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Calcitonin Gene-Related Peptide Is an Important Regulator of Cutaneous Immunity: Effect on Dendritic Cell and T Cell Functions

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Some cutaneous inflammations are induced by percutaneous exposure to foreign Ags, and many chemical mediators regulate this inflammation process. One of these mediators, calcitonin gene-related peptide (CGRP), is a neuropeptide released from nerve endings in the skin. CGRP binds to its receptors composed of receptor activity-modifying protein 1 and calcitonin receptor-like receptor to modulate immune cell function. We show that CGRP regulates skin inflammation under physiological conditions, using contact hypersensitivity (CHS) models of receptor activity-modifying protein 1-deficient mice. CGRP has different functions in CHS responses mediated by Th1 or Th2 cells; it inhibits Th1-type CHS, such as 2,4,6-trinitrochlorobenzene-induced CHS, but promotes Th2-type CHS, such as FITC-induced CHS. CGRP inhibits the migration of Langerin⁺ dermal dendritic cells to the lymph nodes in 2,4,6-trinitrochlorobenzene-induced CHS, and upregulates IL-4 production of T cells in the draining lymph nodes in FITC-CHS. These findings suggest that CGRP regulates several types of CHS reactions under physiological conditions and plays an important role in cutaneous immunity. *The Journal of Immunology*, 2011, 186: 6886–6893.

Recent investigations have shown that the immune system and nervous system are linked through several mediators, such as hormones, cytokines, and neurotransmitters (1, 2). As one of those molecules, calcitonin gene-related peptide (CGRP) and its immunoregulatory functions have been studied. CGRP, a 37-aa neuropeptide, is stored in the endings of sensory nerve fibers and is widely expressed in the central and peripheral nervous systems. CGRP binds to its specific receptor composed of receptor activity-modifying protein 1 (RAMP1) and calcitonin receptor-like receptor (CLR) (3). Upon CGRP binding to the receptor, intracellular cAMP levels are increased, leading to vasodilation (4, 5). CGRP receptors are also expressed on immune cells, and CGRP modulates the immune responses through receptor binding. For example, CGRP inhibits T cell functions, macrophage Ag presentation, and B cell Ab secretion (6–8). We have also reported that CGRP upregulates IL-4 production from

CD4⁺ T cells through the activation of the cAMP/protein kinase A pathway and inhibits the production of proinflammatory cytokines from LPS-stimulated bone marrow-derived dendritic cells (BMDCs) (9–11). These findings suggest that CGRP is an important regulator of immune responses, although most of these effects were studied in *in vitro* experiments. The effect of CGRP on immune responses under physiological conditions is not yet known.

Many CGRP-containing nerve fibers in the skin (12) are in direct contact with epidermal cells such as Langerhans cells (LCs) and keratinocytes (13) that express CGRP receptors (14). Because CGRP inhibits the Ag-presenting activities of LCs (15), as well as the differentiation of and cytokine production by Th cells *in vitro*, we hypothesized that CGRP is an important regulator of T cell-mediated skin inflammation, such as the contact hypersensitivity (CHS). CHS response is induced by epicutaneous sensitization and elicitation with chemical reagents like haptens, which directly bind to carrier proteins. After epicutaneous hapten application, APCs in the skin show enhanced expression of surface MHC class II molecules, migrate from the skin to the draining lymph nodes (LNs), and present hapten–MHC class II complexes to Th cells. Subsequent challenge with the hapten induces cutaneous infiltration and activation of the primed Th cells, resulting in the production of proinflammatory cytokines. The edema, characteristic of CHS, peaks 24–48 h after the hapten challenge.

To investigate the immunoregulatory effects *in vivo*, we studied the physiological roles of CGRP in skin inflammation, using the two CHS model systems—2,4,6-trinitrochlorobenzene (TNCB)-induced and FITC-induced CHS—in RAMP1-deficient mice (RAMP1^{-/-} mice). We report that RAMP1^{-/-} mice exhibit an enhanced and a suppressed CHS response to TNCB and FITC, respectively. Moreover, CGRP inhibits migration of skin APCs in TNCB-induced CHS and increases IL-4 production of Th cells in FITC-induced CHS.

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Abbreviations used in this article: BMDC, bone marrow-derived dendritic cell; CGRP, calcitonin gene-related peptide; CHS, contact hypersensitivity; CLR, calcitonin receptor-like receptor; DC, dendritic cell; dDC, dermal dendritic cell; DNFB, 2,4-dinitrofluorobenzene; LN, lymph node; RAMP1, receptor activity-modifying protein 1; sDC, splenic dendritic cell; TNCB, 2,4,6-trinitrochlorobenzene; VIP, vasoactive intestinal peptide; WT, wild-type.

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Materials and Methods

Mice

Female BALB/c mice and C57BL/6 mice (6–10 wk old) were purchased from Charles River Japan (Kanagawa, Japan). IL-4-deficient mice were obtained from the Laboratory Animal Center, Yamagata University School of Medicine (Yamagata, Japan). RAMP1^{-/-} mice were generated (11) and backcrossed for more than eight generations with BALB/c or C57BL/6 strains to generate RAMP1^{-/-} strains with a BALB/c or C57BL/6 background. We used BALB/c background mice in all experiments except for those shown in Fig. 6B and 6C. All procedures, including acquisition and treatment of experimental animals, were approved by the Experimental Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences, Osaka University.

Abs and reagents

Anti-mouse IFN- γ mAb (R4-6A2) and biotinylated anti-mouse IFN- γ mAb (XMG1.2) were purchased from BD Pharmingen (San Diego, CA). PE-conjugated anti-mouse CD103 mAb (2E7) was purchased from BioLegend (San Diego, CA); allophycocyanin-conjugated anti-mouse CCR2 mAb (475301), from R&D Systems (Minneapolis, MN); and PE-Cy5-conjugated anti-mouse CCR7 mAb (4B12), PE-conjugated anti-mouse CD80 mAb (16-10A1), PE-conjugated anti-mouse CD86 mAb (GL1), biotinylated anti-mouse MHC-II mAb (M5/114.15.2), and biotinylated anti-mouse IL-4 mAb (VBD6-24G2), from eBioscience (San Diego, CA). Biotinylated anti-mouse F4/80 mAb (1:A3-2) was purchased from Serotec (Oxford, U.K.); anti-Langerin mAb (929F3.01), from Dendritics (Lyon, France); and streptavidin/RPE, from Dako (Carpinteria, CA). Alexa Fluor 488-labeled goat anti-rat Ig was purchased from Molecular Probes (Eugene, OR). Rat α CGRP was obtained from the Peptide Institute (Osaka, Japan) and mouse MIP-3 β /CCL19, from R&D Systems.

Sensitization and elicitation of CHS

Mice were sensitized epicutaneously at day 0 by applying 100 μ l 1% TNCB diluted in acetone/olive oil (4:1) or 1% 2,4-dinitrofluorobenzene (DNFB) diluted in acetone on the abdominal skin and challenged at day 5 by applying 20 μ l 1% TNCB or 0.2% DNFB on the ear. In CGRP treatment experiments, the mice were intradermally injected with 50 μ l 10⁻⁵ M CGRP into one ear at 3 and 8 h before sensitization, then sensitized by hapten application on the ear skin, and challenged on the other ear as described above. In FITC-induced CHS, mice were sensitized daily from day 0–4 by applying 400 μ l 0.5% FITC diluted in acetone/dibutyl phthalate (1:1). At 5 d later (day 9), the mice were challenged by applying 20 μ l 0.5% FITC. Before and after challenge of chemicals, ear thickness was measured using a dial thickness gauge (Peacock; Osaka, Japan). Ear swelling was calculated by subtracting the prechallenge value from the value recorded at the corresponding time. For histological analysis, ears were harvested 48 h after challenge, and 6- μ m cryosections were prepared and stained with H&E.

Detection of emigrated LCs in draining LNs after sensitization

Mice were sensitized with 20 μ l 1% TNCB or 0.5% FITC on their ears. After 24 h, single-cell suspensions were prepared from subauricular draining LNs. The cells were fixed and permeabilized using BD Cytotfix/Cytoperm kit (BD Biosciences, San Diego, CA) and stained with anti-Langerin, anti-CD103 mAbs and anti-CCR7 mAbs. After washing, the cells were analyzed by a FACSCalibur analyzer and CellQuest software (BD Biosciences).

Skin explant culture assay

Only dorsal skin of the excised ears was used. Skins were cultured in 24-well plates, as described (16), in the presence of CGRP and/or MIP-3 β for 72 h, and the cells that migrated into the culture medium (RPMI 1640 supplemented with 10% FCS, 0.02 μ M kanamycin, and 5 μ M 2-ME) were counted.

Preparation of dendritic cell subpopulations and analysis of CCR expression

To isolate skin-derived dendritic cells (DCs), mouse skin was treated with 1 μ g/ml dispase (Life Technologies) in HBSS for 4 h. After dispase treatment, the epidermis was separated from the dermis, and epidermal single-cell suspensions were prepared by pipetting. The dermal sheet was treated with 1% collagenase (Wako, Osaka, Japan) for 1 h to prepare dermal single-cell suspensions. LCs were sorted as MHC class II⁺ cells from epidermal cells; dermal Langerin⁺ DCs (dDCs), as CD11c⁺CD103⁺ cells

from dermal cells; and splenic DCs (sDCs), as CD11c⁺ cells from splenocytes, using FACSaria II (BD Biosciences). BMDCs were generated by culturing in RPMI 1640 medium supplemented with 10% FCS and 20 ng/ml mouse GM-CSF for 10 d, as previously described (11). For mRNA expression analysis, sorted cells were stimulated with LPS (0.1 μ g/ml) for 6 h, and mRNA was extracted. For FACS analysis, cells were cultured with or without stimulation with LPS (0.1 μ g/ml) for an appropriate length of time. After culturing, the cells were stained with anti-CCR2 or anti-CCR7 mAbs. In all experiments, cells were treated with 1 nM α CGRP.

RNA extraction and RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was reverse transcribed into cDNA with PrimeScript RT reagent kit (TaKaRa, Kyoto, Japan), according to the manufacturer's instructions. The primers used were RAMP1 forward, 5'-GGGCTCTGCTTGCATGG-3'; RAMP1 reverse, 5'-CACCTGGGATACCTACAC-3'; CLR forward, 5'-GGACGGATGGCTATGC-3'; CLR reverse, 5'-CAGTAAACG-CCTTCAC-3'; Langerin forward, 5'-CCAAGGACTACAGAACAGCTT-3'; Langerin reverse, 5'-ACAGAAAGACCACAGGCT-3'; CCR2 forward, 5'-AGGCCATGCAGGTGACAG-3'; CCR2 reverse, 5'-AGGCAAACCTG-TCACTTACT-3'; CCR7 forward, 5'-AGAGAGACAAGAACCA-3'; CCR7 reverse, 5'-AGGTAGGTATCCGTCA-3'; GAPDH forward, 5'-CATGCCATCACTGCCACCC-3'; and GAPDH reverse, 5'-GGTAGGAACACG-GAAGGCC-3'. PCR products were visualized by ethidium bromide staining and analyzed on a 2% agarose gel.

Measurement of cytokine concentrations

Subauricular draining LNs of the mice with induced CHS were harvested 24 h after challenge, and single-cell suspensions of lymphocytes were prepared. Lymphocytes (10⁶ cells/ml) were stimulated with plate-coated anti-CD3 mAb (145-2C11) and soluble anti-CD28 mAb (PV-1, 1 μ g/ml). Culture supernatants were collected after 48 h, and cytokine concentrations were measured by ELISA, as described (10).

Statistics

The statistical significance of the differences in the groups was examined by the unpaired Student *t* test or Student–Newman–Keuls test after non-repeated-measures ANOVA; *p* < 0.05 was considered significant.

Results

CGRP regulates the CHS reaction

A previous study showed that injection of CGRP inhibits the induction of CHS in response to TNCB or DNFB (17). To confirm this, BALB/c mice were injected with 50 μ l of 10⁻⁵ M CGRP into the ear 3 and 8 h before sensitization with TNCB or DNFB. At 5 d after the challenge, ear swelling was measured, and histological analysis was performed. As seen in Fig. 1A, CGRP significantly suppressed both TNCB- and DNFB-induced CHS. The analysis showed suppression of inflammatory cell infiltration (Fig. 1B). To reveal the physiological roles of CGRP signaling in the CHS response, RAMP1^{-/-} mice were used. Because no difference was observed between the suppression of TNCB- and DNFB-induced CHS responses by CGRP, we used the TNCB-induced CHS model in the following experiments. Ear swelling in TNCB-induced CHS was significantly increased in RAMP1^{-/-} mice compared with wild-type (WT) mice (Fig. 1C, 1D). These results suggest that CGRP physiologically suppresses the TNCB-induced CHS, although it is still unclear how CGRP inhibits this response.

CGRP inhibits Langerin⁺ cell migration in the TNCB-CHS response

Using Langerin-DTR mice, Bennett et al. (18) showed that Langerin⁺ cells play an important role in CHS induction. After applying a hapten, activated LCs migrated from the epidermis to the dermis and then to the draining LNs, wherein they induced T cell sensitization (16). To determine whether CGRP could affect Langerin⁺ cell migration at the sensitization phase of CHS, we examined the effect of CGRP on the number of Langerin⁺ cells that migrated to the draining LNs. LNs were harvested 24 h after

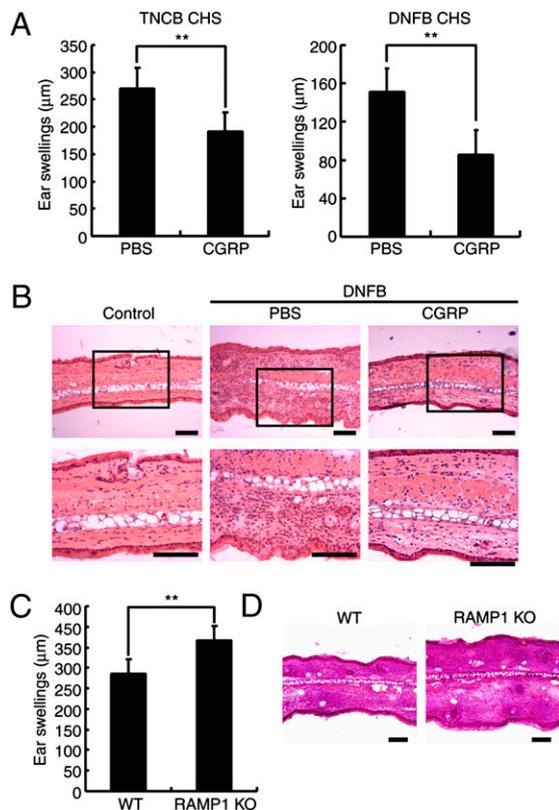


FIGURE 1. CGRP physiologically inhibits TNCB-induced CHS. *A*, BALB/c mice intradermally injected with 50 μ l of 10^{-5} M CGRP into the ear at 3 and 8 h before sensitization. Ear swelling was measured 24 h after TNCB or DNFB challenge. Values are represented as mean \pm SD ($n = 8$ or 9 per group). *B*, Ears from the mice harvested 48 h after the challenge; 6- μ m cryosections were prepared and stained with H&E. Scale bars, 100 μ m. A representative of three independent experiments is shown. *C*, WT and RAMP1^{-/-} mice were sensitized with TNCB, and ear swelling was measured 48 h after challenge. Values are represented as mean \pm SD ($n = 7$ or 9 per group). *D*, Ear cryosections were stained with H&E, as in *B*. Scale bars, 100 μ m. A representative of two independent experiments is shown. ** $p < 0.01$.

TNCB sensitization, and cells were stained with anti-Langerin mAb for FACS analysis. In WT mice, TNCB application increased the number of Langerin⁺ cells in the LNs, and CGRP treatment inhibited this increase (Fig. 2*A*, 2*B*). The number of Langerin⁺ cells was significantly higher in the LNs from TNCB-sensitized RAMP1^{-/-} mice than in those from WT mice (Fig. 2*C*). These findings suggest that CGRP inhibits the migration of Langerin⁺ cells to the LN *in vivo*.

Langerin⁺ cells in the skin tissue were classified into two subpopulations: epidermal LCs and Langerin⁺ dDCs. Therefore, we identified the DC population that migrates into the LNs after TNCB sensitization in our experiment. As shown in Fig. 2*D*, after TNCB application, the number of Langerin⁺CD103⁺ dDCs increased in the LNs of RAMP1^{-/-} mice compared with WT mice. In contrast, the number of Langerin⁺CD103⁻ LCs was comparable. These results show that CGRP regulates the migration of Langerin⁺ dDCs into the LNs after TNCB sensitization.

Inhibitory effect of CGRP on activated Langerin⁺ cell migration in skin explant culture assay

To study the direct effect of CGRP on Langerin⁺ cell migration, we performed a skin explant culture assay, using mouse ear skin. Skin tissue samples from WT or RAMP1^{-/-} mice were cultured in the

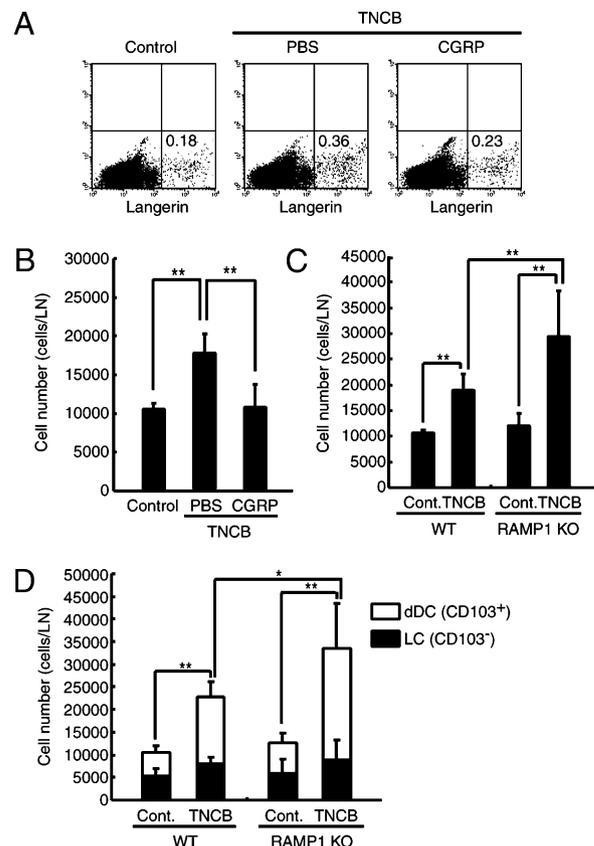


FIGURE 2. CGRP inhibits Langerin⁺ cell migration to the LNs. Draining LNs were collected 24 h after TNCB sensitization, and total LN cells were stained with anti-Langerin and anti-CD103 Abs before analysis by FACSCalibur. *A* and *B*, BALB/c mice were treated with CGRP 3 h before TNCB application. The number of Langerin⁺ cells in the LNs was determined by multiplying the total LN cells by the percentage of Langerin⁺ cells. Values are represented as mean \pm SD ($n = 5$). *C* and *D*, WT and RAMP1^{-/-} mice were sensitized with TNCB. Nontreated mice were used as negative control. Values are represented as means \pm SD ($n = 7$ or 8 per group). The statistical significance of differences between groups was examined by ANOVA followed by the Student–Newman–Keuls test. Statistical analysis was performed to compare differences among each cell population, as shown in *D*. Significant differences among the dDC populations are shown in the figure. No statistically significant differences were observed among the LC populations. * $p < 0.05$, ** $p < 0.01$.

assay medium for 72 h, and the number of migrated cells in the medium was counted. As shown in Fig. 3*A*, all the migrated cells expressed CD11c, Langerin, CCR7, and CD103. Therefore, these cells were considered to be activated Langerin⁺ dDCs, because CCR7 is expressed in activated DCs (19). Addition of CGRP to the culture medium significantly reduced the number of migrated cells in the medium in a dose-dependent manner (Fig. 3*B*); this effect was not observed with the skin tissue of RAMP1^{-/-} mice. Migration into the LNs is induced by chemotactic molecules, such as MIP-3 β /CCL19, one of the CCR7 ligands (20). To investigate the effect of CGRP on chemotactic activity, MIP-3 β was added to the culture medium. The number of migrated cells was increased by MIP-3 β addition, and CGRP completely suppressed this MIP-3 β effect (Fig. 3*C*). These findings suggest that CGRP inhibits CCR7 expression or CCR7 signal transduction on Langerin⁺ dDCs, which results in the suppression of their migration from the dermis to LNs.

CGRP inhibits CCR expression in several DC subpopulations

Because CGRP inhibited CCR7-dependent migration *in vitro*, the effect of CGRP on the expression of CCR7 in Langerin⁺ dDCs was

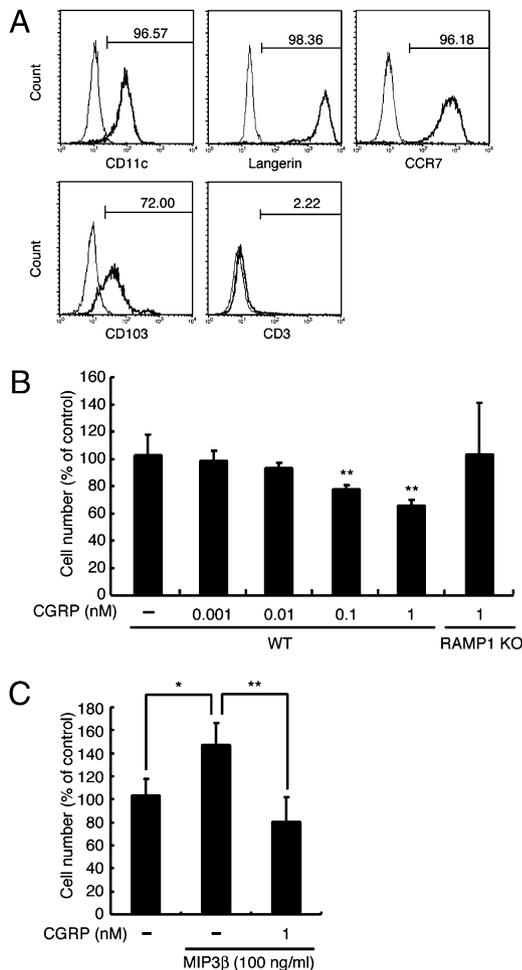


FIGURE 3. Effects of CGRP on the migration of Langerin⁺ cells in vitro, studied using the skin explant culture assay. BALB/c mice-derived ear skins were incubated in the assay medium for 72 h, and the number of migrated cells into the medium was determined. *A*, Migrated cells were almost activated Langerin⁺CD103⁺ dDCs. A representative of three independent experiments is shown. *B*, CGRP reduces the number of cells that have migrated into the medium. Values are represented as mean \pm SD ($n = 4$ or 5). *C*, CGRP inhibits MIP-3 β (100 ng/ml)-induced migration. Values are represented as mean \pm SD ($n = 3$). The data are shown as the ratio of the control to the presence of CGRP. Values are represented as mean \pm SD ($n = 4$). * $p < 0.05$, ** $p < 0.01$.

investigated. Recently, it has been reported that CCR2 plays an important role in DC migration in CHS (21). Therefore, we also investigated the effect of CGRP on CCR2 expression in LCs and dDCs, which were sorted from the epidermal cells and the dermal cells, respectively (Fig. 4A). The sDCs were sorted from the splenocytes. We could detect the mRNA expression of the CGRP receptor components, CLR and RAMP1, in all DC populations (Fig. 4B). In RT-PCR analysis, CGRP suppressed CCR2 expression in all LPS-stimulated DC populations in vitro (Fig. 4C, 4D) and suppressed CCR7 expression in dDCs (Fig. 4C).

Unfortunately, we could not detect CCR2 expression in LPS-stimulated DCs in a protein level (data not shown). It is reported that CCR2 expression is downregulated by stimulation with LPS (22). Then, we examined the effect of CGRP on CCR2 expression in nonstimulated DCs. BMDCs cultured with CGRP showed lowered expression of CCR2 than did nontreated BMDCs (Fig. 4E). This suppressive effect of CGRP was also observed in dDCs (Fig. 4F). These results suggest that CGRP might inhibit CCR2 expression and suppress DC maturation in naive mice.

In contrast to CCR2, CCR7 is upregulated by various stimulations, and it contributes to DC migration. As shown in Fig. 4G, CGRP inhibited CCR7 expression in LPS-stimulated dDCs, but not in LCs, so that the effect of CGRP on DC migration is attributed to the suppression of CCR2 and/or CCR7 expression.

To determine whether CGRP regulates CCR7 expression by dDCs that have migrated into draining LNs, cells from the TNCB-treated mice were stained with anti-CCR7 mAb and analyzed by FACS (Fig. 4H). The number of CCR7⁺ dDCs was increased in the LNs of RAMP1^{-/-} mice compared with that in the LNs of WT mice (Fig. 4I). Moreover, the dDCs from RAMP1^{-/-} mice showed a higher level of CCR7 expression than did those from WT mice (Fig. 4H, 4J). These data indicate that CGRP physiologically inhibits CCR7 expression by dDCs in vivo.

CGRP augments the FITC-CHS reaction in a Langerin⁺ cell-independent manner

The CHS response can be classified into two types: Th1 type, which is induced by TNCB or DNFB, and Th2 type, which is induced by FITC or trimellitic anhydride (23, 24). Because CGRP enhances Th2 responses (10), our findings suggest that CGRP exhibits different effects or mechanisms in the CHS response. In fact, pretreatment with the CGRP antagonist CGRP (8–37) inhibits FITC-induced CHS (25). This observation is in contrast to TNCB-induced CHS, as shown in Fig. 1. To better understand the role of CGRP in FITC-induced CHS, the latter was examined in RAMP1^{-/-} mice. As seen in Fig. 5A, FITC-induced CHS was significantly suppressed in RAMP1^{-/-} mice. Then, we investigated whether CGRP affects the migration of Langerin⁺ cells after FITC application. As shown in Fig. 5B, the number of Langerin⁺ cells in the draining LNs did not increase in WT or RAMP1^{-/-} mice. Furthermore, FITC⁺ cells, which incorporated the FITC-conjugated molecules applied, expressed almost no Langerin (Fig. 5C), and the number of FITC⁺ cells in the draining LNs did not differ between WT and RAMP1^{-/-} mice (Fig. 5D). These findings suggest that Langerin⁺ cells contribute little to FITC-induced CHS.

CGRP regulates the FITC-induced CHS response via regulation of T cell function

In the FITC-induced CHS response, Langerin⁺ cells did not migrate to the draining LNs. Therefore, we decided that Langerin⁺ cells are not the target cells for CGRP in FITC-induced CHS. To determine how CGRP augments the FITC-induced CHS, we studied the regulatory effect of CGRP on T cells in vivo. It has been reported that FITC-induced CHS involves response to Th2-mediated inflammation (26, 27) and that IL-4 is essential for this response (27). Consistent with this report, we observed that the FITC-induced CHS response was remarkably suppressed in IL-4-deficient mice (data not shown); therefore, IL-4 appears to play a critical role in FITC-induced CHS. We previously reported that CGRP downregulates IL-2 and IFN- γ production, but upregulates IL-4 production and promotes Th2 differentiation in vitro (10). To prove the hypothesis that CGRP augments FITC-CHS by upregulation of IL-4 production, cytokine production in the lymphocytes from FITC-induced CHS mice was measured by ELISA. The production of IL-4, a Th2-type cytokine, increased in FITC-induced CHS (Fig. 6A). Moreover, IL-4 production in the lymphocytes of RAMP1^{-/-} mice was lower than in those of WT mice. These results suggest that CGRP stimulates an increase in IL-4 production, leading to an enhanced FITC-induced CHS response in vivo. In contrast, IFN- γ production was not induced by stimulation with either TNCB or FITC in the background of BALB/c mice (Fig. 6A). It is well known that a greater Th2 bias exists in BALB/c mice compared with C57BL/6 mice. To examine the effect

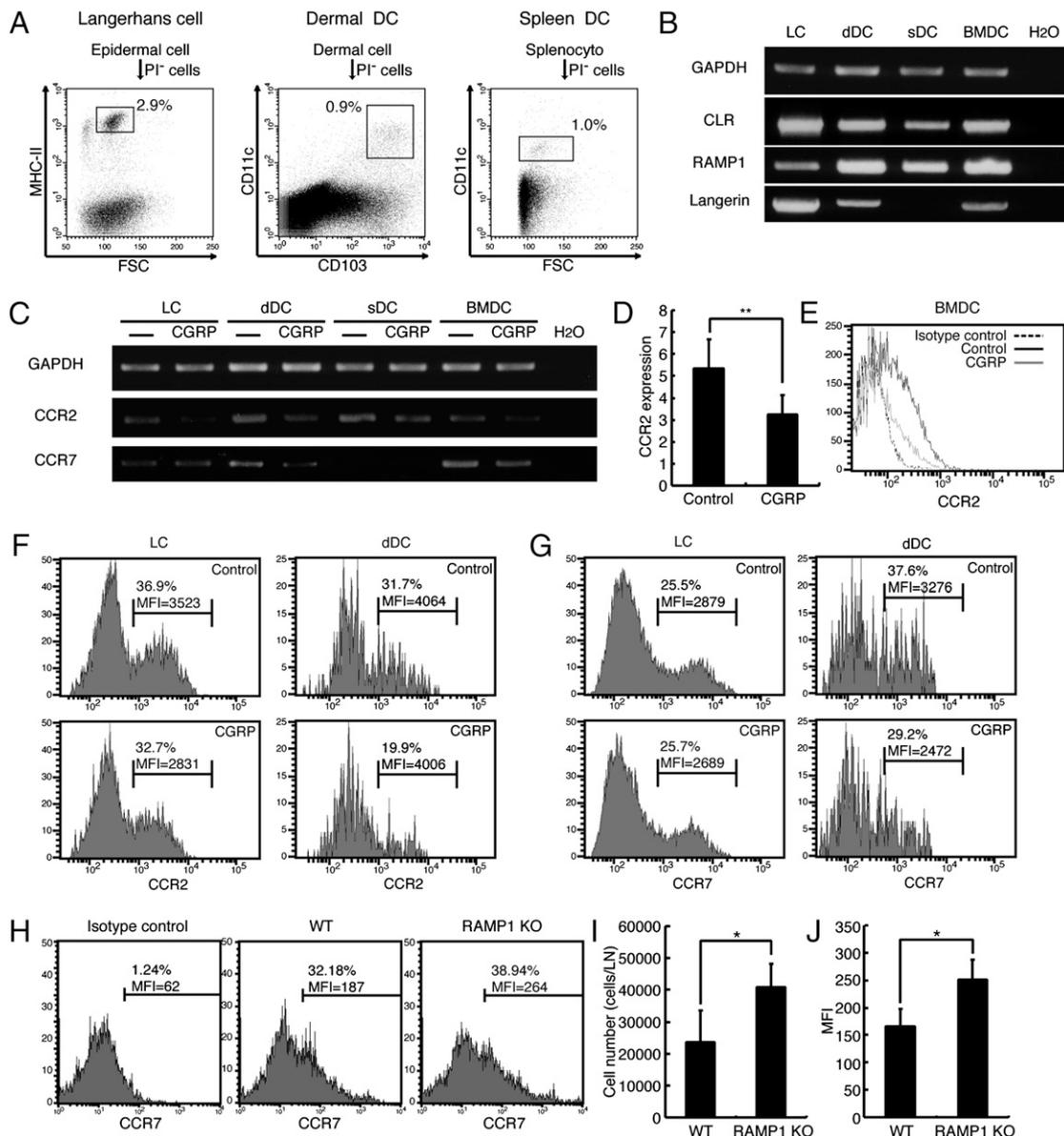


FIGURE 4. CGRP inhibits CCR2 and CCR7 expression. *A*, LCs, Langerin⁺ dDCs, and sDCs sorted by FACSaria II. *B*, Expression of CGRP receptor subunits, CLR and RAMP1, as detected by RT-PCR. *C*, RT-PCR analysis of the expression of CCR2 and CCR7 analyzed by RT-PCR. All DC populations were stimulated with LPS (0.1 μ g/ml) for 6 h. *D*, RT-PCR analysis of CCR2 expression performed using BMDCs. Values are represented as mean \pm SD ($n = 3$). *E*, FACS analysis of CCR2 expression performed using nonstimulated BMDCs. CGRP was added beginning at day 0 of the BMDC culture, and the analysis was performed on day 6. *F*, FACS analysis of CCR2 expression on LCs and dDCs was performed using FACSaria II. A representative of two independent experiments is shown. *G*, FACS analysis of CCR7 expression on dDCs was done using FACSaria II. Cells were stimulated with LPS (0.1 μ g/ml) for 24 h. A representative of two independent experiments is shown. *H*, FACS analysis of CCR7 expression in dDCs that migrated to draining LNs was performed using FACSCalibur. *I*, The number of CCR7⁺ dDCs in the LNs was determined. Values are represented as mean \pm SD ($n = 3$). *J*, The MFI of CCR7⁺ dDCs in the LNs was determined. Values are represented as mean \pm SD ($n = 3$). ** $p < 0.01$, * $p < 0.05$. MFI, mean fluorescent intensity.

of CGRP on the Th1-type CHS response, we used mice with C57BL/6 background. As shown in Fig. 6*B*, IFN- γ production was increased after induction of TNCB-induced CHS in C57BL/6 mice and RAMP1^{-/-} mice backcrossed with C57BL/6 mice. Moreover, RAMP1^{-/-} mice showed higher IFN- γ production than did WT mice in TNCB-induced CHS. We confirmed that IFN- γ production was reduced in the lymphocytes from mice treated with CGRP 3 h before TNCB challenge (Fig. 6*C*). Our findings show that CGRP affects cytokine production by Th cells to amplify the Th2 reaction and regulates Th cell-mediated inflammation.

Discussion

The effects of CGRP in skin inflammation have been reported in some studies. Girolomoni et al. (28) showed that neuropeptide

depletion by capsaicin treatment attenuated the CHS responses induced by oxazolone and DNFB. Gutwald et al. (29) reported that CGRP administration at both the sensitization and elicitation phases enhanced oxazolone-induced CHS. In contrast, the administration of a CGRP antagonist was shown to reduce FITC-induced CHS (25). These findings suggest that CGRP plays the role of a promoter in CHS reactions. However, it was demonstrated that CGRP administration in the sensitization phase suppressed both TNCB- and DNFB-induced CHS (17, 30). This result is inconsistent with the results of the other studies described above. Despite substantial research, a consensus on the regulatory effect of CGRP in CHS has not yet emerged. In addition, because the local concentrations of CGRP vary depending on the patient's physical and mental condition, the physiological importance of

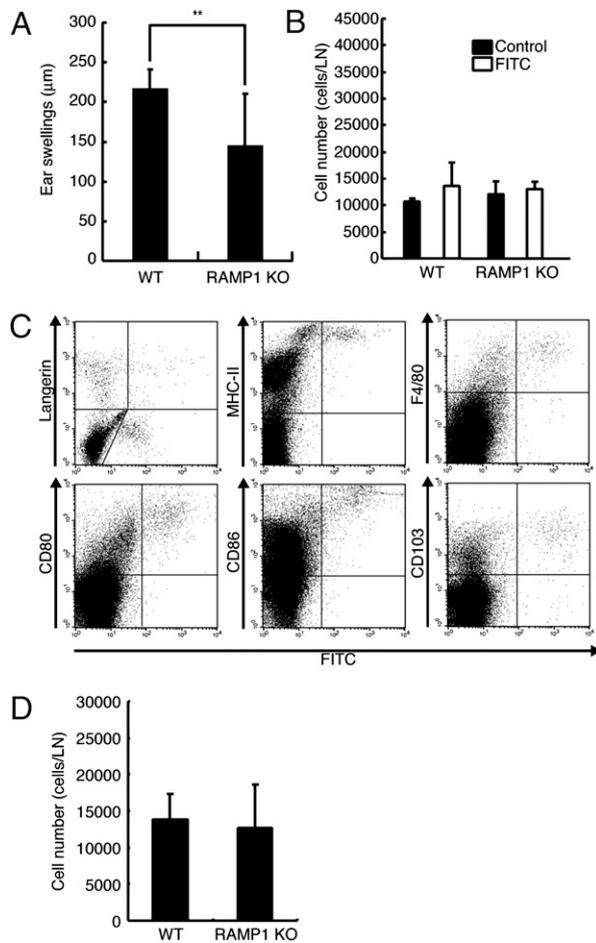


FIGURE 5. CGRP augments the FITC-induced CHS reaction without regulation of Langerin⁺ cells. *A*, WT and RAMP1^{-/-} mice sensitized with FITC; ear swelling measured 12 h after the challenge. Values are represented as mean \pm SD ($n = 8$ or 13 per group). *B*, Counting of the number of migrated Langerin⁺ cells into the draining LNs, as described for Fig. 2. *C*, FACS analysis of cell surface molecules expressed on FITC⁺ cells. A representative of two independent experiments is shown. *D*, Counting of the number of migrated FITC⁺ cells into the draining LNs. Values are means \pm SD ($n = 3$). ** $p < 0.01$.

CGRP in CHS is unclear. In the current study, we examined the physiological function of CGRP in CHS using RAMP1^{-/-} mice.

We found that CGRP exhibited different effects, depending on the type of skin inflammation; it acted as an inhibitory regulator for TNCB-induced CHS and an enhancing regulator for FITC-induced CHS (Figs. 1, 5). The CHS response can be classified into several types according to the differences in functional APC or T cell populations. In fact, our results indicated that the major APC population clearly differs between TNCB-induced CHS and FITC-induced CHS. In the skin, CGRP is released from nerve endings following stimulation with heat, protons, capsaicin treatment, or stress exposure (31, 32). Langerin⁺ cells are associated with CGRP-containing nerve fibers in the skin (13), which suggests that the effects of CGRP on Langerin⁺ cells are important for the development of cutaneous immune reactions. Langerin⁺ cells are considered the major APCs in skin tissue, and they play an important role in the formation of immunological memory against hapten application. In TNCB- or DNFB-induced CHS, Langerin⁺ cells migrate to the LNs and present Ags to the T cells (33, 34); the number of activated Langerin⁺ cells increases in the skin-draining LNs after TNCB application (16). The recently identified Langerin⁺ dDCs, unlike epidermal LCs, express CD103 in the

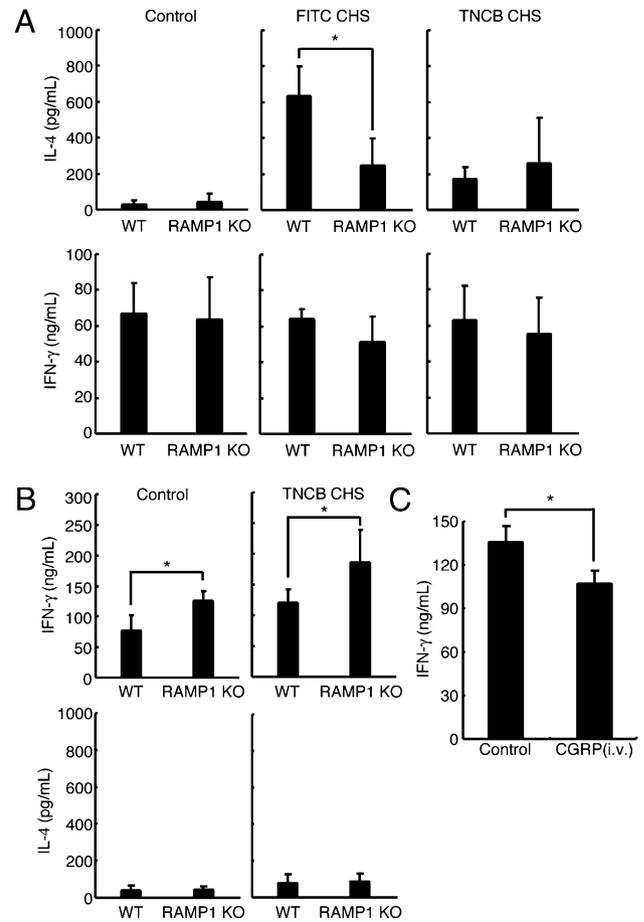


FIGURE 6. CGRP regulates cytokine production of T cells in the CHS reaction. *A*, CHS was induced in BALB/c background mice, WT mice, or RAMP1^{-/-} mice with TNCB or FITC. LNs were collected 24 h after challenge, and total lymphocytes were restimulated with anti-CD3 and anti-CD28 Abs for 48 h. IL-4 and IFN- γ concentrations in the supernatants were measured by ELISA. Values are represented as mean \pm SD (the number of mice used in control, FITC-induced CHS, or TNCB-induced CHS is $n = 3$, $n = 4-6$, or $n = 7$, respectively). *B*, C57BL/6 background WT and RAMP1^{-/-} mice with TNCB-induced CHS; cytokine production evaluated, as described for *A*. Values are represented as mean \pm SD ($n = 6$). * $p < 0.05$. *C*, CGRP treatment inhibits IFN- γ production in the elicitation phase. C57BL/6 mice were injected i.v. with PBS or 10 μ M CGRP (50 μ l) 3 h before challenge. After 24 h, LNs were collected and lymphocytes were stimulated for 48 h. Data are shown as mean \pm SD ($n = 3$). * $p < 0.05$.

dermis and skin-draining LNs (35). In addition, using Langerin⁺ cell-depleted mice, a study showed that Langerin⁺ dDCs, but not LCs, function as the major APCs for the CHS response (33). Our results also indicate that the major APCs for TNCB-induced CHS are Langerin⁺CD103⁺ dDCs.

Our *in vitro* experiments demonstrate that CGRP inhibits CCR7 expression in LPS-stimulated dDCs at both the mRNA and protein levels (Fig. 4C, 4G). Moreover, migrated dDCs after TNCB application show higher CCR7 expression in RAMP1^{-/-} mice (Fig. 4J). CCR7 plays a critical role in DC migration in the CHS response, because APCs fail to migrate into the draining LNs in CCR7-deficient mice (19). The effects of the neuropeptide vasoactive intestinal peptide (VIP) on chemokine receptor expression have been studied. VIP has potent immunomodulatory activity and seems to upregulate CCR1 and downregulate CCR7 expression in LPS-stimulated BMDCs (36). However, these effects of VIP were observed in *in vitro* experiments, and the physiological functions

of VIP are unclear. Our findings show that CGRP is a potent physiological regulator that directly suppresses APC migration. Moreover, we found that CGRP suppresses CCR2 expression in several DC populations under nonstimulated conditions (Fig. 4C–F). Recently, using CCR2-deficient mice, studies demonstrated that CCR2 enhanced DC migration by promoting DC maturation (21, 37). Therefore, CCR2 could play an important role in DC maturation before its activation, and CGRP could inhibit DC maturation in the steady state. These CCR2-dependent mechanisms might contribute to the inhibitory effect of CGRP on DC migration. In LCs, CGRP regulates cellular functions such as cytokine production and Ag presentation in vitro (15). Ding et al. (38) showed that CGRP treatment inhibited LC Ag presentation for a Th1 response but augmented Ag presentation for a Th2 response. They also showed that CGRP inhibits the production of the Th1-type chemokines CXCL9 and CXCL10 but induces the production of the Th2-type chemokines CCL17 and CCL22 in LCs (38). These results suggest that CGRP has effects on dDCs similar to those observed on LCs, and that it inhibits Th1-type inflammatory responses such as TNCB-induced CHS.

In contrast to TNCB-induced CHS, Langerin⁺ cell number did not increase in FITC-induced CHS. We also demonstrated that FITC⁺ cells were found in the draining LNs after FITC application, but they were Langerin[−] MHC class II⁺ APCs (Fig. 5C). These FITC⁺ cells expressed CD80, CD86, CD103, and F4/80. These findings suggest that FITC may pass through the epidermis and be captured by the dermal macrophages to be presented to the T cells as an Ag. It is known that CGRP affects several macrophage functions such as cytokine production (7, 39); however, our results suggest that CGRP has no effect on macrophage migration in vivo.

TNCB-induced CHS is mediated by the Th1-type immune response, whereas FITC-induced CHS is mediated by the Th2-type immune response (23, 24). We previously reported that CGRP downregulates IL-2 and IFN- γ production, but upregulates IL-4 production (10). These observations suggest that CGRP promotes the Th2-type immune response and exerts regulative effects on cutaneous immunity. In fact, IL-4 production from TCR-stimulated lymphocytes was reduced in RAMP1^{−/−} mice with FITC-induced CHS (Fig. 6), but no difference in the T cell population between WT and RAMP1^{−/−} mice (data not shown) was found. These observations indicate that CGRP promotes IL-4 production by T cells in vivo and regulates T cell-mediated inflammation under physiological conditions.

We also evaluated the effects of CGRP on IFN- γ production by T cells in TNCB-induced CHS. TNCB-induced CHS is regarded as a Th1-type response, and IFN- γ is an important effector cytokine in CHS (40). IFN- γ mRNA expression after challenge with TNCB was observed in C57BL/6 mice, but not in BALB/c mice, which are genetically prone to Th2-dominant responses (23). At the protein level, increased production of IFN- γ was observed in TNCB-induced CHS in C57BL/6 mice, but not BALB/c mice. Although IFN- γ upregulation was not noted in RAMP1^{−/−} mice of the BALB/c background, the mice showed more severe skin inflammation than did WT mice. Therefore, we concluded that this severe phenotype may not depend on IFN- γ .

Using RAMP1^{−/−} mice, we demonstrated that CGRP affects skin inflammation in several ways. This observation is very important for understanding the effects of CGRP as a critical regulator of skin inflammation. Our findings also show that chemical allergens induce various types of skin inflammation, depending on their properties, such as carrier protein binding, immunogenic potential, or cutaneous permeability. It is necessary to determine which cells recognize Ags and which cytokines are produced by

cells against Ags. It was reported that in human dermatitis, several Ags, such as metals and methylisothiazolinones, induced a mixed response of Th1 and Th2 cytokines, although they have different propensities to the Th1/Th2 balance (41–45). In fact, both Th1 and Th2 cytokines have been detected in an allergic contact dermatitis lesion (46). These findings suggest that both types of Th cells are involved in human contact dermatitis. Therefore, to reveal how the Th1/Th2 balance is regulated, it is necessary to determine the appropriate therapy for contact dermatitis. It is also essential to reveal what kinds of APCs contribute to the mechanisms underlying the Th1/Th2 balance. Further, to understand human skin diseases, it is important to consider the possibility that neurotransmitter concentration is controlled by a patient's mental condition and that an Ag can have a direct effect on the nervous system. In fact, CGRP treatment inhibits Ag presentation by human peripheral mononuclear cells and human DCs (47, 48). Determination of the mechanisms underlying CGRP-mediated immune regulation will open up a new avenue for understanding various types of skin inflammation and skin diseases.

Disclosures

The authors have no financial conflicts of interest.

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