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Glucagon-like peptide 1 signaling inhibits allergen-induced lung IL-33 release and reduces group 2 innate lymphoid cell (ILC2) cytokine production in vivo

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Abstract

Background: IL-33 is one of the most consistently associated gene candidates for asthma identified by GWAS. Studies in mice and in human cells have confirmed the importance of IL-33 in inducing type-2 cytokine production from both group 2 innate lymphoid cells (ILC2) and Th2 cells. However, there are no pharmacologic agents known to inhibit IL-33 release from airway cells.

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Objective: To determine the effect of glucagon like peptide receptor-1 GLP-1R signaling on aeroallergen-induced airway IL-33 production and release and on innate type-2 airway inflammation.

Methods: BALB/c mice were challenged intranasally with *Alternaria* extract for 4 consecutive days. GLP-1R agonist or the vehicle was administered starting either 2 days before the first *Alternaria* extract-challenge or 1 day after the first *Alternaria* extract-challenge.

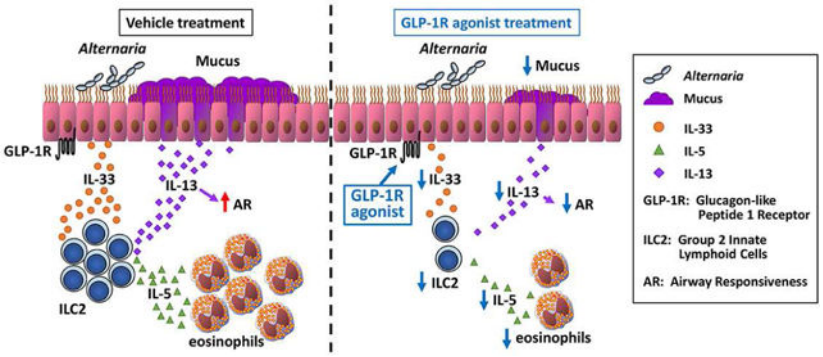
Results: GLP-1R agonist treatment starting 2 days before the first *Alternaria* extract-challenge decreased IL-33 release in the BAL fluid and DUOX1 mRNA expression 1 h after the first *Alternaria* extract-challenge, and IL-33 expression in lung epithelial cells 24 h after the last *Alternaria* extract-challenge. Further, GLP-1R agonist significantly decreased the number of ILC2 expressing IL-5 and IL-13, the lung protein expression of type-2 cytokines and chemokines, the number of perivascular eosinophils, mucus production, and airway responsiveness compared with vehicle treatment. GLP-1R agonist treatment starting one day after the first *Alternaria* extract-challenge also significantly decreased eosinophilia and type-2 cytokine and chemokine expression in the airway after 4 days of *Alternaria* extract-challenge.

Conclusion: These results reveal that GLP-1R signaling may be a potential therapy to reduce IL-33 release and inhibit the ILC2 response to protease-containing aeroallergens, such as *Alternaria*.

Capsule Summary

GLP-1R signaling inhibited IL-33 release and innate allergic airway inflammation, including eosinophilia, mucus hypersecretion and AR following *Alternaria* extract-challenge. The GLP-1R agonists may constitute a novel therapeutic approach for allergic asthma mediated by ILC2 activation.

Graphical Abstract



Keywords

Glucagon-like peptide-1 receptor (GLP-1R); Liraglutide; Group 2 innate lymphoid cells (ILC2); IL-33; *Alternaria*

Introduction

IL-33 is one of the most consistently associated gene candidates for asthma identified by genome wide association studies (GWAS) in diverse ethnic groups.[1–4] IL-33 is predominantly produced by epithelial cells in response to protease containing aeroallergens and its release is mediated by dual oxidase 1 (DUOX1).[5] IL-33 activates group 2 innate lymphoid cells (ILC2) to produce the type-2 cytokines IL-5 and IL-13 that initiate innate immunity-driven allergic responses.[6, 7] In addition, IL-33 polarizes naïve CD4 T cells to differentiate into effector T helper 2 (Th2) cells, which produce IL-4, IL-5, and IL-13 that are responsible for adaptive immunity-mediated allergen-induced responses.[8] Therefore, IL-33 is a central mediator of both innate and adaptive immunity regulated allergic inflammation in the lung that have a role in the pathogenesis of conditions such as asthma, and IL-33 has been deemed to be an important therapeutic target in inhibiting allergic diseases.[9] However, to our knowledge, there have been no reports identifying pharmacologic agents which inhibit lung IL-33 protein release or expression.

Glucagon-like peptide-1 (GLP-1) is a peptide hormone synthesized and released by enteroendocrine L-cells in the ileum and large intestine following oral food intake.[10] GLP-1 has a role in glycemic control by inducing glucose-dependent insulin secretion from β-cells and inhibiting glucagon release from α-cells in the pancreas.[11, 12] GLP-1 also induces weight loss by promoting satiety.[13] GLP-1 receptor (GLP-1R) agonists, such as liraglutide and exenatide are approved by the Food and Drug Administration (FDA) for treatment of type 2 diabetes (T2D).[14, 15] Several studies report that GLP-1R agonists had anti-inflammatory effects in multiple disorders including T2D. For instance, GLP-1R agonist administration decreased TNFα and IL-6 production by peripheral blood mononuclear cells (PBMC) of obese patients with T2D[16] and diabetic mouse adipose tissue.[17] In addition, the GLP-1R agonist, exendin-4, reduced serum inflammatory cytokines during LPS-induced endotoxemia,[18] liver inflammation, and aortic atherosclerosis in a rodent model.[19] These data indicate that GLP-1R agonists down-regulate innate inflammatory responses to endotoxins or endogenous inflammatory mediators. Further, it has been reported that liraglutide attenuated bleomycin-induced pulmonary fibrosis[20] and OVA-induced chronic airway inflammation.[21] These results suggest the possibility of novel therapeutic strategies for chronic lung diseases using GLP-1R agonists. However, no studies have reported the effect of GLP-1R agonists on lung IL-33 expression or release, or the effect of GLP-1R agonists on the innate allergic inflammatory response that is mediated by ILC2.

In this study, we performed airway challenge in mice with an extract of *Alternaria alternata*, an aeroallergen which has protease activity and which is associated with severe asthma exacerbations.[22, 23] GLP-1R agonist or the vehicle treatment was started either 2 days before the first *Alternaria* extract-challenge or 1 day after the first *Alternaria* extract-challenge. We found that treatment with the GLP-1R agonist, liraglutide, starting 2 days before the first *Alternaria* extract-challenge inhibited IL-33 expression and release in the lung or into the BAL fluid. Further, GLP-1R agonist treatment starting 2 days before the first *Alternaria* extract-challenge inhibited lung DUOX1 expression, providing a potential mechanism by which GLP-1R signaling inhibits lung IL-33 release in response to aeroallergen challenge. In addition, we found that GLP-1R agonist treatment starting 2 days before the first

Alternaria extract-challenge inhibited IL-5 and IL-13 production from lung ILC2, blunted airway mucus and responsiveness, and reduced lung eosinophilia. GLP-1R agonist treatment starting 1 day after the first *Alternaria* extract-challenge also reduced the eosinophilia and type-2 cytokines and chemokines in the airway. This report is the first to identify an FDA approved pharmacologic agent that inhibits lung IL-33 release, providing an alternative to biologic therapies that target IL-33-mediated diseases.

Methods

Mice

Nine- to twelve-week old female wild type (WT) BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, ME). IL-33$^{Citrine/+}$ reporter mice were generated by crossbreeding WT BALB/c mice and IL-33$^{Citrine/Citrine}$ (IL-33 KO) mice that are the kind gift of Dr. Andrew N.J. McKenzie.[24] Transgenic mice expressing mApple protein under the control of the GLP-1R promoter were generated as previously described.[25] Animal experiments were approved by the Institutional Animal Care and Use Committee at Vanderbilt University, 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.

GLP-1R agonist treatment and *Alternaria* extract- or house dust mite extract-challenge in a mouse model of allergic inflammation

In the protocol where GLP-1R agonist treatment was started 2 days before the first *Alternaria* extract-challenge (Fig 2, A), the GLP-1R agonist, liraglutide (Novo Nordisk Inc., Bagsvaerd, Denmark) or 50 μl 0.1 % BSA/PBS as vehicle was administered subcutaneously 4 h before and 4 h after *Alternaria* extract-, house dust mite (HDM) extract-(*Dermatophagoides pteronyssinus,* GREER, Lenoir, NC), or PBS-challenge from day −2 to day 3 (Fig 2, A, Fig 3, A, Fig E5, A and C). In the protocol where GLP-1R agonist was started 1 day after the first *Alternaria* extract-challenge, the vehicle was administered from day −2 to day 0, and then either GLP-1R agonist or the vehicle was administered from day 1 to day 3 (Fig 7, A). From day 0 to day 3, either 5 μg (protein amount) of *Alternaria* extract in 100 μl PBS, 25 μg (protein amount) of HDM extract in 100 μl PBS, or 100 μl of PBS as vehicle was used as an intranasal challenge to mice anesthetized with ketamine/xylazine. The mice were sacrificed and bronchoalveolar lavage (BAL) fluid and whole lungs were harvested 1 h after the first challenge of *Alternaria* extract or the vehicle (for IL-33 release), or 24h (for cytokine measurement) or 48 h (for histopathology and airways responsiveness measurement) after the last challenge of *Alternaria* extract, HDM extract, or PBS.

Flow cytometry

The harvested lungs were minced and digested with collagenase IV (1 mg/ml, SIGMA-Aldrich, St. Louis, MO) and DNase I (50 IU/ml, SIGMA-Aldrich) in RPMI1640 with 5% FBS for 45 minutes at 37 °C and a single cell suspension was obtained by grinding and passing the digested lung through a 70 μm cell strainer. The cells were stimulated with PMA (10 ng/ml), ionomycin (1 μM) and BD GolgiStop™ (BD Biosciences, San Jose, CA) at the manufacturer's recommended concentration for 5 hours. Then both cell surface and intracellular cytokine staining were performed. Antibodies used for flow cytometry are

shown in Table E1. All samples were measured on a BD LSR II Flow Cytometer and analyzed using FlowJo Software.

ELISA and LDH activity assay

DuoSet ELISA kits (CCL11, CCL17, CCL22, CCL24, IL-4, IL-9, and IL-33) from R&D Inc. (Minneapolis, MN) and Ready-Set-GO!® ELISA kits (IL-5 and IL-13) from Affymetrix eBioscience (San Diego, CA) were used to measure the concentration of cytokines and chemokines in BAL fluid and lung homogenates according to manufacturer's instructions. Cysteinyl Leukotriene ELISA kit and Prostaglandin D_2 ELISA Kit from Cayman chemical (Ann Arbor, MI) were used to measure cysteinyl leukotrienes (CysLTs), which include LTC_4, LTD_4, and LTE_4, and Prostaglandin D_2 (PGD_2) in BAL fluid. LDH cytotoxity assay kit (Thermo scientific, Waltham, MA) was used to evaluate *Alternaria* extract-induced cell damage associated with IL-33 release. Values of ELISA below the detection limit were assigned a value at half of the lower limit of detection to allow for statistical analysis.

Statistical analysis

All data were analyzed with GraphPad Prism 5 (GraphPad Software, La Jolla, CA). The p values were calculated by one-way analysis of variance (ANOVA) with Bonferroni-multiple pairs comparisons test. Values of $p < 0.05$ were considered significant between two groups.

Additional detail on the methods is available in an Online Repository

Results

GLP-1R expression on lung epithelial cells

We sought to examine if GLP-1R expression was present on lung epithelial cells to determine whether GLP-1 could directly modulate epithelial cell function. GLP-1R mRNA expression was detected in normal human lung as well as mouse lung (Fig 1, A and B). Further, GLP-1R protein expression was also detected on mouse lung epithelial cells and endothelial cells (Fig 1, C and D) using GLP-1R/mApple reporter mice. These results provide a rationale for investigating the effect of GLP-1R signaling in response to airway *Alternaria* extract-challenge.

GLP-1R agonist treatment starting 2 days before the first *Alternaria* extract-challenge suppresses *Alternaria* extract-induced IL-33 expression in lung epithelial cells

IL-33 is a key activator of the innate allergic airway response, and in particular IL-33 stimulates ILC2.[6] Therefore, we tested if GLP-1R agonist treatment starting 2 days before the first *Alternaria* extract-challenge decreases IL-33 protein expression following airway *Alternaria* extract-challenge for 4 consecutive days (Fig 2, A). To confirm the localization of IL-33 expression and the effect of GLP-1R agonist on a per cell IL-33 expression level, we used IL-$33^{Citrine/+}$ reporter mice. We found that *Alternaria* extract-challenge significantly increased mean fluorescence intensity (MFI) of citrine fluorescence as a marker of IL-33 expression by lung epithelial cells ($CD45^-$ $EpCAM^+$), and the percentage and the number of the $citrine^+$ epithelial cells. GLP-1R agonist treatment significantly decreased the *Alternaria* extract-induced MFI of citrine in the lung epithelial cells, the percentage and the

number of citrine^{+} epithelial cells compared with the vehicle treatment (Fig 2, B-E). These results indicate that GLP-1R agonist treatment reduced the number of lung epithelial cells expressing IL-33 and the level of IL-33 expression by individual cells following *Alternaria* extract-challenge.

GLP-1R agonist treatment starting 2 days before the first *Alternaria* extract-challenge suppresses *Alternaria* extract-induced IL-33 release, but not LDH activity in the BAL fluid

Airborne allergens, such as *Alternaria* have proteolytic activity which induces IL-33 release in BAL fluid 1 h after airway administration through PAR2 signaling.[26] We therefore measured IL-33 protein level in BAL fluid 1 h after first *Alternaria* extract-challenge following GLP-1R agonist treatment or vehicle treatment starting 2 days before the first *Alternaria* extract-challenge (Fig 3, A). *Alternaria* extract-challenge significantly increased the protein level of IL-33 in BAL fluid compared with PBS-challenged groups. The *Alternaria* extract-induced IL-33 in BAL fluid was significantly decreased by GLP-1R agonist treatment compared with the vehicle treatment (Fig 3, B). Next, we measured lactate dehydrogenase (LDH) activity in BAL fluid to assess the effect of GLP-1R agonist treatment for lung cell damage during IL-33 release. *Alternaria* extract-challenge significantly increased LDH activity in BAL fluid compared with PBS-challenged groups (Fig E1, B). Although the *Alternaria* extract-induced LDH activity was not decreased in GLP-1R agonist treatment group compared with vehicle treatment group (Fig E1, B), *Alternaria* extract-induced IL-33 protein level was significantly decreased in BAL fluid from the GLP-1R agonist treatment group compared with the vehicle treatment group (Fig E1, A). These results indicate that the effect of GLP-1R signaling on IL-33 release pathway is independent of *Alternaria* extract-induced lung cell damage.

A previous study reported that *Alternaria* extract directly and quickly induced mast cell degradation and activation deriving CysLTs and PGD_2.[27] Therefore, we measured CysLTs and PGD_2 in BAL fluid to assess an effect of GLP-1R agonist treatment for *Alternaria* extract-induced mast cell or the other CysLTs and PGD_2 productive cell activation. *Alternaria* extract-challenge significantly increased the CysLTs and PGD_2 in BAL fluid compared with PBS-challenged groups; however, GLP-1R agonist treatment did not decrease the *Alternaria* extract-induced CysLTs and PGD_2 compared with vehicle treatment (Fig E1, C and D). In addition, we measured GLP-1R protein expression on bone marrow-derived cultured mast cells (BMCMC) using GLP-1R/mApple reporter mice. The BMCMC gating strategy was shown in Fig E1, E. GLP-1R/mApple expression was not detected on the BMCMC (Fig E1, F), supporting that GLP-1R agonist does not have a direct effect on mast cell biology. Further, the induction of LDH activity, CysLTs and PGD_2 by *Alternaria* extract-challenge was observed in IL-33 KO mice as well as WT mice (Fig E1, B-D). Therefore, the *Alternaria* extract-induced cell damage and production of the lipid mediators are independent of biological effects of endogenous IL-33. Taken together, these results reveal that GLP-1R agonist treatment suppresses acute IL-33 release after *Alternaria* extract-challenge, but does not suppress lung cell damage and production of CysLTs and PGD_2 from lung resident cells such as mast cells.

GLP-1R agonist treatment starting 2 days before the first *Alternaria* extract-challenge suppresses DUOX1 mRNA expression in the *Alternaria* extract-challenged lung

DUOX1 is a critical mediator of IL-33 release from lung epithelial cells following aeroallergen challenge.[5] We therefore measured mRNA expression level of DUOX1in the lung 1 h after *Alternaria* extract-challenge, the time of peak IL-33 release, following GLP-1R agonist or vehicle treatment. Lung DUOX1 mRNA expression was significantly decreased by GLP-1R agonist treatment compared with vehicle treatment (Fig 3, C). This result suggests a possible mechanism by which GLP-1R agonist suppressed IL-33 release.

GLP-1R agonist treatment starting 2 days before the first *Alternaria* extract-challenge suppresses the number of mouse lung ILC2 expressing IL-5 and IL-13 in response to airway *Alternaria* extract-challenge.

Since IL-33 is an important stimulus for the innate allergic immune response, we sought to determine if GLP-1R signaling inhibited ILC2 function. First, we measured GLP-1R expression on lung ILC2 using GLP-1R/mApple reporter mice to reveal whether the GLP-1R agonist directly regulates the ILC2. The gating strategy for ILC2 was shown in Fig E1. ILC2 were identified as lineage (lin)$^-$CD3$^-$CD45$^+$CD25$^+$CD127$^+$ cells by cell surface staining. In contrast to epithelial cells and endothelial cells, GLP-1R/mApple expression was not detected on the lung ILC2 (Fig 4, A). Next, we enumerated the number of total, IL-5$^+$ and IL-13$^+$ lung ILC2 following *Alternaria* extract-challenge for 4 consecutive days to determine the effect of IL-33 reduction by GLP-1R agonist treatment. The gating strategy for ILC2 and representative flow cytometry dot plots of IL-5 and IL-13 intracellular staining are shown in Fig E2. ILC2 were identified as lineage (lin)$^-$CD3$^-$CD45$^+$CD25$^+$ and ICOS$^+$. *Alternaria* extract-challenge significantly increased the number of total, IL-5 $^+$ and IL-13$^+$ lung ILC2 compared with PBS-challenged mice (Fig 4, B-D). GLP-1R agonist treatment significantly suppressed the number of *Alternaria* extract-induced total, IL-5$^+$, and IL-13$^+$ lung ILC2 (Fig 4, B-D) compared with vehicle treatment.

To compare the contribution of CD4 T cells to IL-5 and IL-13 expression, intracellular cytokine staining was performed by flow cytometry. The gating strategy and dot plots of CD4 T cell expressing IL-5 and IL-13 are shown in Fig E3. The number of IL-5$^+$ and IL-13$^+$ CD4 T cell were significantly increased in *Alternaria* extract-challenged groups compared to PBS-challenged groups. GLP-1R agonist treatment significantly suppressed the number of *Alternaria* extract-induced CD4 T cells expressing IL-13, but not IL-5. In the *Alternaria* extract-challenged groups, there was a statistically significant 10-fold increase in the number of IL-5$^+$ ILC2 (Fig 4, C) compared to IL-5$^+$ CD4 T cells (Fig E3, C), and a 2.4-fold increase in the number of IL-13$^+$ ILC2 (Fig 4, D) compared to IL-13$^+$ CD4 T cells (Fig E3, D). The fold change between the number of ILC2 and CD4 T cells expressing IL-5 and IL-13 are similar to our previous report.[28] Therefore, these results indicate that ILC2, but not CD4 T cell, are the major source of IL-5 and IL-13 in response to this 4 consecutive day airway *Alternaria* extract-challenge protocol, and that GLP-1R signaling likely inhibits this early innate allergic immune response by the reduction of IL-33 release in the lung.

GLP-1R agonist treatment starting 2 days before the first *Alternaria* extract-challenge suppresses *Alternaria* extract-induced cytokine and chemokine expression in the lung and airway.

Next, we measured the protein level of type-2 cytokines and chemokines in BAL fluid and lung homogenate. *Alternaria* extract-challenge significantly increased the protein expression of IL-4, IL-5, IL-13, CCL11 (eotaxin), CCL17 (TARC), CCL22 (MDC), and CCL24 (eotaxin-2) in both the BAL fluid and lung homogenates compared with PBS-challenged groups. IL-9 was significantly increased in the lung homogenate, but not BAL fluid following *Alternaria* extract-challenge. We found a statistically significant decrease in *Alternaria* extract-induced protein expression of IL-5, IL-13, CCL17, CCL 22 and CCL24 in both the BAL fluid and lung homogenates from GLP-1R agonist treated mice compared with vehicle treated mice (Fig 5). In addition, *Alternaria* extract-induced IL-4, IL-9, and CCL11 protein expression was also decreased in the lung homogenates from GLP-1R agonist treated mice compared with vehicle treated mice.

GLP-1R agonist treatment starting 2 days before the first *Alternaria* extract-challenge suppresses eosinophilia, mucus production, and airway responsiveness in response to airway *Alternaria* extract-challenge.

Based on our finding that GLP-1R agonist treatment significantly decreased protein expression of *Alternaria* extract-induced IL-5, CCL11, and CCL24 that are associated with eosinophil recruitment, we hypothesized that GLP-1R agonist treatment decreases eosinophil accumulation in the lung following *Alternaria* extract-challenge. To test this hypothesis, we measured BAL cell counts and differentials, and assessed perivascular eosinophils by immunohistopathology. *Alternaria* extract-challenge significantly increased the number of total BAL cells, macrophages, eosinophils, lymphocytes and neutrophils compared with PBS-challenged groups. GLP-1R agonist treatment significantly decreased the number of *Alternaria* extract-induced total BAL cells, macrophages, eosinophils, and lymphocytes compared with vehicle treatment (Fig 6, A). Further, we found that GLP-1R agonist treatment resulted in a significant decrease of *Alternaria* extract-induced perivascular eosinophils in the lung compared with vehicle treatment by immunohistopathology (Fig 6, B and C).

IL-13 is a critical inducer of airway mucus production.[29] We found that GLP-1R agonist treatment decreased protein level of IL-13 in the lung. Therefore, we tested the hypothesis that GLP-1R agonist treatment suppresses mucus production in the airway following *Alternaria* extract-challenge. There was no mucus or mucous producing cells in the lungs of mice challenged with PBS. *Alternaria* extract-challenge significantly increased mucus production on large airway epithelial cells. GLP-1R agonist treatment significantly decreased the *Alternaria* extract-induced mucus production compared with vehicle treatment (Fig 6, D and E). These results indicate that GLP-1R agonist treatment suppresses acute eosinophilic lung inflammation and airway mucus production in innate allergic immune responses to *Alternaria* extract-challenge.

We found that GLP-1R agonist treatment significantly decreased asthma-like airway inflammation following *Alternaria* extract-challenge for 4 consecutive days compared

with vehicle treatment. We next tested the hypothesis that GLP-1R agonist treatment decreases airway responsiveness (AR) in *Alternaria* extract-challenged mice. *Alternaria* extract-challenge significantly increased AR compared with PBS challenge. GLP-1R agonist treatment significantly decreased the *Alternaria* extract-induced AR compared with vehicle treatment (Fig 6, F).

GLP-1R agonist treatment starting 2 days before the first HDM extract-challenge suppresses HDM extract-induced IL-5, IL-13 and IL-33 in the lung.

To assess the effects of GLP-1R agonist treatment in a different antigen-induced innate allergic inflammation model, we used HDM extract because HDM is one of common antigens associated with allergic asthma and atopic dermatitis.[30] First, we measured IL-33 protein level and LDH activity in BAL fluid 1 h after first HDM extract-challenge following GLP-1R agonist or vehicle treatment (Fig E5, A). However, IL-33 protein was not detected in either the BAL fluid from HDM extract-challenged groups or the PBS-challenged groups (Fig E5, B); and there was no difference in BAL fluid LDH activity between HDM extract- and PBS-challenged groups (Fig E5, B). These results indicate that HDM extract has no cytolytic activity to induce IL-33 release. Meanwhile, 4 consecutive days of HDM extract-challenge increased protein level of IL-5, IL-13 and IL-33 in the lung homogenates 24 h after the last HDM extract-challenge compared to the PBS-challenge (Fig E5, D). The HDM extract-induced IL-5, IL-13 and IL-33 were significantly decreased in GLP-1R agonist treatment group compared with vehicle treatment group (Fig E5, D). This result revealed that GLP-1R agonist treatment decreased type-2 cytokine and IL-33 expression caused by HDM extract-induced innate inflammation.

GLP-1R agonist treatment starting 1 day after the first *Alternaria* extract-challenge as well as treatment starting 2 days before the first *Alternaria* extract-challenge suppresses *Alternaria* extract-induced eosinophilia, and type-2 cytokine and chemokine expression in the airway.

To determine the effect of GLP-1R agonist treatment as a potential therapeutic use, we compared the effect of GLP-1R agonist treatment starting 1 day after the first *Alternaria* extract-challenge against treatment starting 2 days before the first *Alternaria* extract-challenge in *Alternaria* extract-induced innate allergic inflammation (Fig 7, A). GLP-1R agonist treatment starting 1 day after the first *Alternaria* extract-challenge significantly decreased the number of *Alternaria* extract-induced total BAL cells, eosinophils, and lymphocytes compared with vehicle treatment similar to GLP-1R agonist treatment starting 2 days before the first *Alternaria* extract-challenge (Fig 7, B). *Alternaria* extract-induced IL-5, CCL11, CCL17, CCL22, and CCL24 in both the BAL fluid and lung homogenates were significantly decreased GLP-1R agonist treatment in both protocols compared with vehicle treatment (Fig 7, C and D). *Alternaria* extract-induced IL-13 in the BAL fluid and IL-9 in the lung homogenates were also significantly decreased in GLP-1R agonist treatment group starting 1 day after the first *Alternaria* extract-challenge compared with vehicle treatment (Fig 7, C and D). These results suggest that GLP-1R agonist treatment after initial antigen challenge also suppresses *Alternaria* extract-induced airway eosinophilia and type-2 cytokine and chemokine expression in the airway and lung.

Discussion

Our results show that a GLP-1R agonist inhibited lung epithelial expression and airway release of IL-33 in response to the clinically relevant, protease-containing, ubiquitous aeroallergen, *Alternaria alternata*. This is the first report of an FDA approved pharmacologic agent inhibiting allergen-induced lung IL-33 expression and release, and this finding has significant implications as it may provide an alternative to biologic therapies such as monoclonal antibodies or receptor antagonists that target IL-33-mediated diseases. In addition, DUOX1 mRNA was also decreased by GLP-1R agonist treatment during IL-33 release following *Alternaria* extract-challenge. Further, we found that GLP-1R agonist treatment 2 days before the first *Alternaria* extract-challenge significantly decreased the number of lung ILC2 expressing IL-5 and IL-13 following 4 consecutive days of *Alternaria* extract-challenge. GLP-1R agonist treatment 2 days before the first *Alternaria* extract-challenge significantly decreased IL-4, IL-5, IL-9, IL-13, CCL11, CCL17, CCL22 and CCL24 protein expression in the lung homogenates, the number of eosinophils in the BAL fluid and perivascular eosinophil accumulation, mucus production, and AR induced by the *Alternaria* extract-challenge. Likewise, GLP-1R agonist starting 1 day after the first *Alternaria* extract-challenge significantly decreased eosinophilia and IL-5, IL-13, CCL11, CCL17, CCL22 and CCL24 in the airway. These results suggest that GLP-1R signaling not only has an effect on the initial allergen-induced IL-33 release in the airway, but also inhibits established airway inflammation.

GLP-1R is abundantly expressed in human and mouse lung, heart, brain and kidney as well as pancreas,[31, 32] and many studies indicate the therapeutic effect of GLP-1R signaling for diseases in multiple tissues. For instance, a GLP-1R agonist, exendin-4, improved atherosclerosis by reducing macrophage adhesion to endothelial cells as well as by reducing serum glucose level.[32] GLP-1 peptide treatment decreased irradiation- or LPS-induced proinflammatory cytokine expression in the brain and astrocytes in a mouse model.[33, 34] In addition, GLP-1R agonist decreased chronic lung inflammation in a mouse model of OVA sensitization combined with 66 days of OVA challenge,[21] and reduced mortality and airway resistance in a mouse model of obstructive lung disease induced by OVA and LPS challenge.[35]

Binding of GLP-1 to its G protein coupled receptor causes adenylate cyclase activation resulting in the formation of cyclic adenosine monophosphate (cAMP). Subsequent activation of protein kinase A (PKA) and the cAMP-regulated guanine nucleotide exchange factor II (cAMP-GEFII) leads to an elevation of intracellular calcium concentrations, and enhanced exocytosis of insulin-containing granules in pancreatic β-cells.[36] Further, β2 adrenergic receptor signaling also activate the cAMP/PKA signaling pathway, and then relaxed airway smooth muscle and reduced airway mucus secretion.[37] Our group reported that prostaglandin I_2 (PGI_2) receptor signaling down-regulated DC, T cell, and ILC2 activation mediated by cAMP induction.[38–40] In addition, GLP-1R agonist reduced inflammatory responses in mouse peritoneal macrophages stimulated with LPS through cAMP signaling pathway.[41] These results suggest that cAMP induction by GLP-1R agonist as well as β2 adrenergic agonist or PGI_2 may have suppressive effects on the activation of immune cells that express GLP-1R.

In this study, we found that a GLP-1R agonist treatment down-regulated *Alternaria* extract-induced IL-33 release and expression. IL-33 plays important roles in type-2 innate immunity following helminth infection or after exposure to protease containing aeroallergens.[26, 42] Biologically active full length IL-33 is released into the extracellular space after cell damage such as necrosis, and then IL-33 activates many types of immune cells, including CD4 T cells, mast cells, basophils, macrophages, DCs, and epithelial cells as well as ILC2.[43, 44] IL-33 is likely to be a first alarm signal due to its constitutive expression in normal epithelial and endothelial cells,[44] and is ready to be released following infection or mechanical cell damage. We found that GLP-1R agonist treatment starting 2 days before the first *Alternaria* extract-challenge reduced IL-33 release and DUOX1 mRNA expression, but not LDH activity after the first *Alternaria* extract-challenge. These results suggest that GLP-1R signaling down-regulates the IL-33 release pathway in lung epithelial cells expressing IL-33 without suppression of the cell damage. Hristova and colleagues reported that *Alternaria* induced IL-33 release in cultured primary human bronchial epithelial cells (HBE) depended critically on DUOX1-mediated activation.[5] Shiraki and colleagues reported that GLP-1R signaling inhibited NADPH oxidase activation in primary human endothelial cells.[45] Taken together, our findings suggest that GLP-1R signaling decreases DUOX1 expression in the lung epithelial cells, thus inhibiting *Alternaria*-induced IL-33 secretion.

The reduction in IL-33 protein level to airway *Alternaria* extract-challenge mediated by the GLP-1R agonist is one possible mechanism by which GLP-1R signaling reduced the number of IL-5 and IL-13 expressing ILC2 and protein levels of CCL11, CCL24, TARC, and MDC in the airway and lung following 4 consecutive days *Alternaria* extract-challenge. These findings are consistent with previous reports that IL-33 induced IL-5 and IL-13 expression by ILC2 in dose-dependent fashion,[46] CCL11 expression by fibroblast,[47] and TARC expression by DCs.[48] Although GLP-1R signaling reduced the number of IL-5 and IL-13 expressing ILC2 following 4 consecutive days *Alternaria* extract-challenge, the percentage of IL-5 and IL-13 expressing ILC2 was not decreased. Intracellular cytokine staining is appropriate to determine the cells that have ability to express IL-5 and IL-13; however, cell activation by such a strong stimulus as PMA and ionomycin might prevent the evaluation of the precise quantitative cytokine expression level in individual cells.

Meanwhile, we found that GLP-1R agonist treatment starting 2 days before the first *Alternaria* extract-challenge significantly decreased IL-5, IL-13, and IL-33 expression in the lung after 4 consecutive days of HDM extract-challenge. This HDM extract-induced innate inflammation was induced without IL-33 release. Further, GLP-1R agonist treatment starting 1 day after the first *Alternaria* extract-challenge also significantly decreased eosinophilia, and type-2 cytokine and chemokine expression in the airway. These in vivo results suggest a mechanism for downstream of IL-33 release by which GLP-1R signaling down-regulates innate allergic inflammation. However, we could not detect GLP-1R protein expression on ILC2 in the lung and BMCMC from naïve GLP-1R/mApple reporter mice, raising a concern about the specificity of the anti-GLP-1R antibody that has been used in immunohistochemistry and flow cytometry. Previous studies reported that GLP-1R was expressed in mouse peritoneal macrophages, the human monocyte cell line THP-1,[41] and the human invariant natural killer T (iNKT) cell line.[49] Since GLP-1R protein expression

level on immune cells is not fully known, further experiments are necessary to determine the mechanisms of the anti-inflammatory effect of GLP-1R signaling.

In our 4 consecutive days of *Alternaria* extract-challenge model, the number of IL-5 and IL-13 expressing CD4 T cells was statistically significantly less than the number of IL-5 and IL-13 expressing ILC2, and our previous report showed that total IgE and antigen specific IgG1 in serum were no different between naïve mice and mice challenged with *Alternaria* extract for 4 consecutive days.[40] This supports the concept that the innate immune system, and not adaptive immunity, initiate and promotes type-2 allergic airway inflammation in this mouse model of allergen challenge. Taken together, our findings reveal that GLP-1R signaling suppresses aeroallergen-driven IL-33 release and inhibits ILC2-dependent innate type-2 airway inflammation. Currently, biological agents are in development to antagonize IL-33 for the treatment of atopic dermatitis, food allergy, and asthma.[9] Our results suggest that a currently FDA approved GLP-1R antagonist inhibits allergen-induced IL-33 release and expression.

In conclusion, we found that GLP-1R agonist treatment down-regulated IL-33 release and expression, and innate allergic airway inflammation, including lung eosinophilia, mucus hypersecretion and AR following 4 consecutive days of *Alternaria* extract-challenge. Our results suggest that GLP-1R agonists may constitute a novel therapeutic approach for allergic asthma induced by protease-containing aeroallergens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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Abbreviations

ILC2	group 2 innate lymphoid cells
GLP-1	glucagon-like peptide-1
GLP-1R	glucagon-like peptide-1receptor
Th2	T helper 2
FDA	Food and Drug Administration
T2D	type 2 diabetes
WT	wild type
HDM	house dust mite

BAL	bronchoalveolar lavage
LDH	lactate dehydrogenase
BMCMC	bone marrow-derived cultured mast cells
DUOX1	dual oxidase 1
MFI	mean fluorescence intensity
AR	airway responsiveness
cAMP	cyclic adenosine monophosphate
PKA	protein kinase A
HBE	human bronchial epithelial cells

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Key message

- GLP-1R agonist inhibits IL-33 release and expression in response to aeroallergen challenge
- GLP-1R signaling inhibited aeroallergen-induced ILC2 cytokine production, eosinophilic inflammation, mucus metaplasia, and airway responsiveness.
- A current FDA approved pharmacologic agent, GLP-1R agonist may be a novel therapeutic approach for allergic airway diseases through a blocking IL-33 release into the airway and an inhibition of type-2 inflammation.

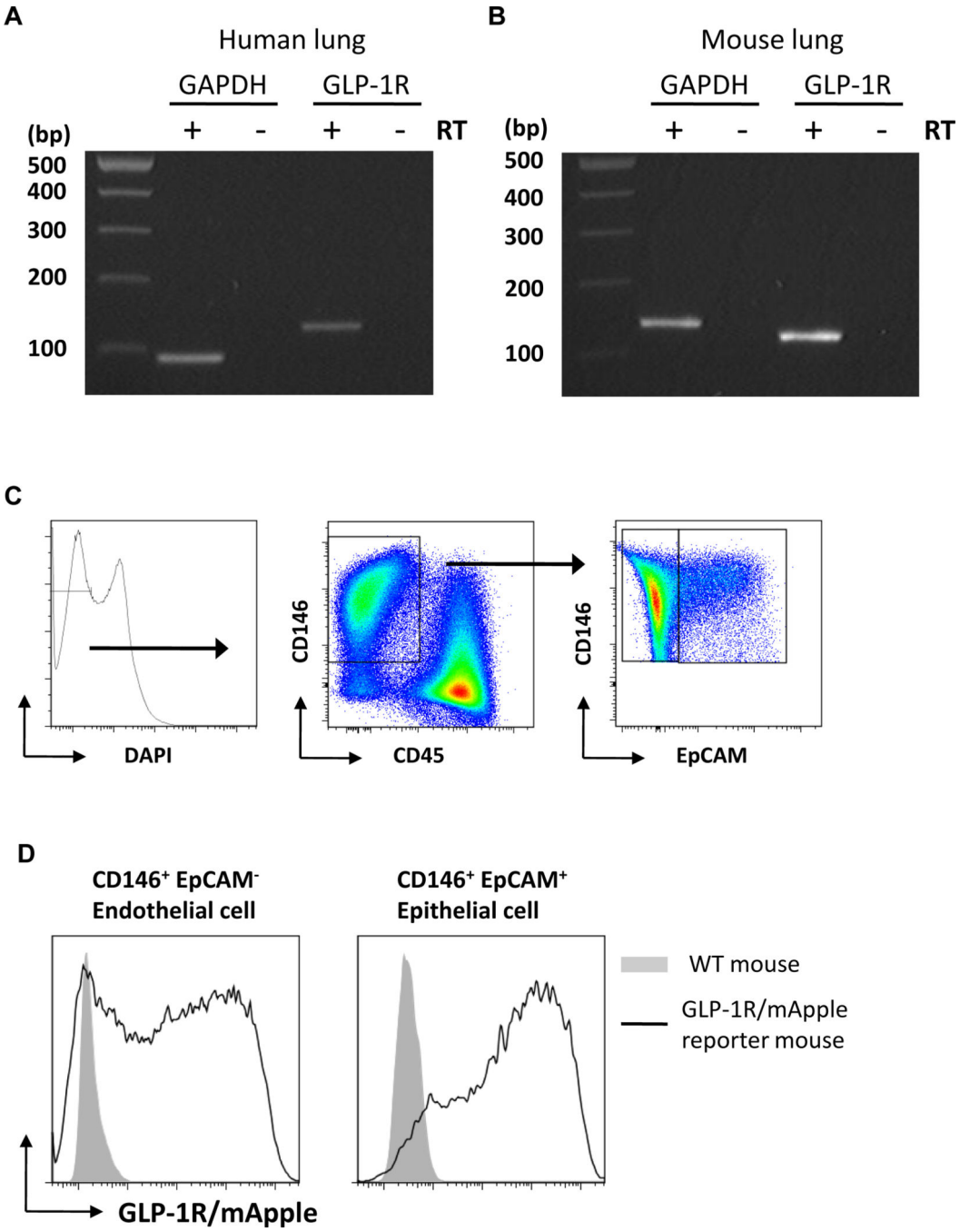


FIG 1.

A and B, RT-PCR of GLP-1R in human lung and mouse lung. Results are representative of 3 different samples. RT= reverse transcriptase. **C,** The gating strategy of epithelial cells and endothelial cells. **D,** Representative histograms of mApple (GLP-1R) expression on $CD45^{-}$ $CD146^{+}EpCAM^{-}$ endothelial cells and $CD45^{-}CD146^{+}EpCAM^{+}$ epithelial cells in the lungs from WT mice (filled gray area) or GLP-1R/mApple reporter mice (black line). Results are representative of 3 different mice.

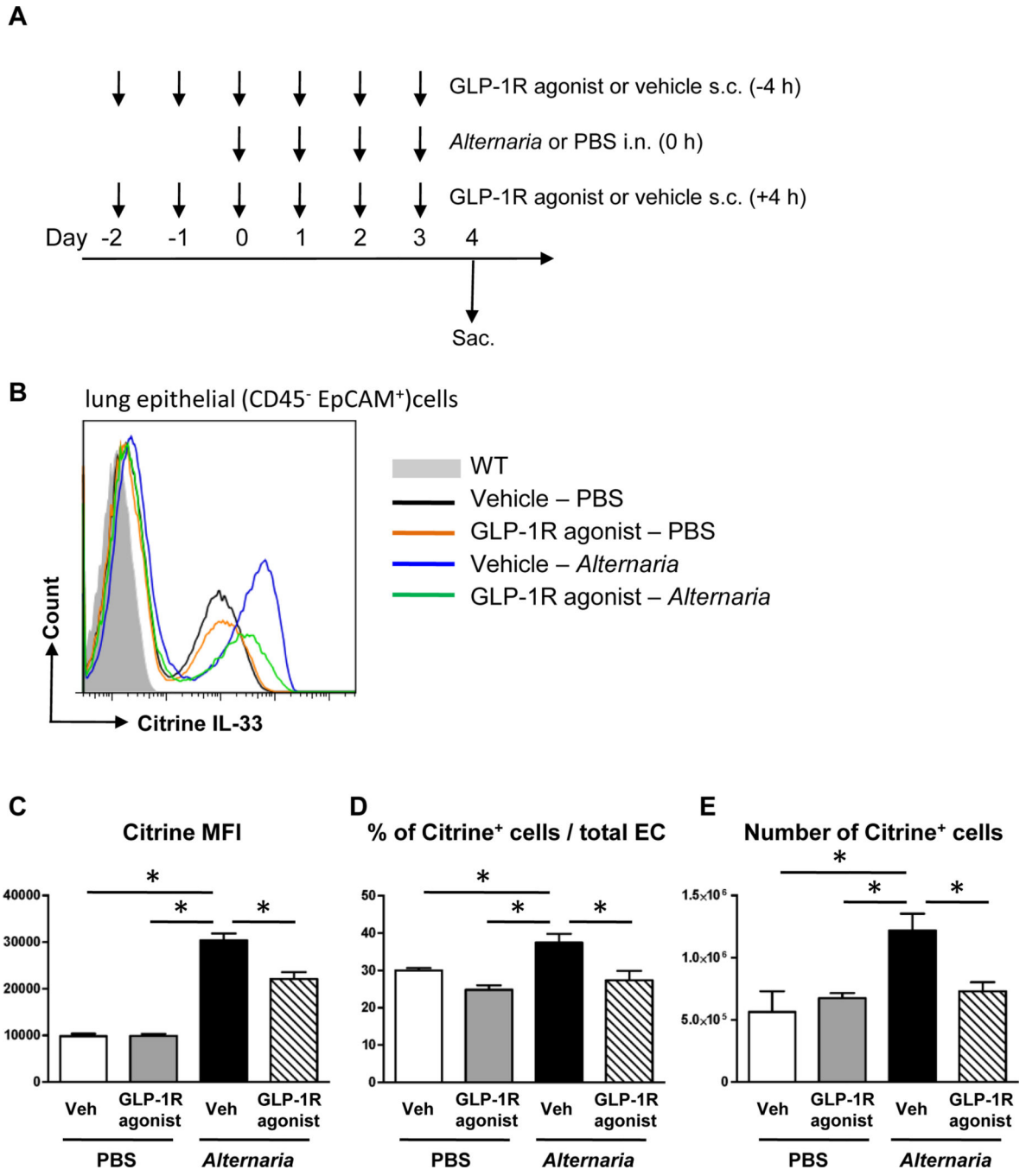


FIG 2.

A, Mice were challenged with *Alternaria* extract intranasally for 4 consecutive days. GLP-1R agonist or its vehicle was administered subcutaneously on day −2 and −1, and then every 4 h before and after *Alternaria* extract-challenge on day 0–3. The dose of GLP-1R agonist was 0.05 mg/kg on day −2, 0.1 mg/kg on day −1, and 0.2 mg/kg on day 0 −3. **B,** Representative histograms of citrine (IL-33) expression on $CD45^{-}EpCAM^{+}$ cells in the lungs from WT mice (filled gray area) or $IL\text{-}33^{Citrine/+}$ reporter mice challenged with vehicle-PBS (black line), GLP-1R agonist-PBS (orange line), vehicle-*Alternaria* extract

(blue line), and GLP-1R agonist-*Alternaria* extract (green line). **C-E,** The MFI, the percentage, and the number of Citrine^{+}CD45^{-}EpCAM^{+} cells in the lung. The results are representative of 2 independent experiments, and shown as mean ± S.E.M. of 3 mice in PBS-challenged groups and 5 mice in *Alternaria* extract-challenged groups. Veh=vehicle. PBS=phosphate buffered saline. EC=epithelial cells * $P< 0.05$

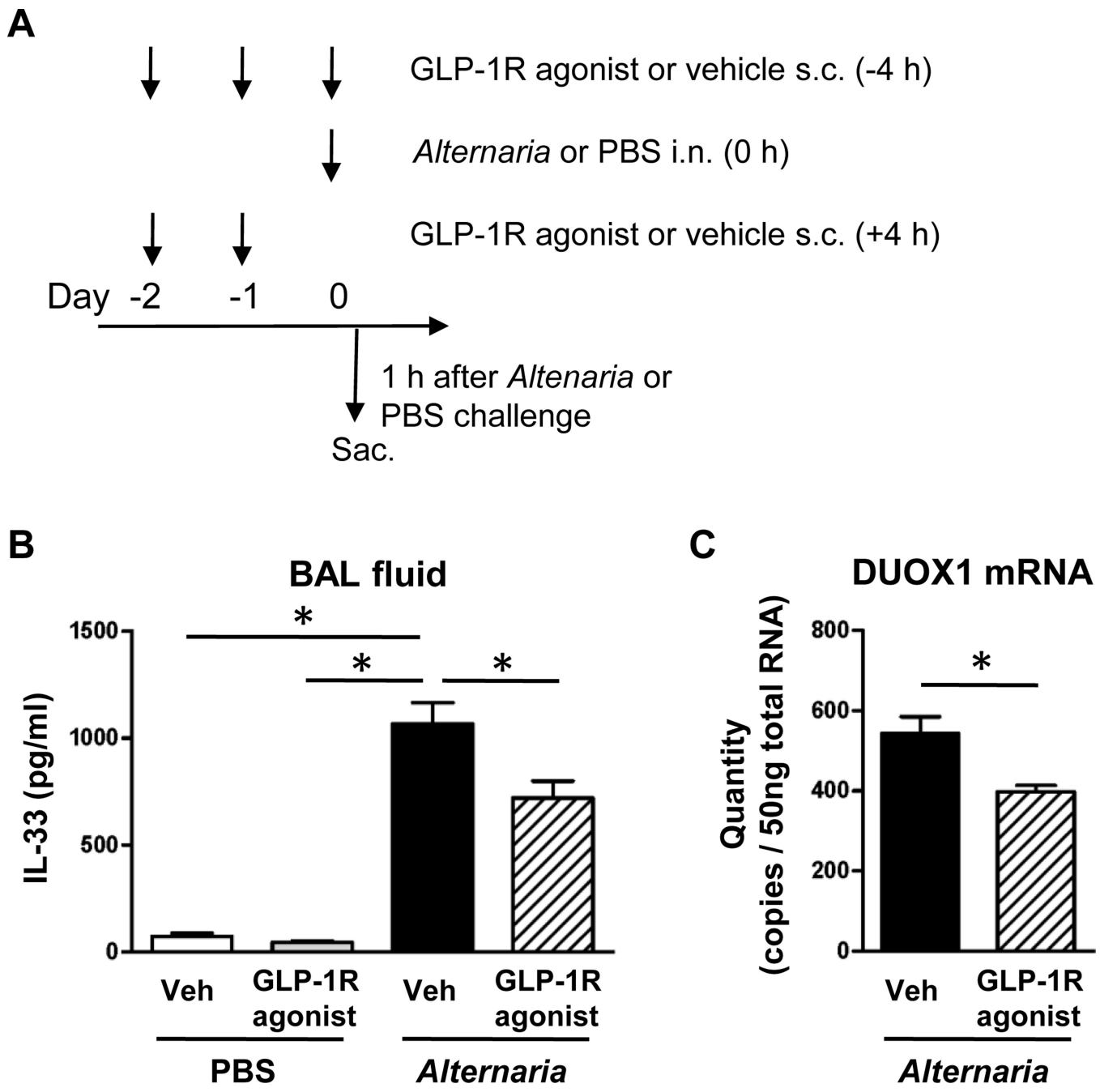


FIG 3.

A, GLP-1R agonist or its vehicle was administered subcutaneously on day −2 and −1, and then 4 h before *Alternaria* extract-challenge on day 0. The dose of GLP-1R agonist was 0.05 mg/kg on day −2, 0.1 mg/kg on day −1, and 0.2 mg/kg on day 0. The BAL fluid was harvested 1 h after the *Alternaria* extract-challenge on day 0. **B,** The protein level of IL-33 in the BAL fluid was measured by ELISA. n=4 for PBS-challenged groups, and n=8 for *Alternaria* extract-challenged groups. **C,** Quantity of DUOX1 mRNA expression in the lung was measured by real-time PCR. n=5 for each groups. The all results are combined

with 2 independent experiments, and shown as mean ± S.E.M. Veh=vehicle. PBS=phosphate buffered saline. * $P < 0.05$

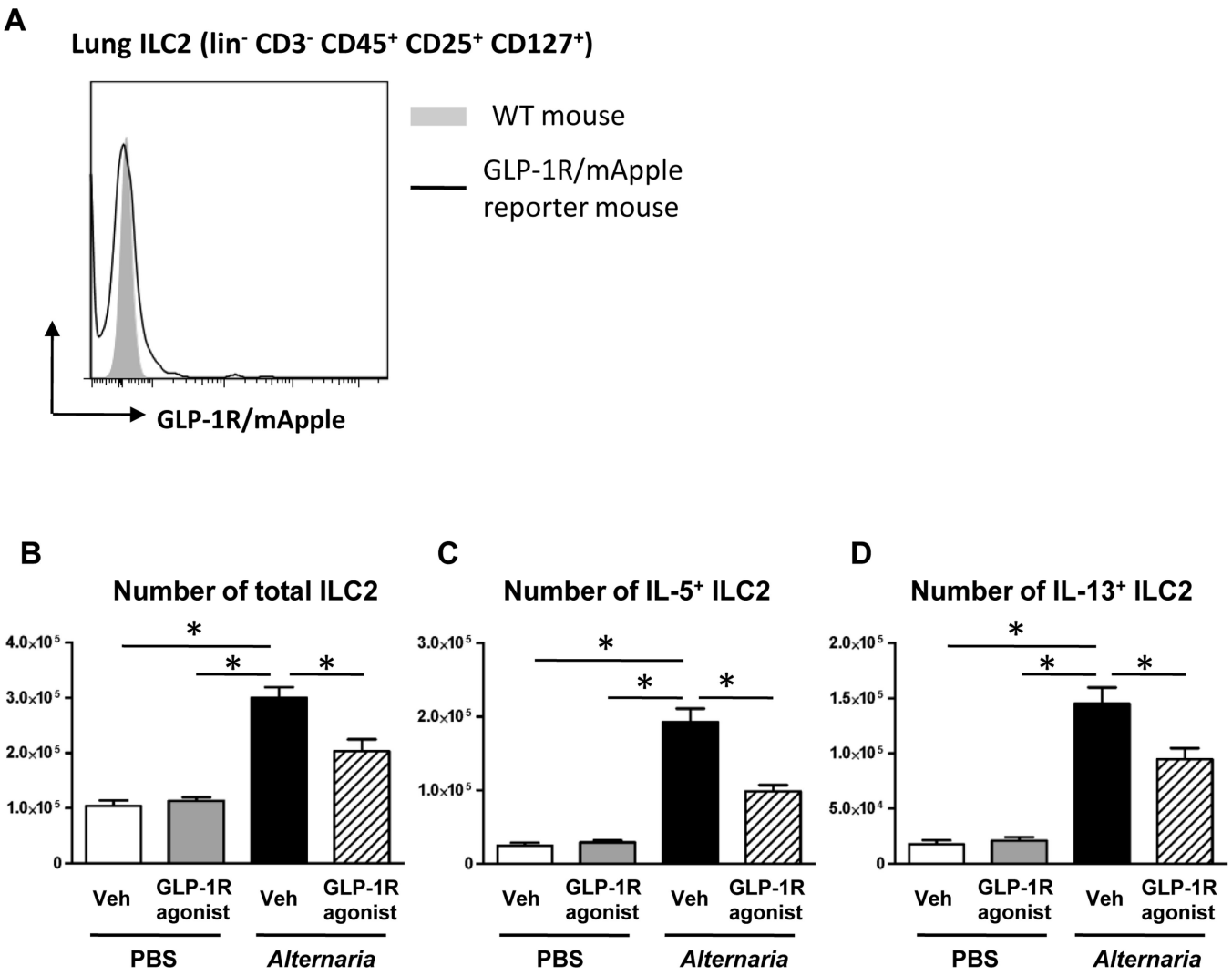


FIG 4.

A, Representative histograms of mApple (GLP-1R) expression on lung ILC2 from WT mice (filled gray area) or GLP-1R/mApple reporter mice (black line). Result is a representative of 3 different mice. **B-D,** Flow cytometric analysis of the number of total, IL-5$^+$, and IL-13$^+$ ILC2 in the lung. The results are combined with 2 independent experiments, and shown as mean ± S.E.M. of 4 mice in PBS-challenged groups and 8 mice in *Alternaria* extract-challenged groups. Veh=vehicle. PBS=phosphate buffered saline. * $P< 0.05$

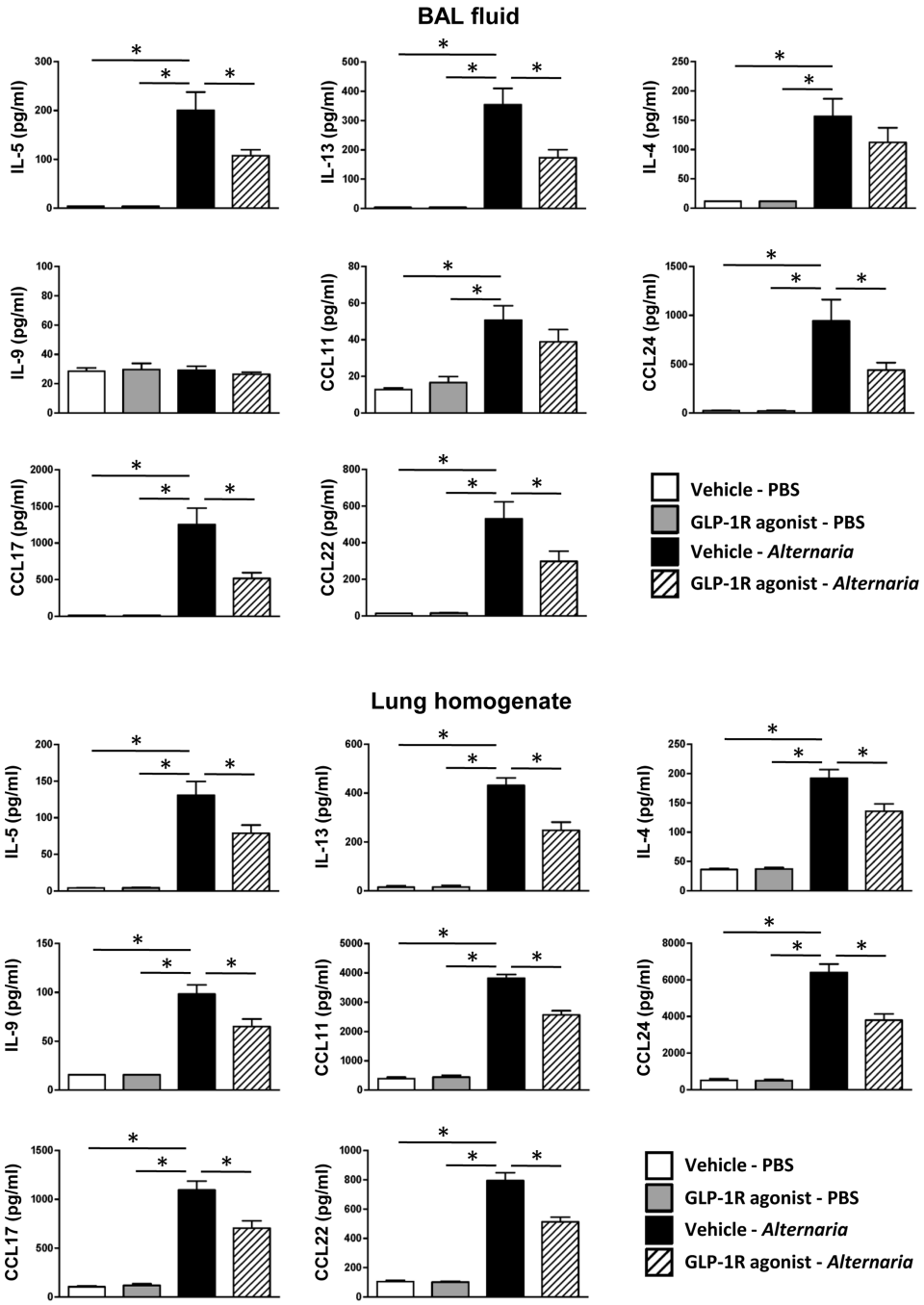
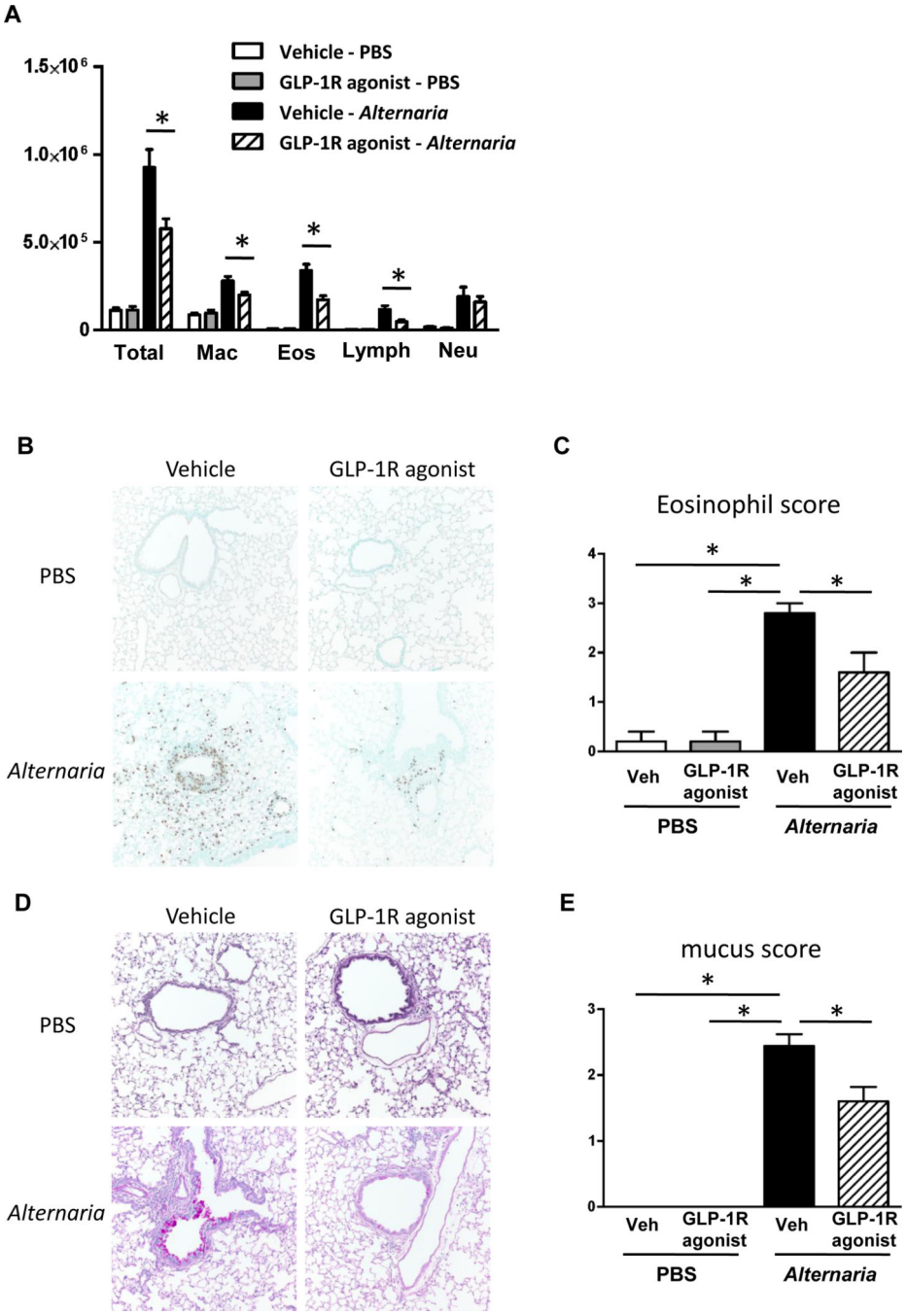


FIG 5.

BAL fluid and lungs were harvested 24 h after the last *Alternaria* extract-challenge to measure the protein expression of IL-4, IL-5, IL-9, IL-13, CCL11, CCL17, CCL22, and CCL24 by ELISA. The results are combined with 2 independent experiments, and shown as mean ± S.E.M. of 6 mice in PBS-challenged groups and 11 mice in *Alternaria* extract-challenged groups. Veh=vehicle. PBS=phosphate buffered saline. * $P< 0.05$



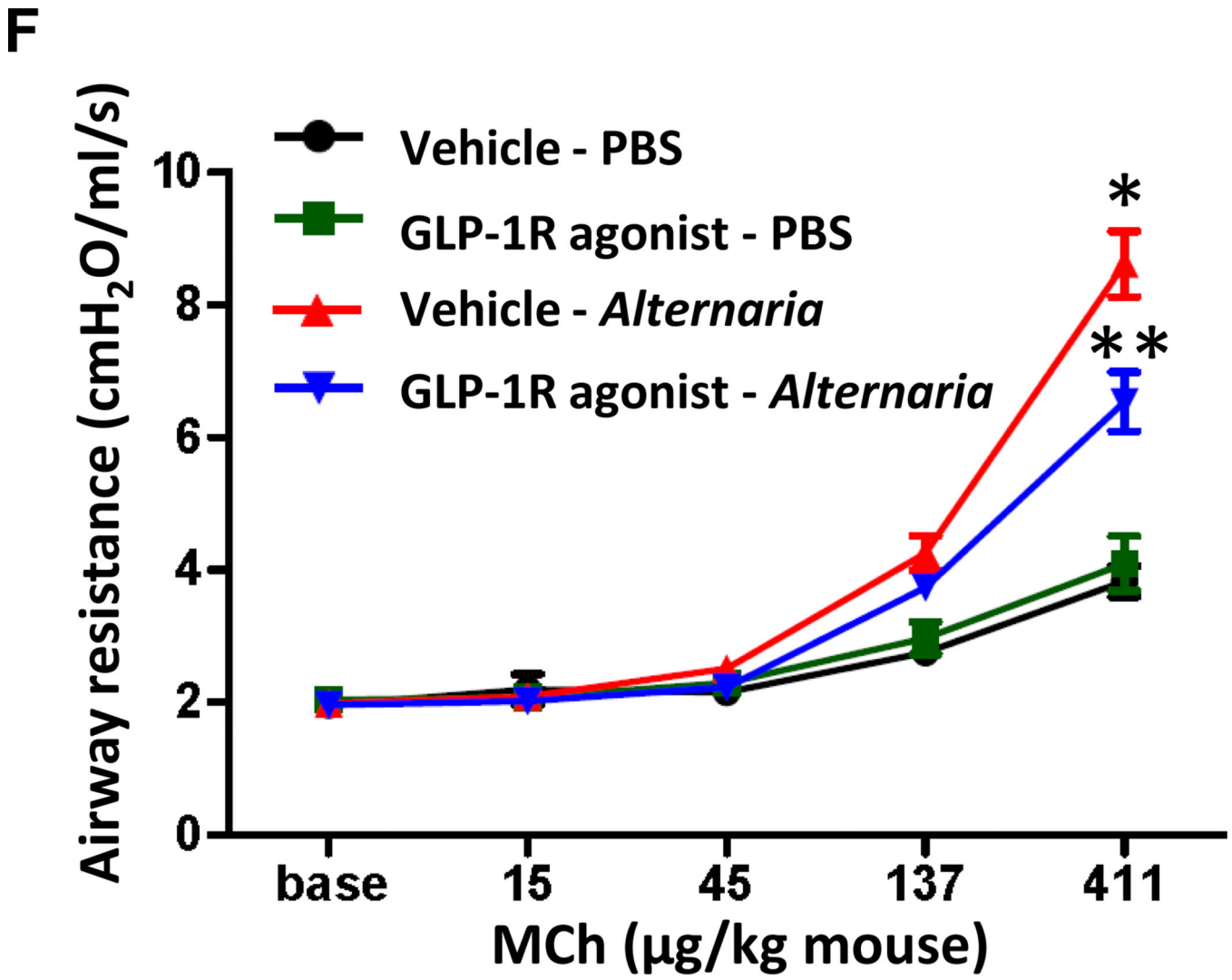
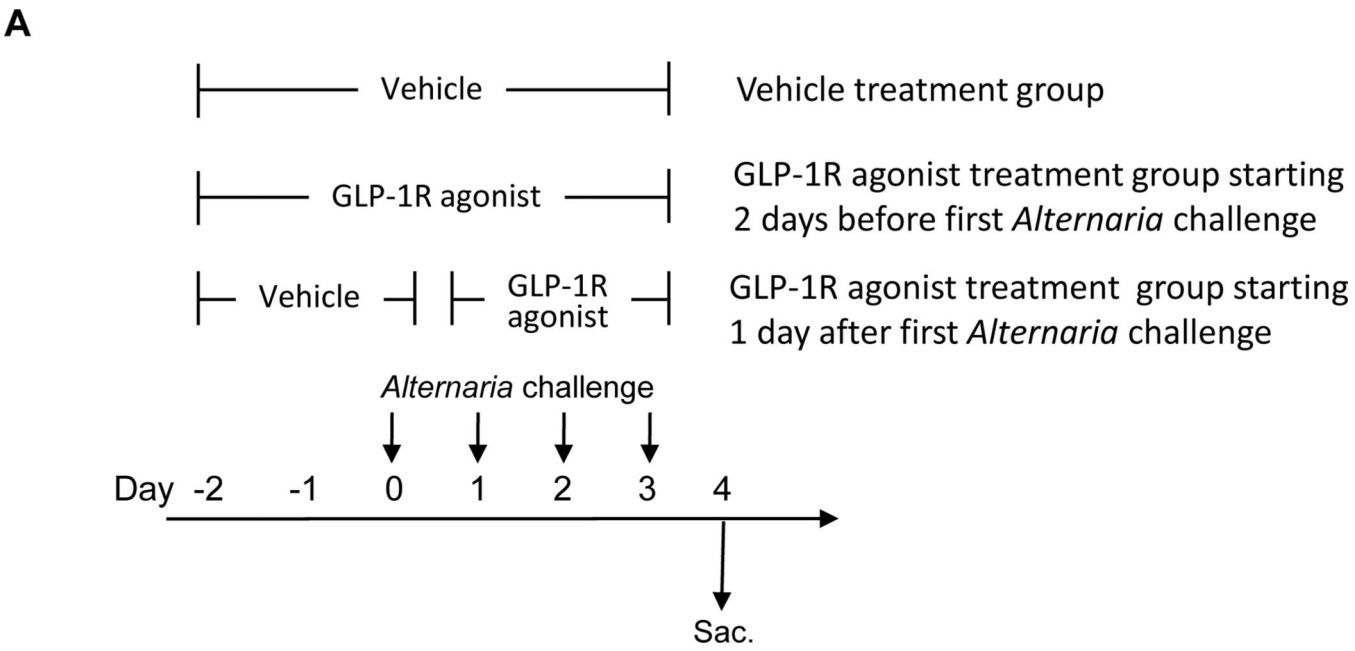
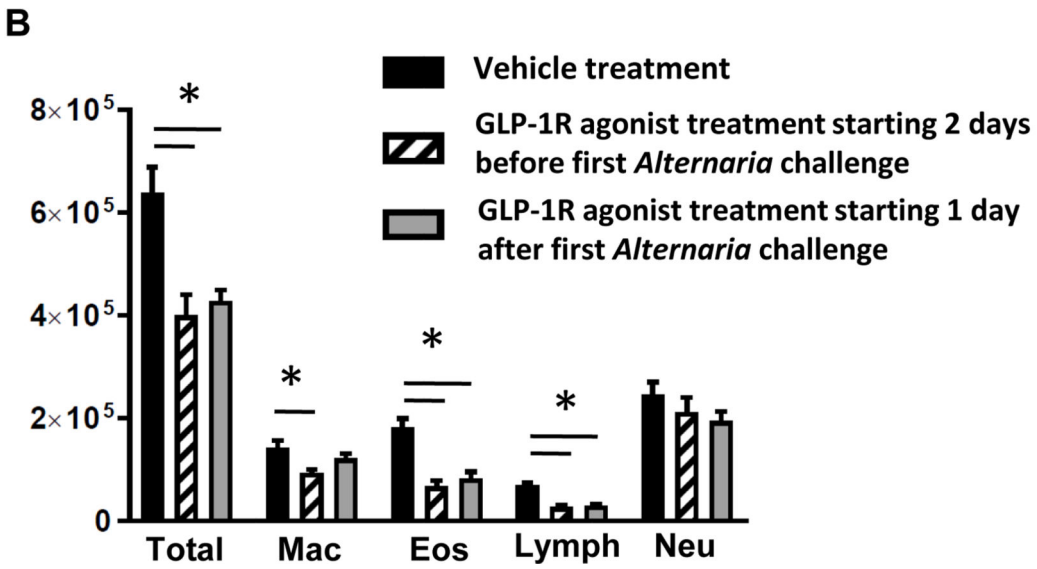


FIG 6.

A, Cell differentials in BAL fluid harvested 24 h after the last *Alternaria* extract-challenge. n=6 for PBS-challenged groups, and n=11 for *Alternaria* extract-challenged groups. **B,** Representative sections and **C,** eosinophil score as determined by anti-MBP antibody staining. n=5 for each group. **D,** Representative sections and **E,** mucus score as determined by PAS staining. n=4 for PBS-challenged groups, and n=10 for *Alternaria* extract-challenged groups. The lungs for histological analysis were harvested 48 h after the last *Alternaria* extract-challenge. **F,** Airway resistance to increasing dose of methacholine challenge. n=7 for PBS-challenged groups, and n=16 for *Alternaria* extract-challenged groups (* $P< 0.05$ compared with vehicle-PBS. ** $P< 0.05$ compared with vehicle-*Alternaria*). The all results are combined with 2 independent experiments, and shown as mean ± S.E.M. Veh=vehicle. PBS=phosphate buffered saline. Mch= methacholine. * $P< 0.05$





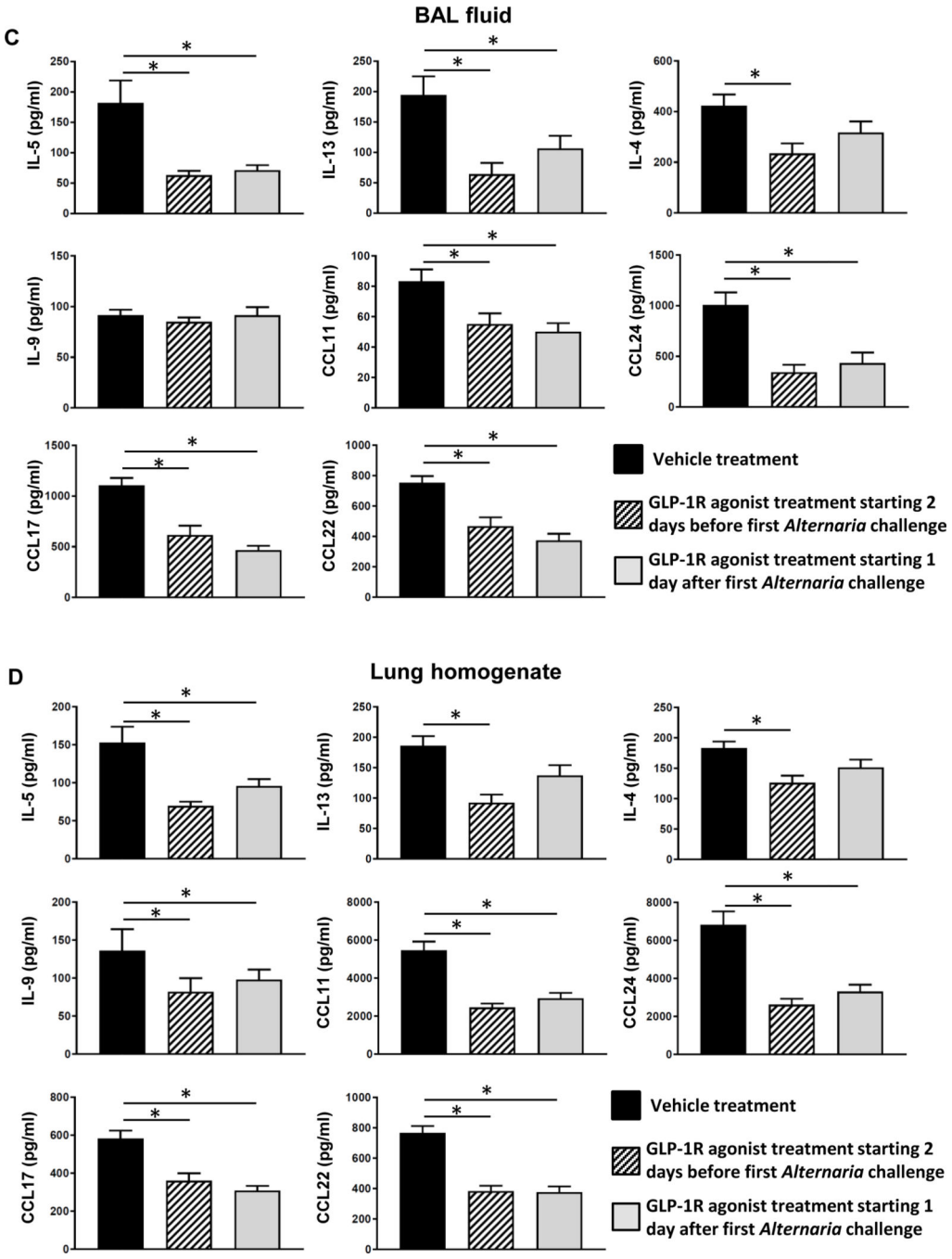


FIG 7.

A, Mice were challenged with *Alternaria* extract intranasally for 4 consecutive days (from day 0 to day 3). The mice of vehicle treatment group and GLP-1R agonist treatment starting 2 days before the first *Alternaria* challenge group were treated with vehicle or GLP-1R agonist respectively from day −2 to day 3. The mice of GLP-1R agonist treatment starting 1 day after the first *Alternaria* challenge group were treated with vehicle from day-2 to day0, and then treated with GLP-1R agonist from day 1 to day 3. **B,** Cell differentials in BAL fluid harvested 24 h after the last *Alternaria* extract-challenge. **C,** BAL fluid and **D,** lungs were

harvested 24 h after the last *Alternaria* extract-challenge to measure the protein expression of IL-4, IL-5, IL-9, IL-13, CCL11, CCL17, CCL22, and CCL24 by ELISA. The results are combined with 2 independent experiments, and shown as mean ± S.E.M. of 10 mice in each groups. * $P < 0.05$