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Endogenous Glucagon-Like Peptide-1 Receptor and Glucose-Dependent Insulinotropic Polypeptide Receptor Signaling Inhibits Aeroallergen-Induced Innate Airway Inflammation

Shinji Toki[1] | Masako Abney[1] | Jian Zhang[1] | Mark Rusznak[1] | Christian M. Warren[2] | Dawn C. Newcomb[1] | Katherine N. Cahill[1] | Daniel J. Drucker[3] | Kevin D. Niswender[2,4] | Ray Stokes Peebles Jr.[1,2]

[1]Division of Allergy, Pulmonary, and Critical Care Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, USA | [2]United States Department of Veterans Affairs, Tennessee Valley Healthcare System, Nashville, Tennessee, USA | [3]Department of Medicine, Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, University of Toronto, Toronto, Ontario, Canada | [4]Division of Diabetes, Endocrinology, and Metabolism, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

Correspondence: Shinji Toki (shinji.toki@vumc.org)

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Keywords: allergy | asthma | GIPR | GLP-1R | incretin

ABSTRACT

Background: Anti-inflammatory effects of incretin signaling through the glucagon-like peptide-1 receptor (GLP-1R) and the glucose-dependent insulinotropic polypeptide receptor (GIPR) in mice have been reported. Therefore, we hypothesized that signaling through the endogenous GLP-1R and the GIPR individually decreases allergic airway inflammation and that the combination of GLP-1R and GIPR signaling together additively inhibits allergen-induced lung and airway inflammation.

Methods: WT (C57BL/6J), GLP-1R knockout (KO), GIPR KO, and GLP-1R/GIPR double KO (DKO) mice were challenged intranasally with *Alternaria alternata* extract (Alt-Ext) or vehicle to evaluate the impact of signaling through these receptors on the innate allergen-induced inflammatory response that is primarily driven by group 2 innate lymphoid cells (ILC2).

Results: Alt-Ext-induced IL-33 release in the bronchoalveolar lavage fluid (BALF) was not different between the mouse strains, but thymic stromal lymphopoietin (TSLP) was significantly increased in GLP-1R/GIPR DKO mice challenged with Alt-Ext compared to the other strains. Furthermore, Alt-Ext-induced protein expression of IL-5, IL-13, CCL11, and CCL24 in the lung homogenates, the number of eosinophils, lymphocytes, and neutrophils in the BALF, and the number of lung GATA3+ ILC2 were significantly increased in GLP-1R/GIPR DKO mice compared to the other 3 strains. Furthermore, ICAM-1 expression on lung epithelial cells was increased in GLP-1R/GIPR DKO mice challenged with Alt-Ext compared to the other 3 strains.

Conclusions: Deficiency of both GLP-1R and GIPR signaling together increased TSLP release, ILC2 activation, and early type 2 innate immune responses to aeroallergen exposure. Combined GLP-1R and GIPR signaling should be explored for the treatment of asthma.

Abbreviations: AGEs, advanced glycation end products; Alt-Ext, *Alternaria alternata* extract; ANOVA, analysis of variance; AUC, area under the curve; BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; DAMP, damage-associated molecular pattern; DKO, double knockout; FBS, fetal bovine serum; GIPR, glucose-dependent insulinotropic polypeptide receptor; GLP-1R, glucagon-like peptide-1 receptor; GTT, glucose tolerance test; HMGB1, high mobility group box 1; ICAM-1, intercellular adhesion molecule 1; ILC2, group 2 innate lymphoid cells; Lin, lineage; MFI, mean fluorescence intensity; PBS, phosphate-buffered saline; RAGE, receptor for advanced glycation end products; T2D, type 2 diabetes; TSLP, thymic stromal lymphopoietin.

1 | Introduction

Asthma is a common heterogeneous disease characterized by variable airflow obstruction and airway inflammation that is manifested by cough, dyspnea, chest tightness, and wheezing [1]. Asthma comorbidities such as obesity and insulin resistance are frequently observed in children [2] and adults [3] and are associated with increased asthma severity and impaired lung function [4]. These comorbidities complicate asthma diagnosis and impair response to first-line inhaled corticosteroid therapy [4–6]. Understanding the mechanisms by which metabolic pathways regulate airway inflammation is critical to develop new pharmacotherapeutic strategies for difficult-to-treat asthma.

Incretin hormones signal through their receptors to induce insulin secretion from pancreatic β-cells thereby regulating glucose homeostasis [7, 8]. Major incretin hormones include glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). GLP-1 and GIP are released by enteroendocrine L-cells and K-cells, respectively, in the small intestine and bind to the GLP-1 receptor (GLP-1R) and GIP receptor (GIPR) on β-cells of the pancreas to augment glucose-dependent insulin secretion [8, 9]. Incretin mimetic drugs are medications for treatment of type 2 diabetes (T2D) and obesity [9]. These drugs mimic the biological functions of endogenous incretin hormones that control glycemia after meals. GLP-1 mimetic drugs and dual GLP-1 and GIP agonist improve glycemic control and also reduce body weight [10, 11].

Previous studies reported that the incretin mimetic drugs exert anti-inflammatory effects in several chronic inflammatory disorders, including asthma and chronic obstructive pulmonary disease (COPD). For instance, GLP-1R agonists decreased the rates of cardiovascular events [12, 13], asthma exacerbations [14], and COPD exacerbations [15] in people with T2D. Taken together, GLP-1R agonists reduce inflammatory responses through direct and indirect pathways reflecting improvement in glucose control, reduction in body weight, and weight loss-independent actions to attenuate inflammation.

Our group reported that treatment with a GLP-1R agonist, liraglutide, significantly decreased aeroallergen-induced airway inflammation in lean and obese mice [16, 17]. Furthermore, the GLP-1R and GIPR dual agonist, tirzepatide, decreased allergic airway inflammation compared to vehicle treatment, and *Alternaria alternata* extract (Alt-Ext)-induced IL-33 expression compared to treatment of GLP-1R agonist or GIPR agonist alone [18]. These pharmacological studies highlight roles for both the GLP-1R and GIPR as transducers of the anti-inflammatory actions of incretin-based therapies. Herein, we determined the role of endogenous signaling through either one or both, of the GLP-1R and GIPR in regulating allergic airway inflammation.

2 | Methods

2.1 | Mice

The generation and characterization of the GLP-1R and the GIPR knockout (KO) mice both on a C57BL/6 background has previously been described [19, 20]. GLP-1R/GIPR double KO (DKO) mice were obtained by crossing the GLP-1R KO and GIPR KO mice. We used 9–13-week-old female mice for all experiments. The animals were maintained in a temperature-controlled room at 22.2°C on a half day light–dark cycle. Mice were fed a regular diet (PicoLab Laboratory Rodent Diet 5LOD), and tap water was available ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center and were conducted according to the guidelines for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council.

2.2 | Innate Allergic Inflammation Model by Alt-Ext-Challenge

In the model of aeroallergen-induced early innate inflammation that we have previously described [16, 17, 21, 22], 6 μg (protein amount) of *Alternaria alternata* extract (Alt-Ext) (Stallergenes Greer, Lenoir, NC) was dissolved in 80 μL of vehicle (phosphate-buffered saline, PBS), or the vehicle alone was administered intranasally to mice anesthetized with ketamine/xylazine. In the first protocol, the mice were analyzed 1 h or 6 h after a single challenge of Alt-Ext or vehicle for detection of IL-33 or thymic stromal lymphopoietin (TSLP), respectively, times that we have previously published represent the peak expression for each of these cytokines in the airway [16, 17, 22]. In the second protocol, Alt-Ext or vehicle was administered intranasally for 4 consecutive days. Whole lungs, bronchoalveolar lavage fluid (BALF), and the sera were harvested 24 h after the last Alt-Ext-challenge or vehicle challenge (day 4) to evaluate cell differentials, lung cytokine and chemokine expression, and lung innate lymphoid cells (ILCs) and CD4 T cells by flow cytometry. We previously reported that group 2 innate lymphoid cells (ILC2) are the principal cells producing type 2 cytokines in this 4 consecutive day aeroallergen challenge model [16, 17, 21, 22].

2.3 | Statistical Analysis

All data were analyzed with GraphPad Prism 9. In metabolism tests, timeline data were analyzed by using two-way analysis of variance (ANOVA) followed by Tukey's multiple paired comparisons test. Statistically significant significances between the experimental groups were assessed by one-way ANOVA with Tukey's multiple paired comparisons test, or the Mann–Whitney test for two group comparison. The *p*-values below 0.05 were considered significant between the two groups.

Further experimental details are provided in the online repository. All antibodies used in this study are listed in Table S1.

3 | Results

3.1 | Single and Double Incretin Receptor Deficiency Slightly Decreased Mouse Body Weight

We compared body weight between WT, GLP-1R KO, GIPR KO, and GLP-1R/GIPR DKO mice when all mice were 9–13 weeks old. Bodyweight was lower in GLP-1R KO, GIPR KO, and GLP-1R/GIPR DKO mice compared to WT mice (Figure 1A). The difference was within 10% of WT body weight. Meanwhile,

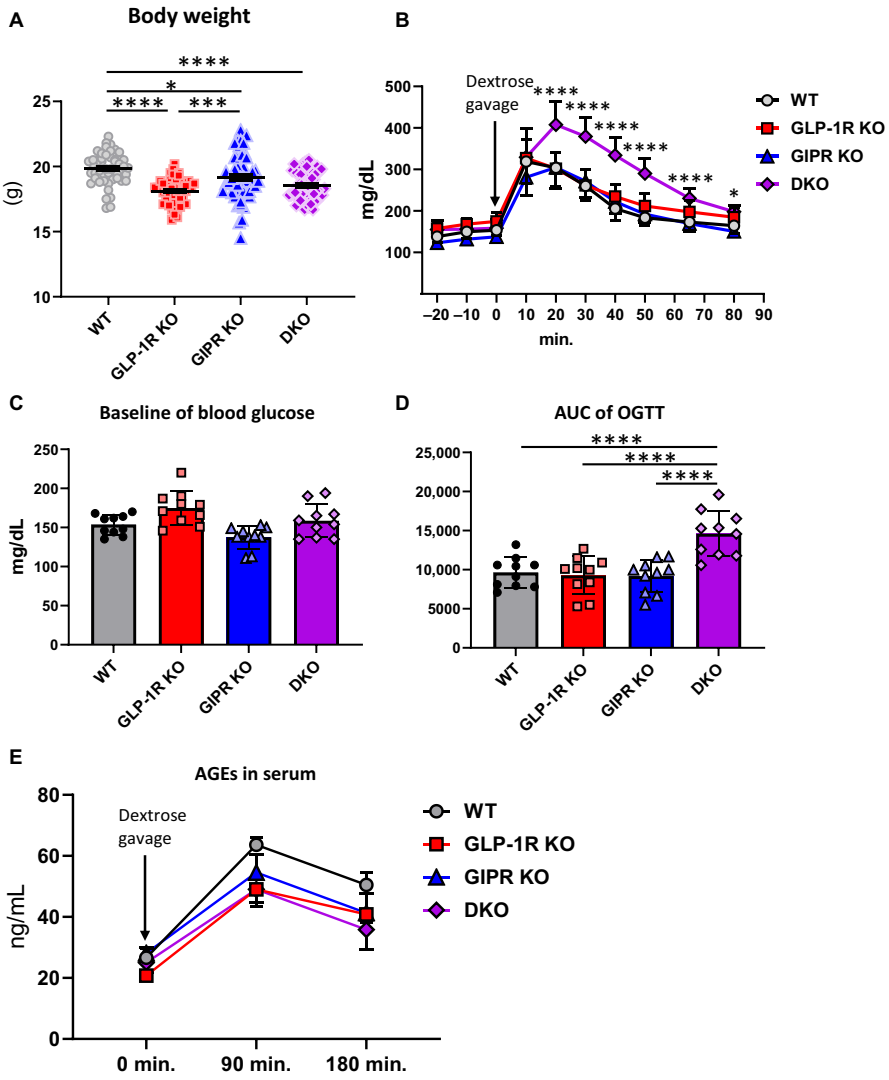

FIGURE 1 | Effects of GLP-1R, GIPR, or GLP-1R/GIPR deficiency on glycemic control. (A) Body weight of each mouse strain ($n = 45–50$). (B) Comparison of blood glucose tolerance after oral dextrose administration ($n = 10$). *$p < 0.05$, ****$p < 0.0001$ vs. WT mice. (C) Baseline of blood glucose levels after 6 h fasting. (D) The area of under the curve (AUC) of oral glucose tolerance test (GTT). (E) Advanced glycation end products (AGEs) in the serum before and after oral dextrose administration ($n = 10$). The results are the mean ± SD. *$p < 0.05$, ***$p < 0.001$ and ****$p < 0.0001$ compared to WT mice.

there was no difference in body weight between GLP-1R KO, GIPR KO, and GLP-1R/GIPR DKO mice (Figure 1A).

3.2 | GLP-1R/GIPR DKO Mice Exhibit Glucose Intolerance

To evaluate the glucoregulatory phenotype arising from loss of incretin receptor signaling, we performed an oral glucose tolerance test (GTT) on WT, GLP-1R KO, GIPR KO, and GLP-1R/GIPR DKO mice. Baseline glucose levels after fasting for 6 h were not different between WT, GLP-1R KO, GIPR KO, and GLP-1R/GIPR DKO mice (Figure 1B,C). After oral dextrose administration, the blood glucose concentration was at its peak within 20 min in all mouse strains (Figure 1B), and GLP-1R/GIPR DKO mice exhibited greater glycemic excursions relative to either of the single incretin receptor knockout or the WT mice from 20 to 80 min (Figure 1B). The area under the curve (AUC) for glucose was significantly increased in GLP-1R/GIPR DKO mice compared to either of the single incretin receptor knockout or the WT mice (Figure 1D).

Next, we examined whether oral dextrose administration-induced greater blood glucose level in GLP-1R/GIPR DKO mice increases advanced glycation end products (AGEs) in the serum compared to the other mouse strains. AGEs were measured in the serum 90 and 180 min after oral dextrose administration. The concentration of AGEs in the serum peaked at 90 min; however, there were no differences in the serum AGEs between the four mouse strains (Figure 1E).

3.3 | GLP-1R/GIPR DKO Mice Exhibit Increased Alt-Ext-Induced TSLP, but Not IL-33, in BALF Compared to the Other Groups

To test the effects of incretin receptor deficiency on allergen-induced inflammation, we first detected mRNA expression of

GLP-1R and GIPR in the lungs from naïve mice of each strain. The mRNA of *Glp1r* was scarcely expressed in the lung from GLP-1R KO and GLP-1R/GIPR DKO mice compared to WT mice, and the mRNA of *Gipr* was extremely low in the lung from GIPR KO and GLP-1R/GIPR DKO mice compared to WT mice (Figure S1A). Meanwhile, other structurally related receptors, glucagon-like peptide-2 receptor (*Glp2r*), and glucagon receptor (*Gcgr*), were detected in the lung from all strains (Figure S1A). Furthermore, lung epithelial cells as CD45− EpCAM+ cells from WT mice expressed *Glp1r* and *Gipr*; however, lung CD45+ cells from WT mice showed very low expression of *Glp1r* and *Gipr* compared to whole lung of WT mice (Figure S1B,C). Therefore, we tested the effects of incretin receptor deficiency for aeroallergen-induced epithelial cell-released mediator detection.

IL-33 and TSLP are key cytokines for type 2 inflammation [23, 24]. These cytokines are released as "alarmins" from damaged or stressed epithelial or endothelial cells [25], and stimulate group 2 innate lymphoid cells (ILC2) [22], CD4 T cells [26], and dendritic cells to skew development of type 2 immunity [27, 28]. Thus, we measured IL-33 and TSLP in BALF to determine the effects of loss of endogenous incretin receptor signaling on IL-33 and TSLP protein release. The mice were challenged with Alt-Ext or vehicle, and the BALF was harvested 1 h and 6 h after the challenge to measure IL-33 and TSLP, respectively (Figure 2A). Alt-Ext-challenge significantly increased IL-33 in BALF content to a similar extent in all mouse strains (Figure 2B). In contrast, TSLP in BALF was higher in GLP-1R/GIPR DKO mice after Alt-Ext-challenge compared to the GLP-1R KO, GIPR KO, or WT mice (Figure 2C).

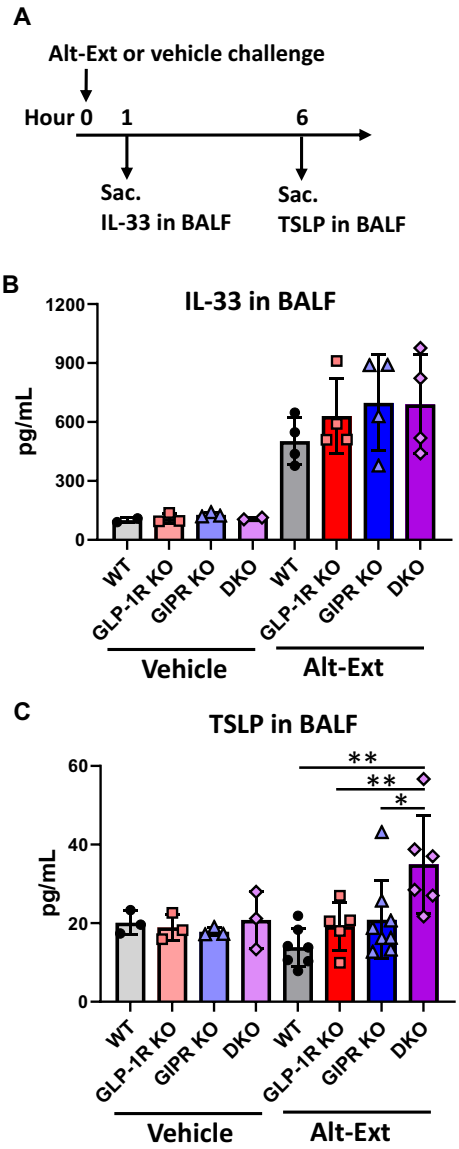

FIGURE 2 | Effects of GLP-1R, GIPR, or GLP-1R/GIPR deficiency on Alt-Ext-induced IL-33 and TSLP release. (A) Experimental protocol. Alt-Ext (6 μg protein/80 μL) or the vehicle (PBS, 80 μL) was intranasally injected to 4 strains of mice. The BALF was harvested 1 h or 6 h after the challenge to measure IL-33 or TSLP, respectively. (B) The protein level of IL-33 in the BALF 1 h after Alt-Ext or vehicle intranasal challenge ($n = 2–4$). (C) The protein level of TSLP in the BALF 6 h after Alt-Ext or vehicle intranasal challenge ($n = 3–8$). The results are the mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$ compared to WT challenged with Alt-Ext.

3.4 | GLP-1R/GIPR DKO Mice Had Increased Lung ILC2, but Not GATA3$^+$ CD4 T Cells, RORγt$^+$ CD4 T Cells, or ILC3 After Alt-Ext-Challenge

We enumerated the lung ILCs and CD4 T cells after 4 consecutive days of Alt-Ext-challenge or vehicle challenge. The lungs were harvested for flow cytometry 24 h after the last Alt-Ext-challenge or vehicle challenge (Figure 3A). The gating strategy of lung ILC2, group 3 innate lymphoid cells (ILC3), GATA3$^+$ CD4 T cells, and RORγt$^+$ CD4 T cells are shown in Figure S2. Alt-Ext-challenge significantly increased the number of lung ILC2 cells compared to vehicle challenge in all strains. The number of Alt-Ext-induced lung ILC2s was greater in GLP-1R/GIPR DKO mice than in the other 3 strains (Figure 3B). In contrast, lung ILC3 was not increased by Alt-Ext-challenge (Figure 3C). Alt-Ext-challenge increased the number of GATA3$^+$ and RORγt$^+$ CD4 T cells in the lungs, but there were no differences in the number of Alt-Ext-induced GATA3$^+$ and RORγt$^+$ CD4 T cells between all 4 mouse strains (Figure 3D,E).

3.5 | GLP-1R/GIPR DKO Mice Exhibit Increased Eosinophil, Lymphocyte, and Neutrophil Recruitment Into Alveolar and Airway Spaces Following Challenge With Alt-Ext

We next characterized leukocyte infiltration by enumerating the cell types and numbers in BALF after 4 consecutive days of Alt-Ext-challenge or vehicle challenge. The number of macrophages, eosinophils, lymphocytes, and neutrophils was significantly increased after Alt-Ext-challenge (Figure 4A–D). Greater numbers of eosinophils, lymphocytes, and neutrophils, but not macrophages, were detected in GLP-1R/GIPR DKO mice challenged with Alt-Ext compared to the GLP-1R KO, GIPR KO, or WT mice (Figure 4A–D).

3.6 | GLP-1R/GIPR DKO Mice Exhibit Greater Alt-Ext-Induced Type 2 Cytokine and Chemokine Protein Expression in the Lung

We next measured the protein levels of inflammatory cytokines and chemokines associated with ILC2 and type 2 inflammation

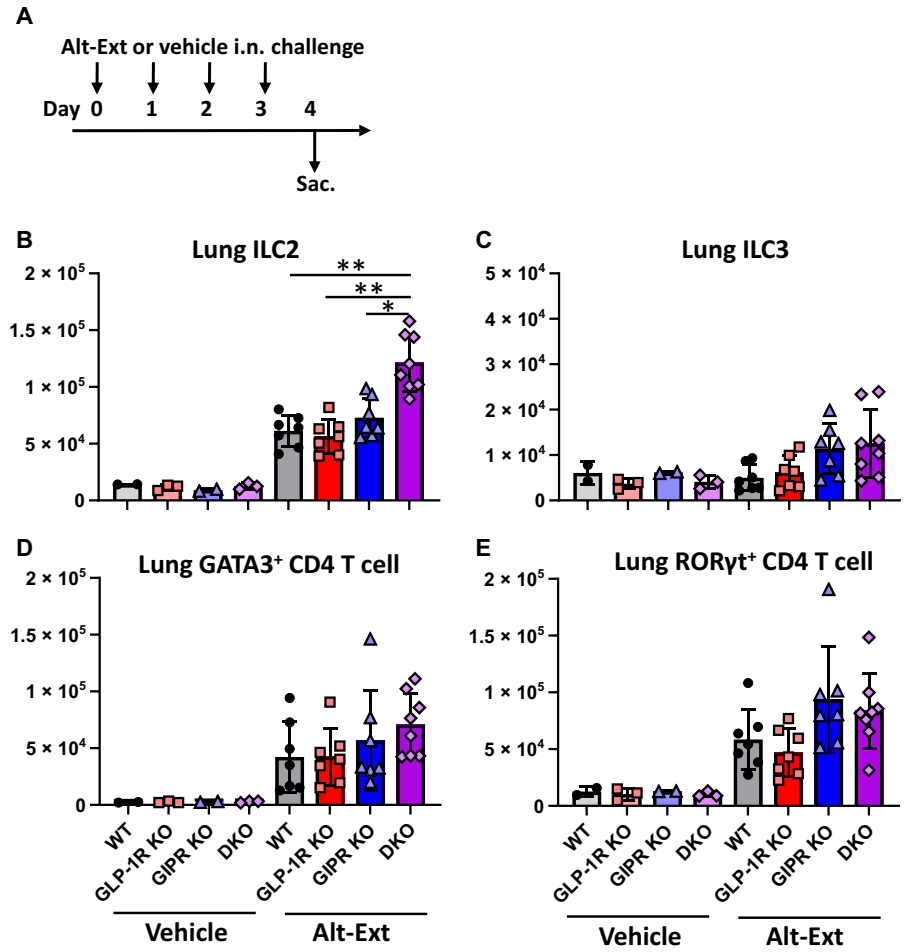

FIGURE 3 | Effects of GLP-1R, GIPR, or GLP-1R/GIPR deficiency on lung ILC2 and CD4 T cells after Alt-Ext-challenge. (A) Experimental protocol. Alt-Ext (6 μg protein/80 μL) or the vehicle (PBS, 80 μL) was intranasally administered to 4 strains of mice for 4 consecutive days. Twenty-four hours after the challenge, the whole lungs were harvested, and the lung cells were collected for flow cytometry analysis. (B) Lung ILC2, (C) Lung ILC3, and (D) Lung GATA3$^+$ CD4 T cells. (E) Lung ROTγt$^+$ CD4 T cells were enumerated. The results are shown as mean ± SD. *$p < 0.05$, **$p < 0.01$ compared to WT challenged with Alt-Ext.

in lung homogenates from the 4 groups of mice challenged with Alt-Ext or vehicle for four consecutive days. Alt-Ext-challenge significantly increased the lung protein expression of IL-5, IL-9, IL-13, IL-33, eotaxin (CCL11), and eotaxin-2 (CCL24) compared to levels detected in the lungs of vehicle-challenged mice (Figure 5A–F). The type 2 cytokines IL-5 and IL-13, and eosinophil-related chemokines CCL11 and CCL24 were higher in the lung homogenates of GLP-1R/GIPR DKO mice challenged with Alt-Ext compared to the GLP-1R KO, GIPR KO, or WT mice (Figure 5A,B,E,F). Meanwhile, IL-25 was not increased by 4 consecutive days of Alt-Ext challenge, and there were no differences in IL-25 between the mouse strains (Figure 5G). The pro-inflammatory cytokine IL-1β and the neutrophil-related chemokine KC (CXCL1) were also increased in Alt-Ext-challenged mice (Figure 5H,I), but the levels were not different between the four mouse strains (Figure 5H,I).

As GLP-1R/GIPR DKO exhibit greater glucose intolerance compared to the single incretin receptor KO mice, we examined circulating levels of AGEs but did not detect differences of AGEs in the serum after Alt-Ext-challenge compared to PBS challenge (Figure 5J). High mobility group box 1 (HMGB1) has a crucial role in inflammation, acting as a damage-associated molecular pattern (DAMP). Four consecutive days of Alt-Ext-challenge significantly increased levels of HMGB1 in BALF in all mouse strains (Figure 5K), and the levels were higher in GIPR KO and GLP-1R/GIPR DKO mice compared to the GLP-1R KO or WT mice (Figure 5K).

3.7 | Neutralization of TSLP Inhibited Alt-Ext-Induced Type 2 Inflammation in GLP-1R/GIPR DKO Mice

We found that TSLP release after Alt-Ext-challenge was significantly greater in GLP-1R/GIPR DKO mice compared to the other 3 strains of similarly Alt-Ext-challenged mice (Figure 2C). To determine whether the TSLP leads to the increased type 2 airway inflammation in GLP-1R/GIPR DKO mice, we performed anti-TSLP antibody (ab) treatment prior to Alt-Ext-challenge in WT and GLP-1R/GIPR DKO mice. Anti-TSLP ab treatment significantly decreased the number of Alt-Ext-induced eosinophils and lymphocytes in the BALF from WT and GLP-1R/GIPR DKO mice compared to isotype ab

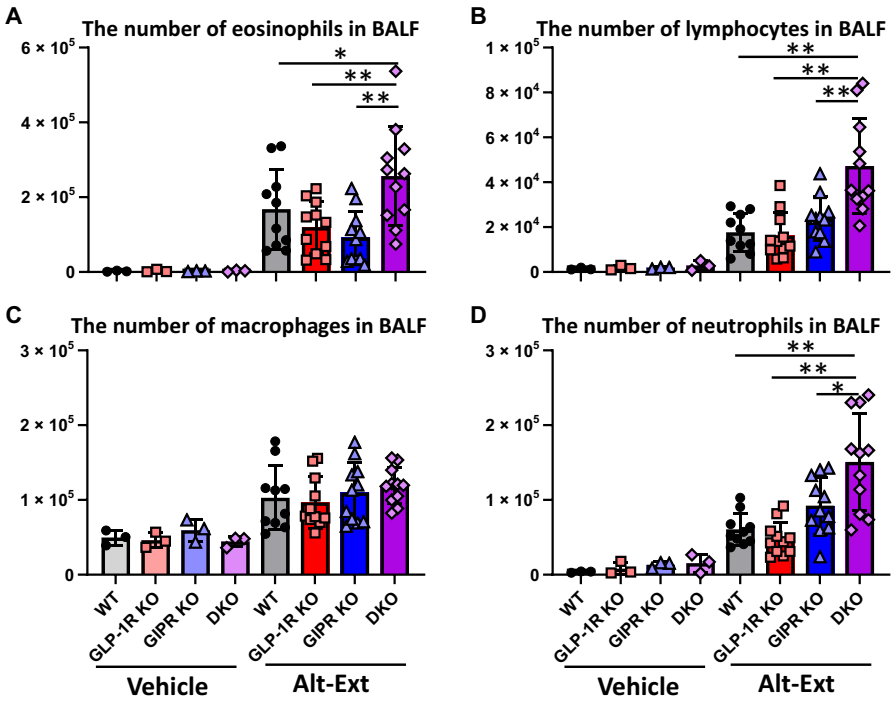

FIGURE 4 | Effects of GLP-1R, GIPR, or GLP-1R/GIPR deficiency on Alt-Ext-induced leukocyte accumulation in the airway or alveolar spaces. The experimental protocol is same as Figure 3A, and the BALF was harvested 24 h after the last Alt-Ext-challenge or vehicle-challenge to evaluate cell differentials, (A) eosinophils, (B) lymphocytes, (C) macrophages, and (D) neutrophils ($n = 3$–10). The results are shown as mean ± SD. *$p < 0.05$, **$p < 0.01$ compared to WT challenged with Alt-Ext.

treatment (Figure S5). Furthermore, anti-TSLP ab treatment significantly decreased Alt-Ext-induced IL-13, CCL11, and CCL24 in lung homogenates from GLP-1R/GIPR DKO mice, but not WT mice (Figure S5). Meanwhile, Alt-Ext-induced IL-33 was significantly decreased by anti-TSLP ab treatment in both WT and GLP-1R/GIPR DKO mice (Figure S5). These results indicated that neutralization of TSLP effectively inhibited allergic inflammation in GLP-1R/GIPR DKO mice compared to WT mice.

3.8 | Tirzepatide Treatment Decreased Alt-Ext-Induced Type 2 Lung Inflammation in WT Mice, but Not in GLP-1R KO, GIPR KO, and GLP-1R/GIPR DKO Mice

To determine the requirements for incretin receptor signaling for the response to exogenous incretin mimetic drug treatment, we treated the 4 groups of mice with the GLP-1R and GIPR dual agonist, tirzepatide and then challenged these groups with Alt-Ext. The tirzepatide treatment significantly decreased the Alt-Ext-induced number of eosinophils and lymphocytes in the BALF, and the protein level of IL-5, IL-33, and CCL24 in the lung homogenates from WT mice compared to vehicle treatment (Figure S6C,D,G,J,L). In contrast, there were no differences in the cell differentials between tirzepatide and the vehicle treatment in GLP-1R KO and GLP-1R/GIPR DKO mice (Figure S6B–L). Meanwhile, the tirzepatide treatment significantly decreased the Alt-Ext-induced number of eosinophils and lung IL-33 in GIPR KO mice compared to vehicle treatment (Figure S6C,J). This suggests the effect of exogenous GLP-1R and GIPR agonist, tirzepatide on allergic inflammation is predominantly mediated through GLP-1R in our mouse models.

3.9 | Induction of ICAM-1 Is Higher in Lung Epithelial Cells of GLP-1R/GIPR DKO Mice After Alt-Ext-Challenge

We next tested whether the deficiency of incretin receptor signaling modifies the basal and Alt-Ext-regulated expression of intercellular adhesion molecule-1 (ICAM-1), ICAM-2, and the receptor for advanced glycation end products (RAGE) on lung endothelial cells and epithelial cells. The gating strategies of lung endothelial cells and epithelial cells are shown in Figure S3. The lung endothelial cells were identified as $CD45^-$ $CD31^+$ $CD146^+$ $EpCAM^-$ cells, and the lung epithelial cells were identified as $CD45^-$ $CD31^-$ $CD146^-$ $EpCAM^+$ cells.

Histograms of ICAM-1, ICAM2, and RAGE expression on the endothelial and epithelial cells are shown in Figure S4. Alt-Ext-challenge did not increase the protein expression of these adhesion molecules on endothelial cells compared to vehicle challenge in each mouse strain (Figure 6A–C). However, Alt-Ext-challenge significantly increased ICAM-1 expression on lung epithelial cells from GLP-1R/GIPR DKO mice compared to the GLP-1R KO, GIPR KO, or WT mice (Figure 6D). In contrast, basal levels of ICAM-2 or RAGE expression on lung epithelial cells were not different across the 4 strains of mice nor was there a difference in the expression of these adhesion molecules after Alt-Ext-challenge (Figure 6E,F).

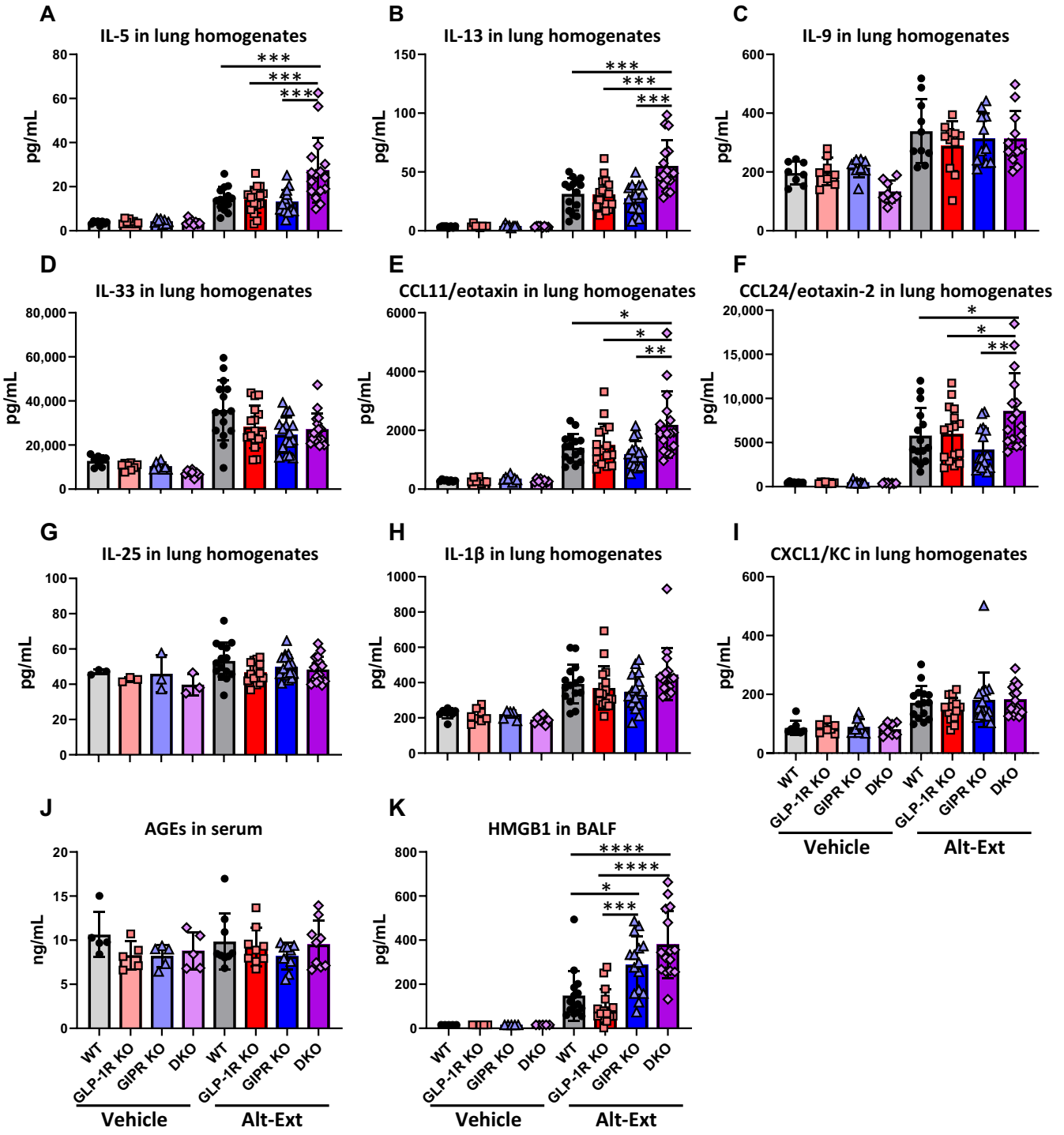

FIGURE 5 | Effects of GLP-1R, GIPR, or GLP-1R/GIPR deficiency on Alt-Ext-induced factors of type 2 inflammation. The experimental protocol is same as Figure 3A. The BALF, whole lungs, and serum were harvested 24 h after the last Alt-Ext-challenge or vehicle challenge. (A–I) IL-5, IL-9, IL-13, IL-33, CCL11 (Eotaxin), CCL24 (Eotaxin-2), IL-25, IL-1β, and CXCL1 (KC) in lung homogenates were measured by ELISA ($n = 8–16$). (J) Advanced glycation end products (AGEs) in serum ($n = 5–9$) and (K) high mobility group box 1 (HMGB1) in BALF ($n = 5–15$) were measured by ELISA. All results are shown as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ compared to WT challenged with Alt-Ext.

4 | Discussion

Genetic deletion of both GLP-1R and GIPR together in an aeroallergen-induced innate allergic lung inflammation model increased TSLP release into the airway. In the 4 consecutive days Alt-Ext intranasal challenge model, loss of both GLP-1R/GIPR signaling increased multiple parameters of inflammation including lung ILC2, eosinophils, lymphocytes, and neutrophils in the airway, expression of type 2 cytokines (IL-5 and IL-13) and chemokines (CCL11 and CCL24) in the lung, and ICAM-1 expression in the lung epithelial cells compared to the other mouse strains. In addition, GIPR KO mice and

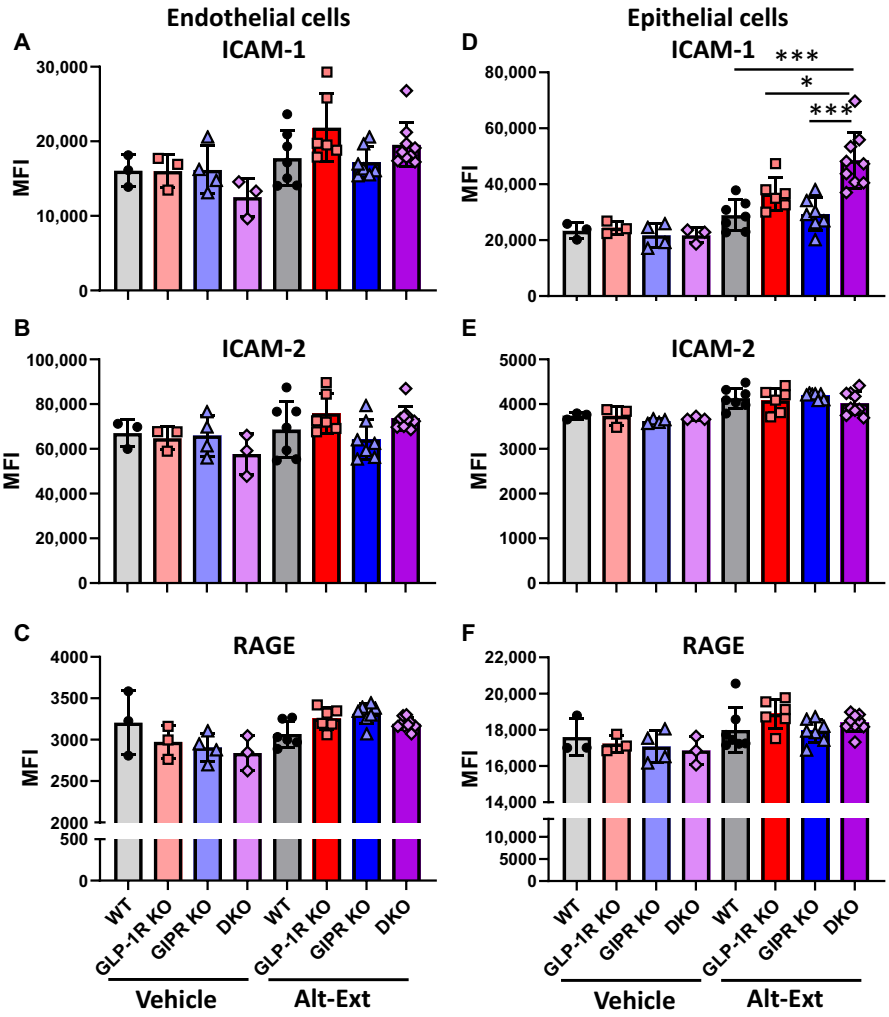

FIGURE 6 | Effects of GLP-1R, GIPR, or GLP-1R/GIPR deficiency on Alt-Ext-induced expressions of ICAM-1, ICAM-2, and RAGE in the lung endothelial (A–C) and epithelial cells (D–F). The MFI (Mean Fluorescence Intensity) was calculated in positive expression area of ICAM-1 (A, D), ICAM-2 (B, E), and RAGE (C, F) compared to fluorescence minus one (FMO) control ($n = 3–9$). The representative histograms are shown in Figure S3. The results are shown as mean ± SD. *$p < 0.05$, ***$p < 0.001$ compared to WT challenged with Alt-Ext.

GLP-1R/GIPR DKO mice had significantly increased Alt-Ext-induced HMGB1 in the BALF compared to WT and GLP-1R KO mice. These data identify that basal incretin signaling has a foundational role in the regulation of airway inflammation in response to allergen exposure and provide plausible mechanisms by which metabolic dysregulation impacts asthma pathobiology.

Alternaria alternata is an important aeroallergen [29]. This allergen has serine protease activity that leads to airway epithelial cell activation and the rapid release of IL-33 from these cells [30, 31]. Our previous studies reported that GLP-1R agonist treatment significantly decreased Alt-Ext-induced IL-33 release [16, 22]. Therefore, we tested the effect of Alt-Ext-challenge in our 4 strains of mice; however, there was no difference in Alt-Ext-induced IL-33 in BALF. In contrast, the protein level of TSLP was significantly increased by Alt-Ext-challenge in GLP-1R/GIPR DKO mice compared to the other mouse strains. Further, our flow data revealed that GLP-1R/GIPR DKO mice had a greater number of lung ILC2 compared to the other strains following Alt-Ext-challenge. Meanwhile, the number of GATA3$^+$ CD4 T cells as identified Th2 was not different between each of the 4 mouse strains after Alt-Ext-challenge. We have previously published that there is an approximate 10-fold increase in the number of IL-5 and IL-13-expressing ILC2 compared to IL-5 and IL-13-expressing CD4 T cells following 4 consecutive days of Alt-Ext-challenge [21, 22]. This reveals that this 4 consecutive days model of Alt-Ext-challenge is truly a model of innate allergic inflammation mediated by ILC2 activation that occurs before effector CD4 T cell proliferation. Furthermore, neutralization of TSLP significantly decreased Alt-Ext-induced type 2 inflammation in GLP-1R/GIPR DKO mice rather than in WT mice. This reveals that TSLP is a key initiator of the aeroallergen-induced innate allergic inflammation mediated by lung ILC2 activation, and both incretin receptor signaling are important to inhibit the TSLP production during aeroallergen exposure.

GLP-1R/GIPR DKO mice had an increased accumulation of airway eosinophils and protein levels of CCL11 and CCL24, both of which are eosinophil-associated chemokines, in response to Alt-Ext compared to the other mouse strains. While GLP-1R/GIPR DKO mice had an increase in the number of airway neutrophils

in response to Alt-Ext, the neutrophilic chemokine KC was not increased in GLP-1R/GIPR DKO mice compared to the other mouse strains. We found that GLP-1R/GIPR DKO mice had increased ICAM-1 expression on lung epithelial cells after Alt-Ext-challenge compared to the other strains. ICAM-1 is an adhesion molecule that promotes neutrophil migration to peripheral tissues [32, 33], and type I alveolar epithelial cells highly expressed ICAM-1 [34]. These previous reports and our finding suggest that loss of GLP-1R/GIPR that induced ICAM-1 on lung epithelial cells may promote increased migration of leukocytes including neutrophils into alveolar spaces.

Signaling through the incretin receptors GLP-1R and GIPR has a critical role in glycemic control which is mediated by increased GLP-1 and GIP driven insulin release after food intake [8, 9]. In our study, an oral GTT showed that GLP-1R/GIPR DKO mice exhibited glucose intolerance compared to WT, GLP-1R KO, and GIPR KO mice. This result is consistent with a previous report and reflects the established compensatory signaling of GLP-1R and GIPR KO [35]. Furthermore, previous studies reported that RAGE was expressed in lung endothelial/epithelial cells [36] and ILC2 [37]. Therefore, we hypothesized that GLP-1R/GIPR DKO mice have greater level of AGEs and the AGEs-RAGE signaling increases allergic inflammation as previous reports [38, 39]. However, there was no difference of AGEs in the serum and RAGE expression on lung epithelial and endothelial cells between each mouse strain with or without Alt-Ext-challenge.

High mobility group box 1 is a nonhistone nuclear protein that enhances inflammatory responses, acting as a DAMP [40] and RAGE is one of the main HMGB1 receptors [41]. Previous studies reported that the HMGB1 promoted allergic inflammatory immune responses [42]. In a mouse model, HMGB1 antagonist or antibody treatment significantly decreased OVA-induced allergic rhinitis or asthma [43–45]. Further, HMGB1 inhibitor treatment significantly decreased neutrophilic inflammation in mouse models using OVA-LPS or OVA-aspergillus protease sensitization and challenge [46, 47]. In addition, HMGB1 treatment increased ILC2 activation in an in vitro model [48, 49], and deficiency of HMGB1 or RAGE decreased hemorrhagic shock-induced ILC2 activation in mice in vivo model [37]. In our mouse model, GIPR KO mice and GLP-1R/GIPR DKO mice had significantly increased Alt-Ext-induced HMGB1 in BALF compared to WT and GLP-1R KO mice. Taken together, the increase of HMGB1 by GLP1R/GIPR double deficiency may augment allergic inflammation.

In this study, we found that tirzepatide treatment decreased Alt-Ext-induced eosinophilia and inflammatory marker IL-33 expression in GIPR KO mice; however, there was no reduction of Alt-Ext-induced type 2 inflammation by tirzepatide treatment in GLP-1R KO and GLP-1R/GIPR DKO mice. These findings suggest that tirzepatide might have limited effects through GIPR in mouse allergic inflammation models, consistent with previously reported reduced potency of tirzepatide at murine GIPR on mouse islets [50]. Our previous studies reported that GLP-1R agonist treatment decreased Alt-Ext-induced innate and adaptive allergic inflammation [16, 17]. The GLP-1R/GIPR dual agonist tirzepatide additively decreased Alt-Ext-induced IL-33 expression compared to GLP-1R agonist or GIPR agonist treatment [18]. However, there were no significant differences in the marker of type 2 inflammation between GLP-1R/GIPR dual agonist treatment and single GLP-1R or GIPR agonist treatment.

Alternatively, tirzepatide is known to be a weaker agonist at the mouse, vs. the human GIPR [50], perhaps contributing to attenuated anti-inflammatory activity in mice. Given the low-affinity of GIPR signaling of the dual GLP-1R/GIPR agonist at the murine GIPR, the anti-allergic inflammatory effects of the dual GLP-1R/GIPR agonist might be limited. In this study, we found that the deficiency of both endogenous GLP-1R and GIPR signaling significantly increased Alt-Ext-induced innate allergic inflammation, compared to deficiency of either receptor by themselves. Therefore, both GLP-1R and GIPR agonists that have high affinity to each receptor signaling may have synergistic effects for inhibition of allergic airway inflammation and this will remain to be studied until such agents are available. Our previous study showed that GLP-1R-mApple reporter protein as GLP-1R expression was detected in lung endothelial and epithelial cells [16]. However, most leukocytes, such as T cells, B cells, granulocytes, and ILCs did not express GLP-1R-mApple reporter protein [16]. Therefore, combined signaling of endogenous incretin hormone GLP-1 and GIP may stimulate lung endothelial or epithelial cells to reduce the release of TSLP and HMGB1, and epithelial expression of ICAM-1. As a consequence, type 2 inflammatory responses may be inhibited through reduction of ILC2 activation and leukocyte accumulation. Our findings in this study demonstrating enhanced expression of pro-inflammatory mediators in the mice lungs deficient in both incretin receptors raise the possibility that simultaneous activation of the GLP-1R and GIPR would be more beneficial than targeting either receptor alone for the attenuation of allergic inflammation in asthma. Clinical trials evaluating the effect of GLP-1R and dual GLP-1/GIPR agonists on airway inflammation in asthma are needed. Our results also suggest that a simultaneous loss of function mutation in both the GLP-1R and GIPR genes may be involved in asthma pathogenesis by increasing airway inflammation by the mechanisms such as TSLP induction that are outlined in this report. A future direction of this work may be to perform a phenome-wide association study (PheWAS) once such mutations in the GLP-1R, and GIPR genes are known to determine whether these mutations are associated with allergic inflammatory diseases and specifically asthma.

Author Contributions

Shinji Toki designed this study and performed experiments, analyzed data, and wrote the manuscript. Masako Abney and Jian Zhang performed experiments. Mark Rusznak and Christian M. Warren supported experiments. Dawn C. Newcomb and Katherine N. Cahill provided scientific advice and edited the manuscript. Daniel J. Drucker provided GLP-1R KO, GIPR KO mice, and scientific advice. Kevin D. Niswender and Ray Stokes Peebles Jr. supervised this study and edited the manuscript.

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Conflicts of Interest

Dr. Drucker has served as a consultant or speaker within the past 12 months to Altimmune, Amgen, AstraZeneca Inc. Boehringer Ingelheim, Kallyope, Merck Research Laboratories, Novo Nordisk Inc. and Pfizer Inc. Neither Dr. Drucker nor his family members hold issued stock directly or indirectly in any of these companies. Dr. Cahill has served as an advisory board member for AztraZeneca, Sanofi, Genentech, Novartis, and Regeneron, and as a consultant for Verantos and Clinical Key, and receives royalties from UpToDate, and research support from NovoNordisk. All other authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.