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Calcitonin Gene-Related Peptide and Cyclic Adenosine 5’-Monophosphate/Protein Kinase A Pathway Promote IL-9 Production in Th9 Differentiation Process

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Th9 cells are a novel Th cell subset that produces IL-9 and is involved in type I hypersensitivity such as airway inflammation. Although its critical roles in asthma have attracted interest, the physiological regulatory mechanisms of Th9 cell differentiation and function are largely unknown. Asthma is easily affected by psychological factors. Therefore, we investigated one of the physiological mediators derived from the nervous system, calcitonin gene-related peptide (CGRP), in asthma and Th9 cells because CGRP and activation of the cAMP/protein kinase A (PKA) pathway by CGRP are known to be important regulators in several immune responses and allergic diseases. In this study, we demonstrated that the CGRP/cAMP/PKA pathway promotes IL-9 production via NFATc2 activation by PKA-dependent glycogen synthase kinase-3β inactivation. Moreover, CGRP also induces the expression of PU.1, a critical transcriptional factor in Th9 cells, which depends on PKA, but not NFATc2. Additionally, we demonstrated the physiological importance of CGRP in IL-9 production and Th9 differentiation using an OVA-induced airway inflammation model and T cell–specific CGRP receptor-deficient mice. The present study revealed a novel regulatory mechanism comprising G protein–coupled receptor ligands and nervous system–derived substances in Th9 cell differentiation and type I hypersensitivity. The Journal of Immunology, 2013, 190: 4046–4055.

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Abbreviations used in this article: 6-bnz-cAMP, N6-benzoyl-cAMP; CGRP, calcitonin gene-related peptide; ChIP, chromatin immunoprecipitation; 8-CPT-cAMP, 8-(4-chlorophenylthio)-2’-0-methyl-cAMP; EPAC, exchange factor directly activated by cAMP; GPCR, G protein–coupled receptor; GSK, glycogen synthase kinase; IRF-4, IFN regulatory factor 4; PACAP, pituitary adenylate cyclase–activating polypeptide; PKA, protein kinase A; RAMP1, receptor activity modifying protein 1; VIP, vasoactive intestinal peptide.

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factors on the pathogenesis of asthma has been well studied (23). Calcitonin gene-related peptide (CGRP) is a 37-aa neuropeptide that is released from sensory neurons by several types of stimulation, such as protons, heat, chemical, and psychological events, and increases the intracellular cAMP level through its specific receptors. We hypothesized that CGRP is a potent Th9 regulator because its expression is increased in asthma (24, 25), and CGRP also has potent regulatory effects on Th cell differentiation (26–28). The CGRP receptor is a compound of receptor activity modifying protein 1 (RAMP1), which has the binding site of CGRP, and calcitonin receptor-like receptor, which is coupled with Gs proteins. CGRP receptors are expressed not only in nerve cells, but also in various types of immune cells, and many effects of CGRP on immune functions are reported in addition to Th cell differentiation. For instance, CGRP inhibits T cell proliferation, Ag presentation, and Ab secretion (29–31). We previously generated RAMP1-deficient mice and investigated the physiological effects of CGRP (32); we found that CGRP suppresses the functions of dendritic and Th1 cells, but promotes Th2 and Th17 cell differentiation in contact hypersensitivity and experimental autoimmune encephalomyelitis, respectively (27, 28, 33). Our investigations also showed that RAMP1-deficient mice were resistant to OVA-induced food allergy, although the regulatory mechanism has not been revealed (34). Moreover, it is reported that αCGRP-deficient mice exhibit less airway inflammation (35). Therefore, CGRP may be a physiological molecule that promotes type I hypersensitivity, and it is possible that CGRP promotes these inflammations by promoting Th9 cell functions.

In this study, we showed that the CGRP/cAMP/protein kinase A (PKA) pathway is important in the regulation of Th9 differentiation. Moreover, our study found that PKA inhibits glycogen synthase kinase (GSK–β) function and enhances NFATc2 binding to the IL-9 promoter region as a new regulatory mechanism in Th9 cells. CGRP/cAMP/PKA pathway also controls the expression of PU.1 via NFATc2-independent manner. Our findings have implications for the roles of CGRP and facilitate investigation of the relationship between the neurotransmitters and airway inflammation.

**Materials and Methods**

**Mice**
C57BL/6 mice and BALB/c mice (6–10 wk old) were purchased from Charles River Japan (Kanagawa, Japan). Lck-Cre transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). RAMP1-deficient mice were generated (32), and heterozygous knockout mice were backcrossed for 12 generations to C57BL/6 or BALB/c mice and used to generate the offspring used in the current study. All procedures, including experimental animals, were approved by the Experimental Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences, Osaka University.

**Abs and reagents**
PE-conjugated anti-mouse CD25 mAb (PC61) was purchased from BD Pharmingen (San Diego, CA). Allophycocyanin-Cy7-conjugated anti-mouse CD4 mAb (GK1.5) and PE-conjugated anti-mouse IL-9 mAb (RM9A4) were purchased from BioLegend (San Diego, CA). FITC-conjugated anti-mouse c-kit mAb (2B8) and PE-conjugated anti-mouse Gr1 mAb (RM9A4) were purchased from BioLegend (San Diego, CA). IL-4, IFN-γ, and IL-10 were measured, as previously described (28).

**Immunocytochemistry**
Stimulated Th cells were collected on glass slides using cytospin and fixed with cooled acetone or 4% paraformaldehyde for 15 min. After blocking with 5% BSA, the cells were stained with anti-NFATc1, anti-NFATc2, anti-p–GSK-3β, or anti-PU.1 Ab at 4°C overnight. After the first staining, the cells were reacted with secondary Abs conjugated with Alexa Fluor 488 or Alexa Fluor 568 (Molecular Probes, Eugene, OR). Specimens were mounted using Vectashield with DAPI (Vector Laboratories, Burlingame, CA), and the signals were recorded photographically using a fluorescence microscope BX51 (Olympus, Tokyo, Japan) equipped with a DP70 CCD camera (Olympus).

**RNA extraction and RT-PCR**
Total RNA was extracted using TRIzol reagent (Invitrogen). Total RNA was reverse transcribed into cDNA using a PrimeScript RT reagent kit (Takara, Tokyo, Japan), according to the manufacturer’s instructions. Semi-quantitative RT-PCR was performed using a Thermal Cycler (Bio-Rad, Hercules, CA), and quantitative real-time RT-PCR was performed using a Lightcycler System (Roche, Basel, Switzerland). Primers used for PCR were as follows: IL-9 forward, 5′-CTGATGGATTTGACACACCGTGC-3′; IL-9 reverse, 5′-GCTTTTGGCATCCTGGTCTTGG-3′; T-bet forward, 5′-TAAACACACTAGCTTTACTACTG-3′; T-bet reverse, 5′-AAGATGGTCATACAGGA-3′; GATA3 forward, 5′-GGGAAAACTCCTTGCAAG-3′; GATA3 reverse, 5′-ACGTCTTGGAGAAGGGG-3′; NFATc1 forward, 5′-GGAGCCGGGAACATCTTGCGG-3′; NFATc2 forward, 5′-GTCCTGCGATACCCAACTACC-3′; c-Maf forward, 5′-CTTCCGGAGCCGTCTTGCAC-3′; Pu.1 forward, 5′-TGGAGAATGAGCTGAGACCTAC-3′; PU.1 reverse, 5′-CTTCCGGAGCCGTCTTGCAC-3′; PU.1; Psi-1 forward, 5′-CTTCCGGAGCCGTCTTGCAC-3′; Pu.1 forward, 5′-AGAGCTTACCAAGCATTAGCTAG-3′; Pu.1 reverse, 5′-TCTTAAACTCTGGGTTGCGG-3′; Foxp3 forward, 5′-CCTCTTTCTCTTTGAAACC-3′; Foxp3 reverse, 5′-GGTGGCACCTTGTGAGGC-3′; and GAPDH reverse, 5′-CGCACAGGAGGCAGCAGGC-3′.
Airway inflammation and ex vivo IL-9 production

To induce the airway inflammation, mice were immunized with 250 μg OVA suspended in 4 mg aluminum hydroxide hydrate [Al(OH)₃.xH₂O] gel on days 0 and 7 (i.p.). To elicit inflammation, mice were challenged daily with 1% OVA in PBS using a nebulizer (Omron, Kyoto, Japan) for 30 min from day 14 to 16. Twenty-four hours after the last challenge, lung tissues and lung lymph nodes were collected. For histology, tissues were fixed with 4% paraformaldehyde and frozen in liquid nitrogen. Transverse cryosections (10 μm) were stained with H&E. The histological scoring was performed according to Table I.

For flow cytometry, lung tissue was homogenized with a frosted glass slide, and the mononuclear cells were then stained with mAbs specific for CD4, FcεR, and c-kit. IL-4 and IL-9 in lung homogenate supernatants were also measured by ELISA.

Lung lymph nodes were collected at 24 h after the last challenge, and mRNA was extracted from sorted Th cells. Spleen Th cells of challenged mice were also harvested at 24 h after the last challenge, and cell suspensions were prepared. The sorted Th cells were stimulated with anti-CD3 and anti-CD28 mAb for 72 h and used for IL-4 and IL-9 ELISA.

Statistics

A significant difference was further examined by unpaired Student t test or Student–Newman–Keuls test after nonrepeated measures ANOVA. A probability of <5% (p < 0.05) was considered statistically significant. We use the means of results from individual mice for the all statistical analysis, and we confirmed the reproducibility of the experiments at least two times.

![Diagram](http://www.jimmunol.org/Downloadedfrom.png)

**FIGURE 1.** CGRP promotes IL-9 production by Th9 cells. (A) The percentage of IL-9+ cells was analyzed by FACS after 48-h CD3/CD28 stimulation in the presence of 1 nM CGRP. Data are representative of four independent experiments. (B) IL-9 mRNA expression was measured by real-time PCR. Data are represented as mean ± SD from three mice. (C) IL-9 production was measured by ELISA. Data are represented as mean ± SD from three mice. (D) Th9 cells were differentiated from naïve Th cells for 4 d or from differentiated Th2 cells with TGF-β for 2 d. These cells were stimulated with anti-CD3 mAb for 24 h, and IL-9 production was measured by ELISA. Data are represented as mean ± SD from three mice. *p < 0.05, **p < 0.01 versus control.

**FIGURE 2.** cAMP/PKA pathway promotes IL-9 production in Th9 cells. (A) Dibutyryl cAMP (dbcAMP) promotes Th9 differentiation. IL-9 production was measured by ELISA. (B) IL-9 production was measured by ELISA in the presence of a PKA-specific (6-bnz) or EPAC-specific (8-CPT) analog. (C) PKA inhibitor completely blocked the promoting effect of CGRP. IL-9 production was measured by ELISA in the presence of 1 nM CGRP and 5 μM H89 (PKA inhibitor). (D) IL-9 production was measured by ELISA in the presence of 1 nM CGRP and 1 μM KT5720 (PKA inhibitor). (E) PGE₂ also promotes IL-9 production via cAMP/PKA pathway. IL-9 production was measured by ELISA in the presence of 1 μM PGE₂ and 5 μM H89. (F) PGD₂ also promotes IL-9 production via cAMP/PKA pathway. IL-9 production was measured by ELISA in the presence of 0.5 μM PGD₂ and 5 μM H89. These data are represented as mean ± SD from three mice. *p < 0.05, **p < 0.01 versus IL-4 + TGF-β.
Results
CGRP upregulates IL-9 production from naive T cells via its receptor

First, we evaluated whether CGRP affects IL-9 production by T cells. Naive splenic Th cells from C57BL/6 mice were sorted and stimulated under the Th9 condition in the presence or absence of CGRP. Because two isoforms of CGRP exist, the effects of both αCGRP and βCGRP were examined in the following experiments. As shown in Fig. 1A, the percentage of IL-9+ cells increased when αCGRP or βCGRP was added to the Th9 conditioning medium. Expression of IL-9 mRNA and IL-9 in the supernatant was also upregulated in the presence of each CGRP isoform through its specific receptor because such an increment was not detected in RAMP1-deficient T cells (Fig. 1B, 1C). Naive splenic Th cells from BALB/c mice produced similar results (data not shown). The dose dependence of CGRP was also confirmed (Supplemental Fig. 1A). CGRP induced the elevation of IL-9 production in a bell-shaped manner, and a high concentration of CGRP rather suppressed IL-9 production due to its inhibitory effects on T cell proliferation or activation (Supplemental Fig. 1B). Incidentally, we estimate that the physiological concentration of CGRP is between 0.1 and 10 nM based on measurements of the CGRP concentration in serum (32). Therefore, the concentration (1 nM) of CGRP for promoting IL-9 seems to be adequate. We also observed the promoting effect of CGRP on IL-9 production even when a Th9 cell-skewed condition was arranged using different concentrations of IL-4 and TGF-β (Supplemental Fig. 1C). These results suggest that CGRP upregulates IL-9 production in Th9 cells through its specific receptor, the RAMP1/calcitonin receptor-like receptor heterodimer on Th cells.

It has been reported that Th9 cells can be generated from Th2 cells in the presence of TGF-β, and that CGRP promotes Th2 differentiation (Supplemental Fig. 1D). Therefore, there is a possibility that CGRP indirectly upregulates IL-9 production by promoting IL-4 production or Th2 differentiation. To clarify this point, we measured IL-4 production in a Th9 cell-skewed condition. CGRP slightly increased IL-4 production in Th9 cells, but the amount of IL-4 in Th9 cells was smaller than that in Th2 cells (Supplemental Fig. 1D). We confirmed that such IL-4 levels (<1 ng/ml) in the Th9-skewed culture did not affect IL-9 production (data not shown). In addition, CGRP showed no effect on IFN-γ and IL-10 productions (Supplemental Fig. 1E, 1F). Furthermore, we investigated whether CGRP affects IL-9 production in differentiated Th9 cells from naive Th cells or Th2 cells. As shown in Fig. 1D, CGRP significantly promotes IL-9 production from differentiated Th9 cells. These results indicate that CGRP directly promotes IL-9 production in Th9 cells independent of other cytokine productions.

CGRP induces IL-9 production through cAMP/PKA pathway

We have reported that CGRP elevates the intracellular cAMP concentration through CGRP receptors in Th cells (28). Therefore, we examined whether CGRP promotes IL-9 production via cAMP pathways in Th9 cells. First, we used a membrane-permeable cAMP analog, dibutyryl cAMP, and observed that the cAMP analog increased IL-9 production in Th9 cells in a dose-dependent manner as well as CGRP did (Fig. 2A). It is well known that the effects of cAMP in immune systems are mediated by activating PKA or exchange factor directly activated by cAMP (EPAC) (36, 37). Therefore, we performed the experiment using PKA- or EPAC-specific cAMP analogs. In the Th9-skewed condition, 6-bnz-cAMP, which is a PKA-specific activator, promoted IL-9 production, but an EPAC-specific activator, 8-CPT-cAMP, had no effect (Fig. 2B). To further examine whether IL-9 production by CGRP is mediated by PKA activation, the PKA inhibitor H89 or KT5720 was added to the Th9-skewed medium with CGRP. As shown in Fig. 2C and 2D, the promoting effect of CGRP disappeared when PKA inhibitor was present. These results demonstrated that CGRP promotes Th9 cell differentiation through the cAMP/PKA pathway.

PGE2 and PGD2 are well known as one of the GPCR ligands. They also upregulate intracellular cAMP level through specific GPCRs and promote IL-4 or IL-17 production through PKA activation (38). Therefore, the effects of PGE2 and PGD2 on IL-9 production were also investigated. As expected, PGE2 and PGD2 promoted IL-9 production in Th9 cells, and these effects were blocked by H89 (Fig. 2E, 2F). Thus, we concluded that the cAMP/PKA pathway is an important factor for IL-9 production in Th9 cells.

CGRP and PKA induce PU.1 and GATA3 expression and NFATc2 activation

To examine the molecular mechanism by which CGRP induces IL-9 production in Th9 cells, we investigated the relationship between CGRP and Th cell–related transcriptional factors in Th9 cells using RT-PCR. As a result, we found that CGRP upregulated mRNA expression of PU.1 and GATA3 in Th9 cells (Fig. 3A). PU.1 is reported to be the major transcriptional factor that induces
Th9 cells and IL-9 expression (14). GATA3 is a putative transcriptional factor because GATA3-null mice exhibit a loss of IL-9 (6). Therefore, we focused on these two factors and confirmed the increased mRNA expression due to a CGRP- or PKA-specific cAMP analog using a real-time PCR system (Fig. 3B). Increases in GATA3 and PU.1 protein levels were also observed by FACS and immunofluorescence staining, respectively (Fig. 3C, 3D). These results suggest that these factors may be central regulators of CGRP signaling in IL-9 production.

Recently, it was also reported that NFATc2 induces IL-9 transactivation in Th cells (16). Moreover, one report demonstrated that IRF-4, which synergizes with NFATc2 in the IL-4 expression process, is also crucial for the development and function of IL-9 production in Th9 cells (17). In contrast, we previously reported that CGRP enhances the transcriptional activity of NFATc2 in Th2 and Th17 cell differentiation (27, 28). Under a Th9-skewed condition, although NFATc2 expression was not changed at either mRNA or protein levels by CGRP (Fig. 3A and data not shown), immunofluorescent staining revealed that CGRP induces the translocation of NFATc2 into nuclei (Fig. 4A). We also investigated the expression of NFATc1, and found that CGRP did not affect the expression and localization of NFATc1 (Fig. 3A, 4A). Therefore, we hypothesized that the translocation of NFATc2 is one of the key mechanisms by which CGRP affects Th9 differentiation. To examine this hypothesis, a ChIP assay was performed. CGRP upregulated the amount of NFATc2 binding to the IL-9 enhancer regions (Fig. 4B, 4C). We also observed that the binding of GATA3 to the IL-9 region was increased in the presence of CGRP (Fig. 4B, 4C). Next, we performed further experiments using a NFAT inhibitor. In the presence of the NFAT inhibitor, CGRP showed no promoting effect on the production of IL-9 (Fig. 4D). These results suggest that NFATc2 activation is also essential in the effect of CGRP on IL-9 production.

**CGRP and PKA induce GSK-3β phosphorylation**

Next, we attempted to elucidate the mechanism by which the CGRP/cAMP/PKA pathway induces the translocation of NFATc2. GSK-3β is well known as a regulator of NFATc2 localization. GSK-3β phosphorylates NFATc2 and inhibits its nuclear localization (39). In contrast, PKA inactivates GSK-3β by phosphorylating it at Ser9 in HEK293 cells (40). Therefore, it is possible that PKA promotes the nuclear localization of NFATc2 by inhibiting GSK-3β activity in Th cells. To investigate this possibility, phosphorylated GSK-3β was detected using its specific Ab. In the Th9-skewed condition, both CGRP and PKA induced GSK-3β (Ser9) phosphorylation compared with nontreated cells (Fig. 4E). In addition, a PKA activator, but not an EPAC activator, induced the phosphorylation of GSK-3β (Fig. 4E). These results suggest that CGRP induced IL-9 production through PKA-dependent GSK-3β phosphorylation.

We next examined IL-9 production in the presence of a GSK-3β inhibitor. As shown in Fig. 4F, nuclear localization of NFATc2

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** CGRP promotes NFATc2 transcriptional activity and GSK-3β phosphorylation. (A) Immunofluorescence staining of NFATc1 and NFATc2 protein was performed after 24-h stimulation. Data are representative of three independent experiments. (B and C) ChIP was performed using anti-NFATc2 or anti-GATA3 Abs. PCR was performed using specific primers for IL-9 promoter. Data are represented as mean ± SD from three mice. (D) IL-9 production under the Th9-skewed condition was measured by ELISA. Data are represented as mean ± SD from three mice. (E) Phosphorylated GSK-3β (Ser9) was determined by immunofluorescence staining. Data are representative of three independent experiments. (F) The effect of GSK-3β inhibitor and NFAT inhibitor on NFATc2 localization was confirmed by immunocytochemistry. Data are representative of two independent experiments. Scale bars, 20 μm. (G–I) IL-9 production was measured by ELISA in the presence of GSK-3β inhibitor, NFAT inhibitor, and CGRP under the Th9-skewed condition. Lithium chloride (LiCl) was used as GSK-3 inhibitor. Data are represented as mean ± SD from three mice. *p < 0.05, **p < 0.01 versus IL-4 + TGF-β. α, 1 nM αCGRP; β, 1 nM βCGRP; 6-bnz, 50 μM 6-bnz-cAMP; 8-CPT, 50 μM 8-CPT-cAMP; dbc, 20 μM dbcAMP.
was increased by treatment with the GSK-3β inhibitor, and this effect was diminished by the NFAT inhibitor. Indeed, the inhibition of GSK-3β induced IL-9 production as well as CGPP or dbcAMP (Fig. 4G), and the NFAT inhibitor suppressed the effect of the GSK-3β inhibitor (Fig. 4H). Furthermore, both the specific GSK-3β inhibitor and lithium chloride (a traditional GSK-3 inhibitor) diminished the effects of CGRP in IL-9 production (Fig. 4I). We confirmed that GSK-3β expression was not affected by CGRP or GSK-3β inhibitor (data not shown). These results suggested that the effect of GSK-3β inhibitor overlapped with the CGRP signaling pathway, and thus the CGRP effect was diminished in GSK-3β inhibitor-treated cells. Taken together, these results indicate that cAMP/PKA activation with CGRP induces the NFATc2 nuclear transition by phosphorylation of GSK-3β (Ser9), which leads to IL-9 production.

**CGRP and PKA induce PU.1 expression via GSK-3β-independent pathway**

Next, we investigated whether mRNA expression of IL-9 depends on NFATc2 or GSK-3β. Both the NFAT inhibitor and the GSK-3β inhibitor blocked CGRP effects on the mRNA expression of IL-9 at 24 h after stimulation (Fig. 5A). However, these inhibitors did not inhibit the CGRP effect on the mRNA expression of IL-9 at 48 h after stimulation, although PKA inhibitor H89 inhibited the effect both 24 and 48 h (Fig. 5B). It suggests that CGRP has other mechanisms in IL-9 production at the late stage of Th9 differentiation independence of GSK-3β/NFATc2 pathway. Thus, we confirmed the time-dependent effect of CGRP and inhibitors on IL-9 production using ELISA. As results, CGRP promotes IL-9 production after 72- and 96-h stimulation (Fig. 5C). Noteworthily, although CGRP could not promote IL-9 in the presence of GSK-3β inhibitor after 48 h (as similar to Fig. 4I), GSK-3β inhibition did not diminish CGRP effects after 72 and 96 h (Fig. 5C). Additionally, PKA inhibitor completely diminished CGRP effect at every time point. Therefore, the presence of the PKA-dependent but GSK-3β independent pathway was suggested.

We also investigated the mRNA expression of GATA3 and PU.1. As shown in Fig. 5D, the NFAT inhibitor also blocked CGRP effects on the mRNA expression of GATA3. In contrast, PU.1 mRNA expression is increased by CGRP even in the presence of the inhibitor of NFAT or GSK-3β, although the PKA inhibitor H89 completely blocked the promoting effect of CGRP on PU.1 expression (Fig. 5E). These results suggest that NFATc2 activated the expression of GATA3 and IL-9 in the CGRP signaling. In contrast, CGRP induced PU.1 expression independent of inactivation of GSK-3β or activation of NFATc2. Therefore, it was suggested that PKA activation occurs through at least two pathways, as follows: the GSK-3β–dependent activation of NFATc2 at

**FIGURE 5.** The effects of CGRP on mRNA expressions in the presence of inhibitors. (A and B) The effect of NFAT, GSK-3β, and PKA inhibitors on the mRNA expression of IL-9 was analyzed by real-time PCR after 24-h (A) and 48-h (B) stimulation under the Th9-skewed condition. (C) The time-dependent effect of CGRP and inhibitors on IL-9 production. IL-9 was measured by ELISA after 48-, 72-, and 96-h stimulation under the Th9-skewed condition. (D) The effect of NFAT, GSK-3β, and PKA inhibitors on the mRNA expression of GATA3 was analyzed by real-time PCR after 24-h stimulation. (E) The effect of NFAT, GSK-3β, and PKA inhibitors on the mRNA expression of PU.1 was analyzed by real-time PCR after 24-h stimulation. These data are represented as mean ± SD from three mice. *p < 0.05, **p < 0.01 versus IL-4 + TGF-β, #p < 0.05 versus IL-4 + TGF-β + inhibitor. α, 1 nM αCGRP; β, 1 nM βCGRP.
an early phase and the GSK-3β–independent expression of PU.1 at a late phase.

**CGRP enhances IL-9 production in vivo**

Finally, we investigated the physiological role of CGRP in Th9 cell development using RAMP1-deficient mice, which indicates a specific deletion of CGRP signaling (32). OVA-induced airway inflammation is a type I hypersensitivity, and this model is characterized by the infiltration of inflammation cells and airway resistance. As results of H&E staining and its scoring (Table I), after the OVA challenge, RAMP1-deficient mice showed less infiltrating cells and lower clinical scores compared with wild-type mice (Fig. 6A, 6B). We also observed a decrease in the number of Th cells and mast cells infiltrated into lung tissue in RAMP1-deficient mice (Fig. 6C). These results suggest that CGRP receptor signaling is necessary for promoting airway inflammation. It has been reported that αCGRP-deficient mice also show resistance to airway inflammation, but that the IgE levels and number of eosinophils in BALF do not change (35). Consistent with this observation, serum IgE levels made no difference in wild-type and RAMP1-deficient mice in our experiments (Fig. 6D).

We next investigated the IL-9 production in these mice. First, we evaluated IL-4 and IL-9 concentrations in spleen Th cells after an OVA challenge. As shown in Fig. 6E, in spleen, only IL-4 (Fig. 6A, 6B). We also observed a decrease in the number of Th cells and mast cells infiltrated into lung tissue in RAMP1-deficient mice (Fig. 6C). These results suggest that CGRP receptor signaling is necessary for promoting airway inflammation. It has been reported that αCGRP-deficient mice also show resistance to airway inflammation, but that the IgE levels and number of eosinophils in BALF do not change (35). Consistent with this observation, serum IgE levels made no difference in wild-type and RAMP1-deficient mice in our experiments (Fig. 6D).

We next investigated the IL-9 production in these mice. First, we evaluated IL-4 and IL-9 concentrations in spleen Th cells after an OVA challenge. As shown in Fig. 6E, in spleen, only IL-4

### Table I. Histological score of airway inflammation

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<th>Score</th>
<th>Score 0</th>
<th>Score 1</th>
<th>Score 2</th>
<th>Score 3</th>
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<td>Around airway</td>
<td>0 cell layers thick</td>
<td>1-4 cell layers thick</td>
<td>5-10 cell layers thick</td>
<td>&gt;10 cell layers thick</td>
</tr>
<tr>
<td>Around vessel</td>
<td>0 cell layers thick</td>
<td>&gt;30% vessels have</td>
<td>&lt;70% vessels have</td>
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Total score is determined as airway score plus vessel score.
production was increased after the challenge. In contrast, the IL-9 concentration in lung tissue was strongly increased, and we found a decreased level of IL-9 in RAMP1-deficient mice compared with wild-type mice (Fig. 6F). We also demonstrated that freshly isolated Th cells derived from RAMP1-deficient lymph nodes exhibited low IL-9 mRNA expression in comparison with wild-type mice (Fig. 6G). However, the IL-4 level in lung tissue was not increased after the challenge, and its expression was not significantly different in control and RAMP1-deficient mice (Fig. 6F). Moreover, the IFN-γ concentration in lung tissue was not detectable even after the challenge (data not shown). These observations suggest that CGRP promotes IL-9 expression in Th9 cells that are activated in the lung-draining lymph nodes and have infiltrated into the lung tissue.

To further clarify the roles of CGRP in Th9 cells, we used T cell–specific RAMP1-deficient mice by crossing Lck-Cre transgenic and RAMP1 flox/flox (f/f) mice. The T cell–specific RAMP1-deficient mice also showed both lower clinical scores and a decrease in infiltrations of Th and mast cells into the lung tissue compared with control mice (Cre-negative RAMP1-f/f mice) after an OVA challenge (Fig. 7A–C), although serum OVA-specific IgE levels in T cell–specific RAMP1-deficient and control mice were similar (Fig. 7D). Moreover, the IL-9 concentration in lung tissue was low in T cell–specific RAMP1-deficient mice (Fig. 7E). The level of IL-4 was not changed in those mice. RAMP1 can also interact with calcitonin receptor, and work as the receptor for other calcitonin family peptides (41). However, both Th0 and Th9 cells do not express calcitonin receptor, and recombinant calcitonin did not affect the production of IL-9 in vitro (data not shown). Moreover, in airway inflammation, the phenotype of αCGRP-deficient mice is similar with that of RAMP1-deficient mice (35). Therefore, these results strongly suggest that CGRP/CGRP receptor signaling promotes Th9 cell differentiation through up-regulation of IL-9 production, which enhances IL-9–mediated inflammation.

**Discussion**

It is well known that IL-9 is mainly secreted by T cells (Th9 cells) and that it plays critical roles in type I hypersensitivities such as asthma. As a cause of airway inflammation and asthma, IL-4 has been considered to be the best therapeutic target in asthma. However, several clinical and fundamental studies have demonstrated that IL-9 and IL-13 are more important cytokines in the onset of allergic asthma than IL-4 (13, 42–44). Moreover, it was also demonstrated that IL-9 induces IL-13 expression in airway epithelial cells and promotes airway inflammation (45). Therefore, the molecular regulation of IL-9 production is one of the great challenges in this research field because IL-9 seems to be directly related to the treatment of asthma.

It is also reported that IL-1 promotes IL-9 production from murine bone marrow–derived mast cells and human eosinophils (46, 47). In mast cells, the LPS/p38 MAPK pathway also promotes...
IL-9 production via NF-κB and GATA1 activation (48). As a novel regulation of IL-9 in Th cells, some cytokines such as IL-25, IFN-γ, and IL-21 can upregulate IL-9 production in Th9 cells (15, 49). These studies show that IL-9 production is regulated by many physiological substances and molecular pathways.

The IL-9 promoter has sites to which several transcription factors, such as c-Jun, IRF-4, NF-κB, NFAT, and GATA3, are able to bind (50). Actually, NF-κB has been shown to bind and activate the IL-9 promoter in mast cells and Th9 cells (15, 48). In addition, it has been also reported that PU.1 and IRF-4 bind to the IL-9 promoter and play essential roles in Th9 cell differentiation (14, 17). Our study also demonstrated that GATA3 and NFATc2 bind to the IL-9 promoter region and contribute to the transcription of IL-9. This observation supports the decreased expression of IL-9 in GATA3-deficient mice or NFATc2-deficient Th cells (6, 16).

In this study, we showed that the neuropeptide CGRP, which is a GPCR ligand, promotes IL-9 production from Th9 cells via the cAMP/PKA pathway. In addition, we demonstrated that CGRP promotes the expression of both GATA3 and PU.1 and the binding of NFATc2 to the IL-9 promoter via nuclear translocation. Our study using a NFAT inhibitor also indicated that CGRP increased GATA3 mRNA expression via NFATc2. The correctness of this result is substantiated by previous reports that showed that the GATA3 promoter also has NFATc2 binding sites and that NFATc2 binding to the GATA3 promoter enhances the expression of GATA3 (51).

It is known that GSK-3β inhibits NFATc2 activity (39, 52) and that GSK-3β activity is inhibited by phosphorylation of its Ser9 in GSK-3β (53). It was also reported that GSK-3β is phosphorylated by PKA (40). In this study, we showed that CGRP inactivates GSK-3β by PKA activation, and thus CGRP may promote NFATc2 activity by inhibiting GSK-3β activity. However, the promoting effect on PU.1 expression is dependent on PKA, but independent of both NFATc2 and GSK-3β. Moreover, the effect of a NFAT or GSK-3β inhibitor on IL-9 expression is restricted in the early stage of IL-9 differentiation. A NFAT or GSK-3β inhibitor suppressed the increase in mRNA expression of IL-9 by CGRP at 24-h stimulation, but IL-9 expression was increased by CGRP even in the presence of a NFAT or GSK-3β inhibitor after 48-h stimulation. These data suggest that regulation of the GSK-3β/NFATc2 pathway is critical in the early stage of Th9 cell differentiation, but that CGRP has other regulatory roles in IL-9 production, for example, PU.1 expression.

In vivo experiments using T cell–specific RAMP1-deficient mice demonstrated the roles of CGRP that enhance OVA-induced airway inflammation. It has been reported that airway inflammation is regulated by several GPCR ligands, such as CGRP, pituitary adenylate cyclase–activating polypeptide (PACAP), and vasoactive intestinal peptide (VIP) (35, 54, 55). Aoki-Nagase et al. (35) demonstrated that CGRP promotes airway inflammation using aCGRP-deficient mice, and our study also demonstrated that RAMP1-deficient mice show a decrease in the number of cells infiltrating into the lung due to a low level of IL-9 production compared with wild-type mice. These data suggest that CGRP physiologically promotes airway inflammation by accelerating the effects on Th9 cell differentiation and IL-9 production.

In contrast to CGRP, PACAP and VIP inhibit the airway inflammation, although they also induce the cAMP/PKA pathway. VIP inhibits the proliferation of smooth muscle and relaxes the airway, resulting in a reduction of airway inflammation (55), and PACAP inhibits neutrophil functions (56). As mentioned above, the cAMP/PKA pathway affects many cell types, such as airway epithelial cells, vascular smooth muscles, and eosinophils. The difference between VIP/PACAP and CGRP might result from the specific expression pattern of these molecules in lymph nodes because only αCGRP mRNA expression could be detected in lymph nodes (data not shown). We have also confirmed that CGRP-positive nerve endings are distributed in lung, skin, and around lymph nodes, but not in spleen (data not shown). Actually, the cytokine expression in spleen Th cells was affected in RAMP1-deficient mice neither in an airway inflammation model, contact hypersensitivity, nor experimental autoimmune encephalomyelitis experiments, although the production in lymph nodes was dramatically regulated (28, 33). To reveal the different effects of CGRP, VIP, and PACAP, it is necessary to investigate whether VIP and PACAP physiologically affect Th cell differentiation in an airway inflammation model like CGRP.

In conclusion, we revealed that CGRP promotes Th9 cell differentiation via the cAMP/PKA pathway and airway inflammation. IL-9 affects many cells, including B cells, mast cells, eosinophils, T cells, and goblet cells; therefore, the regulation of IL-9 production will be directly targeted in the treatment of airway inflammation. Because Th9 cells produce most of the IL-9, targeting the CGRP pathway might open new prospects for designing therapeutic strategies for the treatment of airway inflammation.

Disclosures

The authors have no financial interests of interest.

References
