida albicans* infection in cutaneous innate immunity,⁹ but inhibit maturation of dermal DCs and their migration to LNs in cutaneous acquired immunity.¹⁴ Another neuropeptide, substance P, is also implicated in enhancing DC antigen presentation, leading to the development of CHS responses.¹⁵ Although these studies suggested that TRPV1⁺ nociceptive sensory nerves were involved in cutaneous acquired immunity, the precise roles and underlying mechanism of sensory nerves in cutaneous acquired immunity remain to be further clarified.

To this end, we used resiniferatoxin (RTX), which specifically ablates TRPV1⁺ nerves by promoting continuous activation of TRPV1, to generate chemically denervated mice and asked whether CHS responses were altered in denervated mice. We found that CHS responses were attenuated in mice denervated prior to sensitization, but not in mice denervated during elicitation. Comprehensive mRNA expression analyses in the DRG revealed that the mRNA expression level of pituitary adenylate cyclase-activating polypeptide (PACAP) was decreased by RTX-induced denervation and increased after hapten application. In addition, PACAP enhanced DC functions *in vivo* and increased the expression of chemokine receptors, essential for DC migration, *in vitro*. These findings indicate that the PACAP neuropeptide may be important for initiating CHS responses by promoting DC functions.

METHODS

Animals

BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). Kikume Green-Red (KikGR)-transgenic mice on a C57BL/6 background,¹⁶ CD11c–diphtheria toxin (DT) receptor mice (CD11c–DTR mice) on a C57BL/6 background,¹⁷ and CD11c–yellow fluorescent protein mice on a C57BL/6 background¹⁸ were generated as described. Female mice ages 4 to 10 weeks were used in all experiments and were bred in the specific pathogen-free facilities at Kyoto University. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Kyoto University Graduate School of Medicine.

Reagents and antibodies

We purchased capsaicin and oxazolone from Wako Pure Chemical Industries (Osaka, Japan); RTX from Alomone Labs (Jerusalem, Israel); 2,4-dinitrofluorobenzene (DNFB), DT, and fluorescein isothiocyanate (FITC) from Sigma-Aldrich (St Louis, Mo); and PACAP, PACAP6-38, and substance P from Peptide Institute (Osaka, Japan). A list of the antibodies used is shown in Table E1 in this article's Online Repository (available at www.jacionline.org). For flow cytometry analysis, an anti-PACAP receptor antibody was purified using IgG Purification Kit-G (Dojindo, Kumamoto, Japan) and labeled with Allophycocyanin Labeling Kit-NH₂ (Dojindo).

Pharmacological denervation

We treated mice with 30, 70, or 100 μ L/kg ethanol as a control for RTX treatment. For RTX treatment, 4-week-old female mice were injected subcutaneously with 30, 70, or 100 μ g/kg of RTX on 3 consecutive days and allowed to rest for at least 3 weeks before experiments. The effect of RTX treatment was confirmed by the absence of wiping movements in response to administration of 0.1% (wt/vol) capsaicin on the eyes.

Hapten-induced CHS model and adoptive transfer

Mice were sensitized by application of 0.5% (wt/vol) DNFB in 4:1 acetone/olive oil (vol/vol), 25 μ L of 2% FITC in 1:1 acetone/dibutyl phthalate (vol/vol), 25 μ L of 3% (wt/vol) oxazolone in ethanol to their shaved abdomens, or by application of 20 μ L of 3% (wt/vol) oxazolone in ethanol to both sides of the ear. The mice were challenged on both sides of each ear with 0.3% (wt/vol) DNFB, 0.5% (wt/vol) FITC, 0.6% (wt/vol) oxazolone at 5 days after sensitization. PACAP (500 ng/mouse/day in PBS), PACAP6-38 (500 ng/mouse/day in PBS), substance P (500 ng/mouse/day in PBS), or PBS were injected intradermally into the abdominal skin on the same day and at 1 and 2 days after sensitization. Ear thickness was measured before and 24 hours after the challenge using a thickness gauge (Teclock, Nagano, Japan). For adoptive transfer experiments, single-cell suspensions were prepared from axillary LNs of mice at 5 days after 3% (wt/vol) oxazolone sensitization. Whole LN cells from 1 mouse were injected intravenously into a naive recipient mouse and 1 hour later, the mice were challenged with 10 μ L of 1% (wt/vol) oxazolone.

RESULTS

Denervation of TRPV1⁺ nociceptors suppresses CHS responses

To investigate the role of nociceptors in cutaneous acquired immune responses, mice were treated systemically with RTX to ablate TRPV1⁺ nociceptors.^{8,9} RTX-treated mice exhibited a lack of nocifensive responses to capsaicin administration, confirming the effective ablation of their TRPV1⁺ nociceptors (see Fig E1 in this article's Online Repository at www.jacionline.org). We observed that CHS responses induced by oxazolone were attenuated after RTX treatment (Fig 1, A and B). Attenuated CHS responses were also observed in RTX-treated mice using 2 alternative haptens, DNFB and FITC, indicating that this

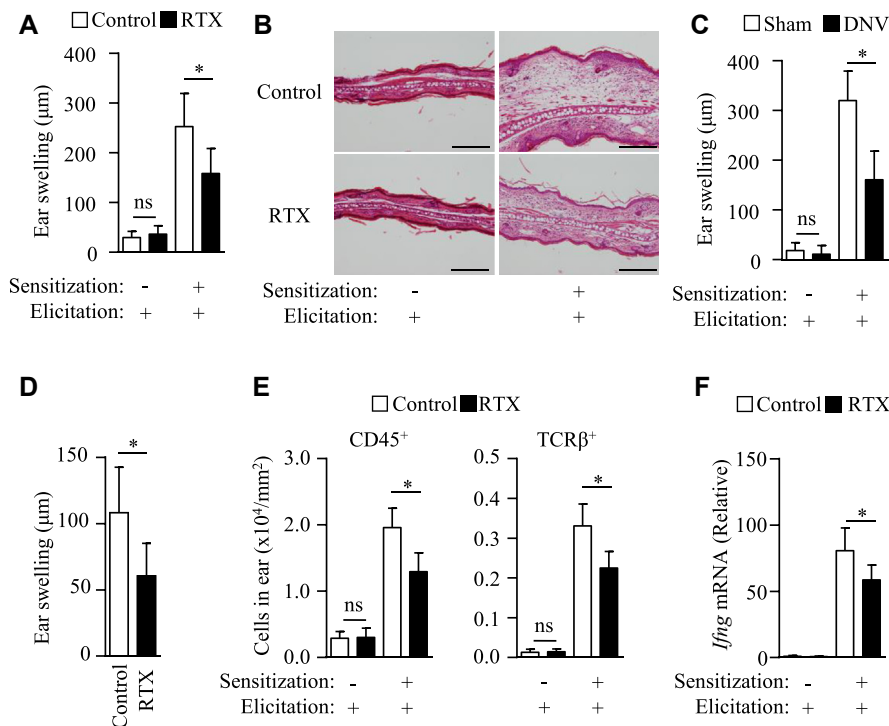


FIG 1. RTX-induced denervation impaired CHS responses. **A**, Ear swelling in RTX-treated and control mice ($n = 5$ -10 per group) at 24 hours after elicitation with or without sensitization. **B**, Histological analysis of the skin at 24 hours after elicitation with or without sensitization. Bars = 200 μ m. **C**, Ear swelling at 24 hours after challenge with oxazolone in mice that underwent surgical denervation (DNV) or sham operation on the ears ($n = 8$ per group). **D**, Ear swelling in RTX-treated or control mice fitted with Elizabethan collars ($n = 5$ per group) after elicitation with oxazolone at 48 hours. **E**, Cell densities of CD45⁺ cells and TCRβ⁺ cells in skin tissue at 24 hours with or without oxazolone sensitization or elicitation ($n = 4$ -8 per group). **F**, *Ifng* mRNA expression in whole skin tissue ($n = 8$ per group) at 24 hours after challenge with oxazolone. All data are presented as mean $\pm$ SD. * $P < .05$ (Student *t*-test or Welch test) versus corresponding mice. *ns*, Not significant.

phenomena is not restricted to oxazolone (see Fig E2 in this article's Online Repository at www.jacionline.org). To evaluate whether sensory nerves are directly involved in CHS responses, we surgically disrupted cutaneous nerves of the ear,^{19,20} and the denervation was confirmed by the attenuation of ear swelling after capsaicin application (see Fig E3 in this article's Online Repository at www.jacionline.org).²⁰ We found that the CHS response was decreased in surgically denervated mice (Fig 1, C). Of note, denervation is also known to suppress dermatitis via inhibition of scratching behavior in a murine model of atopic dermatitis.²¹ Therefore, to exclude the possibility of behavior alteration, we repeated the CHS experiment in surgically denervated mice fitted with Elizabethan collars, which physically prevent mice from scratching the ears,²² and found that the ear swelling remained attenuated in the absence of scratching (Fig 1, D).

Next, we analyzed how TRPV1⁺ nociceptive sensory nerves affect cutaneous immune responses in this model. Flow cytometric analysis revealed that RTX treatment reduced the numbers of CD45⁺ hematopoietic cells and T-cell receptor (TCR) β⁺ cells in the ears after CHS (Fig 1, E). In addition, RTX treatment reduced IFN-γ, a key cytokine in CHS, which is produced by CD4⁺ T_H1 and CD8⁺ T cytotoxic 1 (T_C1) cells²³ (Fig 1, F). In contrast, RTX treatment did not affect the expression of T_H2/T_H17-cell cytokines or TNF (see Fig E4 in this article's Online Repository at www.jacionline.org). Taken together, these results suggest that

TRPV1⁺ nociceptive sensory nerves are involved in the induction of T_H1/T_C1 in CHS responses.

Denervation acts in the sensitization phase of CHS responses

To investigate whether sensory nerves affect either the sensitization or elicitation phase of CHS responses, we severed the nerve innervating either side of the ear, sensitized the mice with hapten on the left ear, and elicited on the right ear at 5 days after sensitization. CHS response was suppressed when the sensitization site was denervated (Fig 2, A).

Next, to verify whether this suppression is due to ablation of TRPV1⁺ nerves, draining LN cells from RTX-treated mice sensitized with oxazolone were adoptively transferred into naive mice that were subsequently challenged with oxazolone to develop CHS. CHS responses were sufficiently induced in RTX-treated and control recipient mice that received LN cells from sensitized mice. In contrast, control and RTX-treated mice that received LN cells from RTX-treated sensitized mice showed reduced responses (Fig 2, B). These results indicate that TRPV1⁺ nociceptive sensory nerves modulate CHS responses during the sensitization phase. To further examine whether denervation affects immune responses in the elicitation phase of CHS, we applied RTX denervation at 5 days after sensitization (when sensitization is established) and elicited CHS. We found that

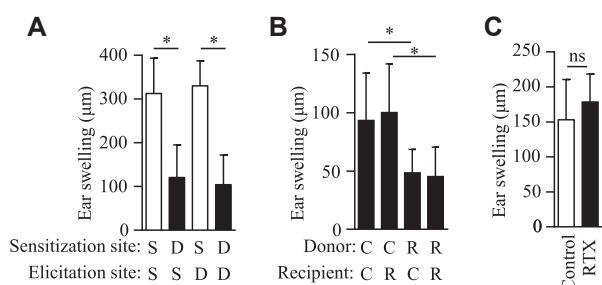


FIG 2. RTX treatment attenuated the sensitization phase in CHS. **A**, Effect of surgical denervation (D) on the sensitization and elicitation sites. Ear swelling in surgically denervated or sham-operated (S) ear ($n = 8$ per group) at 24 hours after challenge with oxazolone at surgically denervated or sham-operated ear. **B**, Ear swelling in RTX-treated mice (R) or control mice (C) ($n = 8$ per group) inoculated with LN cells from donor mice treated with or without RTX, assessed at 24 hours after application of oxazolone to the recipients. **C**, Effect of RTX treatment on elicitation. Ear swelling at 6 hours after elicitation with oxazolone in mice ($n = 8-9$ per group) treated with (RTX) or without (control) RTX during the elicitation phase. Data are presented as mean $\pm$ SD. * $P < .05$ (Student *t*-test or Dunnett test) versus corresponding mice.

the CHS responses were not impaired by RTX denervation after sensitization (Fig 2, C). These results indicate that TRPV1⁺ nociceptive sensory nerves are involved in the establishment of the sensitization phase but not the elicitation phase of CHS.

Cutaneous DC migration to skin-draining LNs is impaired in RTX-treated mice

To further investigate how TRPV1⁺ nociceptive sensory nerves modulate CHS responses, we first analyzed skin-draining LN cells at 3 days after sensitization. The numbers of B220⁺ B cells, TCR β ⁺ T cells, CD44⁺ CD62L⁺ central memory CD4⁺ and CD8⁺ T cells, and CD44⁺ CD62L⁻ effector memory CD4⁺ and CD8⁺ T cells in the skin-draining LNs of RTX-treated mice were lower than those in control mice (Fig 3, A). To evaluate the IFN- γ productivity of antigen-specific LN cells, the skin-draining LN cells from sensitized RTX-treated and control mice were restimulated with oxazolone *in vitro*. The levels of IFN- γ in the culture supernatants in the presence of oxazolone were markedly reduced in the LN cells with RTX treatment (Fig 3, B). These results indicate that antigen-specific T_H1/T_C1 induction in the skin draining LNs is impaired by RTX treatment during sensitization.

The essential steps in the sensitization phase of CHS include activation of keratinocytes, maturation and migration of hapten-bearing cutaneous DCs into skin-draining LNs, and antigen presentation to naive T cells by the mature DCs in the LNs.³ First, we investigated the influence of RTX-induced denervation on the initial local immunological event by measuring the whole skin mRNA expression of *Il1a*, *Il1b*, and *Tnf*, which were reported to be important for the initiation of CHS.³ Contrary to our expectations, the expression of these molecules was increased in RTX-treated mice; therefore the enhanced immunological effects of denervation cannot explain the reduction in CHS on RTX-induced denervation (Fig E5).

We next evaluated the effect of RTX-induced denervation on DC functions. We evaluated the number of cutaneous DCs in the skin-draining LNs at 2 days after oxazolone application and found that the number of cutaneous DCs migrating from the skin to the

draining LNs was significantly decreased by RTX treatment (Fig 3, C, and see Fig E6 in this article's Online Repository at www.jacionline.org). It has been reported that migrated mature cutaneous DCs tend to die via apoptosis at about 48 hours after arriving in the draining LNs.²⁴⁻²⁶ Therefore, we investigated the possibility that RTX treatment may affect the survival of migrated DCs in the draining LNs. We analyzed the viability of migrated DCs using viability dye and annexin V staining by flow cytometry and calculated the percentage of survival cells, early apoptotic cells, and late apoptotic/necrotic cells. We found no differences in the survival rates between RTX-treated and control mice (Fig E6).

To evaluate whether denervation caused a reduction in DC migration, we applied a photolabeling system using KikGR transgenic mice.¹ KikGR is a photoconvertible fluorescence protein that changes from green (KikGR-green) to red (KikGR-red) on exposure to 436 nm violet light for 10 minutes. Following this noninvasive labeling, oxazolone was applied to the photoconverted abdomens and the draining LNs were harvested to quantify photoconverted cells that had migrated from the skin. We found that the number of migrated cutaneous KikGR-red⁺ DCs in the draining LNs was decreased by RTX treatment. In addition, the expression levels of the costimulatory molecules CD80 and CD86 on migrated DCs in RTX-treated mice were lower than those in control mice (Fig 3, D). Moreover, we analyzed the migration of hapten-bearing DCs using a FITC-induced cutaneous DC migration model.^{27,28} The number of FITC⁺ CD11c⁺ migrated DCs from the skin to the draining LNs was similarly decreased in RTX-treated mice compared with control mice (Fig 3, E). These results suggest that TRPV1⁺ nociceptive sensory nerves promote the migration and maturation of cutaneous DCs, and thereby enhance T-cell stimulating capacity and T-cell responses in the draining LNs.

PACAP modulates DC migration

Because cutaneous DC migration on hapten sensitization was affected by RTX-induced denervation, we sought to characterize the spatial relationship between DCs and TRPV1⁺ nociceptive sensory nerves in the skin. As reported previously,⁸ 2-photon microscopy for whole-mount skin imaging revealed that CD11c⁺ DCs were in either direct contact or close proximity to protein gene product 9.5 (PGP9.5⁺) peripheral nerves at the steady state (Fig 4, A, and see Video E1 in this article's Online Repository at www.jacionline.org).

Peripheral nerves can affect surrounding cells by releasing neuropeptides and/or growth factors.²⁹ We hypothesized that these factors from peripheral nerves may modulate DC functions. To address this hypothesis, we sought to compare the mRNA expression levels of growth factors and neuropeptides in DRGs extracted from both denervated and control mice. Initially, we validated the mRNA expression levels in the DRGs by evaluating the expression of the neuronal receptors targeted by RTX. We confirmed that the mRNA expression of *Trpv1*, *Trpa1*, and *Trpm8* was decreased in the DRG on denervation with RTX (see Fig E7 in this article's Online Repository at www.jacionline.org) as reported previously.⁸ We also screened for 16 neuropeptides (Fig 4, B) and 5 nerve-related growth factors (Fig 4, C), which were reported to be expressed by DRGs,^{9,30} and found that the mRNA levels of *Tac1* (substance P) and *Adcyap1* (PACAP) were significantly decreased in the DRG after

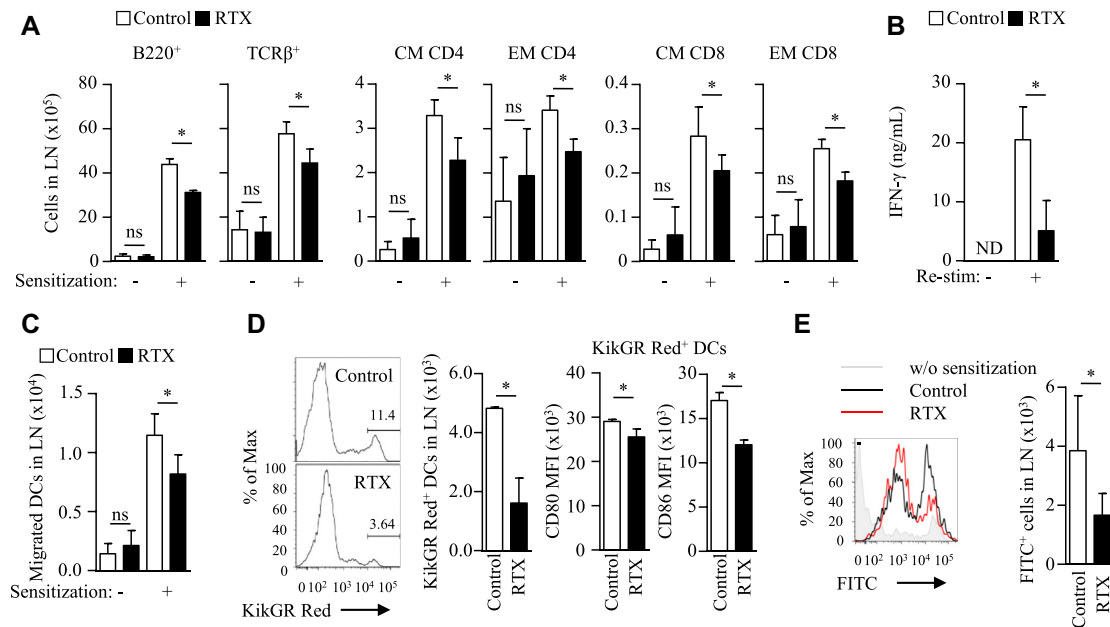


FIG 3. RTX treatment suppressed DC migration and maturation. **A**, Numbers of cells in axillary LNs ($n = 6$ per group) on day 3 with or without sensitization with oxazolone. **B**, IFN- γ production in culture supernatants of LN cells ($n = 6$ per group) after 72 hours of culture. **C**, Migrated DC numbers on day 2 with or without sensitization with oxazolone ($n = 8$ per group). **D**, Representative flow cytometric histograms of KikGR-red expression on migrated DCs. The numbers of KikGR-red⁺ DCs and the expression of CD80 and CD86 on day 2 after sensitization with oxazolone ($n = 3$ per group). **E**, Representative flow cytometric histograms of FITC⁺ cells in migrated DCs and numbers of FITC⁺ cells from control or RTX-treated mice ($n = 5$ per group) at 48 hours after sensitization with FITC. Data are presented as mean $\pm$ SD. * $P < .05$ (Student t -test or Welch test) versus corresponding mice. CM, CD44⁺CD62L⁺ central memory cells; EM, CD44⁺CD62L⁺ effector memory cells; ND, not detected; Restim, re-stimulation.

denervation (Fig 4, B). On the other hand, mRNA expression levels of growth factors and other neuropeptides, including *Ngf*, *Bdnf*, *Gdnf*, *Ntf3*, *Ntf5*, *Calca*, *Vip*, *Sst1*, *Npy*, *Cck*, *Nmb*, *Nmu*, *Nms*, *Tac2*, *Tac4*, *Nts*, *Grp*, *Gal*, and *Galp*, were unchanged after RTX treatment (Fig 4, B and C). In addition, the *Tac1* and *Adcyap1* mRNA expression levels in the DRG were increased by hapten application (Fig 4, D), suggesting that substance P and/or PACAP may play a role in CHS responses.

To determine the effect of substance P and PACAP on the CHS responses, substance P and PACAP were intradermally administered to the sensitization site of RTX-treated mice. PACAP rescued the attenuated CHS responses after denervation, while substance P did not (Fig 4, E). Consistently, administration of *Adcyap1* and *Vipr2* PACAP receptor antagonist, PACAP6-38,³¹ reduced the CHS responses to a similar extent to those in mice denervated with RTX (Fig 4, F). In addition, using KikGR-transgenic mice, we demonstrated that the PACAP antagonist reduced the DC migration from the skin to draining LNs, and that the PACAP administration restored DC migration from the skin to the draining LNs after denervation during CHS (Fig 4, G). Finally, we evaluated the presence or absence of the PACAP receptors, *Adcyap1* and *Vipr2*, on CD4⁺ T cells (TCRβ⁺, CD4⁺), CD8⁺ T cells (TCRβ⁺, CD8⁺), B cells (B220⁺) in the LNs, and skin-derived DCs. We found that these receptors were expressed on DCs (see Fig E8 in this article's Online Repository at www.jacionline.org). Taken together, these results suggest that PACAP modulates DC functions in CHS.

PACAP directly upregulates chemokine receptors responsible for DC migration

Next, we evaluated the effect of the DRG on the maturation of DCs *in vitro*. We found that coculture of Flt3L-induced bone marrow-derived DCs (BMDCs) with the DRG from control mice, but not with the DRG from RTX-treated mice, caused significant increases in the expression levels of costimulatory molecules CD80 and CD86 on BMDCs (Fig 5, A). The enhancement of costimulatory molecule expression of BMDCs by DRG was inhibited by the PACAP antagonist PACAP6-38 (Fig 5, A).

It is known that the expressions of IL-1 receptor and TNF receptor on DCs are essential for their maturation in CHS, and CCR7 and CXCR4 are essential for their migration to the draining LNs.^{4,32-34} Thus, we evaluated the expression levels of these genes and found that PACAP treatment increased the mRNA expression levels of *Ccr7*, *Cxcr4*, *Il1r1*, *Il1rap*, and *Tnfrsf1a* (Fig 5, B). Furthermore, we performed transwell migration assays to evaluate the effect of PACAP on BMDC migration toward the CCR7 ligand CCL21 or CXCR4 ligand CXCL12. PACAP treatment induced significant increases in DC migration toward to these chemokines (Fig 5, C). In addition, we evaluated the chemokinetic and chemotactic effects of PACAP on BMDCs. BMDCs did not show increased chemokinesis on PACAP administration and were not attracted toward PACAP (ie, chemotaxis) (see Fig E9 in this article's Online Repository at www.jacionline.org), suggesting that PACAP does not have chemokinetic or chemotactic activities.

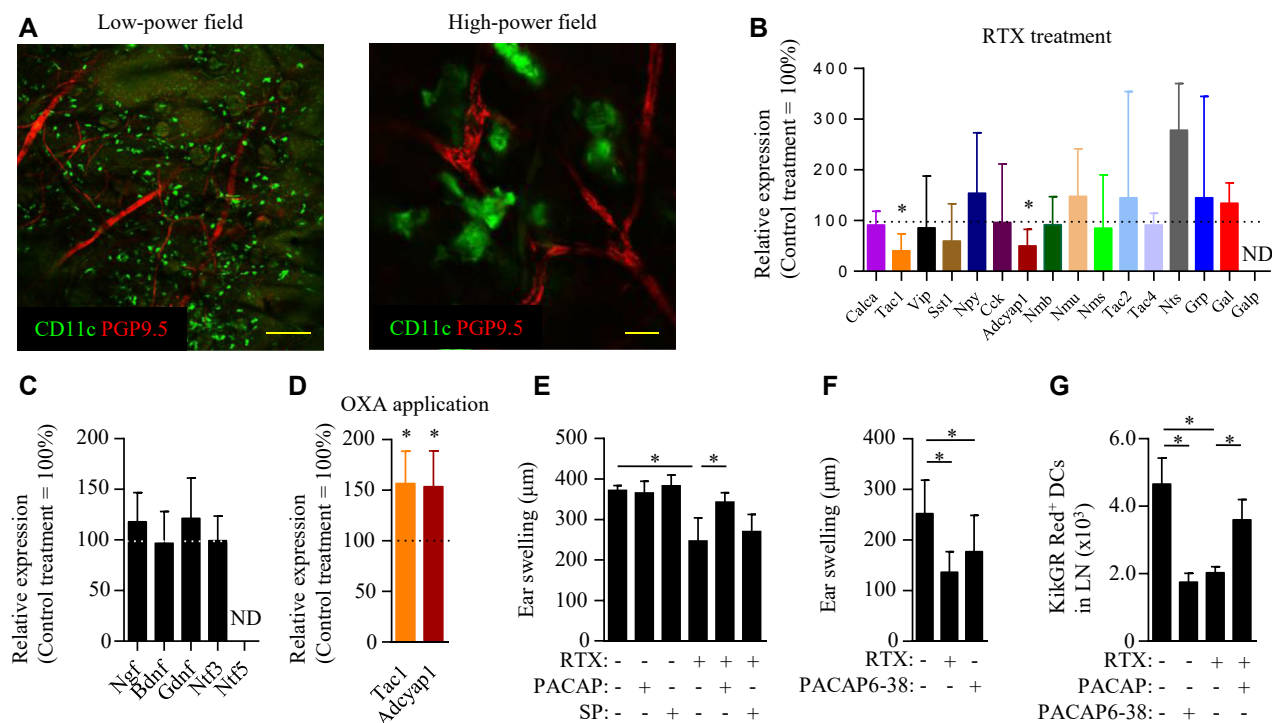


FIG 4. PACAP neuropeptide rescued hapten-induced DC migration and CHS responses impaired by RTX-induced denervation. **A**, Representative confocal micrographs of skin samples stained for protein gene product 9.5 (PGP9.5) (red) in CD11c–yellow fluorescent protein (green) mice. Bars = 50 μm (low-power field) and 10 μm (high-power field). **B**, Relative neuropeptide mRNA expression levels in DRGs from RTX-treated mice (n = 8 per group). **C**, Relative nerve-related growth factor mRNA expression levels in DRGs from RTX-treated mice (n = 8 per group). **D**, *Tac1* and *Adcyap1* expression in DRGs after oxazolone (OXA) application compared with control mice (n = 8 per group). **E**, Effect of substance P (SP) and PACAP treatment on reduced ear swelling by RTX treatment at 24 hours after OXA elicitation (n = 5–10 per group). **F**, Effect of PACAP antagonist PACAP6-38 on CHS responses at 24 hours after OXA elicitation (n = 8 per group). **G**, Migration of DCs derived from mice after single or combined treatment with RTX, PACAP, or PACAP6-38 at 24 hours before OXA application (n = 3 per group). All data are presented as mean ± SD. Dotted horizontal lines indicate control treatment set at 100%. **P* < .05 (Student *t*-test, Welch test, or Dunnett test) versus corresponding sample.

PACAP signaling pathway is known to be mediated through G protein-coupled receptors.^{35,36} Because all PACAP receptors have Gs signaling downstream, we investigated whether Gs signaling was essential in the promotion of DC migration induced by PACAP. Cyclic adenosine monophosphate-protein kinase A inhibitor H-89 did not affect the chemotaxis of DCs toward CCL21 and CXCL12 (see Fig E10 in this article's Online Repository at www.jacionline.org); however, the enhanced DC migration by PACAP toward CCL21 and CXCL12 was significantly suppressed by H-89 (Fig 5, D).³⁷ H-89 also inhibited the PACAP-induced increase in CD80 and CD86 expression and gene expression changes of CCR7 and CXCR4 (Fig 5, E, and see Fig E11 in this article's Online Repository at www.jacionline.org). These results suggest that PACAP promotes the migration of DCs by upregulating chemokine receptors via Gs signaling pathway.

PACAP exerts its effects via *Adcyap1r1*

To determine whether *Adcyap1r1* or *Vipr2* receptor affects DCs, we used BMDCs that lack *Adcyap1r1* or *Vipr2* receptor using small, interfering RNA. First, we confirmed that the expression of *Adcyap1r1* and *Vipr2* receptors were

downregulated in BMDCs (Fig 6, A). Next, we demonstrated that the increased mRNA expression levels of *Ccr7* and *Cxcr4* by PACAP treatment was reproduced in *Vipr2*-knockdown BMDCs but not in *Adcyap1r1*-knockdown BMDCs (Fig 6, B). These results suggest that *Adcyap1r1* is a main receptor of PACAP to modulate DC functions.

To address whether *Adcyap1r1* on DCs play an essential role in the CHS response, we transferred *Adcyap1r1*-knockdown BMDCs into CD11c-DTR mice, which can be depleted of DCs on DT treatment.¹⁷ Intradermal administration of DT reduced the number of CD11c-positive cells in the skin by about 50% as reported previously³⁸ (see Fig E12 in this article's Online Repository at www.jacionline.org). BMDCs were transferred into DT-treated CD11c-DTR mice, sensitized with oxazolone, and elicited with oxazolone 5 days later. In CD11c-DTR mice, intradermal administration of DT reduced the CHS response. Control negative small, interfering RNA-treated BMDCs transfer to DT-treated CD11c-DTR mice recovered CHS response, but this recovery was not fully achieved by transfer of *Adcyap1r1*-knockdown BMDCs (Fig 6, C). Finally, we compared the expression levels of PACAP receptors in migrating DCs in the LNs of RTX-treated mice and control-treated mice. The results showed that the expression level of *Adcyap1r1* was significantly decreased

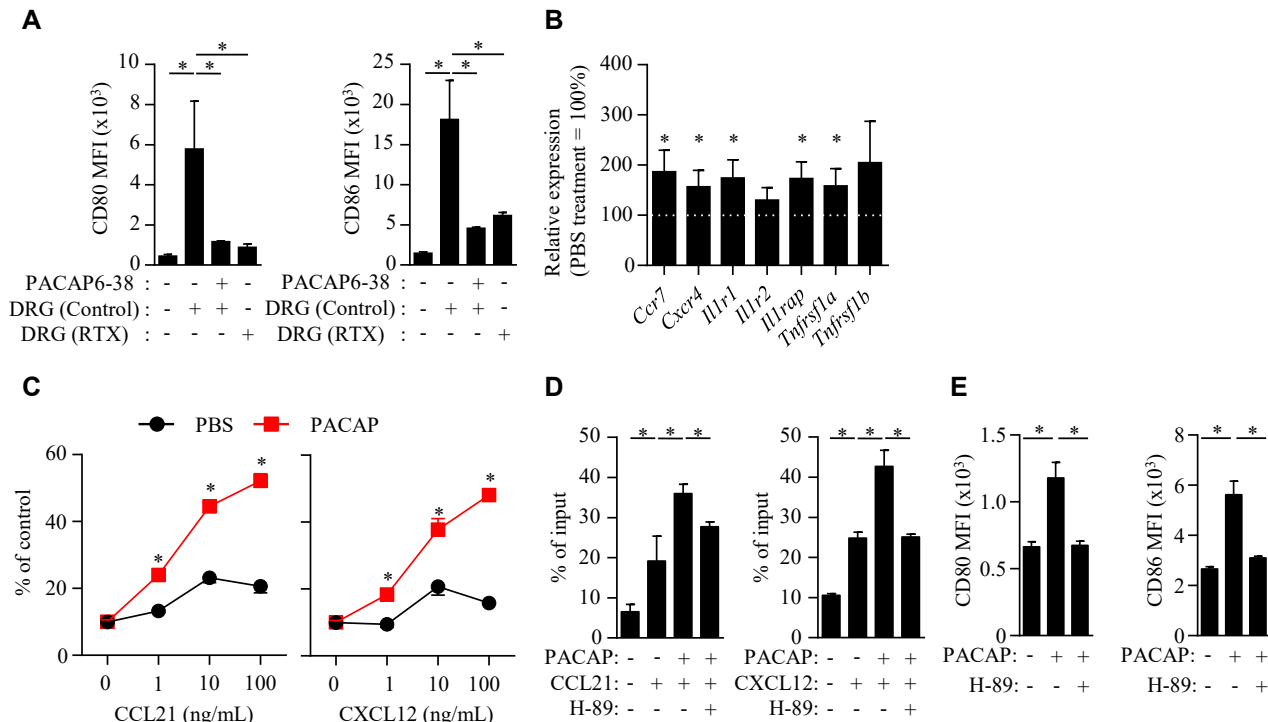


FIG 5. PACAP enhances BMDC maturation and drives BMDC migration. **A**, Expression of CD80 and CD86 on BMDCs cocultured with DRGs from RTX-treated or control mice in the presence or absence of PACAP antagonist PACAP6-38 at 10 μ mol/L ($n = 6$ per group). **B**, Relative mRNA expression in 1 μ mol/L PACAP-treated BMDCs ($n = 4$ per group). Dotted horizontal lines indicate PBS treatment set at 100%. **C**, Transwell migration assay for BMDCs. The numbers of PACAP- or PBS-treated DCs that migrated into the lower chamber containing 1, 10, or 100 ng/mL CCL21, CXCL12, or PBS were measured after 3 hours ($n = 3$ per group). **D**, Effect of protein kinase A inhibitor, H-89, on the transwell migration assay for BMDC. The numbers of PACAP- or PBS-treated DCs with or without H-89 that migrated into the lower chamber containing 10 ng/mL CCL21, CXCL12, or PBS were measured after 3 hours ($n = 3$ per group). **E**, Expression of CD80 and CD86 on PACAP-treated BMDCs in the presence or absence of H-89 at 10 μ mol/L ($n = 3$ per group). Data are presented as mean $\pm$ SD. * $P < .05$ (Student *t*-test, Welch test, or Dunnett test) versus corresponding samples.

in RTX-treated mice while the expression of *Vipr1* and *Vipr2* did not change. These results suggest that PACAP-DC interaction is mediated via *Adcyap1r1* *in vivo*.

DISCUSSION

In this study, we demonstrated that mice whose sensory nerves had been ablated by RTX-treatment displayed significantly reduced CHS responses. By dissecting how sensory nerves modulate CHS responses, we revealed that denervation-mediated reductions in DC maturation and migration during the sensitization phase seemed to be the key events that led to reduced T-cell activation and skin inflammation. We further determined that the PACAP neuropeptides played an essential role by promoting upregulation of chemokine receptors and costimulatory molecules on cutaneous DCs via *Adcyap1r1* and Gs signaling pathway. These findings suggest that PACAP can promote the chemotactic and antigen-presenting ability of dermal DCs in CHS via neuroimmune communication (see Fig E13 in this article's Online Repository at www.jacionline.org).

A recent study revealed interactions of CGRP⁺ nerves and DCs in a murine psoriasis model involving T_H17 cell-mediated immune responses initiated by the innate immunity ligand imiquimod.⁸ It was also reported that sensory neurons detected *C*

albicans and released CGRP to drive IL-23 production from dermal DCs to protect against $\gamma\delta$ T-cell-mediated fungal infection.⁹ In this study, we addressed the role of sensory nerves in cutaneous acquired immune responses and obtained evidence that PACAP was induced by peripheral nerves and promoted DC functions in the skin to establish the sensitization phase of CHS, a prototype of delayed-type hypersensitivity.

Oxazolone has been reported to be a ligand for TRPA1, which activates sensory nerves.³⁹ It has also been shown that sensory nerves are activated by a variety of stimuli, such as heat, mechanical stimuli, and infection (such as *Staphylococcus aureus* and *C albicans*) in addition to haptens. *S aureus* induces calcium flux and action potentials in nociceptor neurons, in part via bacterial N-formylated peptides and the pore-forming toxin α -haemolysin.⁴⁰ *C albicans*-derived β -glucan stimulated nociceptors via the Dectin-1-mediated PLC-TRPV1/TRPA1 axis in primary sensory neurons.⁴¹ Various neuropeptides are released from peripheral nerves to regulate DC functions, which may be related to not only contact dermatitis but also other skin immune responses such as tumor immunity.⁴²

PACAP is a neuropeptide that mediates diverse physiological processes from neuroprotection to thermoregulation and is thought to be a master regulator of the stress response.^{43,44} The roles of PACAP have been revealed in term of the central nervous

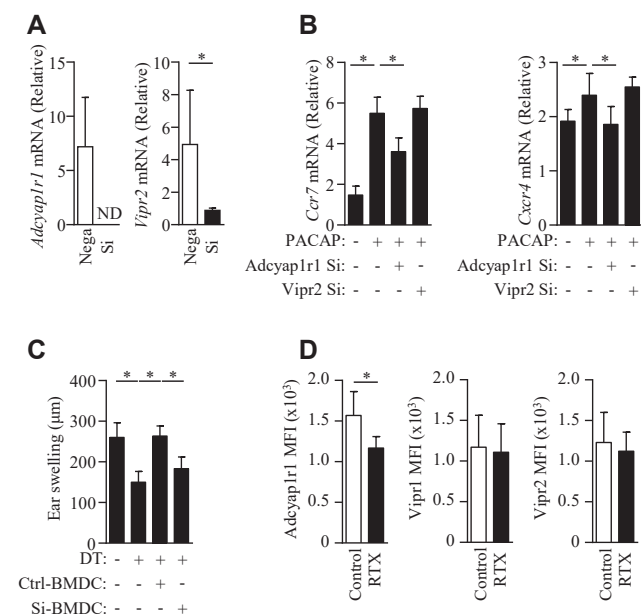


FIG 6. Effect of Adcyap1r1 knockdown on BMDC activation and CHS response. **A**, Expression of Adcyap1r1 and Vipr2 on BMDCs electroporated with 300 nmol/L Adcyap1r1-, Vipr2-, or control (Ctrl) negative (Nega)-small, interfering RNA (Si) ($n = 3$ per group). **B**, *Ccr7* and *Cxcr4* mRNA expression in 1 μ mol/L PACAP-treated BMDCs electroporated with 300 nmol/L Adcyap1r1-, Vipr2-, or negative-small, interfering RNA ($n = 3$ per group). **C**, Ear swelling in Adcyap1r1-knockdown BMDC- or control BMDC-treated CD11c-DTR mice with or without DT pretreatment ($n = 3$ per group). **D**, The expression of Adcyap1r1, Vipr1, or Vipr2 in migrated DCs from control or RTX-treated mice ($n = 8$ per group). Data are presented as mean $\pm$ SD. * $P < .05$ (Student *t*-test, or Welch test) versus corresponding samples.

system, however, the functions of PACAP in the peripheral nervous system remained limited. Kemény et al⁴⁵ reported that oxazolone-induced CHS response was exacerbated using PACAP-deficient mice. Because the infiltration of inflammatory cells in the skin was not significantly enhanced, the investigators considered that the exacerbation might be due to enhanced edematous reaction in the dermis. Regarding the effect of PACAP on antigen presenting cells, the other study by Kodali et al⁴⁶ demonstrated that intradermal administration of PACAP prior to application of hapten at the injected site inhibited the induction of CHS via inducing IL-10 production by activation of epidermal Langerhans cells that are considered to have immune-suppressive roles *in vivo*; however, current understanding is that CHS is induced predominantly by dermal DCs but not by Langerhans cells.⁴⁷ In line with this, our study demonstrated that PACAP induces activation of dermal DCs to establish the sensitization of CHS.

Of note, there are technical differences between the study by Kodali et al⁴⁶ and our study: the mouse strains, application doses of PACAP, and number of days of administration. We performed intradermal administration of PACAP prior to application of hapten at the injected site, but we did not find the attenuation of CHS in our model (Fig 4, E). On the other hand, we demonstrated that attenuated CHS induced by denervation was rescued by PACAP application. Although it still remains unclear precisely, the discrepancy may be derived from the different mouse strain, PACAP doses, and/or immunological contexts.

Several reports have suggested that other neuropeptides, most notably CGRP and substance P, can regulate CHS responses. For example, intradermal injection of a substance P agonist promoted

CHS induced by conventional but not optimal doses of hapten.¹⁵ But we demonstrated that the attenuated CHS responses after denervation were rescued by PACAP, but not by substance P. It remains unclear how the discrepancy occurred. Although we cannot exclude the possibility that other neuropeptides may regulate the properties of DCs in CHS,²⁹ we provided evidence that PACAP played a physiologically relevant role in the modulation of DC functions in the sensitization phase of CHS responses.

In conclusion, we have clarified a novel mechanism whereby sensory nerves affect cutaneous acquired immune responses. The PACAP neuropeptide promotes cutaneous DC functions to establish the sensitization phase of CHS responses. Taken together, the present study suggests an emerging new paradigm whereby TRPV1⁺ nociceptive fibers integrate environmental signals to modulate cutaneous immune responses.

We thank Ms. Hiromi Doi for her technical assistance.

Key messages

- Nociceptive sensory nerves are important for the development of CHS responses in the sensitization phase.
- PACAP promotes DC functions via upregulation of CCR7 and CXCR4.

REFERENCES

1. Tomura M, Honda T, Tanizaki H, Otsuka A, Egawa G, Tokura Y, et al. Activated regulatory T cells are the major T cell type emigrating from the skin during a cutaneous immune response in mice. *J Clin Invest* 2010;120:883-93.
2. Egawa G, Kabashima K. Skin as a peripheral lymphoid organ: revisiting the concept of skin-associated lymphoid tissues. *J Invest Dermatol* 2011;131:2178-85.
3. Honda T, Egawa G, Grabbe S, Kabashima K. Update of immune events in the murine contact hypersensitivity model: toward the understanding of allergic contact dermatitis. *J Invest Dermatol* 2013;133:303-15.
4. Otsuka A, Kubo M, Honda T, Egawa G, Nakajima S, Tanizaki H, et al. Requirement of interaction between mast cells and skin dendritic cells to establish contact hypersensitivity. *PLoS One* 2011;6:e25538.
5. Nakajima S, Igyarto BZ, Honda T, Egawa G, Otsuka A, Hara-Chikuma M, et al. Langerhans cells are critical in epicutaneous sensitization with protein antigen via thymic stromal lymphopoietin receptor signaling. *J Allergy Clin Immunol* 2012;129:1048-55.e6.
6. Usoskin D, Furlan A, Islam S, Abdo H, Lönnerberg P, Lou D, et al. Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. *Nat Neurosci* 2015;18:145-53.
7. LaMotte RH, Dong X, Ringkamp M. Sensory neurons and circuits mediating itch. *Nat Rev Neurosci* 2014;15:19-31.
8. Riolo-Blanco L, Ordoñez-Montanes J, Perro M, Naval E, Thiriot A, Alvarez D, et al. Nociceptive sensory neurons drive interleukin-23-mediated psoriasis-like skin inflammation. *Nature* 2014;510:157-61.
9. Kashem SW, Riedl MS, Yao C, Honda CN, Vulchanova L, Kaplan DH. Nociceptive sensory fibers drive interleukin-23 production from CD301b⁺ dermal dendritic cells and drive protective cutaneous immunity. *Immunity* 2015;43:515-26.
10. Joseph T, Kurian J, Warwick DJ, Friedmann PS. Unilateral remission of psoriasis following traumatic nerve palsy. *Br J Dermatol* 2005;152:185-6.
11. Meng J, Ovsepian SV, Wang J, Pickering M, Sasse A, Aoki KR, et al. Activation of TRPV1 mediates calcitonin gene-related peptide release, which excites trigeminal sensory neurons and is attenuated by a retargeted botulinum toxin with anti-nociceptive potential. *J Neurosci* 2009;29:4981-92.
12. Engel MA, Izdyorczyk I, Mueller-Tribbenese SM, Becker C, Neurath MF, Reeh PW. Inhibitory CB1 and activating/desensitizing TRPV1-mediated cannabinoid actions on CGRP release in rodent skin. *Neuropeptides* 2011;45:229-37.
13. Scholzen T, Armstrong CA, Bunnett NW, Luger TA, Olerud JE, Ansel JC. Neuropeptides in the skin: interactions between the neuroendocrine and the skin immune systems. *Exp Dermatol* 1998;7:81-96.

14. Mikami N, Matsushita H, Kato T, Kawasaki R, Sawazaki T, Kishimoto T, et al. Calcitonin gene-related peptide is an important regulator of cutaneous immunity: effect on dendritic cell and T cell functions. *J Immunol* 2011;186:6886-93.
15. Niizeki H, Kurimoto I, Streilein JW. A substance p agonist acts as an adjuvant to promote hapten-specific skin immunity. *J Invest Dermatol* 1999;112:437-42.
16. Tomura M, Hata A, Matsuoka S, Shand FH, Nakanishi Y, Ikebuchi R, et al. Tracking and quantification of dendritic cell migration and antigen trafficking between the skin and lymph nodes. *Sci Rep* 2014;4:6030.
17. Jung S, Unutmaz D, Wong P, Sano G, De los Santos K, Sparwasser T, et al. In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. *Immunity* 2002;17:211-20.
18. Lindquist RL, Shakhar G, Dudziak D, Wardemann H, Eisenreich T, Dustin ML, et al. Visualizing dendritic cell networks in vivo. *Nat Immunol* 2004;5:1243-50.
19. Ostrowski SM, Belkadi A, Loyd CM, Diaconu D, Ward NL. Cutaneous denervation of psoriasisform mouse skin improves acanthosis and inflammation in a sensory neuropeptide-dependent manner. *J Invest Dermatol* 2011;131:1530-8.
20. Siebenhaar F, Magerl M, Peters EM, Hendrix S, Metz M, Maurer M. Mast cell-driven skin inflammation is impaired in the absence of sensory nerves. *J Allergy Clin Immunol* 2008;121:955-61.
21. Shiratori-Hayashi M, Koga K, Tozaki-Saitoh H, Kohro Y, Toyonaga H, Yamaguchi C, et al. STAT3-dependent reactive astrogliosis in the spinal dorsal horn underlies chronic itch. *Nat Med* 2015;21:927-31.
22. Inan S, Dun NJ, Cowan A. Inhibitory effect of lidocaine on pain and itch using formalin-induced nociception and 5'-guanidinonaltrindole-induced scratching models in mice: behavioral and neuroanatomical evidence. *Eur J Pharmacol* 2009;616:141-6.
23. Mori T, Kabashima K, Yoshiki R, Sugita K, Shiraishi N, Onoue A, et al. Cutaneous hypersensitivities to haptens are controlled by IFN-gamma-upregulated keratinocyte Th1 chemokines and IFN-gamma-downregulated Langerhans cell Th2 chemokines. *J Invest Dermatol* 2008;128:1719-27.
24. De Smedt T, Pajak B, Klaus GG, Noelle RJ, Urbain J, Leo O, et al. Antigen-specific T lymphocytes regulate lipopolysaccharide-induced apoptosis of dendritic cells in vivo. *J Immunol* 1998;161:4476-9.
25. Winzler C, Rovere P, Rescigno M, Granucci F, Penna G, Adorini L, et al. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J Exp Med* 1997;185:317-28.
26. Angeli V, Ginhoux F, Llodra J, Quemeneur L, Frenette PS, Skobe M, et al. B cell-driven lymphangiogenesis in inflamed lymph nodes enhances dendritic cell mobilization. *Immunity* 2006;24:203-15.
27. Otsuka A, Kabashima K. Mast cells and basophils in cutaneous immune responses. *Allergy* 2015;70:131-40.
28. Sawada Y, Honda T, Hanakawa S, Nakamizo S, Murata T, Ueharaguchi-Tanada Y, et al. Resolvin E1 inhibits dendritic cell migration in the skin and attenuates contact hypersensitivity responses. *J Exp Med* 2015;212:1921-30.
29. McMahon SB, La Russa F, Bennett DL. Crosstalk between the nociceptive and immune systems in host defence and disease. *Nat Rev Neurosci* 2015;16:389-402.
30. Hoyer D, Bartfai T. Neuropeptides and neuropeptide receptors: drug targets, and peptide and non-peptide ligands: a tribute to Prof. Dieter Seebach. *Chem Biodivers* 2012;9:2367-87.
31. Xu Z, Ohtaki H, Watanabe J, Miyamoto K, Murai N, Sasaki S, et al. Pituitary adenylate cyclase-activating polypeptide (PACAP) contributes to the proliferation of hematopoietic progenitor cells in murine bone marrow via PACAP-specific receptor. *Sci Rep* 2016;6:22373.
32. Sanchez-Sanchez N, Riol-Blanco L, Rodriguez-Fernandez JL. The multiple personalities of the chemokine receptor CCR7 in dendritic cells. *J Immunol* 2006;176:5153-9.
33. Kabashima K, Shiraishi N, Sugita K, Mori T, Onoue A, Kobayashi M, et al. CXCL12-CXCR4 engagement is required for migration of cutaneous dendritic cells. *Am J Pathol* 2007;171:1249-57.
34. Kish DD, Gorbachev AV, Fairchild RL. IL-1 receptor signaling is required at multiple stages of sensitization and elicitation of the contact hypersensitivity response. *J Immunol* 2012;188:1761-71.
35. May V, Lutz E, MacKenzie C, Schutz KC, Dozark K, Braas KM. Pituitary adenylate cyclase-activating polypeptide (PACAP)/PAC1HOP1 receptor activation coordinates multiple neurotrophic signaling pathways: Akt activation through phosphatidylinositol 3-kinase gamma and vesicle endocytosis for neuronal survival. *J Biol Chem* 2010;285:9749-61.
36. Roy A, Derakhshan F, Wilson RJ. Stress peptide PACAP engages multiple signaling pathways within the carotid body to initiate excitatory responses in respiratory and sympathetic chemosensory afferents. *Am J Physiol Regul Integr Comp Physiol* 2013;304:R1070-84.
37. Engh RA, Girod A, Kinzel V, Huber R, Bossemeyer D. Crystal structures of catalytic subunit of cAMP-dependent protein kinase in complex with isoquinolinesulfonyl protein kinase inhibitors H7, H8, and H89. Structural implications for selectivity. *J Biol Chem* 1996;271:26157-64.
38. Hanakawa S, Kitoh A, Shibuya R, Dainichi T, Nomura T, Honda T, et al. Percutaneous sensitization is limited by in situ inhibition of cutaneous dendritic cell migration through skin-resident regulatory T cells. *J Allergy Clin Immunol* 2019;144:1343-53.e8.
39. Liu B, Escalera J, Balakrishna S, Fan L, Caceres AI, Robinson E, et al. TRPA1 controls inflammation and pruritogen responses in allergic contact dermatitis. *FASEB J* 2013;27:3549-63.
40. Chiu IM, Heesters BA, Ghasemlou N, Von Hehn CA, Zhao F, Tran J, et al. Bacteria activate sensory neurons that modulate pain and inflammation. *Nature* 2013;501:52-7.
41. Maruyama K, Takayama Y, Sugisawa E, Yamanoi Y, Yokawa T, Kondo T, et al. The ATP transporter VNUT mediates induction of dectin-1-triggered Candida nociception. *iScience* 2018;6:306-18.
42. Peters EM, Ericson ME, Hosoi J, Seiffert K, Hordinsky MK, Ansel JC, et al. Neuropeptide control mechanisms in cutaneous biology: physiological and clinical significance. *J Invest Dermatol* 2006;126:1937-47.
43. Rudecki AP, Gray SL. PACAP in the defense of energy homeostasis. *Trends Endocrinol Metab* 2016;27:620-32.
44. Shioda S, Takenoya F, Wada N, Hirabayashi T, Seki T, Nakamachi T. Pleiotropic and retinoprotective functions of PACAP. *Anat Sci Int* 2016;91:313-24.
45. Kemény A, Reglodi D, Cseharovszky R, Hashimoto H, Baba A, Szolcsányi J, et al. Pituitary adenylate cyclase-activating polypeptide deficiency enhances oxazolone-induced allergic contact dermatitis in mice. *J Mol Neurosci* 2010;42:443-9.
46. Kodali S, Friedman I, Ding W, Seiffert K, Wagner JA, Granstein RD. Pituitary adenylate cyclase-activating polypeptide inhibits cutaneous immune function. *Eur J Immunol* 2003;33:3070-9.
47. Honda T, Nakajima S, Egawa G, Ogasawara K, Malissen B, Miyachi Y, et al. Compensatory role of Langerhans cells and langerin-positive dermal dendritic cells in the sensitization phase of murine contact hypersensitivity. *J Allergy Clin Immunol* 2010;125:1154-6.e2.

Yamamoto et al

1

2 **METHODS**

3

4 **Surgical denervation**

5 We adapted the cutaneous denervation surgery from a previous study.¹ The upper and lower
6 main trunks of the ear were incised and the innervating nerves were exposed. A 1-mm piece
7 of each trunk was removed along with the innervating nerves. For controls, nerve trunks were
8 exposed, but not dissected. The success of skin denervation was assessed and confirmed by
9 ear swelling after painting with 0.25% (wt/vol) capsaicin solution.

10

11 **Lymphocyte re-stimulation assay**

12 Single-cell suspensions were prepared from axillary LNs of mice at 5 days after sensitization
13 with oxazolone. A total of 1×10^6 LN cells were cultured with or without oxazolone-
14 conjugated bovine serum albumin (BSA) at 1 mg/mL. Oxazolone was conjugated to BSA as
15 previously described² and with some modifications. For measurement of cytokine production,
16 culture supernatants were collected after 72 h of incubation. The amount of IFN- γ was
17 measured by a mouse IFN- γ ELISA kit (BD Biosciences) according to the manufacturer's

instructions.

Hematoxylin and eosin staining

Paraffin-embedded skin specimens were prepared using routine methods. Five-micrometer sections were deparaffinized and hydrated by washing in xylene followed by a series of graded alcohol concentrations. Hematoxylin and eosin (Wako Pure Chemical Industries) staining was performed using standard protocols. Images were acquired using a polarizing microscope (BX51-P; Olympus, Tokyo, Japan), a 20 × lens (numerical aperture 0.7; Olympus), and cellSens Software (Olympus).

Photoconversion and *in vivo* DC migration assay

Photoconversion of the skin was performed as previously described.³ After 436 nm violet light exposure, mice immediately received an application of 25 µL of 3% (wt/vol) oxazolone to their photoconverted abdomens. For application of PACAP and its antagonist, PACAP (500 ng/mouse/day in PBS), PACAP6-38 (500 ng/mouse/day in PBS), or PBS was injected intradermally into the abdomen for 3 days, beginning at 1 day before sensitization. Skin samples and axillary LNs were collected from mice at 48 h after sensitization.

35

36 FITC-induced cutaneous DC migration

37 Mice were sensitized by application of 100 μ L of 2% (wt/vol) FITC to the shaved abdominal
38 skin. Axillary LNs were collected from mice at 48 h after sensitization.

39

40 Whole-mount skin immunostaining

41 After euthanasia, ears of CD11c-YFP mice were dissected and split into dorsal and ventral
42 sheets. The cartilage-free dorsal sheets were fixed in 4% (wt/vol) paraformaldehyde
43 overnight at 4°C. A rabbit anti-PGP 9.5 primary antibody and anti-rabbit Alexa Fluor 594-
44 conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA) were used. Images
45 were acquired using a confocal microscope (A1RMP; Nikon, Tokyo, Japan), a 10 $\times$ lens
46 (numerical aperture 0.45; Nikon), a 60 $\times$ lens (numerical aperture 1.40; Nikon), and NIS-
47 Elements AR Software (Nikon). Images were analyzed with Imaris Version 8.2.0. software
48 (Bitplane, Concord, MA).

49

50 DRG cultures

51 DRG isolation was performed as previously described⁴ with some modifications. Briefly, the

bilateral DRGs at the C3-T10 levels were removed from BALB/c female mice, and the cells were dissociated with 0.25% collagenase (Wako Pure Chemical Industries) and 0.25% Trypsin-EDTA (Thermo Fisher Scientific). Myelin debris was removed by centrifugation at $1,000 \times g$ for 30 min at 25°C in 20% (wt/vol) BSA (Nacalai Tesque) in DMEM (Thermo Fisher Scientific). The cell suspension was plated in a 24-well culture dish coated with 500 µg/mL poly-D-lysine (Sigma-Aldrich). After 24 h, the culture medium was replaced with DMEM containing 10% heat-inactivated fetal calf serum (FCS) (GE Healthcare, Little Chalfont, UK), 25 µM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Thermo Fisher Scientific), X1 minimal essential medium (MEM) non-essential amino acids solution (Thermo Fisher Scientific), 50 U/mL penicillin (Thermo Fisher Scientific), and 50 µg/mL streptomycin (Thermo Fisher Scientific) supplemented with 10 µM cytosine arabinofuranoside (Sigma-Aldrich) for 3–7 days. For oxazolone stimulation, DRGs cultured for 6 days were stimulated with 300 µM oxazolone in culture medium for 24 h.

BMDC culture

For BMDC cultures, 1×10^7 hemolyzed BM cells generated from BALB/c mice were cultured in 10 mL of RPMI 1640 (Thermo Fisher Scientific) containing 10% heat-inactivated

FCS, 50 μ M 2-mercaptoethanol (Wako Pure Chemical Industries), X1 MEM non-essential amino acids solution, 1 mM sodium pyruvate (Thermo Fisher Scientific), 50 U/mL penicillin, and 50 μ g/mL streptomycin supplemented with 100 ng/mL recombinant murine Flt3L (PeproTech, Rocky Hill, NJ) for 7–9 days. For DRG co-cultures, cultured DRGs were prepared 5 days previously. BMDCs and DRGs were co-cultured for 24 h with 10 μ M PACAP6-38 or PBS. For PACAP stimulation experiments, BMDCs were stimulated with 1 μ M PACAP or PBS. The cells were harvested after 8 h for quantitative real time polymerase chain reaction (qRT-PCR) and 24 h for flowcytometry. For PKA inhibition experiment, BMDCs were incubated for 8 h or 24 h with 10 μ M H-89 in addition to 1 μ M PACAP or PBS. For knockdown experiment, cells were electroporated with 300 nM small interfering RNA (siRNA) for *Adcyap1r1*, *Vipr2*, or control siRNA (Thermo Fisher Scientific) by using Amaxa[®] Mouse Dendritic Cell Nucleofector[®] Kit (Lonza, Basel, Switzerland) according to the manufacturer's instructions.

Transmigration assay

Single-cell suspensions of BMDCs were incubated for 24 h with 1 μ M PACAP or PBS and tested for transmigration across uncoated 5- μ m transwell filters (Corning, Corning, NY)

toward 1 μ M PACAP, 1, 10, or 100 ng/mL CCL21 or CXCL12 (R&D Systems, Minneapolis, MN), or medium for 3 h. For detection of chemokinetic effects, BMDCs with 1 μ M PACAP were tested for transmigration using 5- μ m transwell filters. For PKA inhibition experiment, BMDCs were incubated for 24 h with 10 μ M H-89 in addition to 1 μ M PACAP or PBS. After stimulation, BMDCs were incubated in RPMI 1640 with 0.5% (wt/vol) BSA for 1 hr. Migrated cells were measured by CellTiter-Glo (Promega, Madison, WI) according to the manufacturer's instructions.

RNA isolation and quantitation

Shaved abdomen skin, ears, and DRG samples were collected at 2 days, 24 h, and 2 days after oxazolone treatment, respectively. Total RNA was isolated from these samples using an RNeasy Kit (Qiagen, Hilden, Germany). For quantitative RT-PCR analysis, reverse transcription was performed using a Prime Script RT Reagent Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The results were normalized by the mRNA levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase, ribosomal protein large P1, or peptidylprolyl isomerase A using the Δ Ct method. All PCR amplifications were performed using a LightCycler 480 Instrument II (Roche, Basel, Switzerland).

103

104 **Flow cytometry**

105 Skin-draining LN-derived single-cell suspensions were prepared from axillary LNs of mice at

106 2 days after sensitization with oxazolone. For preparation of skin-derived single-cell

107 suspensions, ears were removed and punched out using disposable 8-mm circular blades

108 (Kai, Tokyo, Japan). The tissue was separated into dorsal and ventral sides and incubated for

109 1.5 h in RPMI 1640 containing 1000 U/mL collagenase II (Worthington Biochemical

110 Corporation, Lakewood, NJ) and 100 µg/mL DNase I (Sigma-Aldrich). To detect DCs in

111 LNs, the LNs were incubated for 0.5 h in RPMI 1640 containing 500 U/mL collagenase II

112 and 100 µg/mL DNase I. The cell suspensions were filtered through a 40-µm cell strainer and

113 stained with appropriate antibodies. For the detection of survival and apoptotic cells, the cell

114 suspensions were stained with Annexin V (Sigma-Aldrich) and fixable viability dye (Thermo

115 Fisher Scientific). Cells were subjected to flow cytometry (Fortessa; BD Biosciences), and

116 the data were analyzed using FlowJo software (FlowJo, Ashland, OR). Cells were quantified

117 by the addition of Flow-Count Fluorospheres (Beckman Coulter, Brea, CA).

118

119 **DT treatment and BMDC transfer**

120 2 ng DT was injected intradermally into the ear of CD11c-DTR mice. 1×10^6 BMDCs were
121 injected intradermally into the same site 24 h after DT treatment.

122

123 **Statistical analysis**

124 Data are expressed as mean $\pm$ standard deviation (SD). All statistical analyses were
125 performed using StatLight software (Yukms, Tokyo, Japan). Differences in values were
126 analyzed for statistical significance by Student's t-test (for homoscedastic data) or Welch's
127 test (for heteroscedastic data) after homoscedasticity analysis by the F-test. For multiple
128 comparisons, Dunnett's test was performed. Values of $P < .05$ were considered significant in
129 all tests.

130

References

1. Siebenhaar F, Magerl M, Peters EM, Hendrix S, Metz M, Maurer M. Mast cell-driven skin inflammation is impaired in the absence of sensory nerves. *J Allergy Clin Immunol* 2008; 121:955-61.
2. van't Erve EH, Wijnand E, Bol M, Seinen W, Pieters RH. The vehicle modulates cellular and humoral responses in contact hypersensitivity to oxazolone. *Toxicol Sci* 1998; 44:39-45.
3. Tomura M, Hata A, Matsuoka S, Shand FH, Nakanishi Y, Ikebuchi R, et al. Tracking and quantification of dendritic cell migration and antigen trafficking between the skin and lymph nodes. *Sci Rep* 2014; 4:6030.
4. Andoh T, Nishikawa Y, Yamaguchi-Miyamoto T, Nojima H, Narumiya S, Kuraishi Y. Thromboxane A2 induces itch-associated responses through TP receptors in the skin in mice. *J Invest Dermatol* 2007; 127:2042-7.

Supplementary Figure Legends**Figure E1. Confirmation of RTX-induced denervation.**

Eye wiping in RTX-treated or control mice ($n = 8$ – 16 per group) with or without capsaicin treatment is shown. Data are presented as mean $\pm$ SD. $*P < .05$ (Student's t -test or Welch's test) versus corresponding mice.

Figure E2. Effect of RTX treatment on DNFB or FITC-induced CHS responses.

Ear swelling in RTX-treated or control mice at 24 h after application of DNFB ($n = 5$ per group) or FITC ($n = 5$ – 8 per group). Data are presented as mean $\pm$ SD. $*P < .05$ (Student's t -test or Welch's test) versus corresponding mice.

Figure E3. Confirmation of surgical denervation.

Ear swelling at 6 h after challenge with 0.25% (wt/vol) capsaicin solution in mice that underwent surgical denervation (DNV) or sham operation (Sham) on their ears ($n = 8$ per group). Data are presented as mean $\pm$ SD. $*P < .05$ (Welch's test) versus corresponding mice.

Figure E4. mRNA profiles in oxazolone-treated denervated mice.

Il4, *Il10*, *Il17a*, *Il17f*, *Tnf*, and, *Il6* mRNA expression in the skin tissue (n = 8 per group) at 24 h after challenge with oxazolone. All data are presented as mean $\pm$ SD.

Figure E5. Gene expression in oxazolone-treated abdominal skin.

The relative expression levels of *Il1a*, *Il1b*, and *Tnf* in the skin of abdomen from control or RTX-treated mice ($n = 8$ per group) were analyzed at 48 h after painting with or without oxazolone. Data are presented as mean $\pm$ SD. $*P < .05$ (Welch's test) versus corresponding mice.

Figure E6. Effect of RTX and oxazolone treatment on DC viability in LNs.

Representative flow cytometric plots of migrated DCs (B220⁻ CD3⁻ CD11c⁺, I-A/I-E^{high}). The percentage of the following cell populations is indicated: Annexin V and viability dye negative stained, indicating viable cells, Annexin V-positive and viability dye-negative stained, indicating early apoptotic cells, and Annexin V and viability dye double-stained cells showing late apoptosis or necrosis. The rates are calculated as the observed number divided total migrated DC number. Data are presented as mean $\pm$ SD.

Figure E7. Confirmation of RTX-induced denervation.

RTX-induced denervation was confirmed by qRT-PCR analysis. Relative *Trpv1*, *Trpa1*, and *Trpm8* expression was evaluated in DRGs from RTX-treated mice ($n = 8$ per group). Data are presented as mean $\pm$ SD. $*P < .05$ (Student's *t*-test) versus corresponding mice.

Figure E8. Flow cytometric histograms of PACAP receptor expression on DCs.

Representative flow cytometric histograms of PACAP receptor (*Adcyap1r1* and *Vipr2*) expression on skin-derived DCs (DC), B220⁺ B cells (B cell), TCR β ⁺ CD4⁺ T cells (CD4), and TCR β ⁺ CD8a⁺ T cells (CD8) are shown.

Figure E9. Chemokinesis and chemotaxis effect of PACAP on BMDCs.

The effects of 1 μ M PACAP treatment on chemokinesis and chemotaxis of BMDCs ($n = 3$ per group). $*P < .05$ (Student's *t*-test) versus corresponding mice.

Figure E10. Effect of PKA inhibitor on BMDC migration towards the CCL21 or CXCL12.

The effects of 10 μ M H-89-treatment for 24 h with 1 μ M PACAP or PBS on BMDC

migration ($n = 3$ per group). The numbers of PACAP or PBS-treated DCs with or without H-89 that migrated into the lower chamber containing 10 ng/mL CCL21, CXCL12, or PBS were measured after 3 h ($n = 3$ per group). Data are presented as mean $\pm$ SD. $*P < .05$ (Student's *t*-test or Welch's test) versus corresponding samples.

Figure E11. Effect of PKA inhibitor on PACAP-induced *Ccr7* and *Cxcr4* upregulations.

The effects of 10 μ M H-89-treatment for 24 h with 1 μ M PACAP or PBS on the expression of *Ccr7* and *Cxcr4* in BMDC ($n = 3$ per group). Data are presented as mean $\pm$ SD. $*P < .05$ (Student's *t*-test) versus corresponding samples.

Figure E12. Effect of intradermal DT treatment on cutaneous CD11c⁺ cells.

The numbers of cutaneous CD11c⁺ cells from CD11c-DTR mice given a single intradermal injection of DT 24 h before analysis. Data are presented as mean $\pm$ SD. $*P < .05$ (Student's *t*-test) versus corresponding samples.

Figure E13. Schematic diagram of the effect of PACAP on DC trafficking from the skin to the lymph nodes.

214 After hapten exposure, TRPV1-positive sensory nerves in the skin are stimulated and release
215 PACAP to the neighboring DCs. PACAP activates cutaneous hapten-bearing DCs via
216 Adcyap1r1- PKA pathway and enhances their migration to the lymph nodes. dLNs: skin-
217 draining lymph nodes.
218

219 Movie E1

220 Representative confocal micrographs of a skin sample stained for PGP9.5 (red) and CD11c
221 (green).

222

1 **Table E1.** List of antibodies used in flow cytometry analyses

Antibody	Fluorochrome	Clone	Supplier
CD11c	APC or Brilliant Violet 605	N418	Thermo Fisher Scientific
CD80	PE or PE-Cy7	16-10A1	Thermo Fisher Scientific
I-A/I-E	FITC or Pacific Blue	M5/114.15.2	Thermo Fisher Scientific
TCRb	PE-Cy7	H57-597	Thermo Fisher Scientific
CD3	PE-Cy7	17A2	BioLegend
CD4	Brilliant Violet 605	RM4-5	BD Biosciences
CD45	BD Horizon V450	30-F11	BD Biosciences
CD45R (B220)	APC or PerCP-Cy5.5	RA3-6B2	BioLegend
CD8a	PerCP-Cy5.5	53-6.7	BioLegend
CD44	PE	IM7	BioLegend
CD62L	FITC	MEL-14	BioLegend
CD86	Brilliant Violet 605	GL-1	BioLegend
Adcyap1r1	-	-	Abcam (Cambridge, UK)
Vipr1	-	-	Alomone Labs
Vipr2	-	-	Abcam

- 2 印刷が困難あるいは不可能な補足資料 (Video E1)
- 3 [https://www.jacionline.org/cms/10.1016/j.jaci.2021.02.005/attachment/6b34c745-8aa9-4636-](https://www.jacionline.org/cms/10.1016/j.jaci.2021.02.005/attachment/6b34c745-8aa9-4636-90b7-27458a88221a/mmc1.mp4)
- 4 [90b7-27458a88221a/mmc1.mp4](https://www.jacionline.org/cms/10.1016/j.jaci.2021.02.005/attachment/6b34c745-8aa9-4636-90b7-27458a88221a/mmc1.mp4)

Figure E1

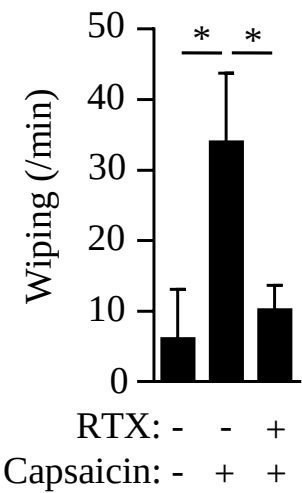


Figure E2

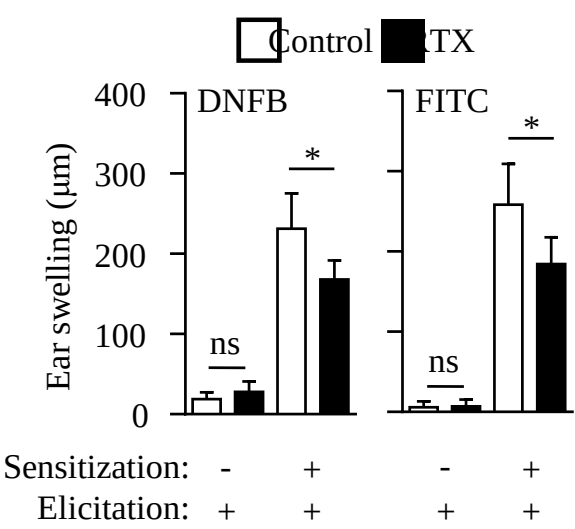


Figure E3

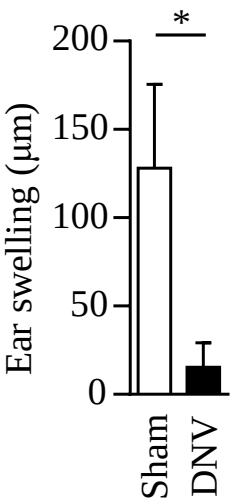


Figure E4

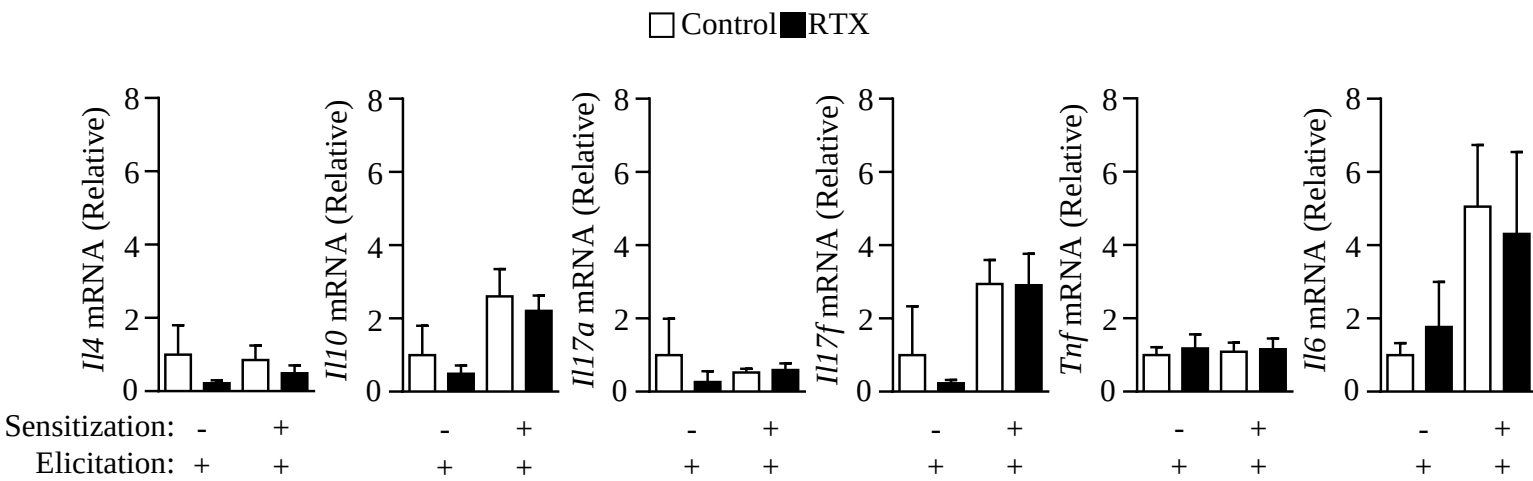


Figure E5

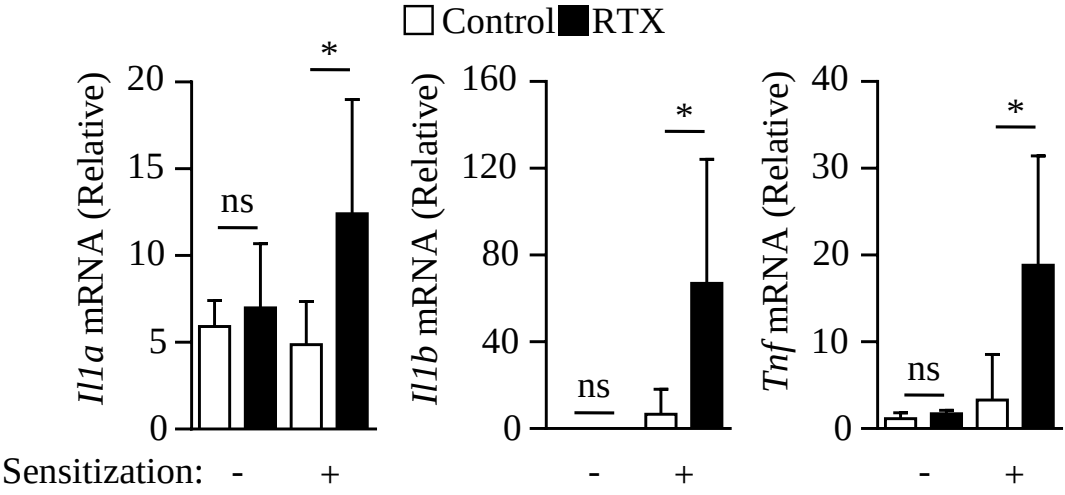


Figure E6

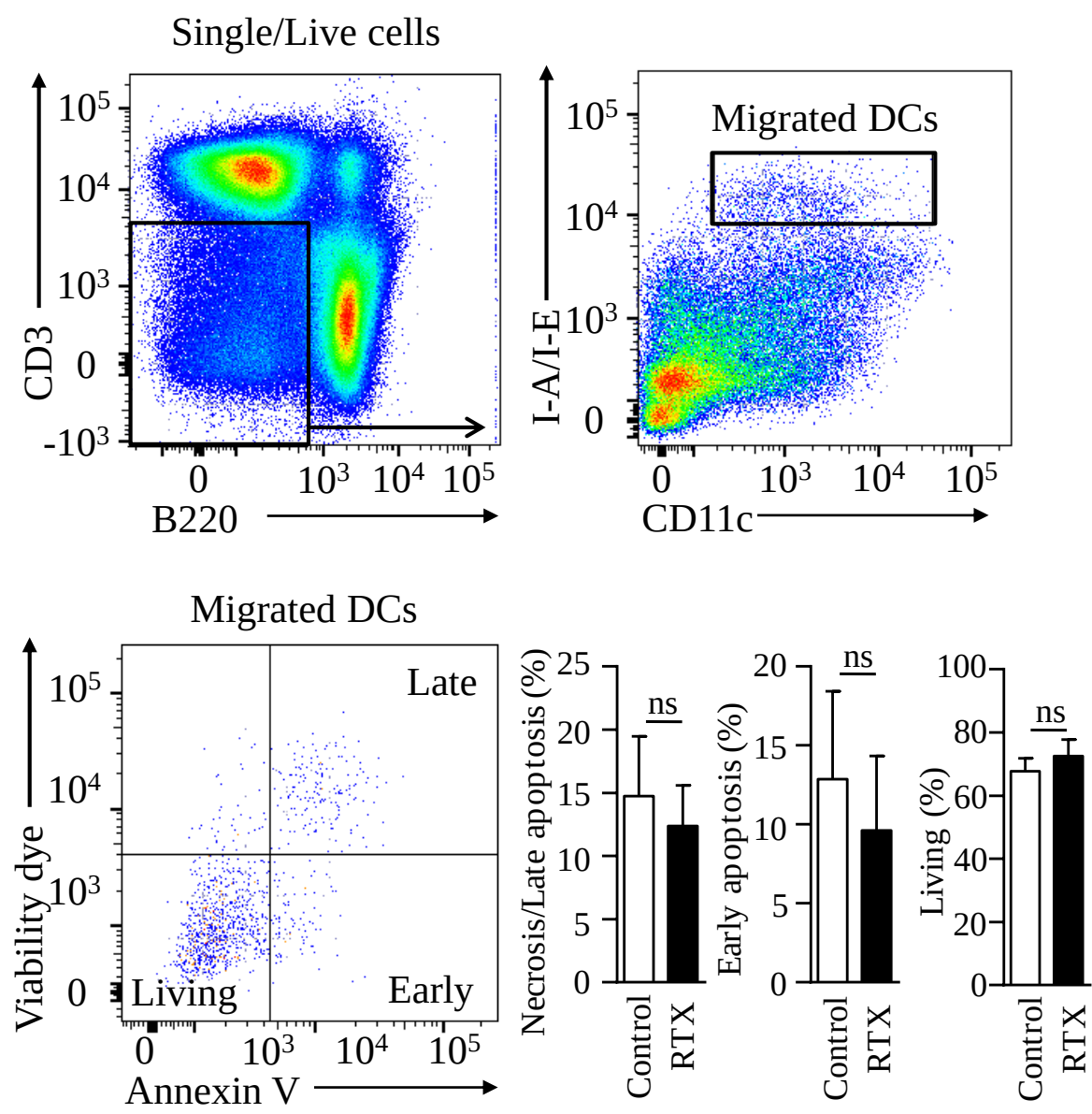


Figure E7

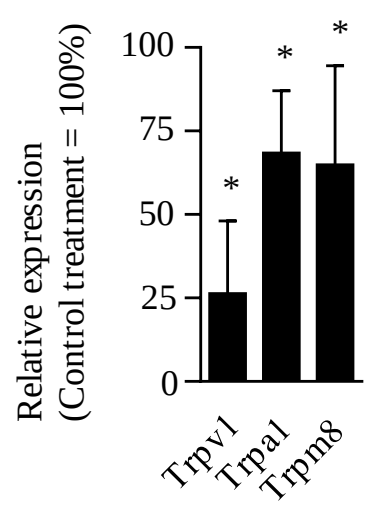


Figure E8

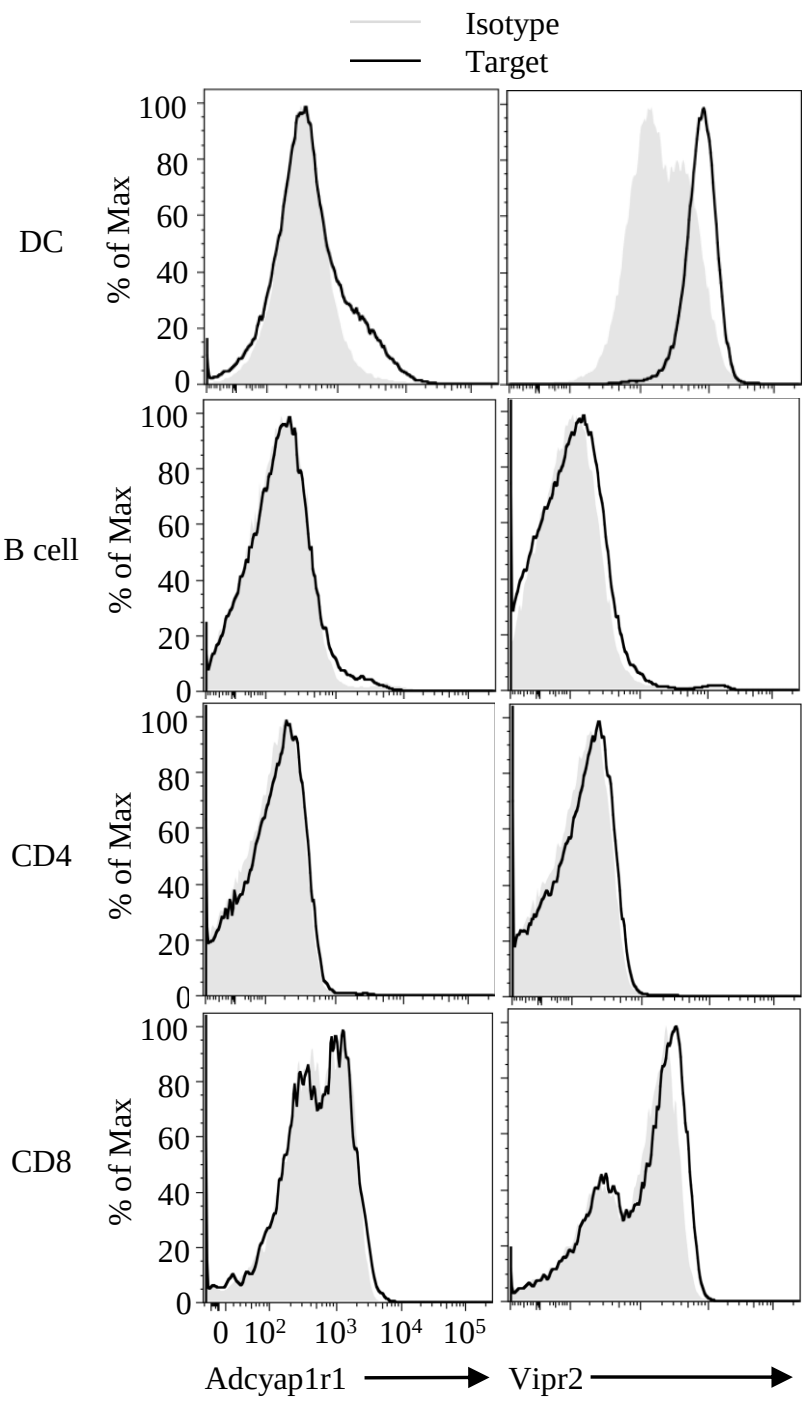


Figure E9

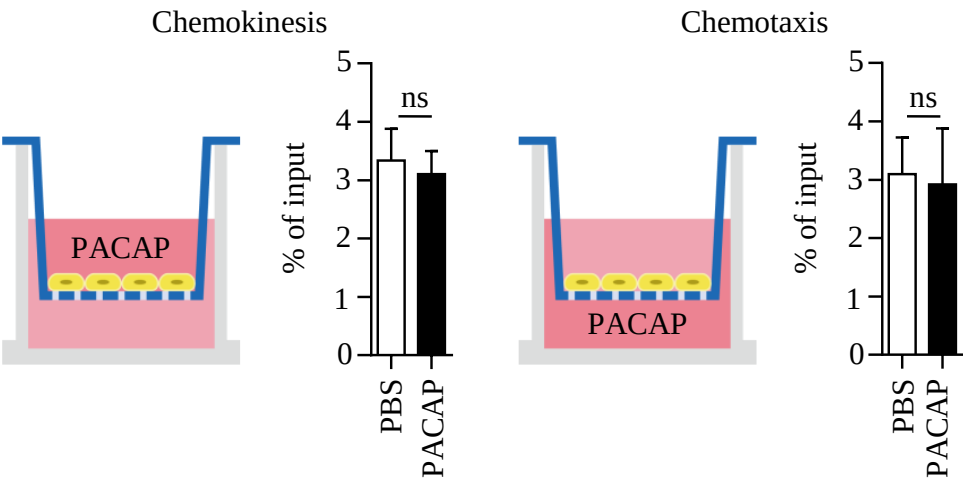


Figure E10

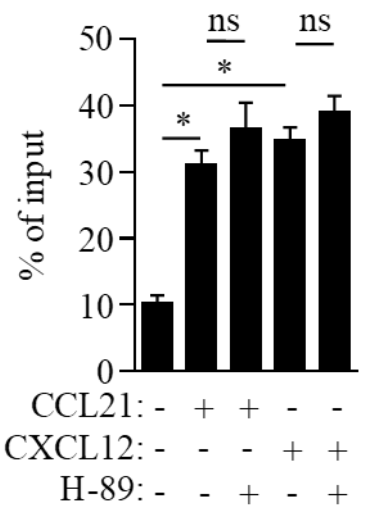


Figure E11

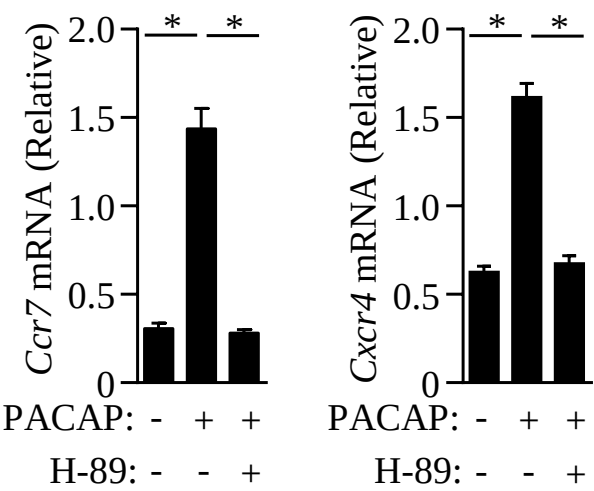


Figure E12

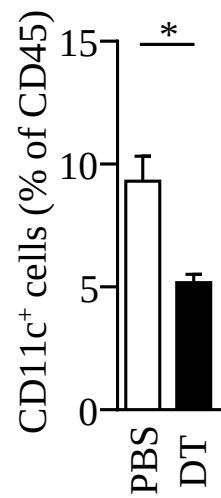


Figure E13

