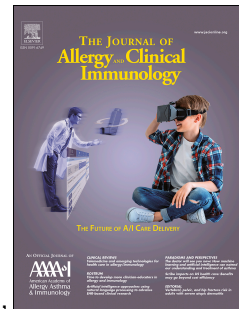


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Pituitary adenylate cyclase-activating polypeptide promotes cutaneous dendritic cell functions in contact hypersensitivity

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Abstract

Background: Sensory nerves regulate cutaneous local inflammation indirectly through induction of pruritus and directly by acting upon 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 (RTX) -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 RTX-treated mice, and pharmacological inhibition of PACAP suppressed CHS. Flowcytometric analysis of skin-draining lymph nodes showed that cutaneous dendritic cell

(DC) migration and maturation were reduced in both denervated mice and PACAP antagonist-treated mice. The expression of chemokine receptors, CCR7 and CXCR4 of DCs 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 DC functions during the sensitization phase.

Key Messages

1. Nociceptive sensory nerves are important for the development of CHS responses in the sensitization phase.

2. PACAP promotes DC functions via upregulation of CCR7 and CXCR4.

Capsule summary: Nervous system is likely to modulate immune response directly, but its mechanism has remained elusive. The authors demonstrate that PACAP is an important neuropeptide that enhances contact hypersensitivity response in the skin.

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

Abbreviations used

BMDC: bone marrow-derived dendritic cell

CGRP: calcitonin gene-related peptide

CHS: contact hypersensitivity

DC: dendritic cell

- 95 DNFB: 2,4-dinitrofluorobenzene
- 96 DT: diphtheria toxin
- 97 DRG: dorsal root ganglion
- 98 FITC: fluorescein isothiocyanate
- 99 IFN: interferon
- 100 IL: interleukin
- 101 KikGR: Kikume Green-Red
- 102 LN: lymph node
- 103 PACAP: pituitary adenylate cyclase-activating polypeptide
- 104 PGP9.5: protein gene product 9.5
- 105 PKA: protein kinase A
- 106 RTX: resiniferatoxin
- 107 SD: standard deviation
- 108 Tc1: T cytotoxic
- 109 TCR: T cell receptor
- 110 Th: T helper
- 111 TNF: tumor necrosis factor

112 TRPA1: transient receptor potential ankyrin 1

113 TRPV1: transient receptor potential vanilloid 1

114 YFP: yellow fluorescent protein

115

INTRODUCTION

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 naïve T cells.^{4,5} In the subsequent elicitation phase, re-exposure 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 subtypes⁶, including non-peptidergic and peptidergic nociceptor clusters, which express nociceptors such as the transient receptor 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 L, et al. demonstrated that DCs reside in close proximity to peripheral nerves in the skin,⁸ and that TRPV1⁺ nociceptive sensory

nerves interact with DCs to regulate the interleukin (IL) -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, in order 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

150 denervated during elicitation. Comprehensive mRNA expression analyses in the DRG
151 revealed that the mRNA expression level of pituitary adenylate cyclase-activating
152 polypeptide (PACAP) was decreased by RTX-induced denervation and increased after hapten
153 application. In addition, PACAP enhanced DC functions *in vivo* and increased the expression
154 of chemokine receptors, essential for DC migration, *in vitro*. These findings indicate that a
155 neuropeptide, PACAP, may be important for initiating CHS responses by promoting DC
156 functions.

157

METHODS

Animals

BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). Kikume Green-Red (KikGR)-transgenic mice on a C57BL/6 background¹⁶, CD11c- diphtheria toxin (DT) receptor mice (CD11c-DTR mice) on a C57BL/6 background,¹⁷ and CD11c- yellow fluorescent protein (YFP) mice on a C57BL/6 background¹⁸ were generated as described. Female mice aged 4–10 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 at www.jacionline.org**. For

flow cytometry analysis, an anti-PACAP receptor ab was purified using an IgG purification Kit-G (Dojindo, Kumamoto, Japan) and labeled with an 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 three 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

192 sensitization. PACAP (500 ng/mouse/day in PBS), PACAP6-38 (500 ng/mouse/day in PBS),
193 substance P (500 ng/mouse/day in PBS), or PBS were injected intradermally into the
194 abdominal skin on the same day and at 1 and 2 days after sensitization. Ear thickness was
195 measured before and 24 h after the challenge using a thickness gauge (Teclok, Nagano,
196 Japan). For adoptive transfer experiments, single-cell suspensions were prepared from
197 axillary LNs of mice at 5 days after 3% (wt/vol) oxazolone sensitization. Whole LN cells
198 from one mouse were injected intravenously into a naive recipient mouse and 1 h later, the
199 mice were challenged with 10 μ L of 1% (wt/vol) oxazolone.

200

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 (**Fig E1**). 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 two alternative haptens, DNFB and FITC, indicating that this phenomena is not restricted to oxazolone (**Fig E2**). 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 (**Fig E3**).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 interferon (IFN)- γ , a key cytokine in CHS, which is produced by CD4⁺ T helper 1 (Th1) and CD8⁺ T cytotoxic 1 (Tc1) cells²³ (**Fig 1, F**). In contrast, RTX treatment did not affect the expression of Th2/Th17 cytokines or tumor necrosis factor (TNF). (**Fig E4**). Taken together, these results suggest that TRPV1⁺ nociceptive sensory nerves are involved in the induction of Th1/Tc1 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

naïve 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 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, TCRβ⁺ 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 re-stimulated 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 Th1/Tc1 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 naïve 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 upon 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 E6**). It has been reported that migrated mature cutaneous DCs tend to die via apoptosis at about 48 h 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) upon exposure to 436 nm-violet light for 10 min. Following this non-invasive 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 co-stimulatory 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 upon 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 two-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 Movie E1**).

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 upon denervation with RTX (**Fig E7**) 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 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 *Adcyap1r1* 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, *Adcyap1r1*, 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 (**Fig E8**). 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 co-culture 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 upon PACAP administration, and were not attracted toward PACAP (i.e., chemotaxis) (**Fig E9**), suggesting that PACAP does not have chemokinetic or chemotactic activities.

PACAP signaling pathway is known to be mediated through G protein-coupled receptors.^{35, 36} Since 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 (PKA) inhibitor H-89 did not affect the chemotaxis of DCs toward CCL21 and CXCL12 (**Fig E10**); 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 E11). 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 siRNA. Firstly, 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 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 upon DT treatment.¹⁷ Intradermal administration of DT reduced the number of CD11c-positive cells in the skin by about 50% as reported previously³⁸ (Fig E12). 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 siRNA-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 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 one of neuropeptides, PACAP, played an essential role by promoting upregulation of chemokine

receptors and co-stimulatory 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 (**Fig E13**).

A recent study revealed interactions of CGRP⁺ nerves and DCs in a murine psoriasis model involving Th 17 cell-mediated immune responses initiated by the innate immunity ligand imiquimod.⁸ It was also reported that sensory neurons detected *Candida 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 *Candida albicans*) in addition to haptens. *Staphylococcus 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 *Candida 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 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 system, however, the functions of PACAP in the peripheral nervous system remained limited. Kemény A et al. 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 authors 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 Dr. Granstein's group⁴⁵ 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 consider to have immune-suppressive roles in vivo; however, current understanding is that CHS is induced predominantly by dermal DCs but not by Langerhans cells.⁴⁶ 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 Granstein's study 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.

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

443

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446

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587

Figure legends

Figure 1. RTX-induced denervation impaired CHS responses. (A) Ear swelling in RTX-treated and control mice ($n = 5-10$ per group) at 24 h after elicitation with or without sensitization. (B) Histological analysis of the skin at 24 h after elicitation with or without sensitization. Scale bars, 200 μm . (C) Ear swelling at 24 h after challenge with oxazolone in mice that underwent surgical denervation (DNV) or sham operation (Sham) 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 h. (E) Cell densities of CD45^+ cells and $\text{TCR}\beta^+$ cells in skin tissue at 24 h 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 h after challenge with oxazolone. All data are presented as mean $\pm$ standard deviation (SD). $*P < .05$ (Student's *t*-test or Welch's test) versus corresponding mice.

Figure 2. RTX treatment attenuated the sensitization phase in CHS. (A) Effect of surgical denervation on the sensitization and elicitation sites. Ear swelling in surgically denervated or sham-operated ear ($n = 8$ per group) at 24 h after challenge with oxazolone at surgically denervated or sham-operated ear. S: sham-operated ear. D: surgically denervated

ear. (B) Ear swelling in RTX-treated or control mice ($n = 8$ per group) inoculated with LN cells from donor mice treated with or without RTX, assessed at 24 h after application of oxazolone to the recipients. C: Control mice; R: RTX-treated mice. (C) Effect of RTX treatment on elicitation. Ear swelling at 6 h 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's t -test or Dunnett's test) versus corresponding mice.

Figure 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. CM: CD44⁺CD62L⁺ central memory cells; EM: CD44⁺CD62L⁻ effector memory cells. (B) IFN- γ production in culture supernatants of LN cells ($n = 6$ per group) after 72 h of culture. Re-stim: re-stimulation. (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). MFI: mean fluorescence intensity. (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 h after sensitization with FITC. Data are presented as mean $\pm$ SD. $*P < .05$ (Student's t -test or Welch's test) versus corresponding mice.

Figure 4. Neuropeptide PACAP rescued hapten-induced DC migration and CHS

responses impaired by RTX-induced denervation. (A) Representative confocal micrographs of skin samples stained for PGP9.5 (red) in CD11c-YFP (green) mice. Scale bars, 50 μ m (Low-power field) and 10 μ m (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 application compared with control mice ($n = 8$ per group). (E) Effect of substance P and PACAP treatment on reduced ear swelling by RTX treatment at 24 h after oxazolone elicitation ($n = 5$ -10 per group). (F) Effect of PACAP antagonist PACAP6-38 on CHS responses at 24 h after oxazolone 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 h before oxazolone application ($n = 3$ per group). All data are presented as mean $\pm$ SD. Dotted horizontal lines

indicate control treatment set at 100%. * $P < .05$ (Student's t -test, Welch's test, or Dunnett's test) versus corresponding sample.

Figure 5. PACAP enhances BMDC maturation and drives BMDC migration. (A)

Expression of CD80 and CD86 on BMDCs co-cultured with DRGs from RTX-treated or control mice in the presence or absence of PACAP antagonist PACAP6-38 at 10 μ M ($n = 6$ per group). MFI: mean fluorescence intensity. (B) Relative mRNA expression in 1 μ M 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 h ($n = 3$ per group). (D) Effect of PKA 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 h ($n = 3$ per group). (E) Expression of CD80 and CD86 on PACAP-treated BMDCs in the presence or absence of H-89 at 10 μ M ($n = 3$ per group). MFI: mean fluorescence intensity. Data are presented as mean $\pm$ SD. * $P < .05$ (Student's t -test, Welch's test, or Dunnett's test) versus corresponding samples.

656

657 **Figure 6.** Effect of Adcyap1r1 knockdown on BMDC activation and CHS response.

658 (A) Expression of Adcyap1r1 and Vipr2 on BMDCs electroporated with 300 nM Adcyap1r1-,

659 Vipr2-, or control negative-SiRNA ($n = 3$ per group). (B) *Ccr7* and *Cxcr4* mRNA expression

660 in 1 μ M PACAP-treated BMDCs electroporated with 300 nM Adcyap1r1-, Vipr2-, or

661 negative-SiRNA ($n = 3$ per group). (C) Ear swelling in Adcyap1r1-knockdown BMDC- or

662 control BMDC-treated CD11c-DTR mice with or without DT pre-treatment. ($n = 3$ per

663 group). (D) The expression of Adcyap1r1, Vipr1, or Vipr2 in migrated DCs from control or

664 RTX-treated mice ($n = 8$ per group). MFI: mean fluorescence intensity. Data are presented as

665 mean $\pm$ SD. * $P < .05$ (Student's t -test, or Welch's test) versus corresponding samples.

666

Figure 1

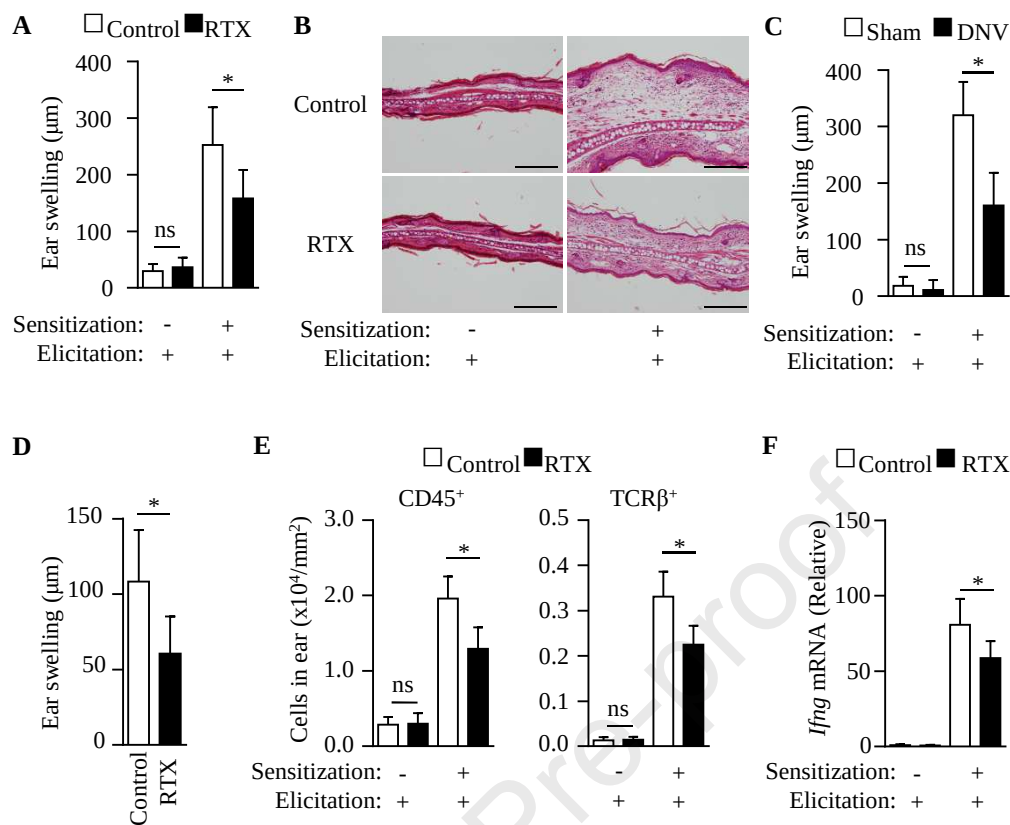


Figure 2

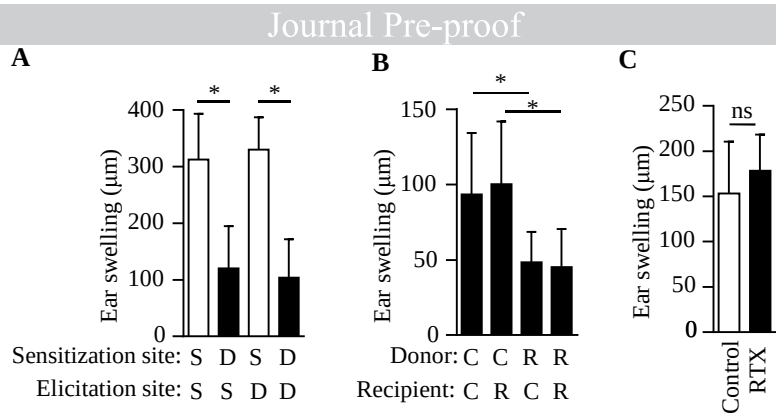


Figure 3

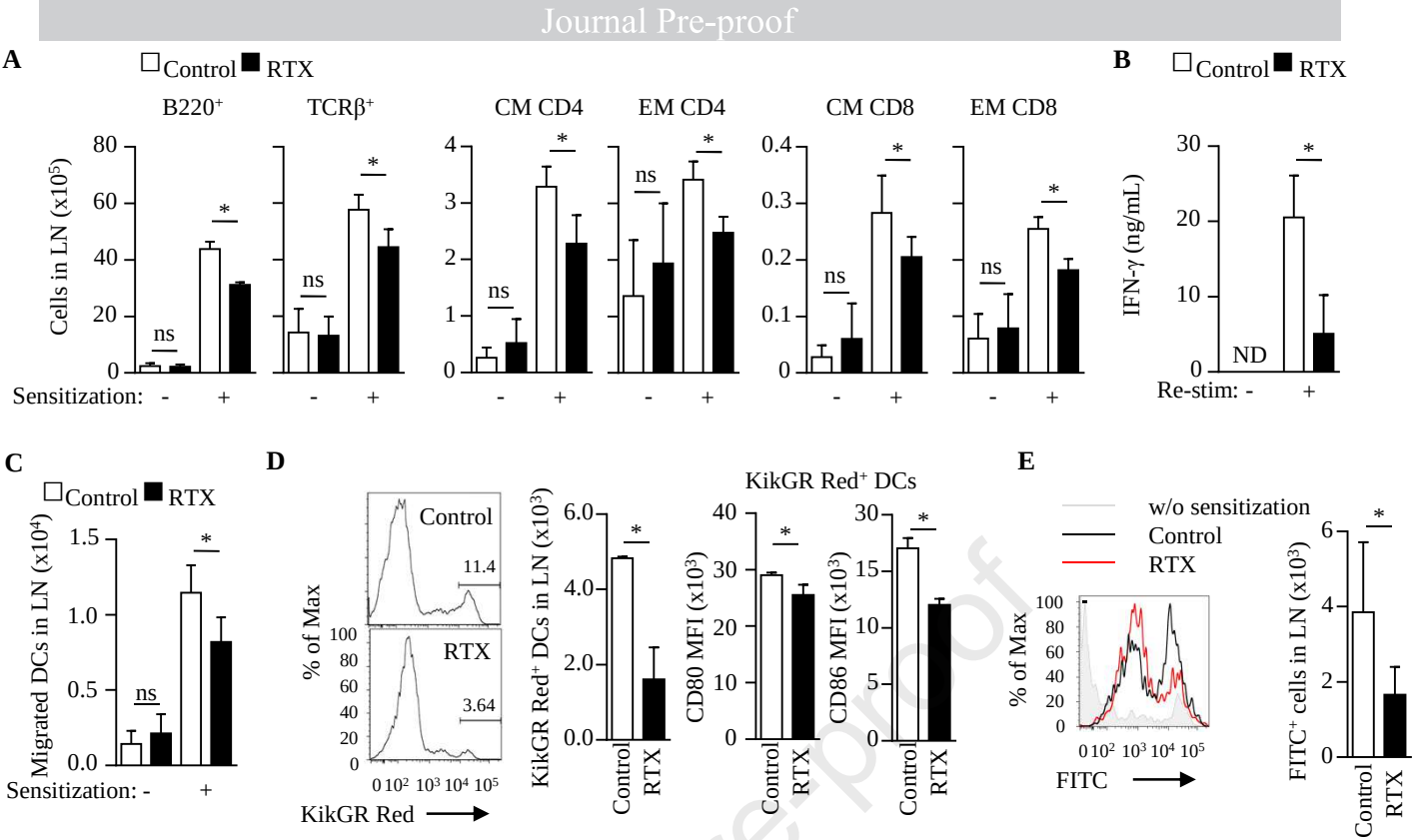


Figure 4

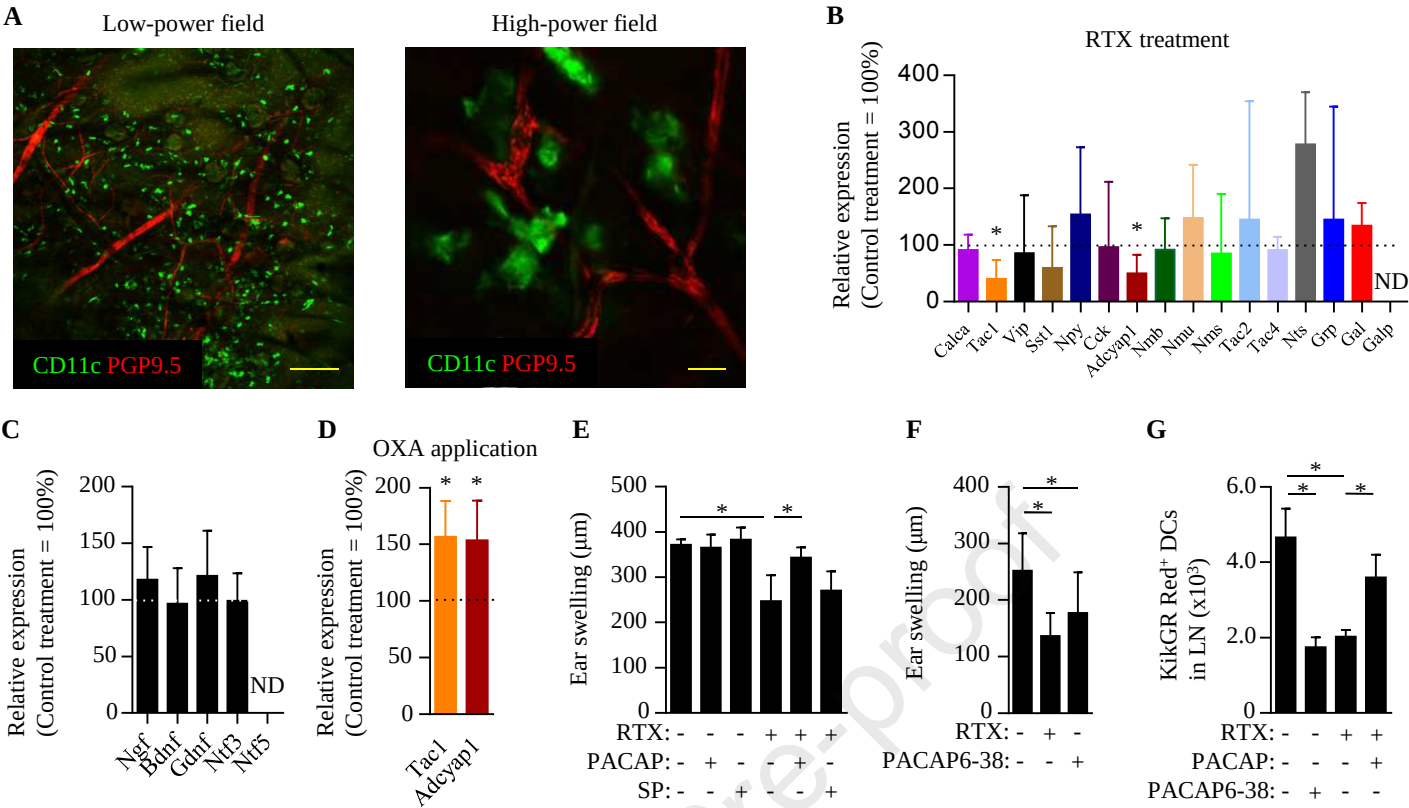


Figure 5

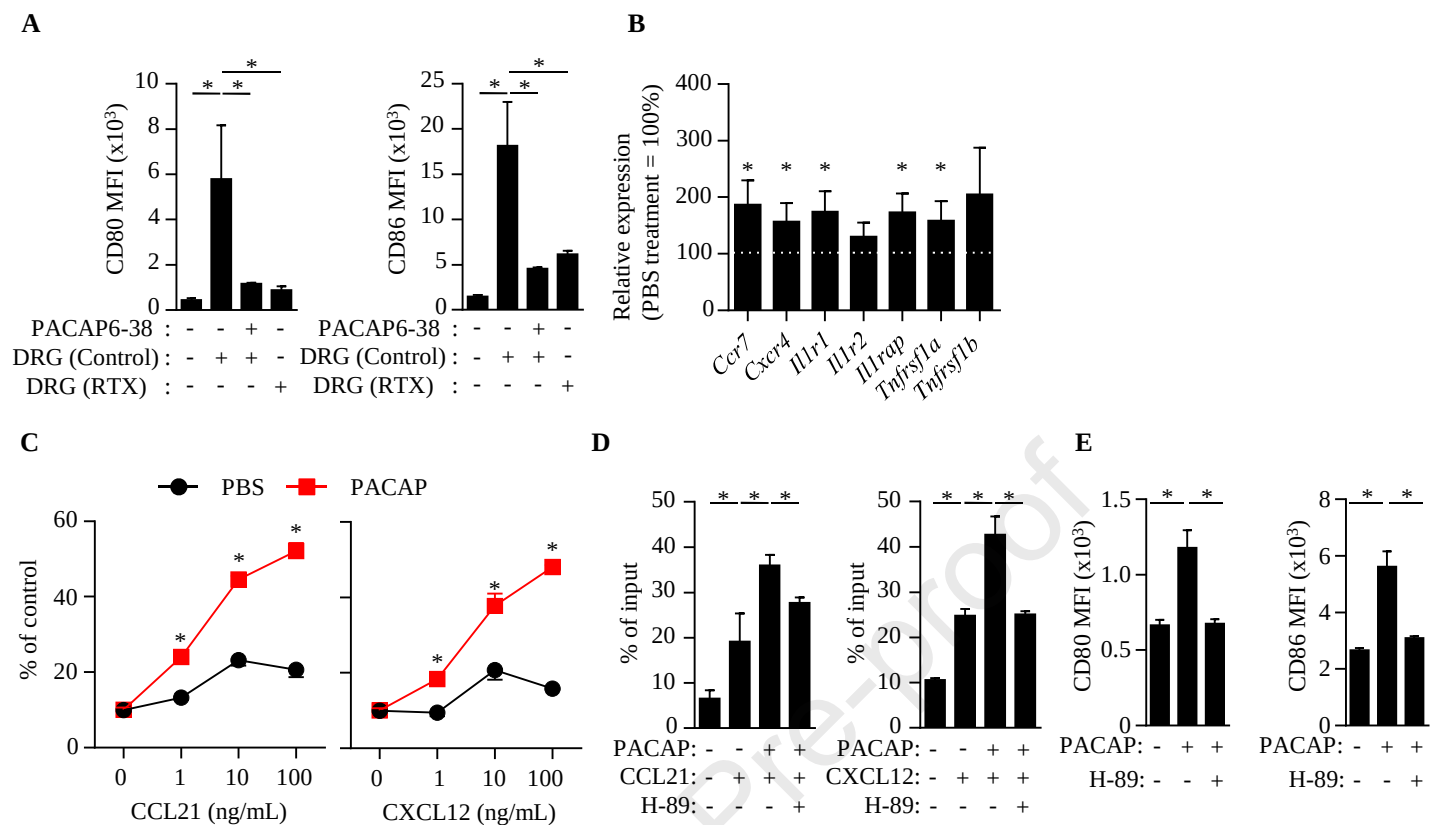


Figure 6

