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Glucagon Like Peptide-1 receptor expression on human eosinophils and its regulation of eosinophil activation.

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ABSTRACT:

Background: Glucagon Like Peptide-1 (GLP-1) and its receptor are part of the incretin family of hormones that regulate glucose metabolism. GLP-1 also has immune modulatory roles.

Objectives: To measure the expression of the GLP-1 receptor (GLP-1R) on eosinophils and neutrophils in normal and asthmatic subjects and evaluate effects of a GLP-1 analog on eosinophil function.

Methods: Peripheral blood samples were taken from 10 normal and 10 allergic asthmatic subjects. GLP-1R expression was measured on eosinophils and neutrophils. Subsequently, the asthmatic subjects underwent allergen and diluent inhalation challenges and GLP-1R expression was measured. Purified eosinophils, collected from mild asthmatic subjects, were stimulated with LPS and a GLP-1 analog to evaluate eosinophil cell activation markers CD11b and CD69 and cytokine (IL-4, IL-5, IL-8 and IL-13) production.

Results: GLP-1R is expressed on human eosinophils and neutrophils. Eosinophil, but not neutrophil, expression of GLP-1R is significantly higher in normal controls compared to allergic asthmatics. The expression of GLP-1R did not change on either eosinophils or neutrophils following allergen challenge. A GLP-1 analog significantly decreased the expression of eosinophil surface activation markers following LPS stimulation and decreased eosinophil production of IL-4, IL-8 and IL-13, but not IL-5.

Conclusion & Clinical Relevance: GLP-1R is expressed on human eosinophils and neutrophils. A GLP-1 analog attenuates LPS-stimulated eosinophil activation. GLP-1 agonists may have additional adjunctive indications in treating persons with concomitant Type 2 diabetes mellitus and asthma.

INTRODUCTION

Asthma is characterized by airway inflammation, reversible airway obstruction, and airway hyper-responsiveness. The large majority of asthmatic patients respond well to conventional inhaled treatments; however, about 5% of asthmatics have severe refractory asthma and account for 50% of the health expenditure on asthma (1). Novel biological therapies that target specific ligands and/or their receptors have demonstrated clinically significant improvements in asthma control (2, 3). Despite the availability of these therapies, there remain asthma phenotypes that are not optimally controlled on conventional treatments or do not fulfill the criteria for the newer biological therapies. This underscores the need to interrogate non traditional cellular pathways and generate a clearer understanding of the multidimensional interplay of epithelial cells, airway smooth muscle cells, hematopoietic cells, and neuronal influences, which will aid in identifying novel therapies and/or therapeutic indications for treating asthma.

Glucagon Like Peptide-1 (GLP-1) is produced by L-cells in the small intestine and is secreted in response to glucose. It is an incretin hormone (4-6), which exerts its effect by stimulating glucose-dependent insulin release from pancreatic β -islet cells and increasing the sensation of satiety (6, 7). GLP-1 binds to its specific GLP-1 receptor (GLP-1R) which is a G protein–coupled receptor. GLP-1 analogs are now frequently prescribed for patients with type II diabetes mellitus (T2DM), who are also overweight, as both an adjunctive treatment for better glycemic control and an appetite suppressant (8). GLP-1R is also expressed in kidney, lung, heart, skin, certain immune cells and the nervous system (9).

GLP-1 can modulate of inflammation (10, 11). The transcription factor, nuclear factor-kappa B (NF- κ B), is an important participant in a broad spectrum of inflammatory networks that regulate cytokine activity in airway pathology. The NF- κ B pathway is implicated in the inflammatory response characteristic of asthma (12). It has anti-apoptotic effects on eosinophils and enables their adherence to the bronchial epithelium (12-14). The anti-inflammatory actions of glucocorticoids (GCs), a mainstay treatment for asthma, involve inhibition of NF- κ B induced gene transcription. GLP-1 analogs can inhibit NF- κ B activation (10, 15, 16). In patients with T2DM, GLP-1 analogs reduce circulating C-Reactive Protein (CRP), intracellular NF- κ B and Tumor Necrosis Factor (TNF) levels, and reduces the frequency of inflammatory macrophages, independent of weight change (17-20). GLP-1 also possesses anti-inflammatory properties through modulation of a Protein Kinase-A (PKA)-dependent signaling pathway (21). Lipopolysaccharide (LPS)-induced macrophage activation *in vitro* and TNF- α expression was significantly reduced following treatment with the GLP-1 analog through inhibition of the PKA/NF- κ B signaling pathway (22).

In an ovalbumin (OVA)-induced murine model of allergic asthma, a GLP-1 analog, Liraglutide, was shown to inhibit OVA-induced airway inflammation, airway mucus hyper-secretion, as well as the up-regulation of E-selectin and MUC5AC, through a PKA-dependent inactivation of NF- κ B (10). In an analysis of database of asthmatic patients aged between 18-85 years, obesity was found to be correlated with a diagnosis of current asthma, and this relationship between was stronger in patients with increasing insulin resistance. The authors concluded that targeting insulin resistance may represent a novel therapeutic strategy for obese patients with asthma (23).

Very little is known about the role of GLP-1 and its receptor may play in human allergic asthma and GLP-1 levels have not been reported in populations of asthmatic subjects. The purpose of this study was to determine whether GLP-1R is expressed on eosinophils and neutrophils and whether allergen inhalation can modulate GLP-1R expression on these cells. In addition, this study examined the effects of GLP-1 analog treatment on *in vitro* LPS-induced eosinophil activation.

METHODS

Subjects

Ten non-smoking subjects with stable allergic asthma were recruited. Subjects (aged 19-60 years) had positive skin prick test responses to common aeroallergens (wheals > 2mm), methacholine PC₂₀ <16 mg/mL, FEV₁ of ≥70% of predicted and a dual phase response to allergen (fall in FEV₁ of ≥20% by 2 h and ≥15% 3-7 h post allergen). Subjects used short-acting bronchodilators for control of asthma and were excluded from the study if they were pregnant or nursing, current smokers or ex-smokers with more than 10 pack-years, had diabetes mellitus or developed lower respiratory infections/exacerbations or used inhaled or oral steroids 4 weeks prior to study. Ten healthy non-atopic, non-asthmatic controls (with both a negative skin prick test and methacholine PC₂₀ challenge greater than 16 mg/mL) were also enrolled. Anti-histamines, caffeine and non-steroidal anti-inflammatory agents were prohibited 48 h before any study visit. All experimental procedures were reviewed, approved by the institutional research ethics board and informed written consent was obtained from every subject.

Study design

Allergic asthmatic subjects underwent either an allergen or diluent challenge that was randomized with a 2-week washout period. On Day 1 a medical history, skin prick test, spirometry, methacholine challenge and peripheral blood collection, were performed. On Day 2 either an allergen or diluent inhalation challenge was performed with peripheral blood collected at the 7 hour (h) time-point. Day 3 entailed a methacholine challenge and peripheral blood collection.

Normal controls were required to attend the laboratory for a single visit, which entailed a detailed medical history, skin prick test, spirometry, methacholine inhalation, and collection of peripheral blood via venipuncture for both *in vivo* and *in vitro* culture studies.

Peripheral blood cells were immunostained with isotype or specific monoclonal antibodies (mAbs) and acquired via flow cytometry to identify eosinophils and neutrophils, and GLP-1R percentage expression. The purity of the isolated eosinophils was assessed with flow cytometry and was 98%, and the viability tested with the trypan blue staining was consistently greater than 96%.

Methacholine inhalation challenge

Methacholine inhalation challenge test was performed using the tidal breathing method of nebulized methacholine, as previously described (24). In brief, doubling concentrations of methacholine chloride (Methapharm, ON, Canada) were inhaled orally from a Hans Rudolph valve every 2 min. The FEV₁ was measured following inhalation at 30 s and 90 s or until it stopped falling. The percent fall was calculated from the post-diluent FEV₁ value. The test was terminated when a fall in FEV₁ of at

least 20% of the lowest post-saline value occurred. The results were expressed as the provocative concentration of methacholine causing a 20% fall in FEV₁ (PC₂₀).

Allergen inhalation challenge

Allergen challenges were conducted by administering inhaled doubling concentrations of allergen extract, as previously described (25). The allergen giving the largest skin wheal was selected for inhalation (or one to historically cause wheezing) and the concentration of allergen required to achieve a 20% decrease in FEV₁ (the allergen PC₂₀) was predicted using the methacholine PC₂₀ and the titration of allergen determined from the skin prick test. Diluent challenges were conducted using 3 inhalations of 0.9% saline for 2 min each. The EAR was the largest percent fall in FEV₁ between 0 and 2 h, and the LAR was the largest percent fall in FEV₁ between 3 and 7 h post-challenge.

Eosinophil *In vitro* Treatment with GLP-1 Analog

Peripheral blood from venipuncture was drawn into heparin (1000 U/mL) and layered on Accu-Prep (Accurate Chemical & Scientific Corp, NY, US) for density gradient sedimentation. The granulocyte layer was harvested and eosinophils were then isolated using a negative selection eosinophil enrichment kit (EasySep™ Human Eosinophil Enrichment Kit, Stemcell Technologies, BC, Canada). Purified eosinophils (1x10⁶/mL) were re-suspended in RPMI-C and cultured in a 96-well plate with or without GLP-1 analog (Sigma, ON, Canada) at concentrations of 1, 5, 10, 15, or 20 µg/mL for 30 min followed by the addition of LPS (10 ng/mL) (Sigma, ON, Canada) for 18 h (5% CO₂ atmosphere at 37°C). After incubation, 1% PFA was added to stop the reaction, and the supernatant was harvested for analysis of IL-4, IL-5, IL-8, and IL-13 cytokine production using a multiplex kit conducted that was

outsourced by Eve Technologies, AB, Canada. The lowest detection thresholds are 0.6 pg/mL for IL-4, 0.1 pg/mL for IL-5, 0.2 pg/mL for IL-8 and 0.4 pg/mL for IL-13.

The cells were also collected and assessed for activation marker expression using flow cytometry.

Flow cytometry staining and analysis

Samples were immunostained with mAbs including CD45-PerCPCy5.5 (BD Biosciences, ON, Canada), CD16-V450 (Ebioscience, CA, US), CCR3-PE (Ebioscience, CA, US), GLP-1R-AF700 (R&D Systems, MN, US), and relevant isotype controls for 30 min at 4°C. For the *in vitro* experiments, the effect of treatment with a GLP-1 analog on LPS-induced eosinophil activation was assessed by immunostaining with specific or isotype control mAbs to assess activation markers with CD69-AF700 (Ebioscience, CA, US) and CD11b-APC (Ebioscience, CA, US). Cells were then re-suspended in 1% PFA and stored at 4°C until flow cytometry could be conducted. The isotype control for the GLP-1R was set to 2%, which was compared to the specific marker to detect the percentage of cells expressing the marker.

Eosinophils were defined as the CD45⁺/CD16⁻/CCR3⁺ population. We first gated around the CD45⁺ population in the SSC vs. CD45 plot, followed by gating around the CD16⁻ population in the SSC vs. CD16 plot, and a final gate around the CCR3⁺ population in the SSC vs. CCR3 plot (Supplementary Fig E1). Conversely, neutrophils were identified as the CD45⁺/CD16⁺/CCR3⁻ population (Fig E1). As described in previous studies, invariant Natural Killer T Cells (iNKT cells) express GLP-1R and were used as a positive control in this study (19, 20, 26). iNKT cells were immunostained with mAbs including CD45-PerCPCy5.5 (BD Biosciences, ON,

Canada), CD3-APC (Ebioscience, CA, US), V α 24J α Q TCR (BD Horizon, Miss, Canada), that is a specific marker for iNKT cells, GLP-1R-AF700 (R&D Systems, MN, US), and relevant isotype controls for 30 min at 4°C (Supplementary Fig E2)

Statistical analysis

Results for GLP-1R expression on eosinophils in allergic asthmatic subjects compared to normal health controls are presented as median with the interquartile range, as are the results for GLP-1R expression following diluent and allergen challenge. The statistical analysis for changes to eosinophil and neutrophil GLP-1R expression was performed using a non parametric 2-way ANOVA and post-hoc Bonferroni test on GraphPad Prism 5 software (GraphPad Software, CA, US). *In vitro* experiments were analyzed using 1-way ANOVA with a post-hoc Tukey test. Significance was accepted at $p < 0.05$.

RESULTS

The two subject groups were age, gender and BMI matched. Allergic asthmatic subjects had a mean baseline FEV₁ (% predicted) of $87.7\% \pm 2.6$ and methacholine PC₂₀ of 4.32 ± 1.5 mg/mL, whereas the healthy normal controls had baseline FEV₁ of $95.9\% \pm 2.9$ and PC₂₀ > 16 mg/ml (Table 1).

The percent of peripheral blood eosinophils expressing GLP-1R at baseline was 1.92% (IQR 1.22-2.69) in allergic asthmatics compared to 8.45% (IQR 6.07-17.28) in healthy controls ($p < 0.05$; Figure 1A, Supplementary Figure E3A/B). Conversely, although neutrophils did express GLP-1R, there was no significant difference in peripheral blood neutrophil expression of GLP-1R between the subject groups (Figure 1B).

In the allergic asthmatic subjects, allergen inhalation induced a maximum early asthmatic response of $34.02 \pm 2.0\%$ and a maximum late response of $20.7 \pm 3.5\%$, compared to no significant change in FEV₁ following diluent inhalation (Supplementary Figure E4). The methacholine PC₂₀ decreased one doubling dose post-allergen challenge, compared to no change post-diluent (Supplementary Figure E4). Allergen challenge did not induce a change in GLP-1R expression at 7 h or 24 h when compared to the diluent challenge, in either peripheral blood eosinophils or neutrophils (Figure 2A and 2B).

Stimulation of eosinophils *in vitro* with LPS (10 ng/mL) for 18h induced a significant increase in eosinophil expression of both CD69 and CD11b ($p < 0.05$; Figure 3A and 3B, Supplementary Figure E5A/B). Treatment with a GLP-1 analog, at a concentration of 1 $\mu\text{g/mL}$, prior to addition of 10 ng/mL of LPS, significantly reduced eosinophil expression of both CD69 and CD11b ($p < 0.05$; Figure 3A and 3B, Supplementary Figure E5A/B), while treatment with a concentration of 5 $\mu\text{g/mL}$ significantly reduced expression of CD69 ($p < 0.05$; Figure 3A), but not CD11b. Concentrations of the GLP-1 analog of 10 $\mu\text{g/mL}$ and 15 $\mu\text{g/mL}$ did not significantly inhibit eosinophil expression of either CD69 and CD11b (Figure 3A and 3B).

Eosinophils stimulated *in vitro* for 18h with LPS also significantly increased supernatant levels of IL-4, IL-8 and IL-13 ($p < 0.05$; Figure 4A, C and D); changes in IL-5 levels were not significant (Figure 4B). All concentrations of the GLP-1 analog significantly attenuated LPS stimulated eosinophil IL-4 and IL-13 production ($p < 0.05$; Figure 4A and D); however, LPS stimulated IL-8 production was only significantly attenuated by the 1 $\mu\text{g/mL}$ concentration of the GLP-1 analog ($p < 0.05$; Figure 4C).

DISCUSSION

GLP-1 analogs have been extensively used as an adjunct treatment in patients with T2DM, who are also overweight. Over the last decade, a body of evidence has emerged that suggests GLP-1 may also have immunomodulatory properties. The current study has demonstrated that the GLP-1R is expressed on both human eosinophils and neutrophils, cells believed to have important roles in the pathogenesis of asthma (27). The expression of GLP-1R on eosinophils was lower in allergic asthmatic subjects compared with normal controls, while no significant differences were demonstrated for neutrophils. In addition, in the allergic asthmatics, allergen inhalation did not alter the expression of GLP-1R on either eosinophils or neutrophils. The functional effects of a GLP-1 analog on human eosinophils was demonstrated by its ability to attenuate LPS stimulated surface expression of CD69, CD 11b and the production of IL-4, IL-8 and IL-13.

The GLP-1/GLP-1R axis possesses anti-inflammatory properties through a PKA-dependent signaling pathway. Arakawa et al. (22) has shown that LPS-induced macrophage activation and TNF- α expression was significantly reduced by a GLP-1 analog through the PKA/NF- κ B signaling pathway. In addition, in a mouse model of allergic asthma, OVA-induced increases in both eosinophils and IL-4, IL-5 and IL-13 in BALF were significantly reduced by GLP-1 (10). The attenuation of LPS-stimulated IL-4, IL-13, as well as IL-8 which is crucial for neutrophil recruitment into the airway (28), from human eosinophils, is consistent with these observations. Interestingly, there was no significant change in IL-5 concentrations from eosinophils following the addition of either LPS or LPS and GLP-1 analog. However, LPS-induced eosinophil recruitment may not be dependent on IL-5 (29).

The induction of a number of eosinophil-surface proteins including CD69 and α M integrin (CD11b) indicate eosinophil activation in asthma (30). CD69 is either absent or expressed at a very low level on unstimulated eosinophils (31). CD69 is induced on blood eosinophils after inhaled allergen (32). CD11b is expressed on the surface of many leukocytes and mediates leukocyte adhesion and migration. CD11b expression is higher on eosinophils from asthmatics compared with healthy controls (33). We have shown that both CD69 and CD11b on human eosinophils are upregulated by LPS. The addition of a GLP-1R analog attenuated this increased receptor expression, although this effect was only significant at lower concentrations of the GLP-1R analog. These results are consistent with evidence from a rat model that a GLP-1 analog reduced neutrophil CD11b surface expression in whole blood and blockade of the GLP-1R abolished this effect (34).

In this study GLP-1R expression on eosinophils was lower in allergic asthmatics, when compared with normal controls. This is a novel finding. Inhaled allergen challenge did not change this expression; however, a GLP-1 analog suppressed markers of eosinophil activation and Th2 cytokine production, which suggests that in the appropriate clinical context (concomitant T2DM, overweight and asthma) the addition of a GLP-1 analog may have beneficial effects.

In summary, this study has identified, for the first time, that a potential immunomodulatory receptor, GLP-1R, is expressed on both human peripheral blood eosinophils and neutrophils and its expression was significantly lower on eosinophils from allergic asthmatics when compared to normal controls. GLP-1R was not changed on eosinophils or neutrophils following inhaled allergen in allergic asthmatics. The expression of GLP-1R on eosinophils has functional consequences, as

the addition of a GLP-1 analog attenuated LPS stimulated expression of two eosinophil activation markers and three cytokines that are implicated in the pathogenesis of asthma. These results suggest that GLP-1 may have immunomodulatory effects in asthma. Further evaluation of this hypothesis will require studies examining the effects of treatment with GLP-1 analogs in allergic asthmatic subjects.

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Statement of Conflicts of Interest:

PDM, BMS, JPO, SGS, EGA, DT, RS, POB, MB and GMG have no conflicts of interest related to this manuscript to declare.

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TABLES

Characteristic	Allergic Asthmatics (n = 10)	Normal Healthy Controls (n=10)
Age, years	31.2 ± 4.0	37.6 ± 3.5
Gender, % male	50%	50%
BMI, kg·m ²	24.71 ± 0.9	25.21 ± 1.3
Pulmonary function:		
FEV ₁ % predicted	87.7 ± 2.6	95.9 ± 2.9
FVC % predicted	92.5 ± 3.6	94.7 ± 3.44
FEV ₁ /FVC	95.1 ± 1.93	101.4 ± 1.86
Methacholine PC ₂ (mg/mL)	4.32 ± 1.5	>16

Table 1: Subject characteristics of normal healthy controls and allergic asthmatic subjects (all p>0.05).

FIGURE LEGENDS

Figure 1. Baseline eosinophil GLP-1R expression is greater in normal healthy controls than asthmatics

Expression of GLP-1R on (A) peripheral blood eosinophils and (B) peripheral blood neutrophils from normal controls (open circles) (N=10) and mild allergic asthmatics (black circles) (N=10). Data are presented as the median and interquartile range (* significantly different between two groups; p<0.05).

Figure 2. Allergen inhalation does not induce changes in GLP-1R expression on peripheral blood eosinophils or neutrophils

GLP-1R expression on (A) Peripheral blood eosinophils, (B) Peripheral blood neutrophils from 10 allergic asthmatics after inhaled diluent (open circles) or allergen (closed circles) inhalation challenge. Data are presented as the median and interquartile range.

Figure 3. *In vitro* LPS-induced eosinophil activation is attenuated following treatment with a GLP-1R analog

Isolated peripheral blood eosinophils from 10 mild allergic asthmatics were stimulated for 18 h *in vitro* with PBS (open bar) or LPS (10 ng/mL, black bars) and then assessed for (A) eosinophil expression of CD69 and (B) CD11b following treatment with GLP-1R-analog (1, 5, 10, 15, µg/mL; grey bars). Data are presented as the mean ± SEM. * Significantly different from positive control (p<0.05).

Figure 4. *In vitro* Eosinophil IL-4, IL-8 and IL-13 production is attenuated by GLP-1R agonist following LPS stimulation, while IL-5 is not.

Isolated peripheral blood eosinophils from 10 mild allergic asthmatics were stimulated for 18 h *in vitro* with PBS (open bar) or LPS (10 ng/mL, black bars) and with a GLP-1R analog (doses 1, 5, 10, 15, µg/mL; grey bars). Levels of (A) IL-4; (B) IL-5; (C) IL-8; and (D) IL-13 in supernatants were measured with and without the addition of increasing concentrations of a GLP-1R analog. Data are presented as the mean ± SEM. * Significantly different from positive control (p<0.05).

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure E1 - Eosinophil and Neutrophil Gating Strategy

Representative whole blood dot plots showing strategic gating used for flow cytometry analysis of eosinophils and neutrophils. Initial granulocyte population was gated through (A) Side Scatter (SSC) vs. CD45 PerCPCy5.5. Through the (B) CD16 Pacific Blue vs. CD45 PerCPCy5.5 plot neutrophils were further identified by gating around the CD16+ population, whereas eosinophils were considered to be the CD16- population. The neutrophils were assessed for CCR3 expression by setting the isotype control to 2% in the (C) PE isotype vs. CD45 PerCPCy5.5 plot. The CCR3- population was considered to be the neutrophils as seen in the (D) CCR3 PE vs. CD45 PerCPCy5.5 plot. Eosinophils were further identified by CCR3 expression by setting isotype control to 2% in the (E) PE isotype vs. CD45 PerCPCy5.5 plot and gating around the CCR3+ population in the (F) CCR3 PE vs. CD45 PerCPCy5.5 plot.

Supplementary Figure E2 – Positive control invariant NK T Cell Gating Strategy

Representative whole blood dot plots showing strategic gating used for flow cytometry analysis of iNK T Cells. Initial lymphocyte population was gated through (A) Side Scatter (SSC) vs. CD45 PerCPCy5.5. Through the (B) CD45 PerCPCy5.5 vs. CD3 APC plot lymphocytes were further identified by gating around the CD45+CD3+ population. Invariant NK T cells were identified by V α 24J α Q TCR expression by setting isotype control to 2% in the (C) BV510 isotype vs. CD45 PerCPCy5.5 plot and gating around the (D) V α 24J α Q TCR + population. Invariant NK T cells were assessed for GLP-1R expression by gating through (E) AF700 isotype at 2% and then (F) GLP-1R AF700 vs. CD45 PerCPCy5.5 plot.

Supplementary figure E3A. A Histogram of GLP-1R expression on eosinophils in a normal healthy control and an allergic asthmatic subject. The pink color represents the isotype. The grey color represents the allergic asthmatic subject and the blue color represents a normal healthy control.

Supplementary Figure E3B. Median fluorescence intensity (MFI) of GLP-1 receptor expression on eosinophils from normal controls and allergic asthmatics. There was significantly greater expression of GLP-1 receptors in the normal group than the allergic asthmatic group. * $p < 0.05$.

Supplementary figure E4. Allergen inhalation induces airway changes in patients with mild allergic asthma.

Diluent (open circles and boxes) and allergen (closed circles and boxes) inhalation challenges in 10 allergic asthmatic subjects. **(A)** Percent change in FEV₁ and **(B)** methacholine PC₂₀ (mg/mL). FEV₁ data expressed as means $\pm$ SEMs, and methacholine PC₂₀, expressed as geometric means $\pm$ geometric SEMs (log 2). * Significantly different ($P < 0.05$).

Supplementary figure E5A. A Histogram of the CD11b expression following LPS and GLP-1 agonist stimulation. The image on the left represents the Isotype (red) and negative control (blue). The image on the right illustrates the isotype (red), negative control (aqua), LPS stimulation (blue) and GLP-1 stimulation at 1 μ g/mL (green) and 5 μ g/mL (orange).

Supplementary Figure E5B. Median fluorescence intensity (MFI) of CD11b and CD69 expression on peripheral blood eosinophils from allergic asthmatics stimulated with PBS (open bar) or LPS (10 ng/mL, black bars) and after treatment with GLP-1R-

agonist (1, 5, 10, 15 and 30 $\mu\text{g/mL}$; grey bars). The isotype control was set at 2%.

Data are presented as the mean $\pm$ SEM. * $p < 0.05$.



