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Original Article

The A118G single-nucleotide polymorphism in *OPRM1* is a risk factor for asthma severity

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SNP, single-nucleotide polymorphism; MOPRs, μ-opioid receptors; S_1PR, sphingosine-1-phosphate receptors; OPRM1, μ-opioid receptor gene; PSS, Perceived Stress Scale; BLN, bronchial lymph node; Teff, effector T cells; Tem, effector memory T cells; Tcm, central memory T cells; Tn, naïve T cells; β-FNA, β-funaltrexamine hydrochloride

ABSTRACT

Background: Although population studies have implicated emotional burden in asthma severity, the underlying genetic risk factors are not completely understood. We aimed to evaluate the genetic influence of a functional single-nucleotide polymorphism (SNP) in the stress-related μ-opioid receptor gene (*OPRM1*; A118G SNP, rs1799971) on asthma severity.

Methods: We initially assessed disease severity in asthmatic outpatients carrying A118G. Using an ovalbumin-induced experimental asthma rodent model harboring the functionally equivalent SNP, we investigated the mechanism by which this SNP influences the allergic immune response.

Results: Among 292 outpatients, 168 underwent airway hyperresponsiveness (AHR) to methacholine testing. Compared with patients carrying the AA and AG genotypes, those carrying the GG genotype exhibited enhanced AHR. The stress levels were presumed to be moderate among patients and were comparable among genotypes. Compared with *Oprm1* AA mice, GG mice demonstrated aggravated asthma-related features and increased pulmonary interleukin-4^+CD4^+ effector and effector memory T cells under everyday life stress conditions. Intraperitoneal naloxone methiodide injection reduced effector $CD4^+$ T cell elevation associated with increased eosinophil numbers in bronchoalveolar lavage fluid of GG mice to the levels in AA mice, suggesting that elevated Th2 cell generation in the bronchial lymph node (BLN) of GG mice induces enhanced eosinophilic inflammation.

Conclusions: Without forced stress exposure, patients with asthma carrying the *OPRM1* GG genotype exhibit enhanced AHR, attributable to enhanced Th2 cell differentiation in the regional lymph node. Further research is necessary to elucidate the role of the *OPRM1* A118G genotype in the Th2 cell differentiation pathway in the BLN.

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Introduction

Asthma is a disorder defined by variable and recurring symptoms, airflow obstruction, and bronchial hyperresponsiveness as a result of inflammation, mainly type-2 inflammation.[1] However, the risk of onset, severity, type of inflammation, and response to treatment in asthma are heterogeneous, and this may be attributed to differences in patients' genetic make-up and environmental influences.[2–5]

Psychological and socioeconomic stress are ubiquitous in everyday life and are strongly associated with poor mental and physical health,[3,6] which are factors that affect disease conditions such as asthma.[7,8] There may be a link between brain activation and the aggravation of airway inflammation, which leads to stress-induced exacerbation of symptoms in patients with asthma.[9] In Puerto Rican populations, a single-nucleotide polymorphism (SNP) of rs34548976 in the pituitary adenylate cyclase-activating polypeptide type I receptor gene increases functional connectivity in brain regions associated with increased anxiety and reduced bronchodilator response.[10] The mechanism underlying the stress response *in vivo* is complex,[11] and there is limited understanding of the genetic risk factors linked to asthma severity triggered by emotional burden.

Endogenous opioids, including β-endorphin, are constantly released, a process that is accelerated during psychological stress[12] and plays an important role in the stress response via binding to μ-opioid receptors (MOPRs).[13] These receptors are involved in allergic reactions *in vivo*[14] and *in vitro*.[15] Addiction to MOPR agonists increases the risk of asthma aggravation.[16] In contrast, MOPR deficiency reduced asthma severity in mice with psychological stress-induced asthma exacerbation.[17]

MOPRs are encoded by the μ-opioid receptor gene (*OPRM1*) and belong to the superfamily of 7-transmembrane-spanning G-protein-coupled receptors. MOPRs are widely distributed in the brain, peripheral sensory neurons, and various types of immune cells.[18] Some SNPs in MOPRs associated with a functional change in amino acid exchange have been reported with a high allelic frequency.[19] Several reports further indicate that an SNP at position 118 of the *OPRM1* sequence (A118G, rs1799971) alters ligand binding and signal transduction of the receptors and their clinical response to opioids.[19,20] This SNP is located in the exon 1 fragment containing an extracellular ligand-binding domain, which exchanges an asparagine for an aspartic acid (N40D) at a putative N-glycosylation site.[19,21] The frequency of the A118G SNP in *OPRM1* is variable according to ethnicity; for example, the G118 MOPR variant is common in 4.7% of African-Americans, 15% of European-Americans, and 36–48% of Asians.[22] With respect to the functional effects of this SNP, vulnerability to heroin and alcohol addiction have been reported in *OPRM1* G118 carriers.[23,24] Additionally, mice carrying an equivalent SNP (A112G) in the *Oprm1* gene show alterations in morphine-mediated conditioned reward and the development of locomotor sensitization.[25]

In the present study, we assessed if and how the A118G SNP in *OPRM1* affects asthma pathogenesis in the context of stress levels encountered in everyday life. We elucidated the associations between the genetic variant of *OPRM1*, the A118G SNP, and asthma severity in patients with asthma. In addition, we investigated the effect of the *OPRM1* A118G SNP on the regulation of asthmatic type-2 immune responses using a mouse model harboring the A112G SNP in the *Oprm1* gene.

Methods

Ethics

The experimental procedures were planned according to the ethical principles for medical research involving human participants in the Declaration of Helsinki. All procedures were approved by Ethics Committee of Tohoku Medical and Pharmaceutical University (2017-8, 2019-10) and Iwate Medical University (HG H24-6). All participants signed the informed consent form before participating.

Patients

All participants were recruited from Iwate Medical University Hospital, Japan. Patients with well-controlled asthma (Asthma Control Test (ACT) score of 20–25) with no other medical disorder and no exposure to environmental hazards were considered for the study[26] (details are provided in the Supplementary Methods). Upon acceptance, patients provided written informed consent according to the ethical protocols of our institution. The demographic characteristics of the study population are shown in Table 1. Of the 292 patients, 168 successfully underwent analysis of airway hyperresponsiveness (AHR) to methacholine, and 84 patients self-reported their perceived psychological stress over the last 3 months using the Japanese version of the Perceived Stress Scale (PSS).[27] Airway methacholine responsiveness was measured using an Astograph (Jupiter 21; CHEST, Tokyo, Japan).[28]

Genotyping

Genomic DNA was isolated from lymphocytes using standard procedures. Subjects were genotyped for the *OPRM1* SNP (rs1799971) using sequence primers covering +118 A/G in exon 1 (7500 Fast Real-Time PCR System Applied Biosystems, CA, USA).[29]

Asthma mouse model

All experimental procedures were approved by the Committee of Animal Experiments at Tohoku Medical and Pharmaceutical University (19,006-cn). *Oprm1* 112 A and *Oprm1* 112G mice were bred and used (see the Supplementary Methods for details).[25] Asthma-related features in asthmatic mice induced by ovalbumin (OVA) were evaluated according to previously published methods.[30–32]

Lung histology

Lungs were isolated from mice three days after secondary OVA inhalation, fixed in 10% buffered formalin, and embedded in paraffin. Sections were cut at a thickness of 4 μm and subjected to periodic acid–Schiff (PAS) staining. Mucin production was estimated as the proportion of PAS-positive cells in the total airway epithelium of each of the five randomly selected bronchioles in three lung sections from each mouse. Perivascular and parabronchial eotaxin-1-producing cells were scored according to a previously published scale.[33] Briefly, the lung sections were stained immunohistochemically with a goat anti-mouse CCL11/eotaxin primary antibody (R&D Systems, Minneapolis, MN, USA). Eotaxin-1-producing cells were scored according to the following scale: 0, no to rare staining; 1, detectable staining; 2, small clusters; 3, moderately-defined aggregates; and 4, well-defined aggregates.

Quantification of the accumulation of eotaxin-1-producing cells in the perivascular area and peribronchial area was based on an average of the staining scores for 22 to 78 veins and 27 to 50 bronchi per mouse, respectively.

Quantitative polymerase chain reaction

qPCR was performed using previously published techniques as described in detail in the Supplementary Methods.[32] The primer sequences used for amplification are shown in Supplementary Table 1.

Flow cytometric analysis

Pulmonary leukocytes and bronchial lymph node (BLN) cells were prepared according to previously published methods.[30,34] One macroscopically swollen BLN in the right paratracheal region of each mouse was collected. Details of the immunofluorescence staining procedures are provided in the Supplementary Methods.

Measurement of plasma corticosterone and β-endorphin levels

The concentrations of corticosterone (Enzo Life Science, Inc., Plymouth Meeting, PA, USA) and β-endorphin in plasma (Novus Biologicals, LL, Littleton, CO, USA) were assayed using enzyme-linked immunosorbent assay (ELISA) kits.

Th2 cell differentiation

Naïve T cells were cultured under Th2 polarizing conditions for 6 days. The details of the cell isolation and culture procedures are provided in the Supplementary Methods.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Intergroup differences were tested using Mann–Whitney *U*-tests and unpaired Student's *t*–tests for data from humans and mice, respectively. Differences among three groups or more were tested using the Kruskal–Wallis *H*-test and analysis of variance (ANOVA) with a *post-hoc* analysis (Tukey's multiple comparison test) for data from humans and mice, respectively. Clinical characteristics were analyzed using the chi-square test. A *p*-value of less than 0.05 was considered statistically significant.

Results

GG homozygotes of rs1799971 with asthma exhibit enhanced airway hyperresponsiveness

Of the 292 patients with asthma, 36%, 41%, and 23% carried the AA wild-type, AG heterozygote, and GG homozygote variants of the *OPRM1* (rs1799971), respectively. These data did not fulfill the conditions for Hardy–Weinberg equilibrium ($p = 0.005$). No significant difference in sex distribution, obesity, smoking history, blood eosinophil counts, or serum immunoglobulin E (IgE) concentrations were observed among the three groups (Table 1). After adjusting for possible confounding factors, including sex, age, and smoking history, GG homozygous patients had a 2.0-fold (95% confidence interval, 1.0–3.9) higher odds ratio of pollen allergies than the other patients, although this result did not reach statistical significance ($p = 0.0517$). Furthermore, among patients in whom AHR to methacholine was successfully analyzed, GG homozygotes of rs1799971 exhibited significantly enhanced AHR compared to patients with the AA+AG genotypes of rs1799971 (Fig. 1). In contrast, there was no significant difference between

Table 1
Clinical characteristics of patients (n = 292).

rs1799971 genotype	n	Total	AA (n = 105)	AG (n = 120)	GG (n = 67)	p
Sex, M/F	292	126/166	45/60	50/70	31/36	0.8
Age, years	292	57.1 ± 16.5	56.2 ± 17.8	57.2 ± 16.3	58.6 ± 14.7	0.6
Height, cm	286	158.7 ± 9.1	158.6 ± 10.1	158.5 ± 8.5	159.3 ± 8.8	0.8
Body weight, kg	286	60.3 ± 11.0	59.3 ± 11.7	61.0 ± 10.7	60.6 ± 10.4	0.5
Body mass index, kg/m^2	286	23.9 ± 3.4	23.5 ± 3.6	24.2 ± 3.3	23.9 ± 3.4	0.3
Serum IgE levels, IU/mL	267	126 (56–473)	134 (60–371)	146 (57–572)	105 (38–538)	0.5
Serum IgE levels >200 IU/mL, %	267	104 (39.0%)	35 (37.2%)	48 (43.2%)	21 (33.9%)	0.4
Proportion of eosinophils, %	282	5.2 ± 4.1	5.3 ± 5.1	5.4 ± 3.6	4.9 ± 3.0	0.8
Smoking history, %	292					0.7
Never		156 (53.4%)	51 (48.6%)	70 (58.3%)	35 (52.2%)	
Stopped		65 (22.3%)	27 (25.7%)	22 (18.3%)	16 (23.9%)	
Current		33 (11.3%)	12 (11.4%)	15 (12.5%)	6 (9.0%)	
Unclear		38 (13.0%)	15 (14.9%)	13 (10.8%)	10 (14.9%)	
Pulmonary function						
Dmin, Units	168	2.64 ± 6.22	2.20 ± 3.15	3.64 ± 8.77	1.34 ± 1.62	0.1
Rrs, cmH_2O/L/sec	173	4.24 ± 1.76	4.38 ± 1.80	4.03 ± 1.40	4.43 ± 2.24	0.4
FEV1, L	285	2.22 ± 0.82	2.26 ± 0.85	2.22 ± 0.85	2.15 ± 0.74	0.7
%FEV1, %	280	98.8 ± 24.2	99.4 ± 22.7	99.4 ± 25.5	97.1 ± 24.3	0.8
FEV1%, %	285	69.8 ± 12.0	71.2 ± 11.7	69.6 ± 11.5	67.9 ± 13.3	0.2
%V50, %	285	46.6 ± 24.0	49.0 ± 24.4	44.9 ± 23.5	45.4 ± 24.4	0.4
%V25, %	265	33.2 ± 17.5	35.3 ± 16.4	32.4 ± 19.1	31.0 ± 16.0	0.3
History of pollen allergy, %	292					0.006
No		196 (67.1%)	64 (61.0%)	85 (70.8%)	47 (70.2%)	
Yes		52 (17.8%)	16 (15.2%)	19 (15.8%)	17 (25.4%)	
Unclear		44 (15.1%)	25 (23.8%)	16 (13.3%)	3 (4.5%)	
Treatment with glucocorticoids, %	292	262 (89.7%)	91 (86.7%)	112 (93.3%)	59 (88.1%)	0.2
Step 4 treatment, %	292	34 (11.6%)	7 (6.7%)	15 (12.5%)	12 (23.0%)	0.08

Data are expressed as the mean ± standard deviation. Serum IgE levels are expressed as the median and interquartile range (25%–75%). M, male; F, female; IgE, immunoglobulin E; Dmin, minimum cumulative dose of methacholine; Rrs, respiratory resistance; FEV1, forced expiratory volume in 1 s; %V50, maximum flow at 50% of the vital capacity; %V25, maximum flow at 25% of the vital capacity.

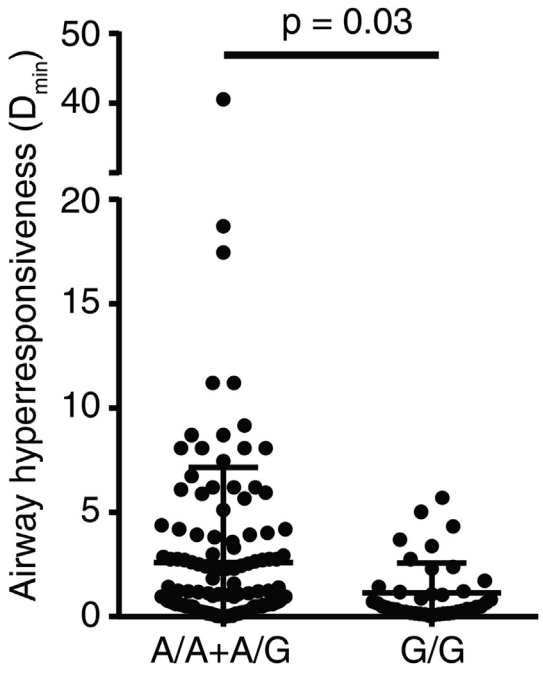

Fig. 1 GG homozygotes of rs1799971 exhibit enhanced airway hyperresponsiveness. Airway hyperresponsiveness was evaluated in terms of the minimum cumulative dose of methacholine (Dmin) required to decrease respiratory conductance from the baseline in asthmatic subjects. Patients carrying the A/A+A/G genotypes are compared to those carrying the GG genotype. Bars indicate mean ± SD.

patients carrying the GG genotype and the AA+AG genotypes in terms of asthma severity based on lung function, the number of eosinophils in sera, IgE levels, or the percentage of those receiving treatment with glucocorticoids or Step 4 treatments, except for the number of patients with a history of pollen allergies (Supplementary Table 2).

The GG genotype exacerbates asthma-related features in mice

To examine the mechanism underlying the effect of the *OPRM1* rs1799971 SNP on asthma severity, we used a mouse model and investigated differences in asthma-related features between WT mice and mice harboring the functionally equivalent SNP, *OPRM1* G118. The number of eosinophils in the bronchoalveolar lavage (BAL) fluid 3 days after secondary OVA inhalation was significantly enhanced in GG mice compared to AA mice when there was an interval of 3 days (Supplementary Fig. 1A) or 7 days (Fig. 2A), but not 4 h (Supplementary Fig. 1B) between the first and second OVA inhalation. OVA-specific serum IgE and IgG_1 levels (Fig. 2B), goblet cell hyperplasia (Fig. 2C), and AHR to methacholine (Fig. 2D) were greater in GG mice than in AA mice 3 days after secondary OVA inhalation. In contrast, when we challenged mice with saline or saline and OVA (Supplementary Figs. 1C- E), there were no differences in eosinophil counts between the two groups.

The GG genotype increases the accumulation of eotaxin-1 producing cells in the lung

Among the eosinophil-recruiting chemokines in the lung, the mRNA levels of CCL11/eotaxin-1 and CCL28/mucosae-associated epithelial chemokine (MEC) were significantly enhanced 18 h after secondary OVA inhalation in GG mice compared to AA mice (Fig. 3A, B). There were no differences in the expression levels of CCR3, a receptor of eotaxin-1 and MEC, or eosinophil activation states in BAL fluid between the genotypes (Supplementary Fig. 2A). Lung histological examination showed inflammatory cell accumulation around blood vessels (Fig. 3C, D, G, H) and airways

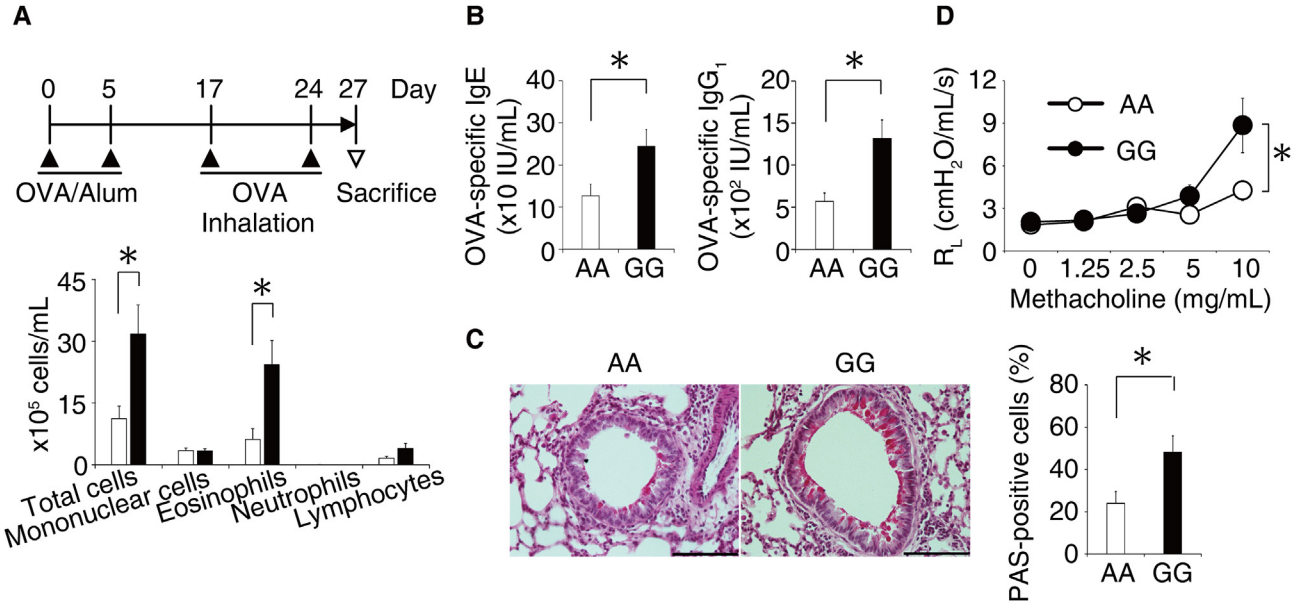

Fig. 2 GG mice exhibit aggravated asthma-related features. **(A)** Mice were sensitized with ovalbumin (OVA)/adjuvant aluminum hydroxide (alum) and challenged with OVA, and then sacrificed at the indicated time points. Cell composition in the bronchoalveolar lavage fluid was evaluated by Diff-Quick staining. Data are shown as the mean ± SEM based on six mice per group. **(B)** Serum levels of OVA-specific immunoglobulin E (IgE) and immunoglobulin G1 (IgG_1) Ab measured by enzyme-linked immunosorbent assay (ELISA). Data are shown as the mean ± SEM based on six to 15 mice per group. **(C)** Mucin production was estimated as the proportion of periodic acid-Schiff (PAS)-positive cells in the total airway epithelium of bronchioles in the PAS-stained section. Representative microscope photographs (original magnification, × 400) of the staining are shown (left). Data are taken from five random bronchioles in the three lung sections from each mouse (right). Data are shown as the mean ± SEM based on six mice per group. Scale bars = 100 μm. **(D)** Lung resistance (R_L) measured under each condition by the Resistance and Compliance System. Data are shown as the mean ± SEM based on five to six mice per group. Experiments were repeated twice with similar results. □, AA mice; ■, GG mice. ▲, time points of sensitization or inhalation; Δ, time points of specimen sampling. *$p < 0.05$ compared to AA mice.

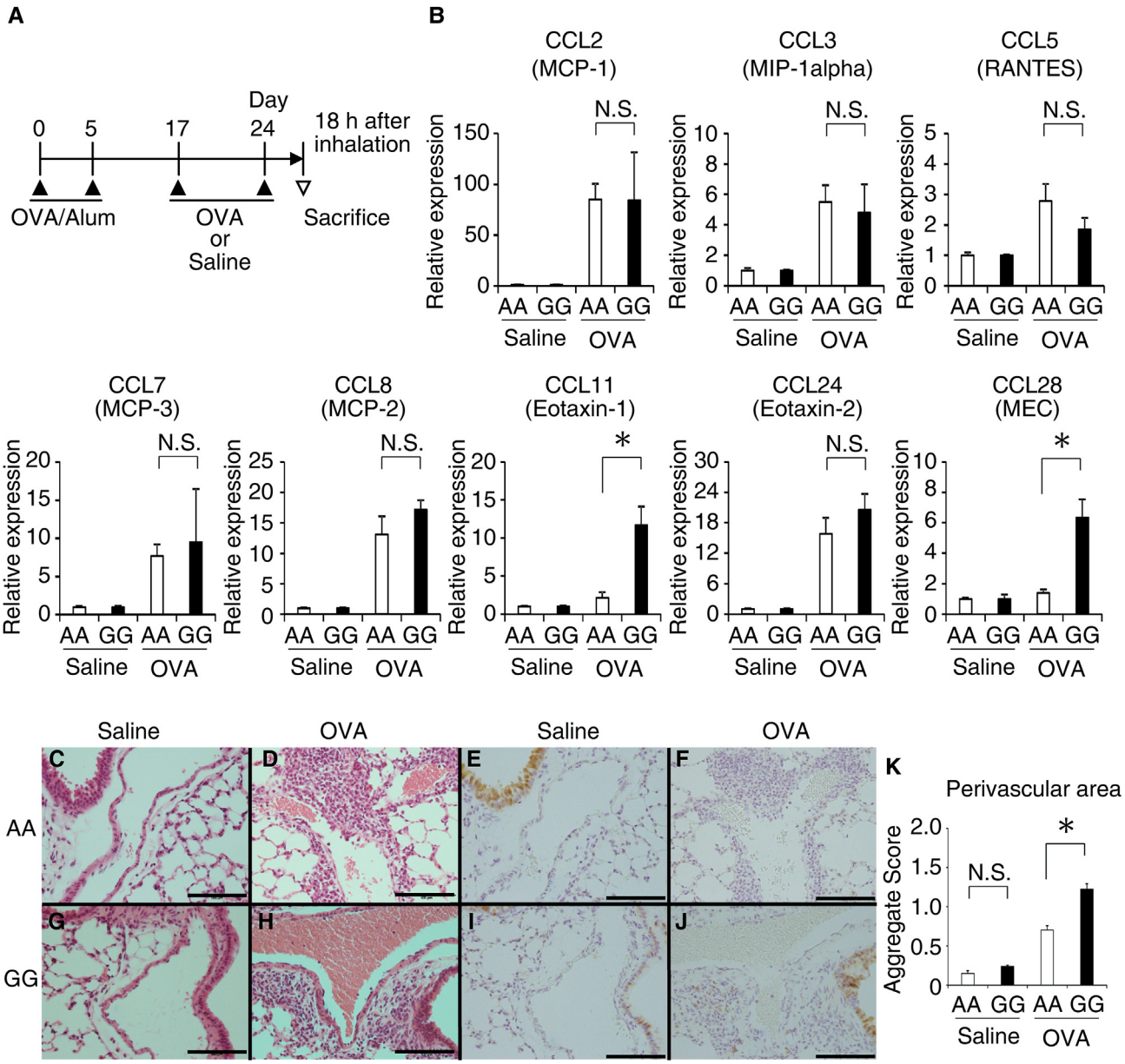

Fig. 3 Enhanced accumulation of eotaxin-1-producing cells in the lungs of GG mice. **(A)** At the indicated time points, mice were sensitized and challenged with ovalbumin (OVA) or saline and sacrificed. **(B)** Chemokine mRNA expression in the lungs was determined by quantitative polymerase chain reaction (qPCR). Data are shown as the mean ± SEM based on five to six mice per group. **(C–K)** Representative lung sections stained with hematoxylin and eosin (**H, E**) or an anti-eotaxin-1 antibody in sensitized AA and GG mice following OVA or saline challenge are shown (original magnification × 400). **(C, D, G, and H)** H&E staining shows inflammatory cell accumulation around blood vessels. (**E, F, I, and J**) Accumulation of eotaxin-1-expressing cells in the perivascular area is shown. **(K)** Aggregate scores of eotaxin-1 producing cells are shown as the mean ± SEM based on the data from 22 to 78 veins per mouse (five to six mice/group). Scale bars = 100 μm. Experiments were repeated twice with similar results. □, AA mice; ■, GG mice. ▲, time points of sensitization or inhalation; Δ, time points for sampling of specimens. *$p < 0.05$ compared to AA mice; **$p < 0.01$ compared to AA mice. NS, not significant.

(Supplementary Fig. 2B, C, F, G) after OVA inhalation in both AA and GG mice. Accumulation of eotaxin-1-expressing cells in the perivascular area (Fig. 3E, F, I, J) at 18 h after OVA inhalation was significantly greater in GG mice than AA mice (Fig. 3K). Although a similar tendency was also observed in the peribronchial area (Supplementary Fig. 2D, E, H, I), the aggregation score did not significantly differ between genotypes (Supplementary Fig. 2J).

Th2 cells are responsible for the enhanced number of eosinophils in the lungs of GG mice

Since the Th2 cytokine is critical for eotaxin-1 production from inflammatory cells[35] and airway epithelial cells,[36] we next examined the number of Th2 cells in the lung (Fig. 4A). The number of eosinophils and lymphocytes in the lung 6 h after secondary OVA inhalation was significantly greater in GG mice than AA mice (Fig. 4B). In lymphocytes, the numbers of interleukin (IL)-4- or IL-5-producing $CD4^+$ effector T cells (Teff), effector memory T cells (Tem), and central memory T cells (Tcm) in the lung, gated as in Figure 4C, were significantly higher in GG mice than in AA mice (Fig. 4D), although IL-4 mRNA levels in Teff and memory phenotype T cells in the lung were comparable between the two genotypes (Supplementary Fig. 3). Although a similar tendency in the numbers of IL-4- or IL-5-producing cells was also observed in $CD8^+$ T cells (Fig. 4E), their numbers were 3–100 times lower than those of $CD4^+$ T cells. Moreover, the increased

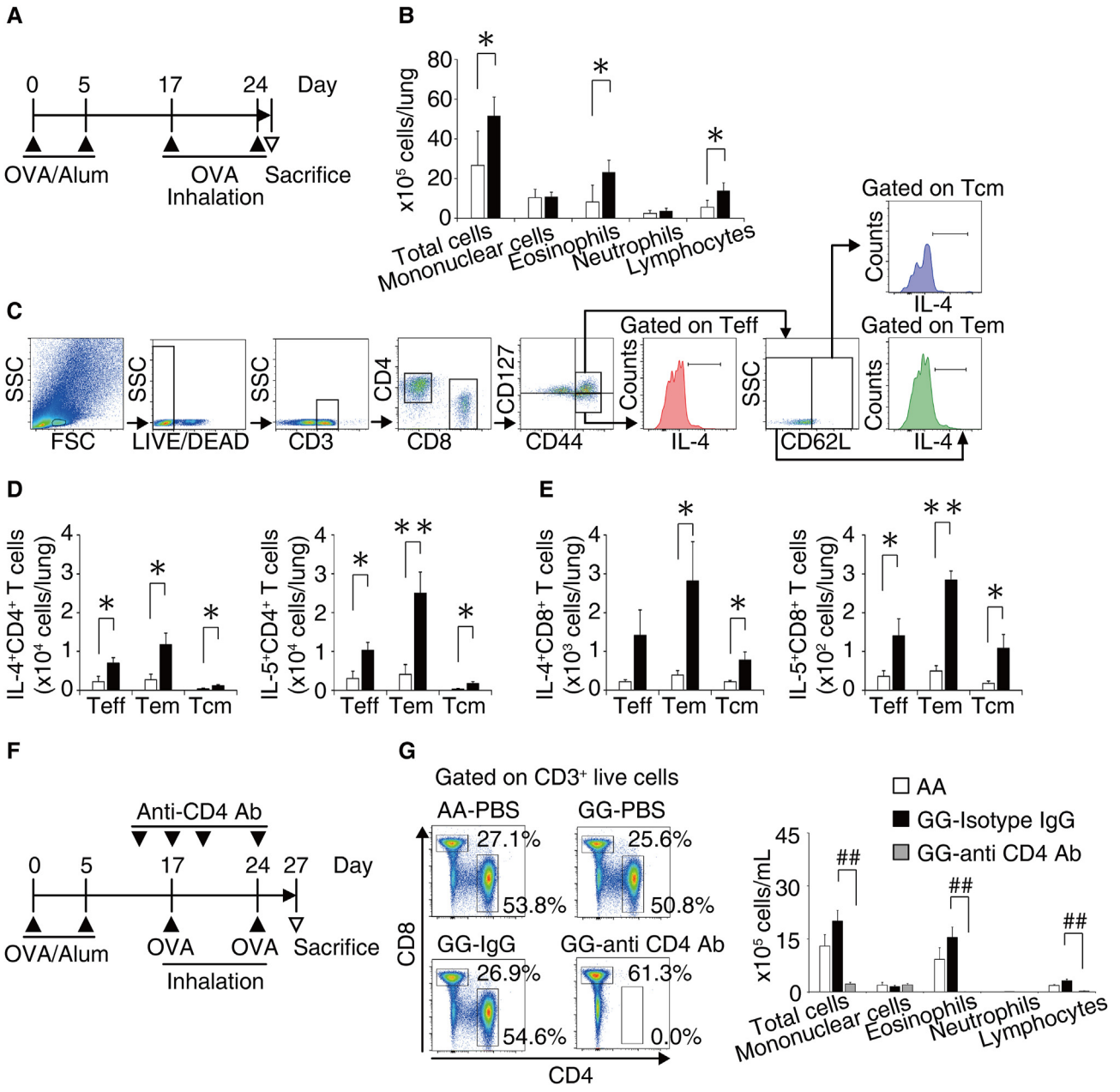

Fig. 4 Increased number of Th2 cells in GG mice is responsible for eosinophilic inflammation in the lung. **(A)** Mice were sensitized with ovalbumin (OVA)/alum on days 0 and 5, challenged with OVA on days 17 and 24, and sacrificed 6 h after OVA inhalation. **(B)** Six hours after the second OVA inhalation, cell composition in the lungs of mice was evaluated by Diff-Quick staining. Data are shown as the mean ± SEM based on five to six mice per group. **(C)** Gating strategies for type-2 effector T cells (Teff), effector memory T cells (Tem), and central memory T cells (Tcm) are shown. The numbers of interleukin 4 (IL-4)- or interleukin 5 (IL-5)-producing subtypes of $CD4^+$ T cells **(D)** and $CD8^+$ T cells **(E)** in the lung were evaluated by flow cytometry. Data are shown as the mean ± SEM based on five to six mice per group. **(F)** An anti-CD4 antibody was injected intraperitoneally on days 14, 17, 20, and 24 during the elicitation phase of asthma. **(G)** Representative plots of $CD4^+$ T cells in the spleen after intraperitoneal injection of the anti-CD4 antibody or phosphate-buffered saline (PBS) are shown on the left. The cell composition in the bronchoalveolar lavage (BAL) fluid of mice treated as in panel F was evaluated by Diff-Quick staining (right). Data are shown as the mean ± SEM based on six mice per group. Experiments were repeated twice with similar results. FSC, forward scatter; SSC, side scatter. □, AA mice; ■ and gray square, GG mice. ▲, time points of sensitization or inhalation; Δ, time points of sampling of specimen. *$p < 0.05$ compared to AA mice; **$p < 0.01$ compared to AA mice; ##$p < 0.01$ compared to immunoglobulin G (IgG)-treated GG mice. NS, not significant.

number of eosinophils and lymphocytes in the BAL fluid of GG mice was attributable to $CD4^+$ T cells (Fig. 4F, G). Even when mice were challenged twice with OVA inhalation with an interval of 3 months between inhalations (Supplementary Fig. 4A), a greater number of eosinophils in the BAL fluid (Supplementary Fig. 4B) and more IL-4^+ Tem and IL-5^+ Tem cells in the lung (Supplementary Fig. 4C) were observed in GG mice. In contrast, the levels of thymus- and activation-regulated chemokine (TARC)/CCL17 and macrophage-derived chemokine (MDC)/CCL22, a chemokine for the recruitment of Th2 cells, in the lung 6 h after secondary OVA inhalation (Supplementary Fig. 4D) were not significantly different between AA and GG mice (Supplementary

Fig. 4E). These results suggest that the increased numbers of Th2 cytokine-producing CD4$^+$ Teff and Tem cells are responsible for the enhanced eosinophilic inflammation in the lungs of GG mice.

The GG genotype of peripheral MOPRs is responsible for the increased number of Th2 cells in the lung

To address the role of peripheral and central MOPRs in the increased numbers of eosinophils and Th2 cells in the lung, we injected a MOPR antagonist in mice during OVA inhalation (Fig. 5A). Intraperitoneal administration of naloxone methiodide, a MOPR antagonist that does not cross the blood–brain barrier, attenuated the increased number of eosinophils in BAL fluid (Fig. 5B) and the number of Teff cells, but not Tem cells, in the lungs (Fig. 5C) of GG mice. In contrast, intracerebroventricular injection of the MOPR antagonist β-funaltrexamine hydrochloride (β-FNA) did not alter the increased number of eosinophils and lymphocytes in the BAL fluid of GG mice compared to AA mice (Supplementary Fig. 5A). In line with these data, the plasma levels of corticosterone produced from neuroendocrine axis activation mediated by central MOPRs[30] were not largely different between genotypes (Supplementary Fig. 5B). However, oral administration of fingolimod (FTY720), which induces internalization of sphingosine-1-phosphate receptors (S_1PR) on immune cells and inhibits the egress of lymphocytes from lymph nodes, blocked the enhanced inflammatory cell infiltration in the BAL fluid in GG mice (Fig. 5D). To rule out the possibility of the effect of this SNP on the expression level of CD69, an inhibitory molecule for S_1PR expression, we confirmed the numbers of CD69 expressing-CD4$^+$ Teff, and CD4$^+$ Tem cells in the BLN after OVA inhalation (Supplementary Fig. 5C). The levels were not significantly different

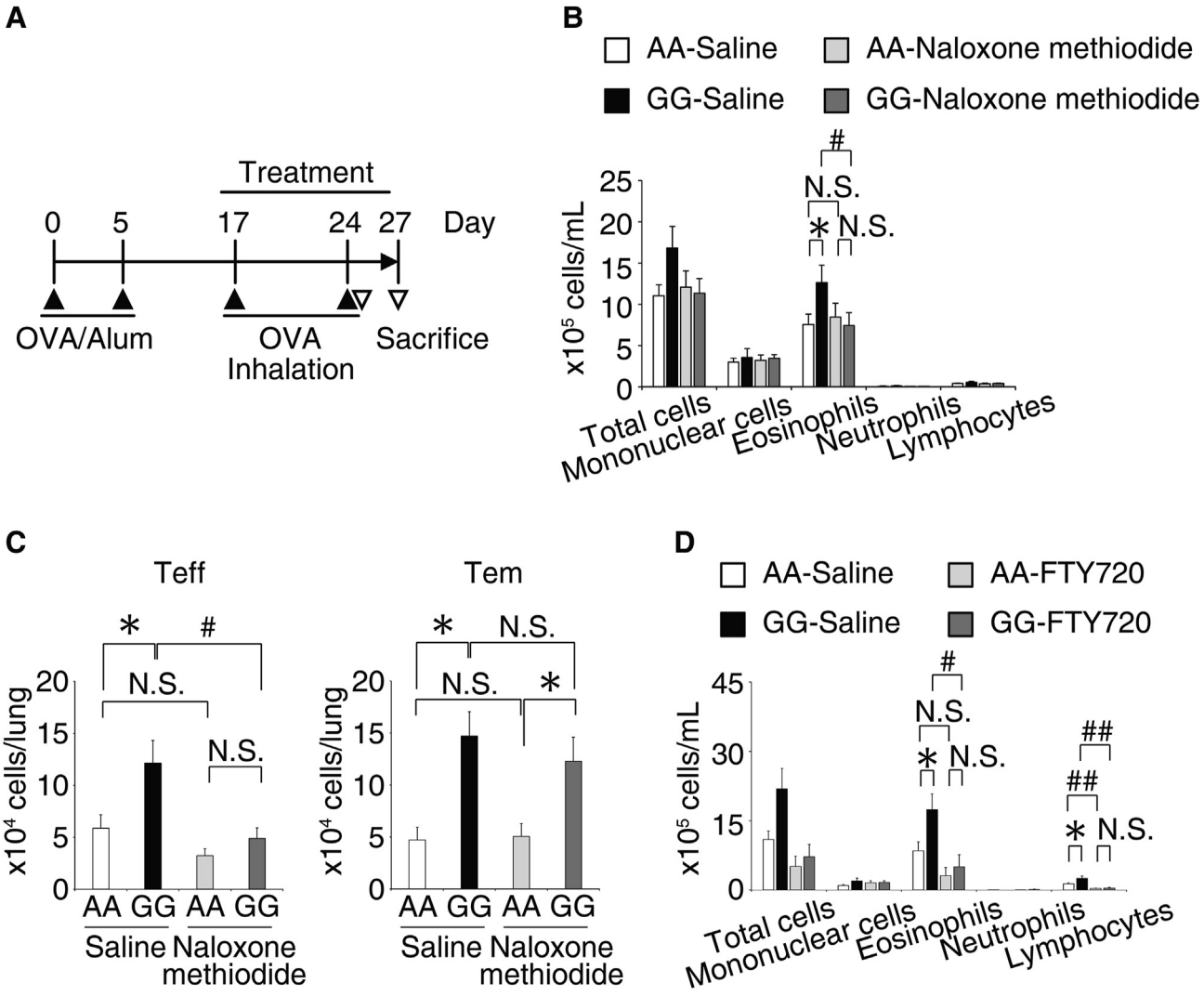

Fig. 5 GG genotype of peripheral μ-opioid receptors is responsible for enhanced effector T cell generation in lymph nodes. **(A)** Mice were sensitized with ovalbumin (OVA)/alum on days 0 and 5, challenged with OVA on days 17 and 24, and sacrificed 6 h or 3 days after OVA inhalation. Daily intraperitoneal injection of naloxone methiodide (10 mg/kg body weight) was performed from day 17 to day 24. Oral administration of FTY720 (1.25 mg/kg body weight) was performed from day 14 to sacrifice. **(B)** The cell composition in the bronchoalveolar lavage (BAL) fluid of mice treated with naloxone methiodide 3 days after the second OVA inhalation, as in panel A, was evaluated by Diff-Quick staining. Data are shown as the mean ± SEM based on three to six mice per group. **(C)** The total numbers of effector T cells (Teff) and effector memory T cells (Tem) in the lungs of mice treated with saline or naloxone methiodide 6 h after the second OVA inhalation were evaluated by flow cytometry. Data are shown as the mean ± SEM based on 11 to 12 mice per group. **(D)** The cell composition in the BAL fluid of mice treated with FTY720 3 days after the second OVA inhalation, as in panel A, was evaluated by Diff-Quick staining. Data are shown as the mean ± SEM based on five to six mice per group. Experiments were repeated twice with similar results. □ and light gray bars, AA mice; ■ and dark gray bars, GG mice. ▲, time points of sensitization or inhalation; Δ, time points of sampling of specimen. *$p < 0.05$ compared to AA mice; #$p < 0.05$ compared to vehicle-treated mice; **$p < 0.01$ compared to AA mice; ##$p < 0.01$ compared to vehicle-treated mice. NS, not significant.

between the AA and GG mice during the interval between OVA inhalations (Supplementary Fig. 5D). These results suggest that the *Oprm1* G112 SNP enhances Teff generation, but does not affect their egress from peripheral lymph nodes during the interval between OVA inhalations, which is responsible for the increased number of Th2 cytokine-producing Teff cells in the lungs of GG mice.

Increased numbers of Th2 cells are generated in the bronchial lymph nodes of GG mice after the first allergen inhalation

We next assessed the effect of the MOPR genotype on the composition of subsets of T cells in the BLN from the first to the second OVA inhalation (Fig. 6A, B). The total number of BLN cells was elevated and peaked 48 h after the first OVA inhalation; the numbers decreased and were maintained thereafter. Similar changes in the numbers of $CD4^+$ Tn, $CD4^+$ Tcm, IL-4^+CD4^+ Teff, and IL-4^+CD4^+ Tem cells were observed (Fig. 6C). In addition, the numbers of IL-4^+CD4^+ Teff, IL-4^+CD4^+ Tem, and $CD4^+$ Tcm 48 h after OVA inhalation were significantly greater in GG mice than in AA mice. Similarly, IL-4 mRNA levels per $CD4^+$ Teff and per $CD4^+$memory phenotype T cells in the BLN were significantly higher in the GG mice than in the AA mice (Supplementary Fig. 6). Nevertheless, the number of $CD4^+$ Tn cells was not largely different between the genotypes (Fig. 6C). One hundred and 20 h after the first OVA inhalation and before the secondary OVA inhalation, the increased numbers of IL-4^+CD4^+ Teff and IL-4^+CD4^+ Tem cells were stored in the lungs of GG mice (Fig. 6D).

In the BLN, 48 h after the first OVA inhalation, low MOPR expression was observed on immune cells such as $CD4^+$ T cells, B cells, and dendritic cells (DCs), but these levels were comparable between genotypes (Supplementary Fig. 7A). In $CD4^+$ T cells, MOPRs were expressed on Teff and Tem, but not on Tn cells (Supplementary Fig. 7B). In addition, the levels of plasma β-endorphin, an endogenous agonist of MOPR, were also comparably

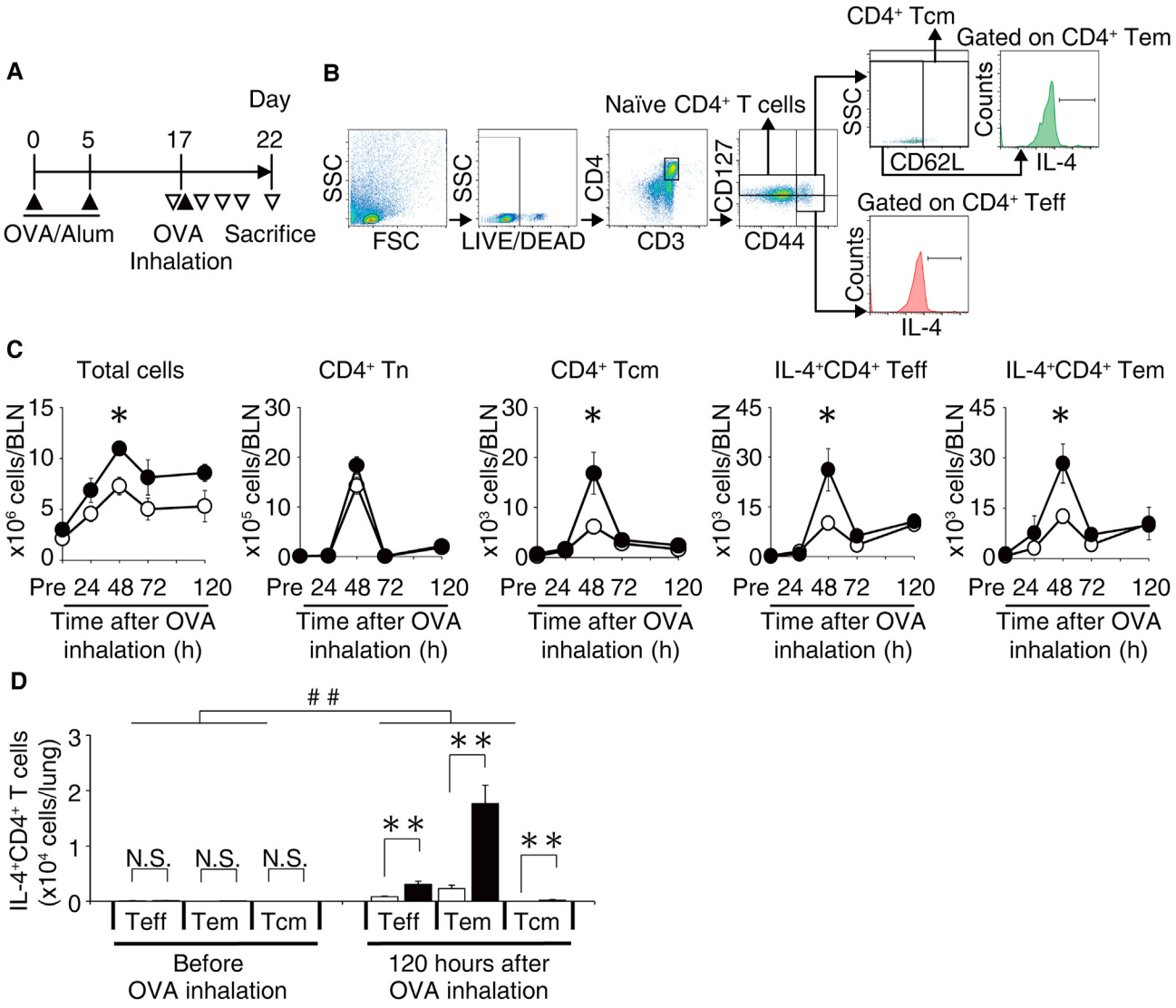

Fig. 6 Increased numbers of Th2 cells in bronchial lymph nodes were observed in GG mice after the first allergen inhalation. **(A)** Mice were sensitized with ovalbumin (OVA)/alum on days 0 and 5, challenged with OVA on day 17, and sacrificed at the indicated time points. **(B)** Gating strategies for $CD4^+$ naïve T cells (Tn), $CD4^+$ effector T cells (Teff), $CD4^+$ effector memory T cells (Tem), and $CD4^+$ central memory T cells (Tcm) in the bronchial lymph nodes (BLNs) are shown. **(C)** The numbers of total cells, $CD4^+$ Tn, $CD4^+$ Tcm, IL-4^+ $CD4^+$ Teff, and IL-4^+ $CD4^+$ Tem in the BLN were counted at the indicated time points after the first OVA inhalation. Data are shown as the mean ± SEM based on five to six mice per group. **(D)** The number of IL-4^+ $CD4^+$ T cells was counted before (pre) and 120 h after the first OVA inhalation. Data are shown as the mean ± SEM based on 6 mice per group. Experiments were repeated twice with similar results. FSC, forward scatter; SSC, side scatter. ○ and □, AA mice; ● and ■, GG mice. ▲, time points of sensitization or inhalation; Δ, time points of sampling of specimen. *$p < 0.05$ compared to AA mice; **$p < 0.01$ compared to AA mice; ##$p < 0.01$ compared to before OVA inhalation. NS, not significant.

different between the genotypes (Supplementary Fig. 7C). These data indicate that genetic differences in MOPR signaling contribute to the increased number of Th2 cytokine-producing Teff in BLNs. The percentage of MOPR$^+$ T cells peaked one day after IL-2 and anti-CD3/CD28 antibody stimulation and decreased thereafter *in vitro* (Supplementary Fig. 8A). However, MOPRs expressed on CD4$^+$ T cells alone played a limited role in the enhanced Th2 cell differentiation in the GG genotype (Supplementary Fig. 8B). These data suggest that cell–cell interactions may be required for the enhanced Th2 cell differentiation in GG mice.

Discussion

This study provides the first evidence showing the involvement of the *OPRM1* A118G SNP (rs1799971) in enhanced AHR via increased Th2-type inflammation. The mouse model showed that the *Oprm1* G112 SNP increased effector Th2 cell generation in peripheral lymph nodes (LNs), which induced enhanced eosinophilic inflammation in the lungs of mice.

Our findings in asthmatic patients carrying the *OPRM1* G118 SNP suggest the involvement of a sustainable pro-inflammatory milieu surrounding the airway smooth muscle and allergic diathesis, as evident by the enhanced AHR. Although a positive correlation between the severity of AHR and the number of eosinophils in the sputum of asthmatics was previously reported,[37] the clinical findings of bronchial asthma, except for AHR, were not largely different among genotypes in the present study, which could be explained by the fact that eosinophils and IgE levels were measured in peripheral blood, rather than in lower airway samples. In line with this possibility, there was a positive correlation between the *OPRM1* G118 SNP and the increased morbidity of pollen allergies, which may have resulted in the increase in these allergic indicators in the airway mucosa. Further investigation will be necessary to address this possible relationship.

The stress levels of patients in the present study are presumed to be moderate, according to the Japanese version of the PSS,[38] and the genetic differences in the *OPRM1* A118G did not alter the stress scores of patients with asthma (Supplementary Table 3). However, we recognize that our findings were inherently limited by the number of patients who were eligible for the evaluation of the genetic influence of MOPR variability on the stress responses to emotional burdens. In asthmatic patients with a stress-related condition modified by obesity or smoking,[3] remarkably, the *OPRM1* G118 SNP was associated with a reduced % forced expiratory volume in 1 s (%FEV1), forced expiratory volume in 1 s/forced vital capacity (FEV1%), or a reduced maximum flow at 50% of the vital capacity (%V50), which corresponded to the aggravation of pulmonary function (Supplementary Tables 4 and 5). Thus, the G118 SNP in *OPRM1* exacerbated asthmatic symptoms not only in everyday life, but also under high-stress conditions.

The mice used in the present study were free from forced stress conditions, such as restraint stress and forced-swimming stress, although they experienced conditions of everyday life stress caused by temporary handling or retention and breeding for experiments. This was confirmed by changes in plasma β-endorphin and corticosterone levels, which were consistent with previously reported circadian patterns[39] and were not largely different between the AA and GG mice (Supplementary Fig. 5B, 7C). Increased asthmatic severity in GG mice may have occurred due to increased ligand affinity and receptor signal transduction.[20] The higher response to stress exposure in terms of eosinophilic inflammation in the GG genotype compared to the AA genotype is also supported by our findings that AA mice exposed to forced stress such as forced-swimming stress and restraint stress between the first and second allergen inhalations exhibit enhanced eosinophilic inflammation after the second inhalation, reaching the levels observed in the GG mice in the present study (data not shown).

In the present study, the increase in eosinophil counts in the BAL fluid 3 days after the first OVA inhalation was minimal (Supplementary Fig. 1F- I), although the numbers of effector and memory phenotype Th2 cells in the BLN peaked at 2 days after the first OVA inhalation (Fig. 6C). Rather, definitive eosinophilic inflammation was observed 3 days after the second OVA inhalation in either genotype (Fig. 2A). To ensure a definite exacerbation of eosinophilic inflammation after the second OVA inhalation, an interval of more than 3 days between the first and second OVA inhalation was essential (Supplementary Fig. 1A). Furthermore, Th2 cells were preliminarily stored in the lungs before the secondary OVA inhalation in both genotypes (Fig. 6D). Therefore, the higher eosinophilic inflammation observed in the GG mice could be due to the higher number of Th2 cells in the BLN, resulting in more abundant Th2 cells in the lung. CD4$^+$ Teff and memory phenotype T cells in the BLN of GG mice may be responsible for the immediate and long-term increases in Th2 cytokine production responses in the lung, respectively. Conversely, there was no difference between the genotypes in the number of Th2 cells in the BLN before OVA inhalation (Fig. 6C). These data suggest that the *OPRM1* A118G polymorphism is associated with enhanced eosinophilic inflammation, which clearly manifests with repetitive antigen exposure.

After the first allergen inhalation, there was a higher number of newly generated CD4$^+$ Teff cells in the BLN of mice carrying the GG genotype as a result of peripheral MOPR signaling (Fig. 5C). MOPRs are not expressed on Tn cells, but are induced after anti-CD3/CD28 antibody- or IL-4-mediated activation of human T cells.[40] Moreover, in the present study, MOPRs were induced on T cells one day after CD3/CD28 stimulation (Supplementary Fig. 8A). The expression levels of MOPRs on differentiated T cells did not largely differ between genotypes *in vivo* (Supplementary Fig. 7E). Furthermore, cytokines previously reported to increase MOPR expression, such as IL-6,[41] IL-1β,[42] IL-4, and tumor necrosis factor alpha (TNF-α),[43] did not modify MOPR expression levels on Tn cells (data not shown), although the cytokine levels *in vivo* were not confirmed in the current study.

In Th2 cell differentiation, the MOPR-dependent signal induces IL-4 mRNA production through the p38 mitogen-activated protein kinase (MAPK) pathway, but not via protein kinase A- and Erk1/Erk2-dependent pathway activation.[15,44] In the present study, IL-4 production by Tn cells after stimulation with an anti-CD3/CD28 antibody was not enhanced in the presence of IL-2, IL-4, and morphine in either genotype (Supplementary Fig. 8B), although we did not confirm IL-4 mRNA expression levels in the T cells. These data suggest that MOPR-mediated signaling in other types of cells modulates the T cell phenotype in the enhanced Th2 cell differentiation that occurs in GG mice. Indeed, MOPR was expressed on B cells and DCs in the BLN 48 h after the first OVA inhalation (Supplementary Fig. 7A). Li *et al.* demonstrated that MOPR expression on activated DCs was mediated through toll-like receptor (TLR) signaling, and their signals augmented Th2 cell differentiation through reduced IL-12 and IL-23 production.[45] This suggests that the reduced Th1-oriented or regulatory T cell-oriented DC response related to the *OPRM1* G118 SNP may orchestrate the enhanced Th2 immune responses.

Since the frequency of heterozygotes was less than the theoretical value, it is necessary to consider the possibility that there may have been several genetically isolated populations in this study, which may have affected the results. According to the

results of recent next-generation sequencing studies,[46] population structuring is unavoidable, even in the Japanese population, which is considered to be relatively homogeneous, and this may also be relevant to our study. However, this effect may be limited considering the consistency of the results between the human studies and animal experiments. On the other hand, our study has the following limitations: (1) the number of patients from whom laboratory data were successfully collected, including pulmonary function and stress levels, was limited; (2) our study is a single-center study; and (3) the patients enrolled in this study were adults over 56 years of age, on average, although the patient background characteristics did not significantly differ among the groups. Therefore, further research is required to verify the results, especially the association between the GG genotype and lung function, in a multicenter study with a larger sample size, considering the structuring of the population.

In summary, our findings provide evidence that the *OPRM1* G118 SNP is potentially associated with disease severity in patients with asthma exposed to stressors during everyday life. The present study also provides evidence that the connection between the *OPRM1* G118 SNP and asthma severity in emotional burden-induced asthma exacerbation is mediated by increased generation of Teff and Tem Th2 cells in the BLN. The types of cells responsible for the generation of those cells in the BLN and the effect of β-endorphin produced by activated lymphocytes[47] on asthma severity require further investigation. Our data could also facilitate the development of more effective strategies for the treatment and/or prevention of disorders worsened by emotional burdens, such as asthma.[6]

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.alit.2021.08.006.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

KaoK., model development, data acquisition, data analysis, data interpretation, and manuscript preparation; TM, data acquisition, data analysis, data interpretation, and manuscript preparation; YN and KY, data acquisition, data analysis, and data interpretation; SM, data interpretation, and revising manuscript; MS, model development, data acquisition, and data analysis; IS and HM, data interpretation and manuscript review; KazK., data analysis, data interpretation, and manuscript revision; JAB. and HS, manuscript review; MT and TT, study conduct and manuscript review; TK, model development and data acquisition; IO, study conception, model development, study design, data analysis, and manuscript review.

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