Online data supplement

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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Supplementary Materials and Methods

RT-PCR

Human lung tissue was obtained from lungs procured from deceased organ donors whose lungs were not used for transplantation. Lungs were resected at the time of operative procurement of other organs for transplantation and transported to the laboratory inflated and cooled to 4°C. Once received, samples of lung tissue were procured from grossly normal areas of the lung and preserved in RNA*later* solution (ThermoFisher Scientific, Waltham, MA) for gene expression analysis. For this analysis, lung tissue was used only from donors with normal or near normal lung histology after review by a clinical pathologist. Mouse lungs were harvested from naïve WT BALB/c mice.

Total RNA was isolated by RNeasy mini kit (Qiagen, Valencia, CA) from frozen human lung tissues using RNA*later* and flesh mouse lungs. cDNA was synthesized using SuperScript III (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. 1 μl of RT product was amplified in the presence of GoTaq DNA polymerase (Pomega, Madison, WI) with QuaniTect Primer Assay. The following primers were purchased from Qiagen: human GAPDH (Hs_GAPDH_1_SG), human GLP1R (Hs_GLP1R_1_SG), mouse GAPDH (Mm_Gapdh_3_SG), and mouse GLP1R (Mm_Glp1r_1_SG). The GAPDH PCR cycling conditions were 95°C for 2 min followed by 37 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 30sec. The GLP1R PCR cycling conditions were 95°C for 2 min followed by 42 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 30sec. PCR products were separated using 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Quantitative real-time PCR

Total RNA was isolated from mouse lungs using the RNeasy Kit (Qiagen); and cDNA was synthesized using SuperScript III (Invitrogen). Quantitative real-time PCR analyses were performed using the StepOnePlus™ Real-Time PCR System (ThermoFisher Scientific) and iQ™ SYBR® Green Supermix (Bio-Rad) with QuaniTect Primer Assay (mouse GAPDH (Mm_Gapdh_3_SG), and mouse DUOX1 (Mm_Duox1_1_SG)) according to manufacturer's instructions. To determine the copy numbers of the DUOX1, the quantified concentrations of PCR amplicon was serially diluted and used as standards. Data of DUOX1 were normalized using the GAPDH expression levels in each sample.

Generation of bone marrow-derived cultured mast cell (BMCMC)

BMCMCs were generated as previously described method with modifications.[1] Briefly, bone marrow cells were obtained by flushing the femurs of GLP-1R/mApple reporter and WT mice. The bone marrow cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 medium with 10 ng/ml IL-3 (PeproTech, Rocky Hill, NJ), 10% FBS, 2 mM L-glutamine, 50 nM β-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. Every 3 or 4 days, two-thirds of floating cell suspension was transferred to new flask to remove adherent cells, and fresh culture media (one-third of total media) was added. After 3 weeks, the floating cells were used for flow cytometry to detect GLP-1R/mApple protein expression.

Cell differential counting

Cytospin preparations of BAL fluid cells were performed for each sample. The slides were stained with a Richard-Allan Scientific Three step stain kit (ThermoFisher scientific) to determine cell differentials. Differential counts were based on counting 200 cells per slide.

Histological analyses of lung sections

48 h after the last challenge of *Alternaria* extract or PBS, mice were sacrificed and the lung tissue was fixed in 10% formalin solution, paraffin-embedded, cut in 5 μm sections, mounted, and stained with anti-major basic protein (MBP) antibody that was generously provided by Dr. James J. Lee (Mayo Clinic, Scottsdale, AZ, USA) to evaluate eosiniophils.[2] Slides were examined by a pathologist blinded to experimental groups. Eosinophil score: 0= no eosinophils observed in the examined sections; 1= rare perivascular eosinophils; 2= few perivascular eosinophils and scattered eosinophils in the interstitium; 3= Moderate number eosinophils in perivascular space and in the interstitium; 4= Marked eosinophils in the perivascular space, in the interstitium and in alveolar spaces.

In addition, the paraffin-embedded lung tissue slices were stained with Periodic Acid Schiff's (PAS) solution to visualize mucus and mucous producing cells. Slides were scored by a pathologist blinded to the experimental groups. PAS score: 0= PAS positive cell are not observed in the examined sections; 1= Less than 10% of cells in medium and small airways are PAS positive; 2= 10-20% PAS positive cells in medium and small airways; 3= Greater than 20% cells in medium and small airways are PAS positive and hyperplasia of PAS positive cells is observed; 4= Greater than 20% PAS positive cells in medium and small airways, hyperplasia of PAS positive cells and mucous plugging of airways.

Airway responsiveness (AR) measurement

Mice were anaesthetized with pentobarbital sodium (105 mg/kg), and a tracheostomy tube was placed and the internal jugular vein was cannulated. The mice were mechanically ventilated in a

plethysmography chamber. Lung resistance was measured following intravenous administration of acetyl-β-methacholine chloride (0–411 μg/kg; SigmaAldrich St. Louis, MO) as previously described.[3]

Supplementary References

1. Kalesnikoff J, Galli SJ. Anti-inflammatory and immunosuppressive functions of mast cells. In: Cutri MC, Anegon I, editors. Suppression and Regulation of Immune Responses, methods and protocols. Methods in Molecular Biology book series (MIMB, volume 677): Humana Press; 2011. p. 207-20

2. Denzler KL, Farmer SC, Crosby JR, Borchers M, Cieslewicz G, Larson KA, Cormier-Regard S, Lee NA, Lee JJ. Eosinophil Major Basic Protein-1 Does Not Contribute to Allergen-Induced Airway Pathologies in Mouse Models of Asthma. *The Journal of Immunology* 2000; 165: 5509-17.

3. Peebles RS, Jr., Sheller JR, Johnson JE, Mitchell DB, Graham BS. Respiratory syncytial virus infection prolongs methacholine-induced airway hyperresponsiveness in ovalbumin-sensitized mice. *J Med Virol* 1999; 57: 186-192.

Supplementary Figure Legend

FIG E1. A, The protein level of IL-33, **B,** LDH activity, **C,** CysLTs, and **D**, PGD_2 in the BAL fluid from WT and IL-33 KO mice 1 h after the first *Alternaria* extract-challenge following GLP-1R agonist or vehicle treatment. n=3 for PBS-challenged groups, and n=5 for *Alternaria* extract-challenged groups. * $P< 0.05$ compared with vehicle-*Alternaria* extract-challenged group. **E,** The gating strategy of BMCMC. BMCMC was identified by flow cytometry as $Fc\varepsilon RI^+$ c-kit^+ CD49b $(DX5)^-$ cells. **F,** The histograms of mApple (GLP-1R) expression on cultured BMCMC from WT mice (filled gray area) or GLP-1R/mApple reporter mice (black line)

FIG E2. A, The gating strategy of ILC2 by cell surface staining. ILC2 were identified by flow cytometry as FSC-A/SSC-A low, $lineage^-$ $CD3^-$ $CD45^+$ $CD25^+$ $CD127^+$ cells.

FIG E3. A, The gating strategy of ILC2 by cell surface and intracellular staining. ILC2 were identified by flow cytometry as FSC-A/SSC-A low, $lineage^-$ $CD3^-$ $CD45^+$ $CD25^+$ $ICOS^+$ cells. **B,** Representative plots of lung ILC2 expressing IL-5 and IL-13 from mice challenged with *Alternaria* extract or PBS, and treated with GLP-1R agonist or the vehicle.

FIG E4. A, The gating strategy of CD4 T cell by cell surface and intracellular staining. CD4 T cell were identified by flow cytometry as FSC-A/SSC-A low, $CD45^+$ $CD3^+$ $CD4^+$ cells. **B,** Representative plots of lung CD4 T cells expressing IL-5 and IL-13 from mice challenged with *Alternaria* extract or PBS, and treated with GLP-1R agonist or the vehicle. **C and D,** The number of IL-5^+ and IL-13^+ CD4 T cells 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. * $P< 0.05$

FIG E5. A, GLP-1R agonist or its vehicle was administered subcutaneously on day -2 and -1, and then 4 h before HDM 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 HDM extract-challenge on day 0. **B,** The protein level of IL-33 and LDH activity in the BAL fluid. The results are shown as mean ± S.E.M. of 2 mice in PBS-challenged groups and 3 mice in HDM extract-challenged groups. **C,** WT mice were challenged with HDM 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 HDM 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. **D,** The protein expression of IL-5, IL-13, and IL-33 in the lung homogenates was measured by ELISA. The results are combined with 2 independent experiments, and shown as mean ± S.E.M. of 4 mice in PBS-challenged groups and 6 mice in HDM extract-challenged groups. * $P< 0.05$. Veh=vehicle. PBS=phosphate buffered saline. N.D.=not detected.* $P< 0.05$

Table E1. Antibodies for flow cytometry

Antigen	Fluorochrome	Clone	Supplier
CD3	Pacific Blue	17A2	BioLegend (San Diego, CA)
CD4	PE	GK1.5	BD Pharmingen™ (San Jose, CA)
CD25	Alexa488	PC61	BioLegend
CD45	redFluor™ 710	30-F11	TONBO Biosciences (San Diego, CA)
CD49b	FITC	DX5	BioLegend
CD117 (c-kit)	APC	2B8	BD Pharmingen™
CD127	APC	A7R34	BioLegend
CD146	PE-Cy7	ME-9F1	BioLegend
CD278 (ICOS)	PE	7E.17G9	eBioscience™ (Waltham, MA)
CD326 (EpCAM)	APC-Cy7	G8.8	BioLegend
FcεRI	PE-Cy7	MAR-1	eBioscience™
IL-5	APC	TRFK5	BD Pharmingen™
IL-13	PE-Cy7	eBio13A	eBioscience™
Lineage cell detection cocktail-biotin (mouse)	(Biotin)		Miltenyi Biotech Inc. (Auburn, CA)
Streptoavidin-APC-Cy7	APC-Cy7		BioLegend
DAPI			BD Pharmingen™
Ghost Dye TM UV450	UV450		TONBO Biosciences
Propidium iodide			BD Pharmingen™

FIG E1.

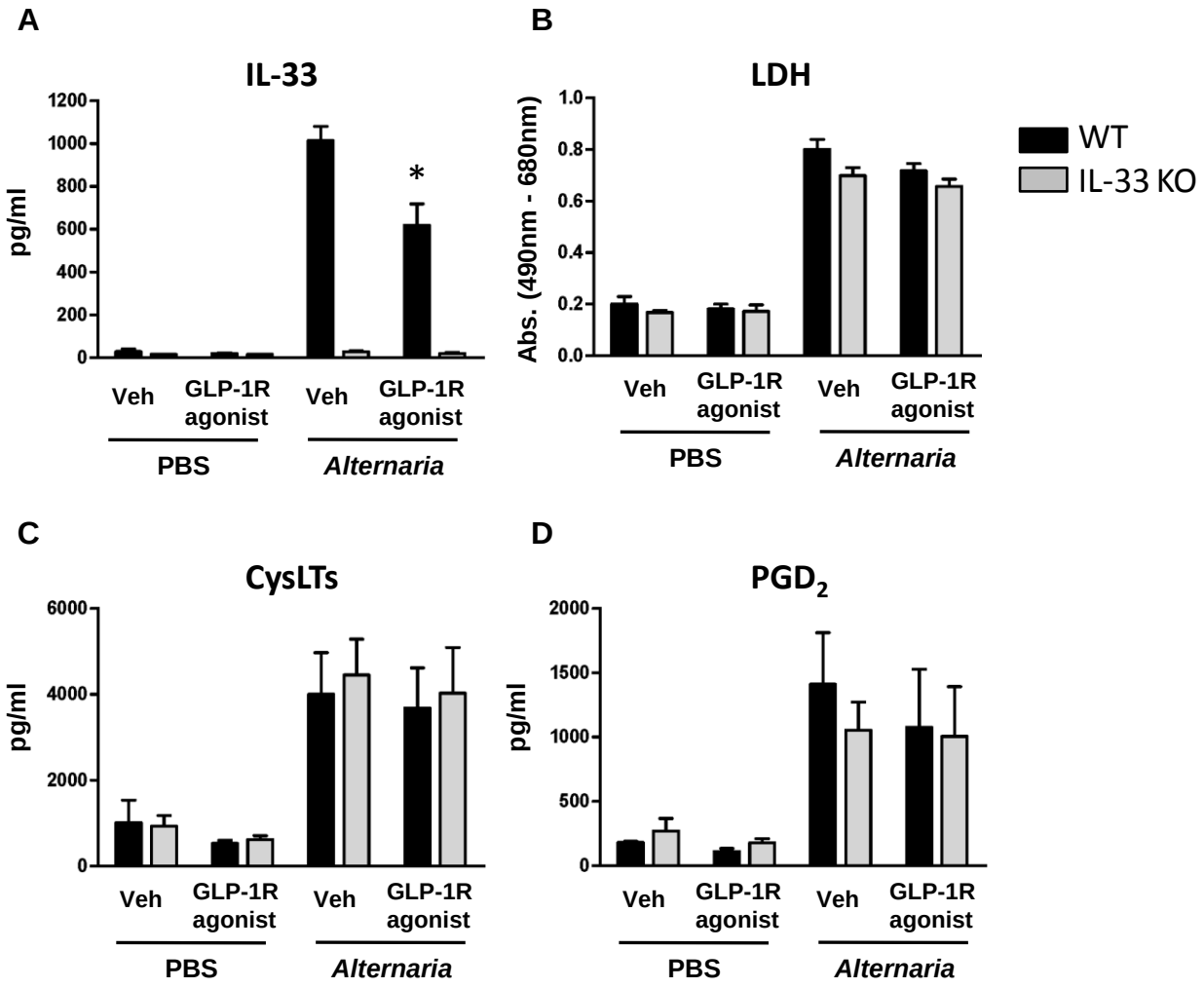
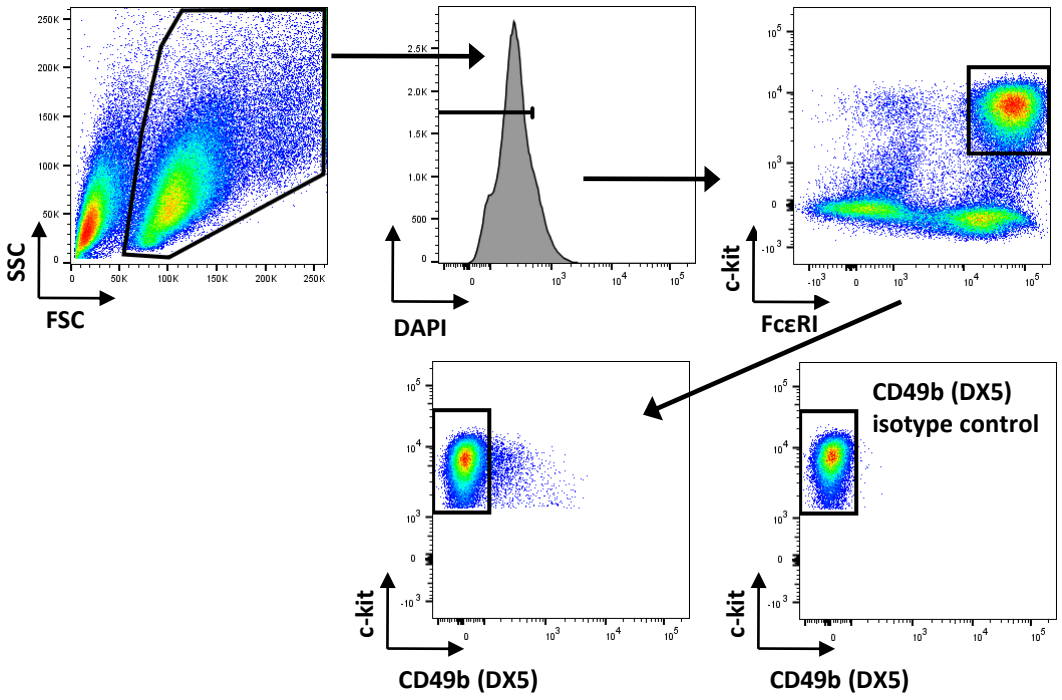

A
IL-33
pg/ml
1200
1000
800
600
400
200
0
*
Veh
GLP-1R agonist
Veh
GLP-1R agonist
PBS
Alternaria
B
LDH
Abs. (490nm - 680nm)
1.0
0.8
0.6
0.4
0.2
0.0
Veh
GLP-1R agonist
Veh
GLP-1R agonist
PBS
Alternaria
WT
IL-33 KO
C
CysLTs
pg/ml
6000
4000
2000
0
Veh
GLP-1R agonist
Veh
GLP-1R agonist
PBS
Alternaria
D
PGD2
pg/ml
2000
1500
1000
500
0
Veh
GLP-1R agonist
Veh
GLP-1R agonist
PBS
Alternaria

FIG E1.

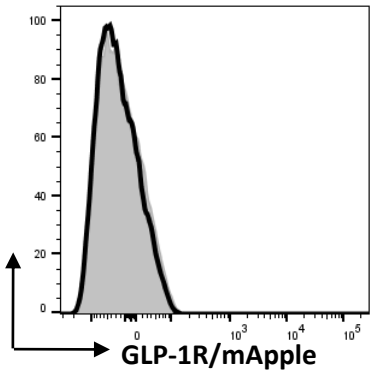
Bone marrow-derived cultured mast cell gating strategy

E



F

Bone marrow-derived cultured mast cells



WT mouse

GLP-1R/mApple reporter mouse

FIG E2.

A

ILC2 gating strategy (cell surface staining)

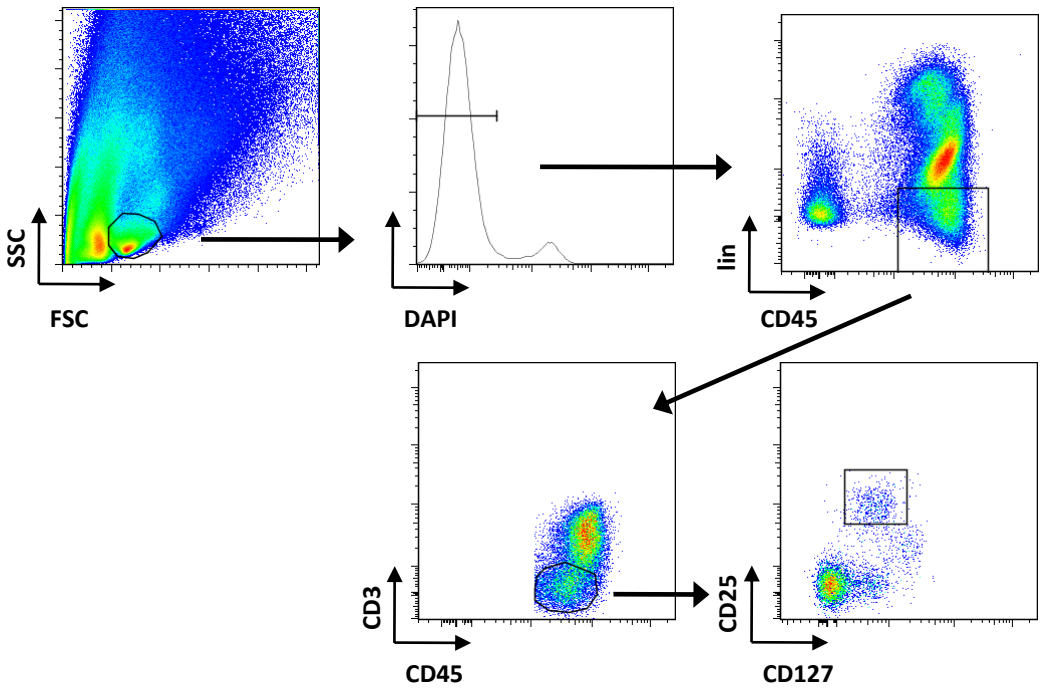


FIG E3.

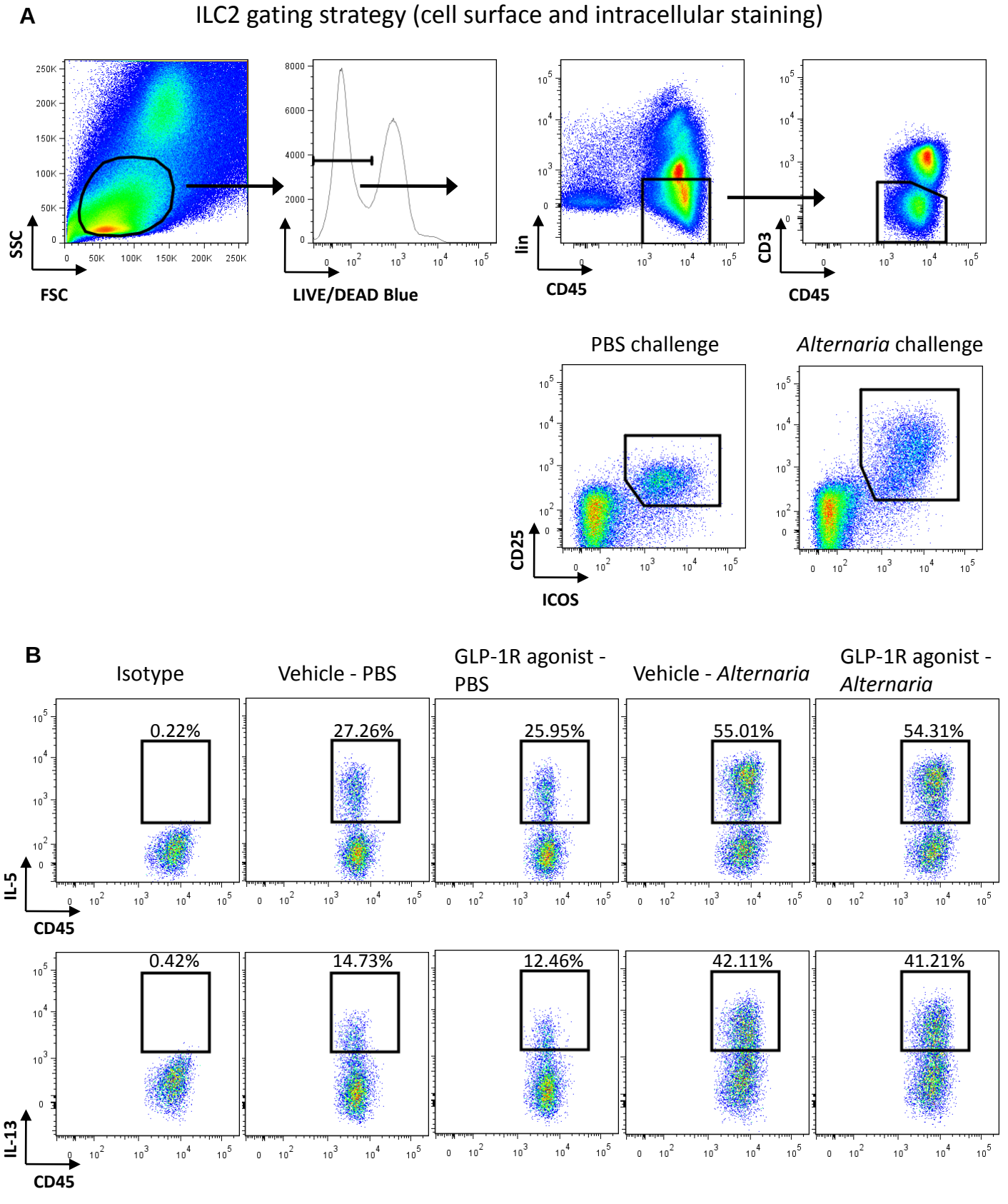
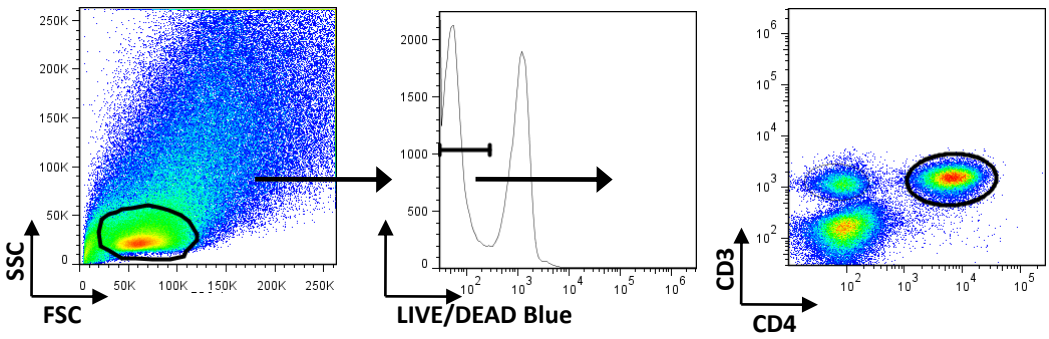

A
ILC2 gating strategy (cell surface and intracellular staining)
SSC
FSC
LIVE/DEAD Blue
lin
CD45
CD3
CD45
PBS challenge
Alternaria challenge
CD25
ICOS
B
Isotype
Vehicle - PBS
GLP-1R agonist - PBS
Vehicle - Alternaria
GLP-1R agonist - Alternaria
0.22%
27.26%
25.95%
55.01%
54.31%
IL-5
CD45
0.42%
14.73%
12.46%
42.11%
41.21%
IL-13
CD45

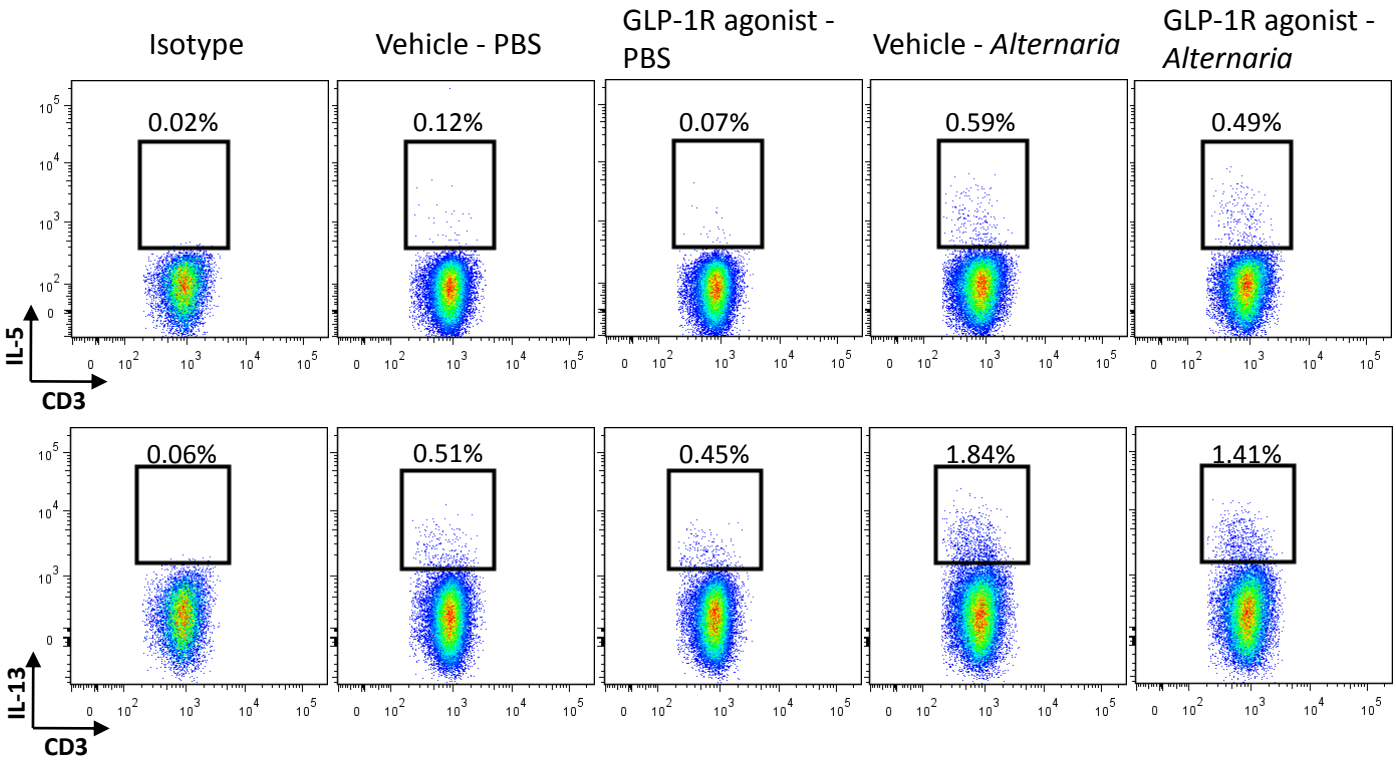
FIG E4.

A

CD4 T cell gating strategy (cell surface and intracellular staining)

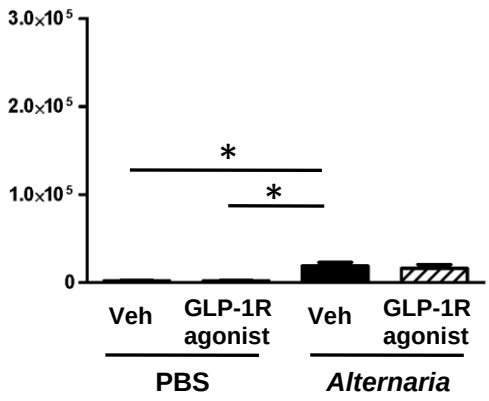


B



C

Number of IL-5⁺ CD4 T cell



D

Number of IL-13⁺ CD4 T cell

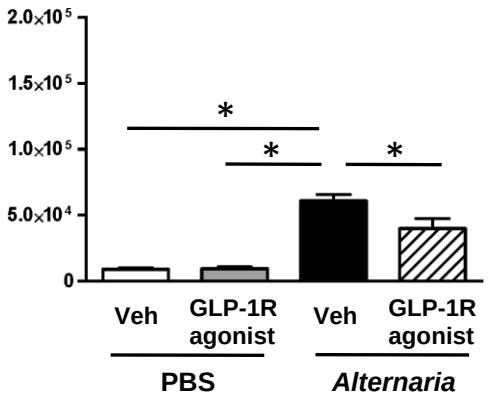
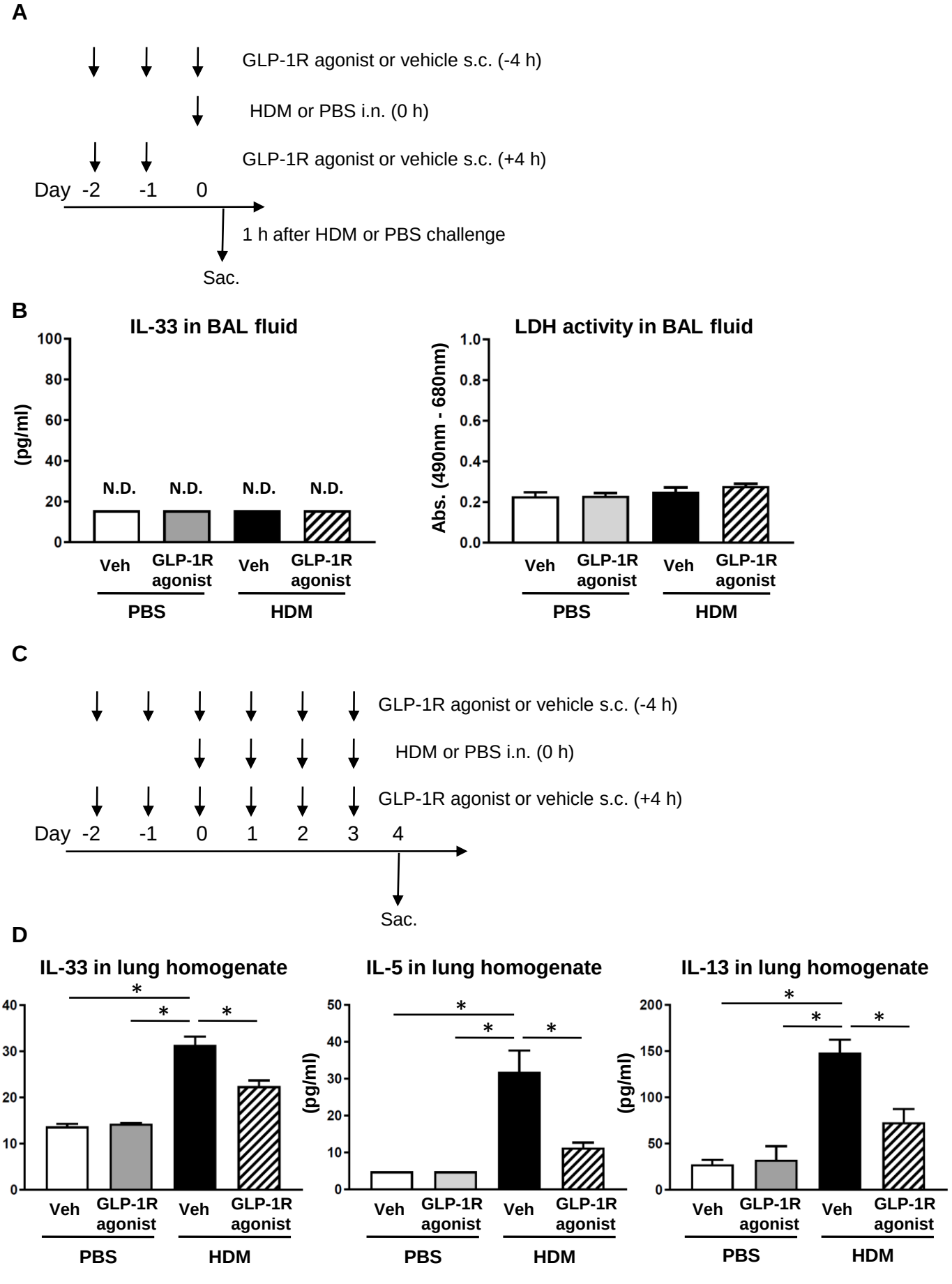


FIG E5.


A
GLP-1R agonist or vehicle s.c. (-4 h)
HDM or PBS i.n. (0 h)
GLP-1R agonist or vehicle s.c. (+4 h)
Day -2 -1 0
1 h after HDM or PBS challenge
Sac.
B
IL-33 in BAL fluid
(pg/ml)
N.D.
N.D.
N.D.
N.D.
Veh
GLP-1R agonist
Veh
GLP-1R agonist
PBS
HDM
LDH activity in BAL fluid
Abs. (490nm - 680nm)
C
GLP-1R agonist or vehicle s.c. (-4 h)
HDM or PBS i.n. (0 h)
GLP-1R agonist or vehicle s.c. (+4 h)
Day -2 -1 0 1 2 3 4
Sac.
D
IL-33 in lung homogenate
(ng/ml)
IL-5 in lung homogenate
(pg/ml)
IL-13 in lung homogenate
(pg/ml)
Veh
GLP-1R agonist
PBS
HDM