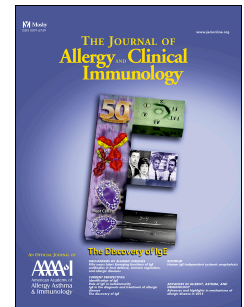


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Ozone exposure induces respiratory barrier biphasic injury and inflammation controlled by Interleukin-33

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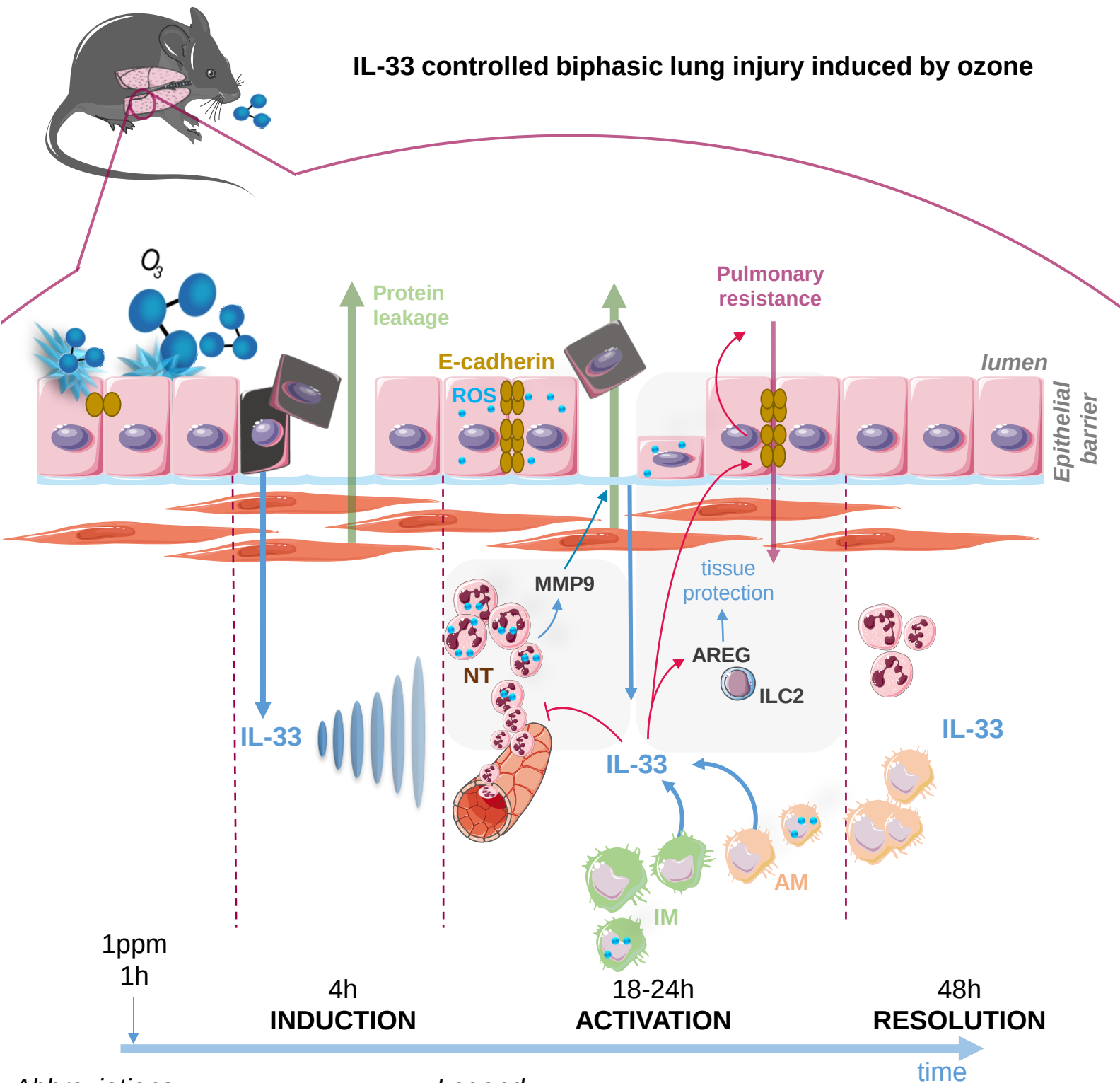
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IL-33 controlled biphasic lung injury induced by ozone



Abbreviations

AM	Alveolar macrophages
IL-33	Interleukine 33
ILC2	Innate lymphoid cells
IM	Interstitial macrophages
MMP-9	Matrix metalloproteinase 9
NT	Neutrophils

Legend

- Activation / Production
- ⊣ Inhibition

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47

48 **Background**

49 IL-33 plays critical role in the regulation of tissue homeostasis, injury and repair. Whether IL-
50 33 regulates neutrophil recruitment and function independently of airways
51 hyperresponsiveness (AHR) in ozone induced lung injury and inflammation is unclear.

52 **Objective**

53 To examine the role of IL-33/ST2 axis in lung inflammation upon acute ozone exposure in
54 mice.

55 **Methods**

56 ST2 and IL-33 deficient mice, IL-33-citrine reporter and C57BL/6 (WT) mice underwent a
57 single ozone exposure (1 ppm for 1h) in all studies. Cell recruitment in lung and broncho-
58 alveolar space, inflammatory parameters, epithelial barrier damage and airway hyperreactivity
59 (AHR) were determined.

60 **Results**

61 We report that a single ozone exposure causes a rapid disruption of the epithelial barrier
62 within 1h followed by second phase of respiratory barrier injury with increased neutrophil
63 recruitment, ROS production, AHR and IL-33 expression in epithelial and myeloid cells in
64 WT mice. In the absence of IL-33 or IL-33R/ST2, epithelial cell injury with protein leak and
65 myeloid cell recruitment and inflammation are further increased, while tight junction proteins
66 E-cadherin and ZO-1, ROS expression in neutrophils and AHR are diminished. ST2
67 neutralization recapitulated the enhanced ozone induced neutrophilic inflammation. However,
68 myeloid cell depletion using GR1 antibody reduced ozone induced lung inflammation,

epithelial cell injury and protein leak, while administration of rmIL-33 reduced neutrophil recruitment in IL-33 deficient mice.

Conclusion

The data demonstrate that ozone causes an immediate barrier injury which precedes myeloid cell mediated inflammatory injury under the control of IL-33/ST2 axis. Thus, IL-33/ST2 signaling is critical for the maintenance of intact epithelial barrier and inflammation.

Capsule Summary

Interleukin-33 has a protective role in ozone induced lung inflammation through its capacity to activate tight junction expression, maintain pulmonary barrier integrity and reduce neutrophil recruitment.

Key Messages

- Immediate lung barrier disruption with upregulation of IL-33 expression precedes inflammation
- Absence of IL-33 or ST2 enhances inflammatory cell recruitment and injury
- ST2 antibody blockade recapitulates the ST2^{-/-} phenotype
- Antibody depletion of neutrophils confers partial protection

Key words

Ozone ; ST2 ; IL-33 ; AHR ; E-cadherin ; inflammation ; neutrophils

91

92 **Abbreviations**

93 AHR : Airway hypereactivity

94 AREG : Amphiregulin

95 BAL : Broncho alveolar lavage

96 GR1 : Ly-6G antibody to neutrophils

97 IL-33 : Interleukin-33

98 CXCL1/KC : Keratinocyte chemoattractant

99 LCN-2 : Lipocalin 2

100 CCL2/MCP-1 : C-C Motif Chemokine ligand 2

101 CXCL2/MIP-2 : Chemokine (C-X-C motif) ligand 2

102 MMP-9 : Matrix Metalloproteinase 9

103 MPO : Myeloperoxidase

104 rmIL-33 : recombinant mouse IL-33

105 ROS : Reactive oxygen species

106 TIMP-1 : Tissue inhibitor metalloproteinase 1

107 WT : Wild type mouse

Introduction

Ozone is a major component of air pollution and causes airways hyperactivity (AHR) independent of adaptive immunity. In fact, ozone inhalation contributes to morbidity and mortality especially in a vulnerable population with asthma or COPD^{1, 2} and is known to be an important trigger of asthma exacerbation^{3, 4}. Ozone causes asthma symptoms, reduces lung function and increases inflammatory cell infiltration and airway hyperresponsiveness. Following days of high environmental ozone exposure, there is an increase of emergency hospital admissions for severe asthma crisis⁵⁻⁷.

Airway epithelium forms a physical barrier which is the first front line defense of mucosal immunity^{8, 9}. The integrity of the epithelial barrier depends not only on tight junctions (TJ) and adherens junctions (AJ), which keep bronchial epithelial cells together and maintain their apicobasal polarity, but also on surface liquid and mucus^{10, 11}. Tight junctions are composed of a number of transmembrane proteins, such as the claudin family, occludin and tricellulin. In addition, several scaffolding proteins, such as zonulae occludens (ZO)-1, ZO-2, ZO-3, multi-PDZ domain protein 1, membrane associated guanylate kinase and cingulin have been identified in the tight junctions^{12, 13}. The expression of E-cadherin, a major adherent junction component is reduced in biopsies from asthmatic patients¹⁴. Disturbed tight junctions function allows the entrance of pathogens, irritants, and allergens into the organism¹⁵. Epithelial barrier is impaired in patients with house dust mite (HDM) induced allergic rhinitis which is associated with lower occludin and ZO-1 expression¹⁶. Ozone exposure impairs the function of crucial molecules that make up the epithelial barrier¹⁷. Ozone-induced lung inflammation in mice, rats and human relies on the production of reactive oxygen species (ROS)¹⁸⁻²⁰ which is characterized by epithelial injury²¹ with disruption of the epithelial barrier, increased inflammatory cytokines, such as IL-17A^{22, 23}, or TNF α ²⁴ and neutrophils recruitment associated with AHR²³ through the activation of toll-like receptors 2 and 4 and

the adaptor protein, MyD88²⁵. Furthermore IL-1 family members are induced and contribute to lung inflammation. IL-1 α and IL-1 β are produced after ozone exposure, *in vivo*²⁶ or *in vitro*²⁷ as recently reviewed²⁸.

IL-33 belongs to IL-1 family cytokines that also includes IL-1 α , IL-1 β , IL-18 and IL-36^{29, 30}. IL-33 binds to ST2 chain known as IL-33R or IL-1RL1³¹ which associates with IL-1RAcP. IL-33 likely plays a role in tissue homeostasis and is released by dying cells upon tissue injury and therefore coined alarmin³². The full length, 35 kDa IL-33 is cleaved by different proteases derived from neutrophils, macrophages or mast cells that process bioactive full length IL-33 to several active forms that have up to 30-fold higher biological activities than the full length form³³⁻³⁵. IL-33 receptor ST2 is expressed abundantly on the cell surface of high endothelial venules in lymphoid tissues in mice³⁶ and most innate immune cells including mast cells³⁷, innate lymphoid cells³⁸, myeloid and dendritic cells³⁹ and to a lesser extent on Th2 cells³⁷. Further, nuclear IL-33 may associate with chromatin *in vivo*³⁶, and may have a transcription factor function.

The alarmin IL-33 is expressed at steady state in tissues and released upon respiratory diseases associated with airway epithelial injury and repair during inflammatory process^{32, 40}. IL-33 bridges innate and adaptive immunity and plays critical roles in the regulation of tissue homeostasis, injury and repair³⁸. Whether IL-33 can regulate neutrophils recruitment and function independently of airways hyper-responsiveness (AHR) is unclear.

Here we report that ozone exposure causes injury of the respiratory epithelium with release of IL-33 which may have a protective effect since lung injury and inflammation are enhanced in the absence of IL-33 or the IL-33R/ST2. However rmIL33 enhanced ozone induced lung inflammation in WT mice, but partially normalized the inflammatory response in IL-33 deficient mice in comparison with WT mice. Depletion of IL-33 expressing myeloid cells attenuates lung injury and inflammation.

Methods

Mice and reagents

ST2-deficient (ST2^{-/-} or IL-1rl1^{-/-}) and IL-33 citrine reporter mice were generated and provided by Andrew N.J. McKenzie (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK)^{41, 42} and IL-33 deficient mice (IL-33^{-/-}) were obtained from Susumu Nakae (Institute of Medical Science, University of Tokyo, Japan)⁴³. Experiments are performed with female mice aged 8-10 weeks.

All gene deficient mice and wild type (WT) littermate controls (C57BL/6J background) were bred and housed in our specific pathogen free animal facility at Transgenesis, Archiving and Animal Models (TAAM- UPS 44, CNRS under agreement D-45-234-6, 2014), Orleans, France. Mice were maintained in a temperature controlled (23°C) facility with a strict 12h light/dark cycles and were given free access to food and water. Animal experiments were performed according to the French Institutional Committee under agreement CLE CCO 2012-047.

The following reagents were used *in vivo* studies:

For the neutralizing ST2, we used rat-IgG1-anti-mouse-ST2 (M955a, obtained from Dirk Smith, Amgen) and isotype IgG1 (BP0088, HRPN, BioXcell), i.p. 200 µg/mouse, 12h and 2h before ozone exposure. To deplete neutrophils, we injected rat-IgG2a-anti-mouse-GR1 (BE0075-1, RB6-8C5, BioXcell) or isotype IgG2a (BR0089, 2A3, BioXcell) at 0,5 mg/mouse 2h before and 6h after ozone exposure. To restore IL-33 we used rmIL-33 (Ser109-Ile266, Biolegend) which was administered by intranasal instillation (i.n.) at 1 µg/mouse 2h before and 6h after ozone exposition.

Ozone induced airway inflammation

Mice were exposed to ozone in a plexiglas chamber (*EMB 104, EMMS®*) at 1 ppm for 1h in all studies as described before ⁴⁴. Ozone is created by ozoniser (*Ozoniser S 500mg, Sander®*) and level of 1.0 ppm was controlled by sensor (*ATI 2-wire transmitter, Analytical Technology®*). Mice were euthanized by progressive CO₂ inhalation until 1h-48h after ozone exposure and BAL was collected. After a cardiac perfusion with ISOTON II (*Acid free balanced electrolyte solution Beckman Coulter, Krefeld, Germany*) lung were collected and sampled for analyses.

Broncho alveolar lavage (BAL)

After ozone exposure, BAL was performed by 4 lavages of lung with 500 µL of saline solution (NaCl 0.9 %) via a cannula introduced into mice trachea. BAL fluids were centrifuged at 2000 rpm for 10 min at 4°C, the supernatants were stored at -20°C for ELISA analysis and pellets were recovered to prepare cytospin (*Thermo scientific, Waltham, USA*) glass slides followed by a staining with Diff-Quik solution (*Merz & Dade A.G., Duding, Switzerland*). Differential cell counts were performed with at least with 400 cells.

Measurement of cytokines and total protein

MPO, LCN-2, MMP9 and TIMP1 in BALF were determined by ELISA (R&D systems, *Abingdon, UK*), while IL-6, CXCL1/KC, CCL2/MCP-1 and CXCL2/MIP-2 from BAL and IL-33 from lung homogenate were measured by Luminex immunoassay using MagPix reader (Bio-Rad) according to the manufacturer's instructions. Data were analyzed with Bio-Plex Manager software (Bio-Rad). Total protein levels in BALF were analyzed with the Bio-Rad DC Protein Assay.

Evans blue leak

Vascular leakage was quantified by protein concentration in the bronchoalveolar space, and Evans blue after intravenous injection of 0.3% of EB measured 45 min later in the supernatant by absorbance at 460 nm as described ⁴⁵.

Histology

Lung tissue was fixed in 4% buffered formaldehyde and paraffin embedded under standard conditions. Tissue sections (3 µm) were stained with haematoxylin and eosin (H&E). Histological changes were determined by a semi-quantitative severity score (0 to 3) for inflammatory cell infiltration and alveolar epithelial injury (0 to 3). The slides were blindly examined by two independent investigators with a Nikon microscope (Nikon eclipse 80i, Country).

FACS analysis

Lungs were removed from mice, minced, digested with DNase (Sigma, 1mg/mL) and Liberase (Roche, 5mg/mL) during 1h at 37°C and red bloods cells were lysed with Lysing buffer (BD Pharm LyseTM - BD Pharmingen). Cells were incubated with the antibodies during 25 minutes at 4°C in FACS buffer (PBS, 2% FCS, 2mM EDTA). The antibodies used are against mouse molecules and described in the table 1 (online supplemental information). Cells were washed with FACS buffer and fixed by lysing buffer 1x (BD Pharmingen). 7-AAD (Pharmingen) was added during the last 5 minutes of incubation. Data were acquired with a flow cytometer (BD FACS Canto II) and analyzed with FlowJo software (TreeStar, Mountain View, CA).

Cellular ROS determination

To quantify ROS positive cells in BAL, cells are incubated at 5 μ M with 2',7'-Dichlorofluorescein diacetate ≥ 97 (H2DCFDA Fisher Scientific). In the presence of H₂O₂, H2DCFDA is cleaved to green fluorescent adduct detected by FACS analysis (Fig. E1).

Immunofluorescence and confocal microscopy

The detailed methods are shown in Online Repository Materials.

Lung function measurement with invasive plethysmography

Airway hyperresponsiveness was measured by using increasing concentrations of methacholine (5-40 mg/mL) using the FinePointe system (Buxco, DSI) as previously described⁴⁶.

Immunoblotting

Lung tissues were homogenized in RIPA buffer (NaCl 0.15M Sigma, EDTA 1mM Sigma, Tris 10mM Sigma, Nonidet p40 1% Sigma, SDS 0.2%, protease inhibitor Roche). The following step and antibodies used for immunoblotting were realized as previously described⁴⁶.

Statistical analysis

Data were analyzed using Prism version 7 (Graphpad Software, San Diego, USA). The non-parametric Mann Withney test or the parametric one-way ANOVA tests with multiple Bonferroni's comparison tests were performed. Values are expressed as mean $\pm$ SEM. Statistical significance was defined at a p-value <0.05 **** <0.0001, *** <0.001, ** <0.01, * <0.05.

Results

1. Biphasic epithelial damage with IL-33 release upon ozone exposure

First, we investigated the time-dependent lung inflammation parameters and cell recruitment after ozone exposure (1 ppm for 1h) in C57BL/6J mice (WT). EpCam⁺ epithelial cells are detected within 1-4h after ozone exposure associated with increased total protein in BALF (**Fig 1, A** and see **Fig E1, A**). Further, IL-6 and the chemokines CXCL1/KC, CXCL2/MIP-2 and amphiregulin (AREG) are increased within 1-6h and further augmented at later time points in BALF (**Fig 1, A** and see **Fig E1, C**). CCL2/MCP-1 and lipocalin 2 (LCN-2) are increased at later time points 18-24h in BALF (**Fig 1, A** and see **Fig E1, C**). Therefore the data suggest an acute respiratory barrier disruption within 1h after ozone exposure.

As evidence of sustained barrier dysfunction, a second increase of total protein at 18-24h BALF is observed (**Fig 1, A**). Neutrophils, MPO, interstitial and alveolar macrophages are increased only between 6h and peaked at 18- 24h (**Fig 1, B**) together with increased TIMP-1 and MMP-9 levels in BALF (**Fig 1, B**). Lymphocytes and eosinophils are increased later at 18-24h (see **Fig E1, B**). Furthermore, an increase of reactive oxygen species (ROS) producing cells, cell death (7-AAD⁺ cells) in BAL (**Fig 1, C** and see **Fig E1, A**) and desquamation of airway epithelial cells as evidenced by E-cadherin staining which is associated to higher inflammation score by histological analysis are observed (**Fig 1, D** and see **Fig E1, E-F**). Therefore, ozone causes a rapid structural and functional damage of the epithelial barrier with protein leak within 1h with increased chemokines followed by a second sustained injury associated with myeloid cell recruitment.

In view of the acute airway injury we asked whether ozone induces the expression and release of the alarmin IL-33. IL-33 protein is detected in lung, significantly increased at 4h and sustained at the later time point 24h-48h upon ozone exposure (**Fig 2, A**). Western blot analysis shows an increased level of full length 35 kDa IL-33 protein 24h after ozone

exposure (**Fig 2, B**). Lung tissue immunostaining reveals IL-33 expressing cells at steady state which increased 24h after ozone exposure representing presumably epithelial and myeloid cells (**Fig 2, C**). We found a significant increase of IL-33 mRNA expression at 24h in the lung upon ozone exposure as compared with air control, while the expression of IL-33R/ST2 is unchanged in lung (see **Fig E1, D**).

We further examined the dose dependence of lung inflammation parameters and cell recruitment in the BAL 24h after ozone exposure (0.3, 1 and 3 ppm for 1h) in C57BL/6J mice (WT). A marked dose-dependent increase of total cell, neutrophils and macrophages, pro-inflammatory cytokines (MPO, LCN-2 and IL-6), chemokines (CXCL1, MCP-1, MIP-2), remodeling parameters (TIMP-1, MMP9 and AREG) and desquamation of airway epithelial cells as evidenced histology analysis was already observed at 0.3 ppm and further increased at 1 ppm (see **Fig E2, A-E**).

To identify the cellular source of IL-33, we used IL-33 citrine reporter mice⁴¹. Lung and BAL cells were characterized using the gating strategy shown in **Fig E1, A**, and IL-33-citrine expression was determined for each cell population at 4h (see **Fig E3, A-C**) and 24h (**Fig 2, D-E** and see **Fig E3, D-E**) after ozone exposure. There was an increase in IL-33-citrine⁺ cells mainly in epithelial cells in BAL and lung at 4h which was sustained at 24h post exposure with an increase in alveolar and interstitial macrophages both in absolute numbers and frequency, but also in MFI, indicating an increased expression of IL-33 per cell (see **Fig E3, E**) in the lung at 24h. Therefore IL-33 expression is induced in macrophages, epithelial cells and neutrophils after ozone exposure, but only alveolar macrophages and epithelial cell IL-33-citrine⁺ positive cells are recruited in the broncho-alveolar space.

Therefore, a single exposure to ozone causes acute lung injury with an increased expression of IL-33 in the lung tissue and recruitment of IL-33⁺ cells in the lung, although extracellularly released IL-33 could not be detected in the BALF.

2. Absence of IL-33 or IL-33R/ST2 enhances lung inflammation

Next, we investigated the role of IL-33/ST2 axis using ST2^{-/-} and IL-33^{-/-} mice. Ozone exposure causes rapid recruitment of total cells and neutrophils in BAL from WT mice at 24h. In the absence of the ST2 receptor and to a lesser extent in the absence of IL-33, neutrophils and macrophages in BAL are further increased (**Fig 3, A**). Consistent with the increased neutrophil numbers in BAL from WT mice, epithelial cell, lymphocyte numbers and overall cell death (7-AAD⁺ cells) were increased in BAL from WT mice, and further in ST2^{-/-} and IL-33^{-/-} mice, while eosinophils seemed reduced (see **Fig E4, A**).

Increased neutrophil recruitment in the absence of ST2 is associated with augmented chemokine CXCL1 (KC), myeloperoxidase (MPO) and lipocalin-2 (LCN-2) expression in BALF, while these parameters were unchanged in BALF from IL-33^{-/-} mice (**Fig 3, B**). Furthermore, MMP-9 is increased in WT mice after ozone exposure and further enhanced in ST2^{-/-} and IL-33^{-/-} mice, while TIMP-1, IL-6 and MCP-1 are not further increased in ST2^{-/-} and IL-33^{-/-} mice (**Fig 3, C** and see **Fig E4, A**). However, MIP-2 is reduced in both ST2 and IL-33 deficient mice, while amphiregulin (AREG) is reduced only in BALF from ST2^{-/-} mice (**Fig 3, C** and see **Fig E4, A**). ROS expressing total cells, alveolar macrophages, neutrophils and epithelial cells and necrotic/apoptotic cells are augmented upon ozone exposure in WT and ST2^{-/-} mice, but were significantly reduced only in neutrophils from IL-33^{-/-} mice (see **Fig E4, B-D**).

To evaluate whether the early barrier disruption is associated with chemokines production and inflammatory cell infiltration in lung, we exposed WT and ST2^{-/-} mice to ozone and found

increased airway inflammation in BALF, 4h after ozone exposure, in ST2^{-/-} mice in comparison with WT mice (see **Fig E4, E**).

Microscopic investigations of lung tissue reveal tissue damage with inflammatory cell recruitment around small airways and alveolar septum upon ozone exposure, which is enhanced in ST2^{-/-} and IL33^{-/-} mice at 24h (**Fig 3, D**).

IL-33 acts on different cell type including dendritic cells³⁹, T_H2¹¹, mast cell⁴⁷, ILC2⁴⁸, and others. IL-33 activates group 2 innate lymphoid cells (ILC2) with the production of IL-5 and IL-13⁴⁷. A recent report demonstrated that ozone activated ILC2, which may mediate airway inflammation and hyperresponsiveness⁴⁸. We do not find any difference in ILC2 frequencies and absolute number upon ozone exposure as compared to air exposed WT mice and no changes in IL-33^{-/-} mice (see **Fig E5, A-B**).

In view of enhanced lung inflammation in ST2 and IL-33 deficient mice, the data suggest that IL-33/ST2 signaling plays a homeostatic role, which is disrupted upon acute ozone induced injury.

3. ST2 blockade enhances airway inflammation in WT mice and rIL-33 in controls neutrophil recruitment IL-33^{-/-} mice

To investigate whether neutralizing ST2 antibody recapitulates enhanced lung inflammation described above in ST2^{-/-}, we used a neutralizing ST2 antibody in ozone exposed WT mice (**Fig 4, A**). Cell recruitment in the BAL was analyzed at 24h after ozone exposure using the cell gating strategy described in **Fig E1, A**. Total cells, neutrophils and macrophages are increased in BAL from WT and isotype control antibody treated mice upon ozone exposure (**Fig 4, B**). By contrast, neutralizing ST2 antibody augmented cell death, epithelial cells, recruitment of neutrophils, CD3ε⁺ T cells in BAL and enhanced tissue injury and inflammation as assessed by lung histopathology analysis (**Fig 4, E** and see **Fig E6, B**).

However, similar CXCL1 (KC), lipocalin-2 (LCN-2), MMP9, TIMP-1, AREG, IL-6 and MIP-2 levels were observed with neutralizing ST2 antibody administration while MPO and MCP-1 increased (**Fig 4, C-D** and see **Fig E6, C**). Similar number and frequency of ILC2 were found in lung after ozone exposure in WT and α ST2 treated mice (see **Fig E5, B**).

The data suggest that IL-33/ST2 axis may have a protective effect as reported in other disease models⁴⁹⁻⁵³. Therefore, we asked whether exogenous rmIL-33 modulates ozone induced inflammation in WT and IL-33 deficient mice (see **Fig E7, A**). Intranasal administration of exogenous rmIL-33 increased ozone-induced inflammation in air and ozone exposed WT mice as reported before³¹ (see **Fig E7, B-D**). However in IL-33^{-/-} mice, rmIL-33 partially reduced neutrophil numbers observed in WT, but did not abrogate heightened neutrophil recruitment in IL-33^{-/-} mice (see **Fig E7, B-D**).

Therefore, the data suggest that IL-33 may have a partial protective effect on ozone-induced lung inflammation.

4. Reactive oxygen species (ROS) expression and the effect of myeloid cell depletion on ozone-induced inflammation

Ozone induces ROS expression in the lung⁵⁴, which is likely involved in the injury and subsequent inflammatory response. Since we demonstrated increased ROS expression in total BAL cells (**Fig 1, C**), we investigated the cellular sources of ROS expression. The gating strategy is shown in **Fig E1, A**. ROS production in BAL is detectable within 18h after ozone exposure and is expressed by epithelial cells, neutrophils and macrophages (see **Fig E8, A**). However, the administration of two different ROS inhibitors, N-acetyl cysteine (NAC) or apocynin, had no effect on acute injury and lung inflammation (see **Fig E8, B**).

Since neutrophils are the main source of ROS, we depleted myeloid cells with GR1 antibody in WT mice exposed to ozone (**Fig 5, A**). GR1 antibody injection depleted total cells and neutrophils (**Fig 5, B**), reduced ROS production in neutrophils, epithelial cells, interstitial macrophages and eosinophils (see **Fig E8, B and D**), but had no effect on ROS production by lymphocytes (see **Fig E8, D**). Furthermore myeloid cell depletion significantly attenuated epithelial desquamation and cell death in BAL (**Fig 5, B**), epithelial cell damage, lung inflammation, protein level and secretion of MPO, MMP-9 and TIMP-1, which are mainly produced by neutrophils, while IL-6, MCP-1, LCN-2 and MIP-2 are unchanged (see **Fig E8, E**). Importantly, GR-1 antibody cell depletion reduced protein leak into the BALF indicating that neutrophils play an important role in ozone mediated disruption of the epithelial barrier (see **Fig E8, F and G**). However, myeloid cell depletion did not modify airways hyperresponsiveness after ozone exposure (**Fig 5, C**). Therefore myeloid and epithelial cells are important sources of ROS and myeloid cell depletion attenuates epithelial cell damage and inflammation in WT mice. ROS expression in neutrophil is IL-33 dependent, but ST2 independent (see **Fig E4, B and C**). Therefore, increased ROS unlikely explains the enhanced lung inflammation in the absence of ST2, but neutrophils are critically involved in ozone-induced pathology.

5. Ozone-induced disruption of epithelial barrier depends on the IL-33/ST2 signaling

Previous studies addressed the role of IL-33/ST2 axis in maintaining of barrier integrity^{38, 40, 55, 56}. In view of the increased protein and enhanced desquamation of epithelial cells in BAL (**Fig 1**) in WT mice, we investigated the integrity of the epithelial barrier by histopathology analysis of small airways from WT and ST2^{-/-} mice. Analysis of the bronchiolar lung

epithelium reveals a single layer of basal cells with no intact epithelial cells (**Fig 6, A**), cell death (see **Fig E9, A**) and epithelial damage after ozone exposure in WT mice. The epithelial injury is augmented and focal with complete denudation of epithelial cells of the airways in the absence of ST2 and in WT mice given neutralizing ST2 antibody (**Fig 4, E** and **Fig 6, A-B**), leading to disruption of the epithelial barrier with increased protein (see **Fig E9, B**) and Evans blue (EB) leakage into the BALF (**Fig 6, C**).

Ozone causes airway hyperreactivity (AHR) as demonstrated before²³. Using invasive plethysmography we find enhanced airways resistance in WT mice, which however is reduced in ST2 deficient mice after ozone exposure in comparison with WT mice (**Fig 6, D**). Reduced airway resistance may be mediated by severe structural damage with loss of function of airway cells including smooth muscle cells or epithelial cells as revealed by morphology and reduced tight junction proteins.

The expression of tight junction proteins is critical for the integrity of the epithelial barrier. We found enhanced expression of E-cadherin (**Fig 6, E** and see **Fig E9, C-D**), zona occludens-1 (ZO-1) (**Fig 6, F** and see **Fig E9, G**) and claudin-4 (Cld-4) (see **Fig E9, F**) by immunofluorescence analysis at 4h or 24h after ozone exposure. However, epithelial E-cadherin, ZO-1 and Cld-4 expression is reduced in ST2^{-/-} mice after ozone exposure (**Fig 6, F**; see **Fig E9, C** and see **Fig E9, F**). Furthermore, we found a reduction of E-cadherin positive cells and fluorescence intensity after ozone exposure in ST2^{-/-} in comparison to WT (**Fig 6, E** and see **Fig E9, D**). Epithelial E-cadherin is also reduced in IL-33^{-/-} mice. Interestingly, administration of rmIL-33 restores E-cadherin expression in IL-33^{-/-} mice consistent with attenuated injury and inflammation (**Fig 6, E**; see **Fig E9, D** and see **Fig E7, D**). At the transcriptional level, we found similar ZO-1 expression and an increased Cld-4 (4h until 6h) or E-Cadherin (at 4h until 48h) expressions after ozone exposure (see **Fig E9, E**).

424 Therefore, IL-33/ST2 signaling may confer a protective effect on the epithelial barrier through
425 its capacity to enhance tight junction function and formation, notably E-cadherin, ZO-1 and
426 Cld-4.

427

428

Discussion

Environmental air pollution plays an important role in chronic respiratory diseases⁵⁷. Ozone is a major air pollutant contributing to the development of allergic and non-allergic asthma and chronic lung diseases such as COPD and emphysema. Recent studies demonstrated the importance of TNF α , IL-1 and IL-17A axis in ozone induced lung inflammation model using gene deficient mice^{22-25, 58, 59}.

This is the first report demonstrating an acute lung epithelium disruption and cell death with the release of IL-33 upon a single ozone exposure (1 ppm for 1h) reminiscent of acute protease or cigarette smoke induced lung injury/inflammation^{46, 60}. We postulated that IL-33/ST2 axis may have a protective effect since genetic absence of IL-33 or ST2 and ST2 antibody blockade in mice augmented lung injury and inflammation.

Previous studies reported a protective effect of exogenous IL-33 in other inflammatory models of lung and systemic diseases such as cerebral malaria⁵⁰, experimental autoimmune encephalomyelitis (EAE)⁵², intestinal mucositis⁶¹, sepsis⁴⁹ and intestinal barrier function^{55, 62}. IL-33 administration induces a Th2 driven inflammation⁶³ and polarization into alternatively activated M2 macrophages, which may confer protection⁶⁴. IL-33 mediated protection could be due to the capacity to modulate IL-17A⁶⁵ production, probably via $\gamma\delta$ T cells⁶⁶, which are one of the main sources of IL-17A in innate immune response.

The time-dependent investigations reveal that ozone causes immediate cell damage at the respiratory barrier with leak of plasma proteins within 1h followed by a second peak of protein at 18-24h in BALF upon ozone exposure. The first phase may be due to direct toxic interaction of ozone with cells of the alveolar and bronchiolar epithelium including endothelial capillary cells, while the second phase is probably driven by activated

macrophages and neutrophils, which are recruited by IL-33 and chemokines released upon the initial cell injury. This biphasic injury and inflammatory response is novel, and requires further investigations.

Since ozone exposure induces ROS expression in the lung and enhances inflammation¹, we thought ROS might be involved in increased barrier permeability, cell death and inflammation. We found a significant increase of ROS positive cells, detectable at 6h and further increase significantly at 18h, mainly produced by neutrophils and epithelial cells. However cellular ROS expression in ST2 deficient mice was similar to WT mice suggesting that ROS is unlikely involved in the increased inflammatory response, since ROS levels are not further increased in the absence of IL-33/ST2 expression. ROS causes mitochondrial and cell injury, release of toxic metabolites and DNA damage²¹. We hypothesized that ROS production is important for epithelial cell death and inflammatory cell recruitment. However, we found that the ROS inhibitors N-acetyl cysteine and apocynin had no effect on acute injury and inflammation consistent with a previous report⁶⁷.

Inflammatory cell recruitment occurs only 6h after ozone exposure with epithelial desquamation, increased CXCL1, CXCL2/MIP-2 and IL-6 production 4h and progressive recruitment of macrophages and neutrophils. At 24h, this response is enhanced in the absence of ST2 or IL-33, where neutrophils and interstitial macrophages are the main recruited cells. Since neutrophils are the major cell population in the lung at 18h and 24h, we investigated their role in the second inflammatory phase upon GR-1 antibody depletion of neutrophils and some myeloid cells. We found a striking reduction of protein leak, myeloid cell recruitment, epithelial cell and tissue damage with reduced MMP9 expression using GR1 antibody to deplete neutrophils in ozone exposed mice. These observations suggest that neutrophils are important for tissue injury and inflammation as reported in other inflammatory models⁶⁸.

Moreover, myeloid cell depletion does not affect airways resistance which is increased upon ozone exposure as reported before²³. Ozone has an irritant effect on the airway cells causing a non-allergic AHR within 24h in wild-type mice which may be due to IL-17A produced by iNKT cells and not Th17 cells²³. Interestingly AHR is reduced in the absence of IL-33/ST2 signaling. This unexpected decrease of AHR may be explained by injured epithelial tight junction proteins that control permeability⁶⁹. Furthermore, enhanced edema and inflammatory cell infiltrates in the small airways together with a likely damage of the smooth muscle cells may contribute to reduce AHR in absence of ST2.

Functional disruption of tight junctions is demonstrated by increased epithelial permeability upon ozone exposure, which further augmented in ST2^{-/-} mice. ROS production and epithelial cells destabilization may contribute to increase injury and permeability⁷⁰. Since ROS production is unchanged in ST2 deficient mice, tight junction regulation by IL-33/ST2 axis could explain in part increased permeability of the respiratory epithelium. In fact, we find increased expression of E-cadherin upon ozone exposure, which is not found in the absence of ST2 likely due to severe epithelial injury. E-cadherin expression is restored by rmIL-33 in IL-33^{-/-} mice, suggesting that IL-33 might control tight junction protein expression and function.

IL-33 is expressed 4h after ozone exposure mainly from airways epithelial cells and from 24h until 48h and can be produced by several cell types as reported before^{40, 41}. At 24h, we show increased IL-33 expression upon ozone exposure in epithelial and myeloid cells such as macrophages and neutrophils in the lung, but in the peribronchiolar area alveolar macrophages and epithelial cell are the main source of IL-33.

IL-33 acts on different cell type like DC³⁹, T_H2¹¹, mast cell⁴⁷, group 2 innate lymphoid cells (ILC2)⁴⁸, and others. IL-33 activates ILC2 with the production of IL-5 and IL-13⁴⁷. A recent report demonstrated that ozone activated ILC2, which may mediate airway inflammation and

hyperresponsiveness⁴⁸. We do not find any difference in ILC2 number upon ozone exposure as compared with air exposed WT mice and no changes with α ST2 injections and in IL-33^{-/-} mice. Absence of detectable Th2 cytokines levels (data not shown) do not support ILC2 activation. Yang Q *et al.*, demonstrated difference between Balb/c and C57BL/6 mice response, notably on eosinophils infiltration and cytokine expression including IL-5. Similar number of ILC2 in air and ozone exposed Balb/c mice was detected, the transfer of activated ILC2s into ILC2 antibody depleted recipient mice restored ozone induced hyperreactivity and IL-5⁴⁸. In addition, ozone induced hyperreactivity and neutrophilia is dependent on IL-13⁷¹, which support that ILC2 are a potent source of IL-13 induced by ozone. By contrast Kumagai *et al.*, found ILC2 cells in nasal mucosa after long term ozone exposure⁷², but their role was not investigated.

The administration of rmIL-33 to air exposed control WT and IL-33^{-/-} mice causes an increase number of neutrophils, interstitial macrophages and epithelial cell. Upon ozone exposure cell recruitment is increased in WT mice, but not further augmented in IL-33^{-/-} mice. The lack of full reversal of IL-33^{-/-} mice by rmIL-33 may be explained by the following facts: first, IL-33 present in lung is composed principally of a full length form, and commercial rmIL-33 is a truncated 20 kDa protein. Second, exogenous rmIL-33 administration cannot reproduce the effects of nuclear IL-33 forms, that is absent in IL-33^{-/-} and likely contribute to ozone induced responses. Further exogenous rmIL-33 administration increase pulmonary soluble ST2, an antagonist, in WT mice, thus this is not seen in IL-33^{-/-} mice (data not shown). Finally, it is possible that the absence of IL-33/ST2 axis or rmIL-33 administration disrupts physiological homeostasis and exogenous rmIL-33 cannot fully restore protective effect.

In conclusion, ozone exposure induces immediate lung barrier injury with protein leak, epithelial cell desquamation and the release of the alarmin IL-33, followed in the later phase

by the recruitment of myeloid cells, notably neutrophils, which enhance respiratory epithelial injury and inflammation. IL-33 is produced by epithelial cells, macrophages and to lesser extent by neutrophils. We show that rmIL-33 induces expression of tight junction proteins and diminishes the neutrophils influx. Neutrophils appear to play an important role in ozone-induced injury and inflammation. Since inflammation is enhanced in IL-33 and ST2 deficient mice, we postulate that endogenous IL-33 is important for the homeostatic control of the respiratory epithelium which may attenuate acute lung inflammation and damage. However the cleaved rmIL-33 20 kDa form restores only partially enhanced neutrophilic inflammation in IL-33 deficient mice.

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Figure legends

Figure 1 : Epithelial damage, chemokines production and cell death in BAL upon ozone exposure

Time course of epithelial cell recruitment, total protein, IL-6 and chemokines (CXCL1, MIP-2 and MCP-1) levels in BALF (**A**). Neutrophils and MPO, macrophages (interstitial and alveolar), TIMP-1 and MMP-9 in BALF (**B**). Total ROS expression and cell death (7AAD⁺) in BAL (**C**). Histological analysis (H&E staining) of the lung tissue for epithelial damage score, scale bar = 100 μ m, arrow indicates desquamation (**D**). The data are from three independent experiments. Values are expressed as mean $\pm$ SEM with n=5-6 mice per group.

Figure 2 : IL-33 expression in lung after ozone exposure

Time course of pulmonary IL-33 protein expression by Luminex (**A**) and cleavage forms by Western blot at 24h in WT (**B**). Immunofluorescence staining of IL-33 24h after ozone exposure, frequency and fluorescence intensity (MFI) of IL-33 positive cells in WT mice, scale bar = 100 μ m (**C**). Absolute numbers of IL-33-citrine⁺ cells in BAL and lung upon ozone exposure using IL-33 citrine reporter mice at 24h (**D, E**). The data are representative of 2 independent experiments. Values are expressed as mean $\pm$ SEM with n=5-6 mice per group.

Figure 3: Ozone-induced lung inflammation is enhanced in IL-33 and ST2 deficient mice 24h after ozone exposure.

Total cell, neutrophils and macrophages (**A**), CXCL1, MPO and lipocalin-2 (LCN-2) (**B**), MMP-9, TIMP-1 and amphiregulin in BALF of WT, ST2^{-/-} and IL33^{-/-} mice (**C**). Histological analysis (H&E staining) of the lung tissue and cell infiltration in peribronchial area and epithelial damage score in ST2^{-/-} or IL-33^{-/-} mice, scale bar = 100 μ m, arrow indicates cell

infiltration (**D**). Data are pooled from 4 experiments. Values are expressed as mean $\pm$ SEM with 4-6 mice per group.

Figure 4: ST2 antibody neutralization enhanced inflammatory cell recruitment 24h after ozone exposure in WT mice

Anti-ST2 or isotype control antibody treatment study scheme (**A**). Total cell, neutrophils and macrophages (**B**). Anti-ST2 or isotype control antibody treatment study scheme (**A**). Total cell, neutrophils and macrophages (**B**), CXCL1, MPO and lipocalin-2 (LCN-2) (**C**), MMP-9, TIMP-1 and amphiregulin in BALF from WT mice (**D**). Histological analysis (H&E staining) with cell infiltration in peribronchial area and epithelial damage score from the lung of WT mice treated with ST2 neutralizing antibody, scale bar = 100 μ m, arrow indicates desquamation and semi-quantitative score (**E**). Experiments are pooled of 2 experiments and repeated twice. Values are expressed as mean $\pm$ SEM with 5-6 mice per group.

Figure 5 : Neutrophils depletion attenuate inflammation

GR-1 antibody administration study scheme (**A**), effect of GR-1 depletion on total cell, neutrophils, macrophages, desquamation (epithelial cell) and cell death (7-AAD⁺) (**B**), and airways hyperresponsiveness to increasing dose of methacholine (**C**). Data are pooled from two experiments. Values are expressed as mean $\pm$ SEM with n=5-6 mice for air and ozone.

Figure 6 : Ozone induced disruption of epithelial barrier is mediated by IL-33/ST2 signaling at 24h

Histological analysis (H&E staining) of lung sections from WT and ST2^{-/-} mice, scale bar = 100 μ m, arrow = desquamation and injury score (**A**), epithelial desquamation (**B**), epithelial barrier permeability with Evans Blue leakage at 24h into the BALF (**C**) and airway resistance (**D**) in WT and ST2^{-/-} mice. E-cadherin expression in BAL from rIL-33 treated WT, ST2^{-/-} and

787 IL-33^{-/-} mice at 24h after exposure (**E**). Experiments data are pooled from 2 experiments.
 788 Values are expressed as mean $\pm$ SEM with 4-6 mice per group. ZO-1 expression after Ozone
 789 exposure at 24h in WT and ST2^{-/-} mice (**F**). Representative images of one experiment are
 790 shown in the upper panel. Quantification of ZO-1 expression, depicted in the lower panel, is
 791 shown as a mean of fluorescence intensity from three independent experiments.

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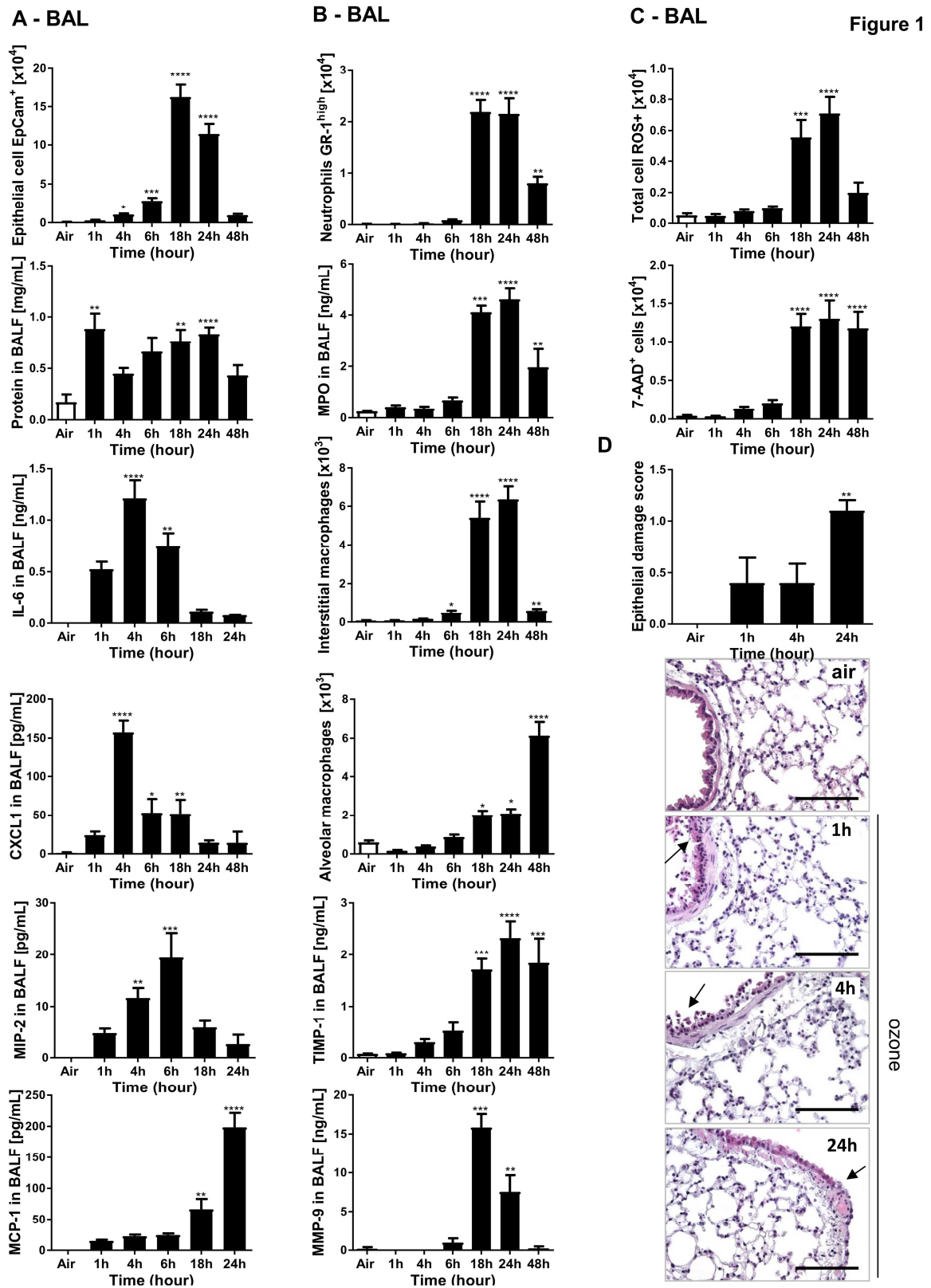


Figure 2

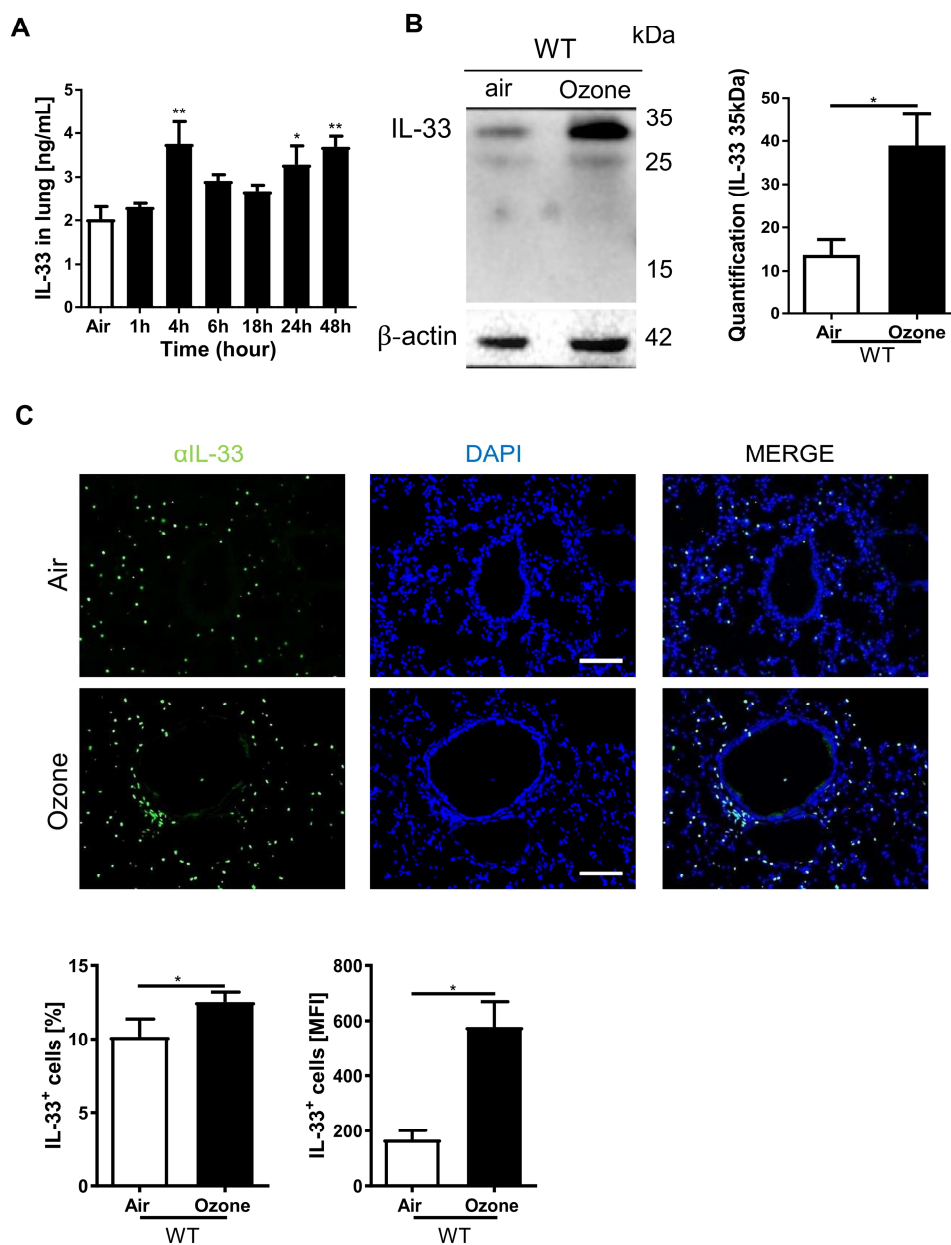


Figure 2

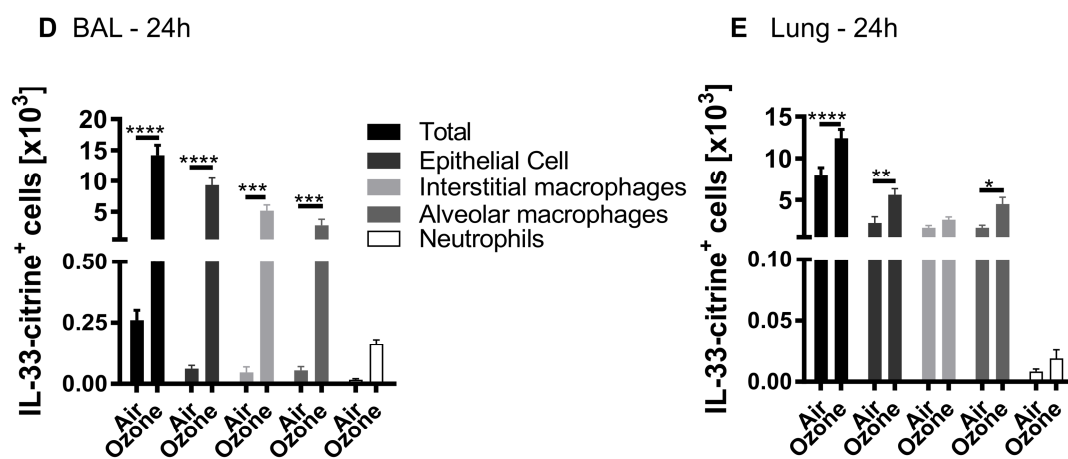


Figure 3

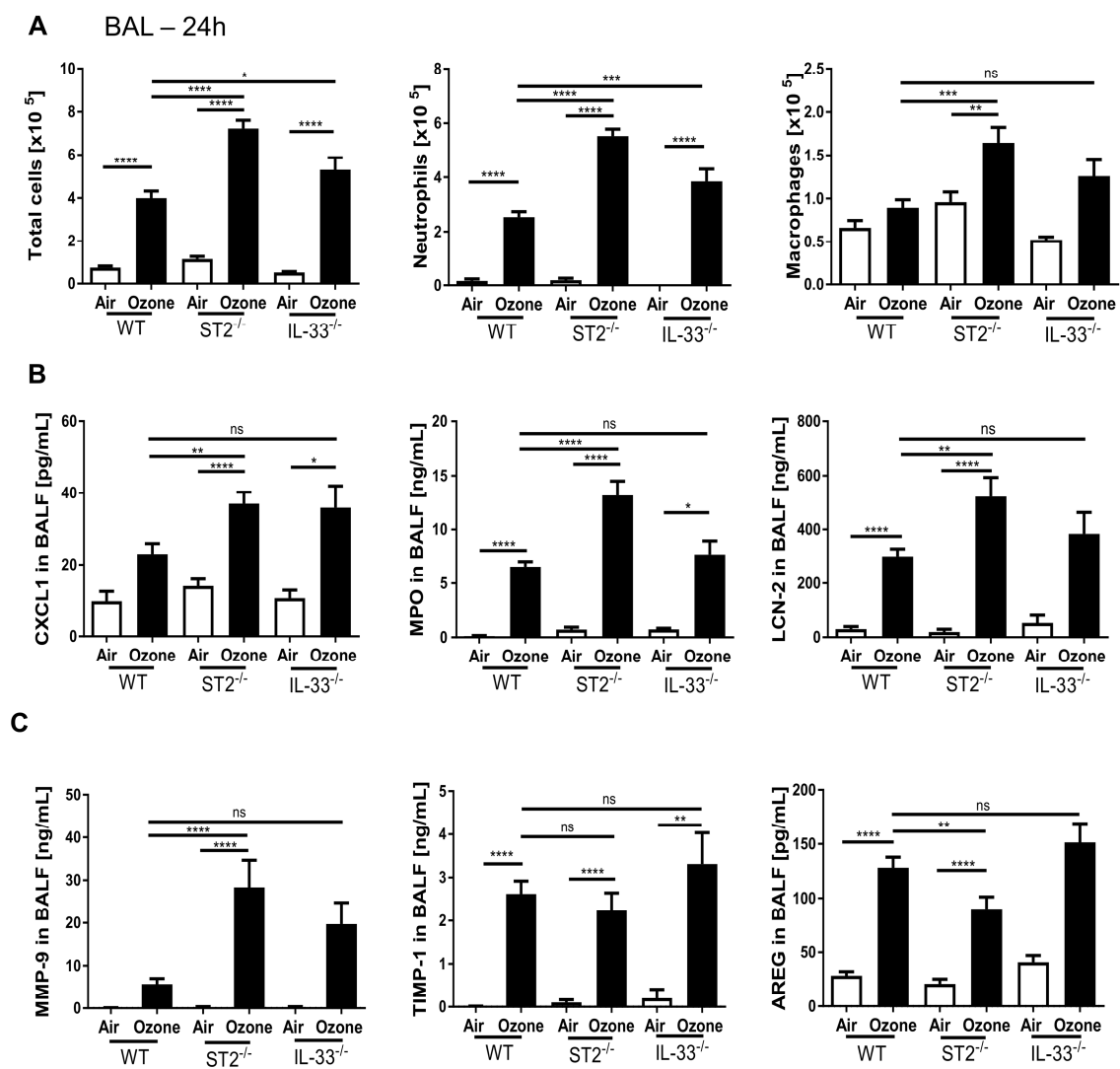


Figure 3

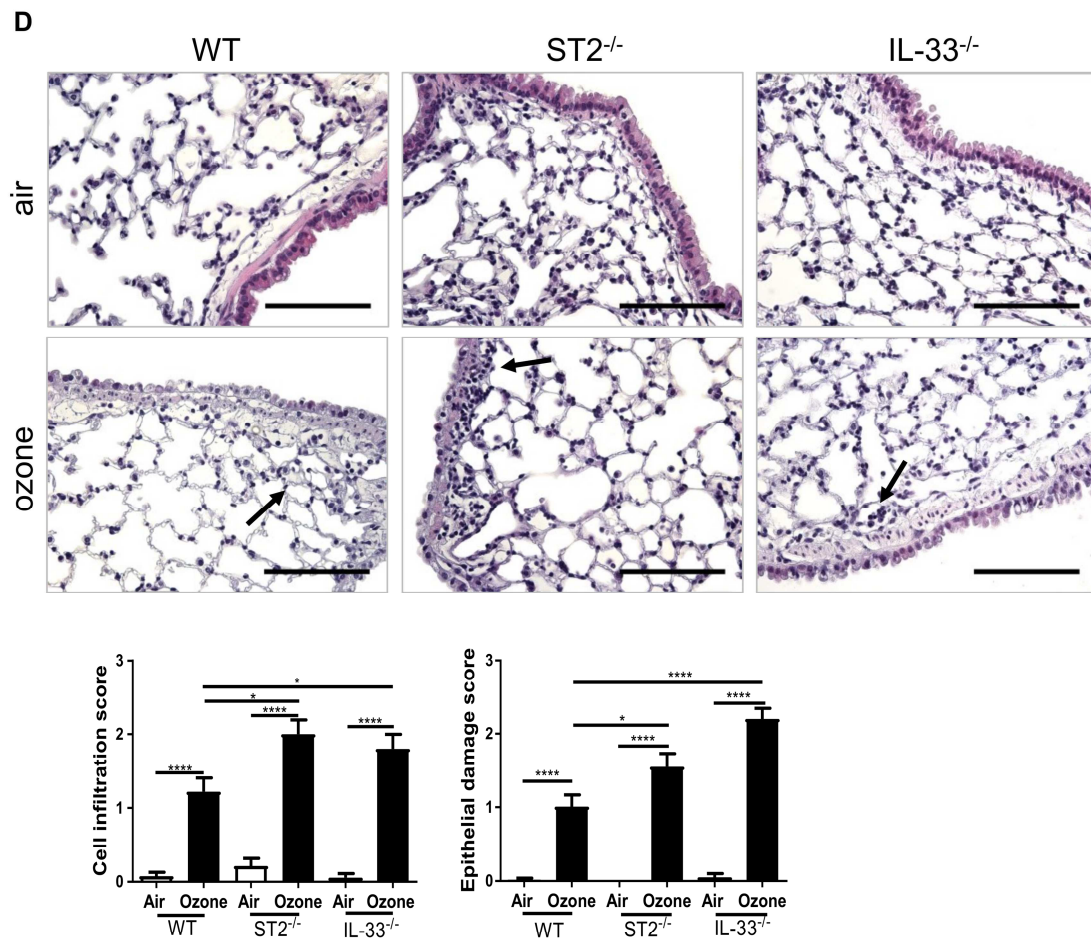


Figure 4

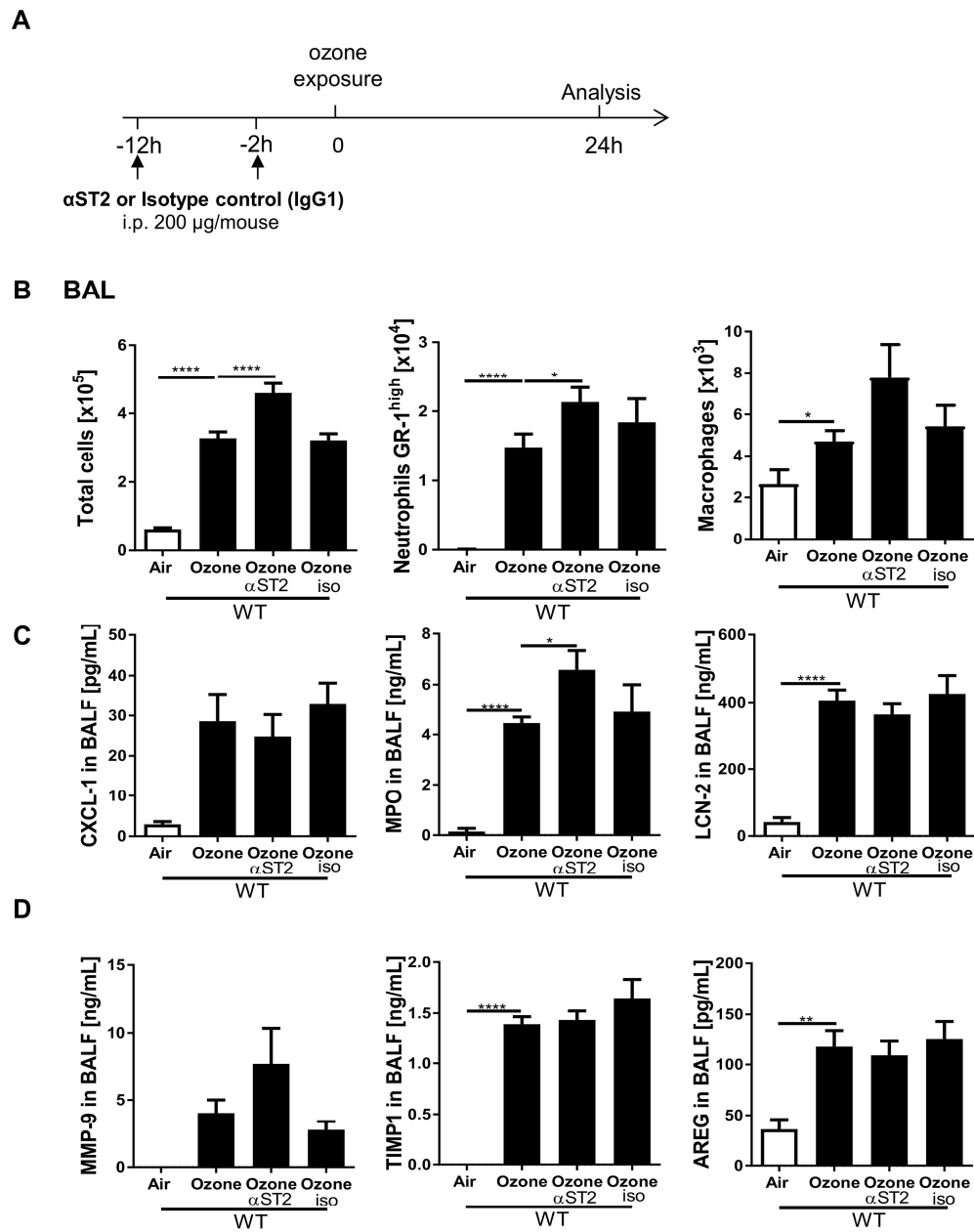


Figure 4

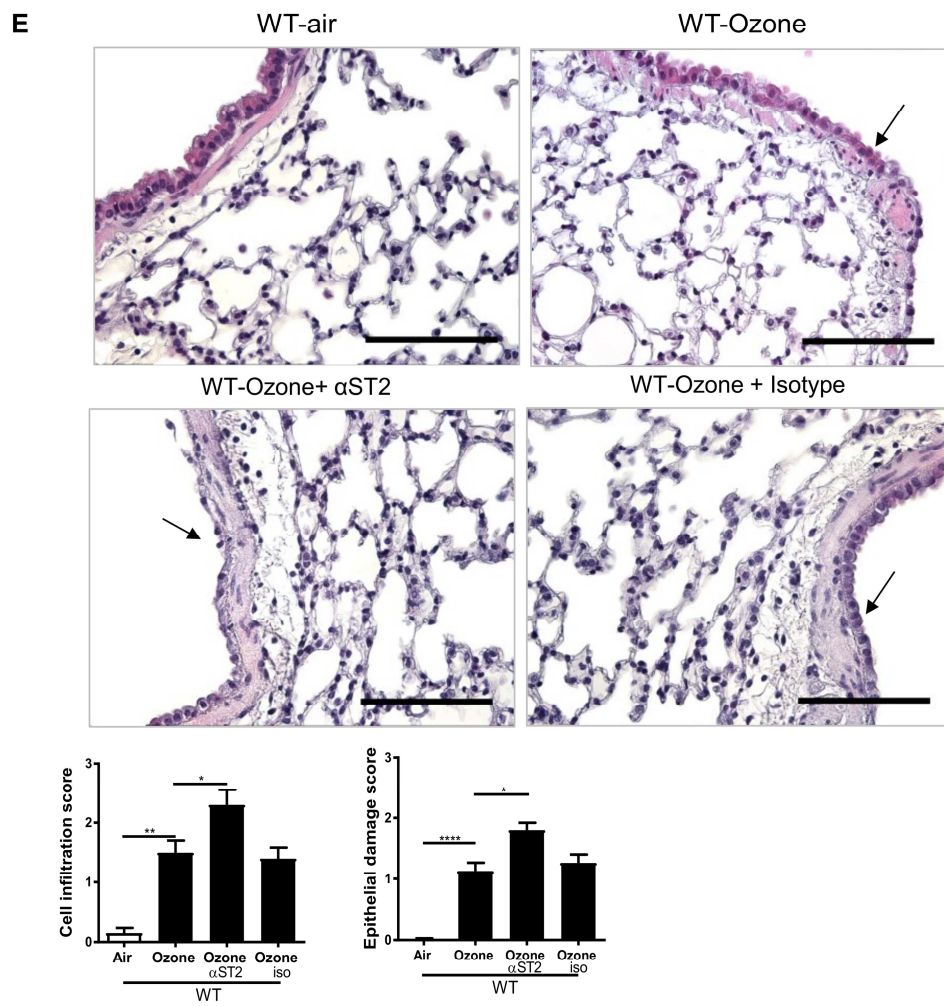


Figure 5

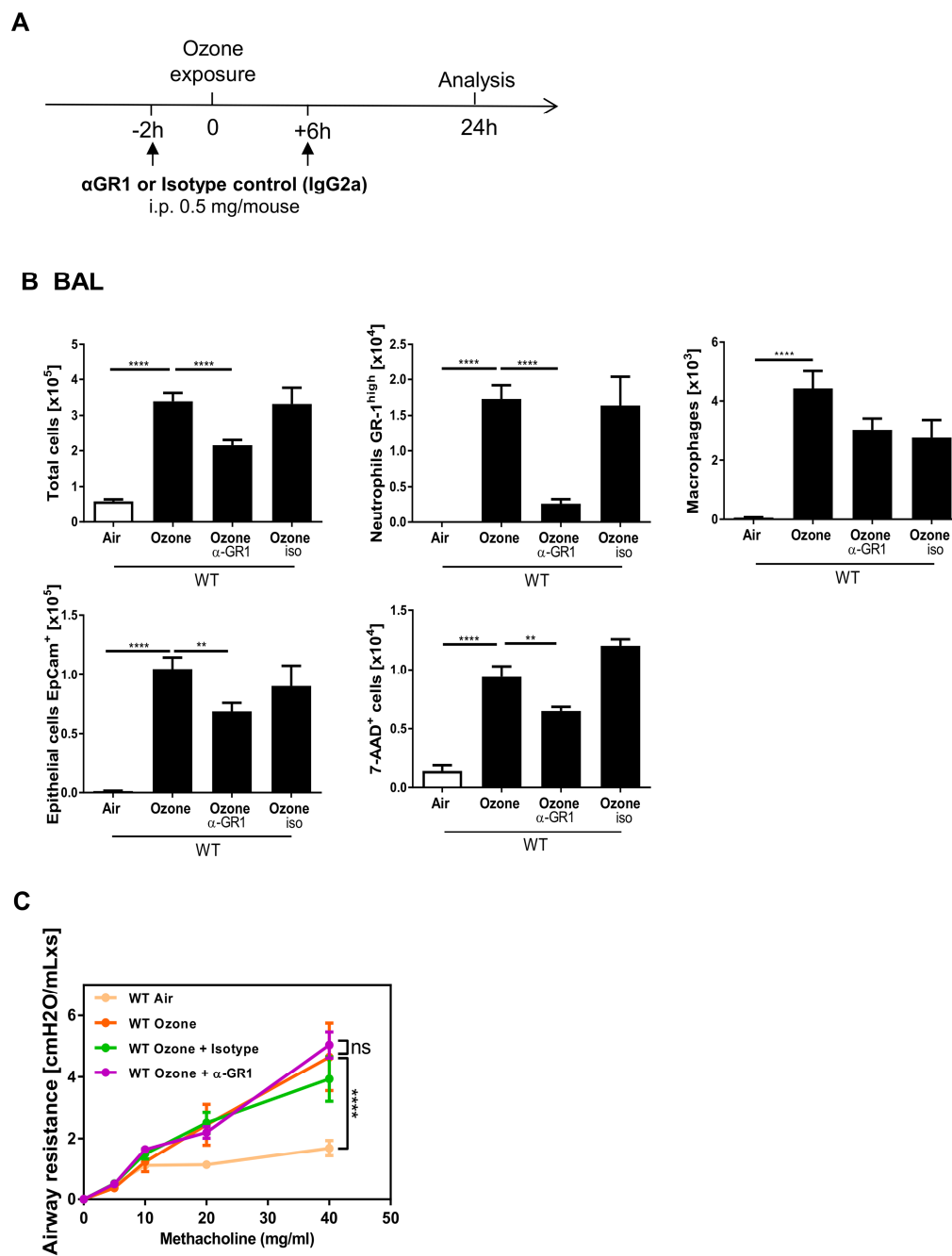


Figure 6

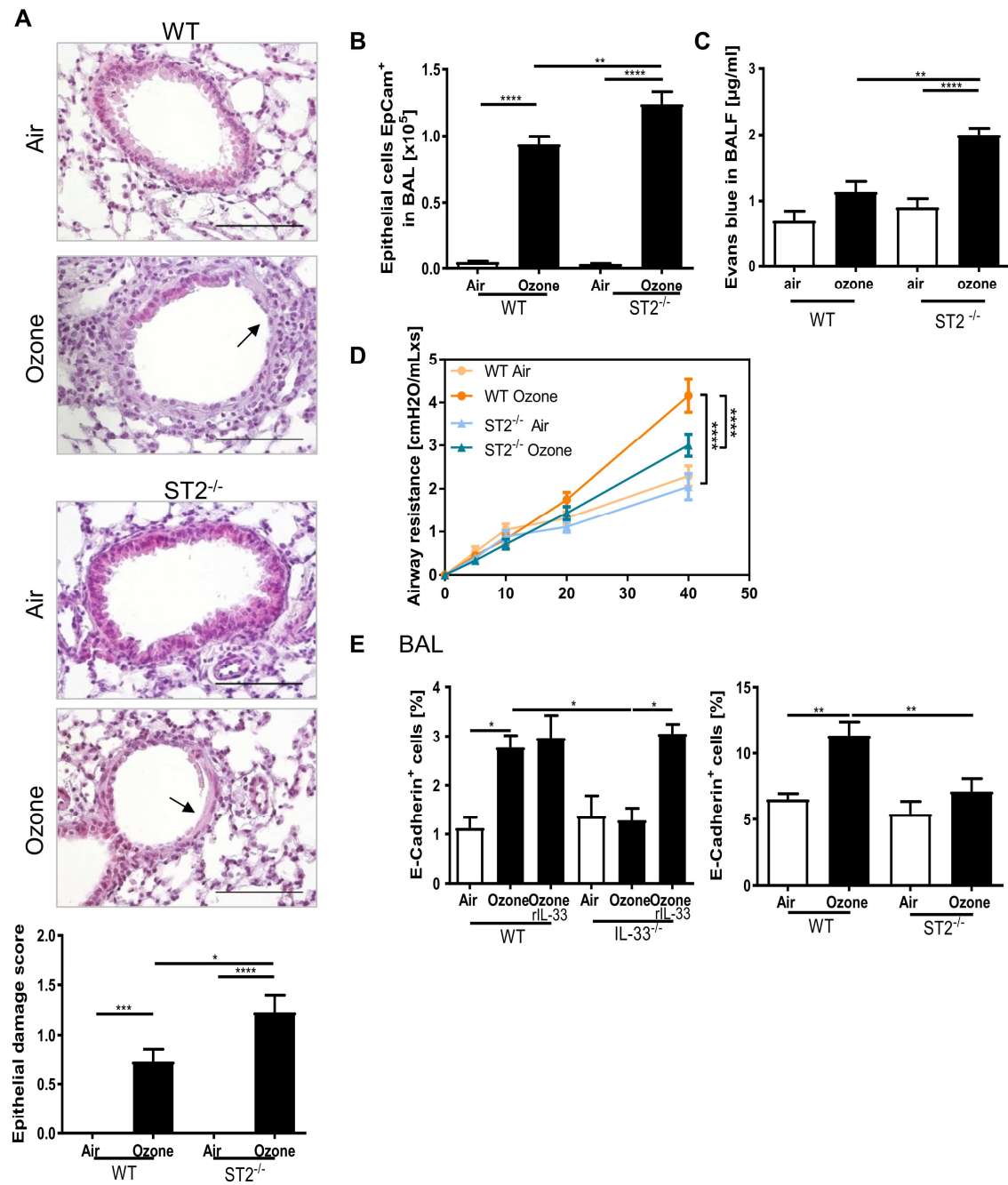
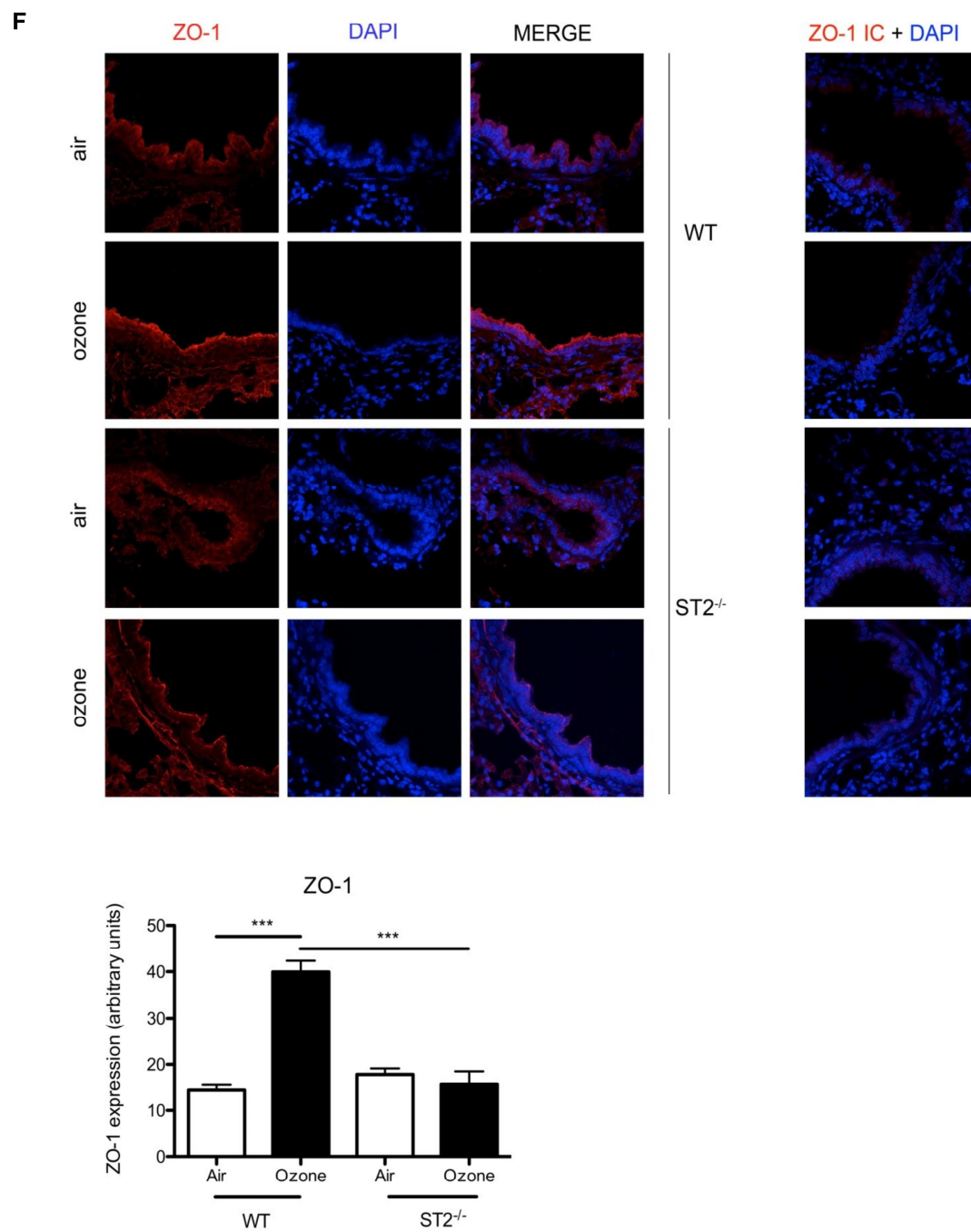


Figure 6



[Supplementary Materials]

**Ozone exposure induces respiratory barrier biphasic injury and
inflammation controlled by Interleukin-33**

Chloé Michaudel, MSc,^a Claire Mackowiak, MSc,^a Isabelle Maillet, BSc,^a Louis Fauconnier, MSc,^b
Cezmi Akdis, MD,^c Milena Sokolowska, MD, PhD,^c Anita Dreher, MSc,^c Hern-Tze Tina Tan, PhD,^{c,d}
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Supplementary Methods (pages 2-5)

Supplementary Figures Legend (pages 6-9)

Supplementary methods

RNA extraction, reverse transcription and Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Sigma) and RNeasy Mini Kit (Qiagen) according to the manufacturer's protocols. Total RNA (1 µg) was reverse transcribed using GoScript™ Reverse Transcription System (Promega). The mRNA levels for genes of interest were examined by quantitative RT-PCR using the GoTaq® qPCR Master Mix according to the manufacturer's protocol. The primer sequences used were: IL-33 (QT00135170), ST2 (IL-1RL1; QT01063062). Relative levels of mRNA expression were normalized to HPRT1 mRNA levels using a comparative method ($2^{-\Delta Ct}$). Non-reverse-transcribed RNA samples and water were included as negative controls.

ROS inhibition with NAC and apocynin

ROS were inhibited by intraperitoneal (i.p.) injections of NAC (Sigma) at 20 mg/kg diluted in PBS and apocynin (Abcam) at 5 mg/kg in DMSO 0.1% by i.p. 1h before and 1h after ozone exposure. Mice were analyzed 24h after ozone exposure.

Immunofluorescence and confocal microscopy

Lungs were treated with paraformaldehyde (PFA) perfusion and tissues were fixed during 3 days in PFA 4% and transferred in sucrose 20% during two weeks. Half of right lungs was included in tissue teck® O.C.T (4583, tissue teck®) and frozen tissue was cut by cryomicrotome (Leica) at 10 µm at -20°C. For immunofluorescence, slides with lungs sections were incubated 30 min in citrate buffer at 80°C. Slides were washed with PBS 1x after all steps followed by 45 min incubation in saturation medium (TBS1X, 1%BSA, 10%

SVF, 0.3% triton x100). The following primary antibodies were used: mouse-anti-mouse-E-cadherin (1 μ g/ml, ab76055, Abcam), -anti-mouse IgM Alexa Fluor 488 (4 μ g/ml, A-21042, Invitrogen) and goat-anti-mouse-IL-33 (2 μ g/mL, AF3626, R&D Systems). After washing, slides were treated with pontamin (0.05%, Chicago sky Blue 6B, Sigma) during 15 min. After washing (PBS 1x), slides were incubated with secondary antibody, rabbit-anti-goat (20 μ g/mL, ab150141 Abcam) during 1 h at room temperature. After washing, slides were incubated with DAPI or DRAQ5 (Fisher Scientific) 1/5000 dilution for 10 min, washed and mounted in fluoromount® (0100-01, SouthernBiotech). Lung sections were observed on a fluorescence microscope Leica (Leica®, CTR6000) at x 200 magnification for counting or x 400 magnification for microphotography and measure of fluorescence intensity (MFI).

For confocal microscopy, after citrate buffer treatment, the slides were washed with 1x TBS, followed by 20 min incubation with blocking buffer (10% goat serum, 1% BSA, 0.2% TritonX 100 in 1x TBS). The slides were washed twice in TBS buffer before 2.5 h incubation with primary antibody. The following primary antibodies were used: rabbit anti-ZO-1 (5 μ g/ml, 40-2200, Invitrogen) and the isotype control rabbit IgG (5 μ g/ml, X0936, Dako) or anti-Claudin 4 (4 μ g/ml, ab15104, Abcam), the isotype control rabbit IgG (5 μ g/ml, X0936, Dako) and the secondary antibody goat anti-rabbit IgG Alexa Fluor 546 (0.5 μ g/mL, A11035, Invitrogen). After washing (three times) with TBS-Tween 20 the slides were incubated with the secondary antibody goat anti-rabbit IgG Alexa Fluor 546 (0.5 μ g/ml, A11035, Invitrogen) for 30 min at room temperature. Following three times washing with 1x TBS-Tween the slides were fixed for three min with 1% PFA in PBS and a final wash for 10 min in TBS was performed before mounting with ProLong Gold antifade reagent with DAPI (P36935, Molecular Probes). The slides were incubated for 24 h at room temperature before analysis on the confocal microscope (Zeiss LSM780). Approximately 10 z-stacks have been obtained per picture. Quantification was performed after combining all z-stacks at the maximum

fluorescence intensity mode, by measuring fluorescence intensity at 10 randomly chosen areas of the similar size within epithelium and subtraction of fluorescence intensity from the isotype control.

FACS analysis

Apoptosis and necrosis are evaluated with AnnexinV-FITC with Annexin V, 7-AAD and binding buffer from BD Pharmingen, according to the manufacturer's instructions.

Other antibodies are used for figures (Table 1) and supplementary figures (Table 2) in extracellular staining for FACS:

Table 1 :

Antibodies	Clones	Concentrations	Supplier
Anti-EpCAM-APC	G8.8	2 µg/mL	BD Pharmingen
anti-GR1-PECy7	RB6-8C5	0.02 µg/mL	BD Pharmingen
anti-CD11c-APC-Cy7	HL3	2 µg/mL	BD Pharmingen
anti-CD4-V500	RMA-4-5	2 µg/mL	BD Pharmingen
anti-CD3ε-PE	145-2C11	2 µg/mL	BD Pharmingen
anti-F4/80-V450	BM8	2 µg/mL	eBioscience
anti-CD11c-APC-Cy7	MEC13.3	2 µg/mL	BD Pharmingen
anti-E-cadherin-PE	DECMA-1	2 µg/mL	Biolegend
Anti-CD45-PE	RA3-6B2	2 µg/mL	BD Pharmingen

Table 2 :

Antibodies	Clone	Concentrations	Supplier
anti-CD3ϵ-PerCPCy5.5	145-2C11	1 μ g/mL	BD Pharmingen
anti-FcER1-PE	MAR-1	1 μ g/mL	eBioscience
anti-B220-PE	RA3-6B2	1 μ g/mL	BD Pharmingen
Anti-Siglec-F-PE	E50-2440	1 μ g/mL	BD Pharmingen
Anti-CD11b-PE	M1/70	1 μ g/mL	BD Pharmingen
Anti-T1/ST2-FITC	7E.17G9	1 μ g/mL	MdBiosciences
anti-iCOS-PE-Cy7	7E.17G9	1 μ g/mL	eBioscience
anti-CD45-APC	30-F11	1 μ g/mL	eBioscience
anti-CD4-V500	RMA-4-5	2 μ g/mL	BD Biosciences
Anti-EpCAM-APC	G8.8	2 μ g/mL	BD Pharmingen
anti-GR1-PECy7	RB6-8C5	0.02 μ g/mL	BD Pharmingen
anti-CD11c-APC-Cy7	HL3-BD	2 μ g/mL	BD Pharmingen
anti-CD8α-APC-Cy7	53-6.7	2 μ g/mL	BD Pharmingen
anti-CD3ϵ-PE	145-2C11	2 μ g/mL	BD Pharmingen
anti-F4/80-eFluor450	BM8	2 μ g/mL	eBioscience
anti-CD11c-APC-Cy7	MEC13.3	2 μ g/mL	BD Pharmingen
anti-E-cadherin-PE	DECMA-1	2 μ g/mL	Biolegend
Anti-CD45-PE	30-F11	2 μ g/mL	BD Pharmingen

Supplementary data – Figure legend

Table 1 : Antibody used for FACS staining in figures

Table 2 : Antibody used for FACS staining in supplementary figures

Figure E1: Time-dependent ozone exposure increased ROS⁺ inflammatory cells and epithelial damage

A, Gating strategy for ROS production and cell differentiation in BAL at 24h after ozone exposure. Leukocytes (CD45⁺), neutrophils (GR-1⁺), T lymphocytes (CD3ε⁺), cell death (7AAD⁺), interstitial (F4/80⁺, CD11c⁻) and alveolar (F4/80⁺, CD11c⁺) macrophages, epithelial cells (EpCam⁺) and ROS expression measured by H2DCFDA. **B,** lymphocyte and eosinophil numbers in BALF; **C,** LCN-2 and AREG levels in BALF. **D,** IL-33 and ST2 gene expressions after ozone exposure. **E,** Time course of cell infiltration score in lung after ozone exposure. **F,** Immunofluorescence staining of E-cadherin 4h after ozone exposure showing epithelial damage together with DRAG5 nuclear staining; representative images and quantification of E-Cadherin depicted in the lower panel, were shown. Experiments are repeated twice. Data are expressed as mean ± SEM with 5-6 mice per group.

Figure E2: Dose response of ozone exposure induced lung inflammation and epithelial damage

A, Total cells, neutrophils and macrophages, **B,** CXCL1, MPO and lipocalin-2 (LCN-2), **C,** MMP-9, TIMP-1 and amphiregulin (AREG), **D,** IL-6, CCL2 (MCP-1) and CXCL2 (MIP-2) in BALF of WT mice and **E,** (H&E staining) of the lung tissue for epithelial injury score, scale bar = 100 μm, arrow indicates desquamation, 24h after 0.3, 1 or 3 ppm ozone exposure. Data are expressed as mean ± SEM with 5 mice per group.

Figure E3: IL-33 expressing cells after 4h or 24h ozone exposure

A, Gating strategy for IL-33 expression in BAL and lung tissue from citrine reporter mice exposed to ozone. Leukocytes (CD45⁺), neutrophils (GR-1⁺), epithelial cells (EpCam⁺), interstitial (F4/80⁺CD11c⁻) and alveolar (F4/80⁺CD11c⁺) macrophages,. **B-C**, Absolute number, frequency and MFI of IL-33 citrine⁺ cells in BAL and lung 4h after ozone exposure. **D**, frequency of IL-33 citrine⁺ cells in BAL; **E**, MFI and frequency of IL-33 citrine⁺ cells in lung at 24h after ozone exposure. Experiments are repeated twice. Data are expressed as mean $\pm$ SEM with 5-6 mice per group.

Figure E4: Inflammatory cell recruitment and death, cytokines and ROS production in ST2^{-/-} or IL-33^{-/-} mice

A, Lymphocyte and eosinophil numbers in BAL, IL-6, MCP-1 and MIP-2 levels in BAL, epithelial cells (EpCam⁺) and cell death (7 AAD⁺) in BAL24h after ozone exposure from WT, ST2 and IL-33 deficient mice. **B-C**, ROS production by different cell populations in BAL by FACS 24h after ozone exposure in ST2^{-/-} and IL-33^{-/-} mice. **D**, cells death (necrosis and apoptosis) from BAL cells 24h after ozone exposure of WT, IL-33^{-/-} and ST2^{-/-} mice by FACS (Annexin V and 7-AAD). **E**, Total cells, neutrophil and macrophage numbers, CXCL-1, MPO, LCN2, IL-6, MCP-1 and MIP-2, levels in BAL 4h after ozone exposure, from WT and ST2^{-/-}. Experiments are repeated twice. Data are expressed as mean $\pm$ SEM with 5-6 mice per group.

Figure E5: ILC2 recruitment after ozone exposure

A, Gating strategy for innate lymphoid type 2 cells (ILC2: CD45⁺ lin⁻ CD3 ϵ ⁻ ICOS⁺ ST2⁺) by FACS. **B**, ILC2 absolute numbers and frequency 24h after ozone exposure in lung from WT,

IL-33^{-/-} mice or after ST2 antibody injections. Experiments are pooled of 2 independent experiments. Values are expressed as mean $\pm$ SEM with 5-6 mice per group.

Figure E6: Effect of ST2 neutralization in WT mice on inflammatory parameters

A, Lymphocyte (CD3 ϵ ⁺CD4⁺ or CD3 ϵ ⁺CD4⁻) and eosinophil numbers in BAL, 24h after ozone exposure. **B**, epithelial cells (EpCam⁺) and cell death (7-AAD⁺ cells) in BAL, 24h after ozone exposure. **C**, IL-6, MCP-1 and MIP-2 levels 24h after ozone exposure in WT mice injected with α ST2. Experiments are repeated twice. Data are expressed as mean $\pm$ SEM with 5-6 mice per group.

Figure E7: Effect of rmIL-33 administration on inflammation in WT and IL-33^{-/-} mice

A, Intranasal (i.n.) recombinant mouse IL-33 (rmIL-33) administration scheme. **B**, Effect of rmIL-33 in WT and IL-33^{-/-} mice on cell recruitment in BAL and **C**, lung inflammation and epithelial damage scores assessed by histology analysis (H&E staining), scale bar = 100 μ m, arrow indicates desquamation. **D**, Effect of intranasal administration of rmIL-33 in air exposed mice. Cell recruitment and E-cadherin expression in BAL 24h after rmIL-33 administration in air control WT and IL-33^{-/-} mice by FACS. Experiments are pooled of 2 independent experiments. Values are expressed as mean $\pm$ SEM with 5-6 mice per group.

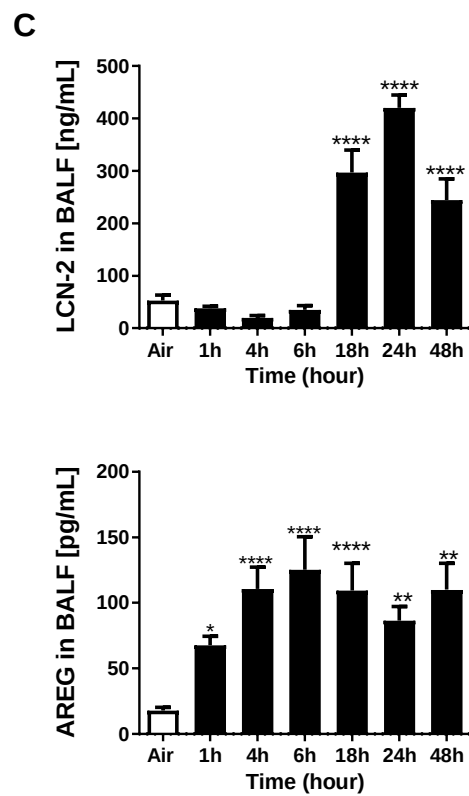
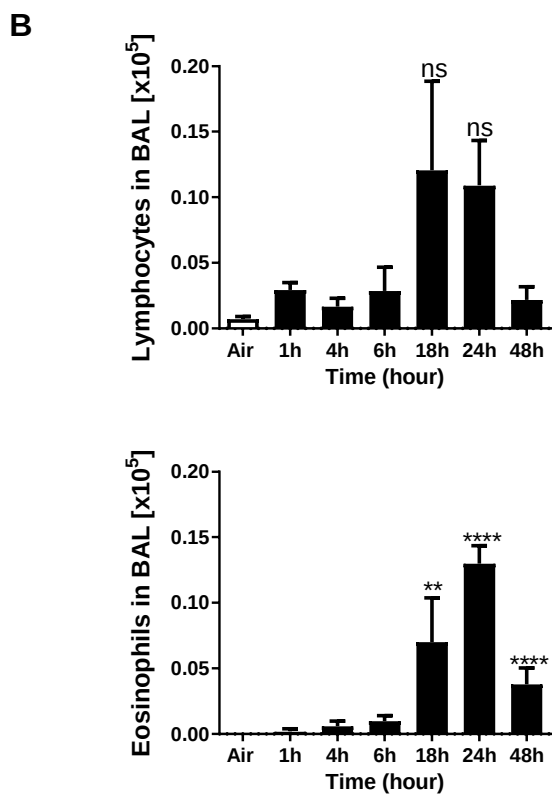
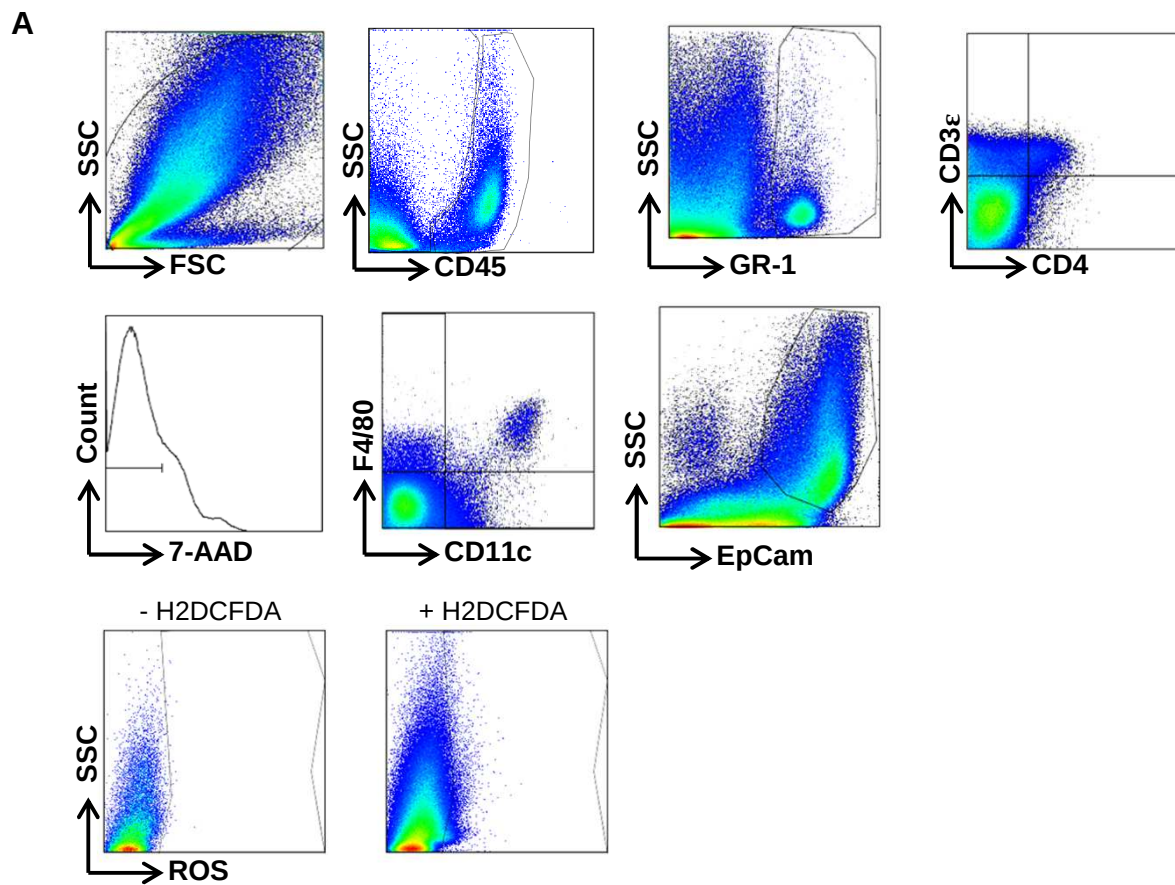
Figure E8: Effect of ROS inhibitors on cell recruitment in lung and protein release after myeloid cells depletion

A, time-dependent ROS expressing cells and epithelial damage in BAL after ozone exposure. **B**, effect of ROS inhibitors on cell recruitment in BAL at 24h after ozone exposure. Cell numbers in BAL by FACS after N-acetyl cysteine (NAC) or apocynin (Apo) injections. **C**,

effect of myeloid cells depletion on ROS expressing cells; **D**, lymphocyte and eosinophil numbers; **E**, CXCL1, MPO, LCN-2, MMP9, TIMP-1, AREG, IL-6, MCP-1, and MIP-2, **F**, total protein, levels in BALF and **G**, lung injury and inflammation by histological analysis with semi-quantitative score, scale bar = 100 μ m, arrow indicates desquamation. Experiments are pooled of 2 independent experiments. Values are expressed as mean $\pm$ SEM with 5-6 mice per group.

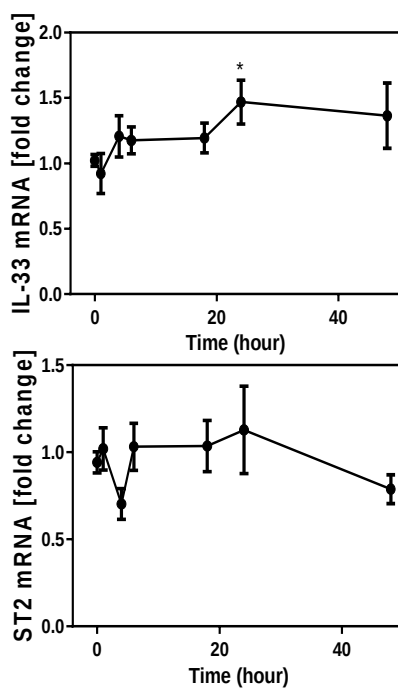
Figure E9: Tissue damage, protein leakage and tight junction expression after ozone exposure **A**, Cell death (7-AAD⁺ cells), **B**, total protein level in BALF, **C**, E-Cadherin expression by immunofluorescence or **D**, mean fluorescence intensity (MFI) by FACS, **E**, time dependent mRNA expression of tight junctions (ZO-1, Cld-4 and E-Cadherin) in WT, IL-33^{-/-}, ST2^{-/-} mice or rmIL-33 administration, and **F**, Claudin-4 (CLD-4) protein expression after ozone exposure, at 24h in WT, and ST2^{-/-} mice and Claudin-4 isotype control (CLD4 IC). **G**, ZO-1 expression in WT and ST2^{-/-} mice 4h after ozone exposure. Representative images of one experiment are shown. Quantification of tight junction expressions is shown as a mean of fluorescence intensity from three independent experiments. Experiments are repeated twice. Values are expressed as mean $\pm$ SEM with 5-6 mice per group.

Supplementary Figure E1

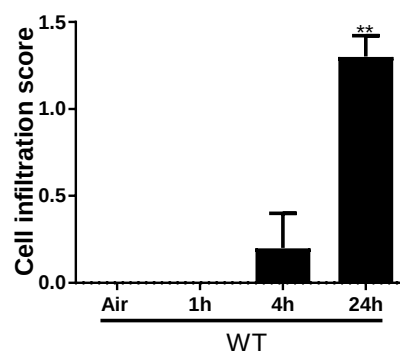


Supplementary Figure E1

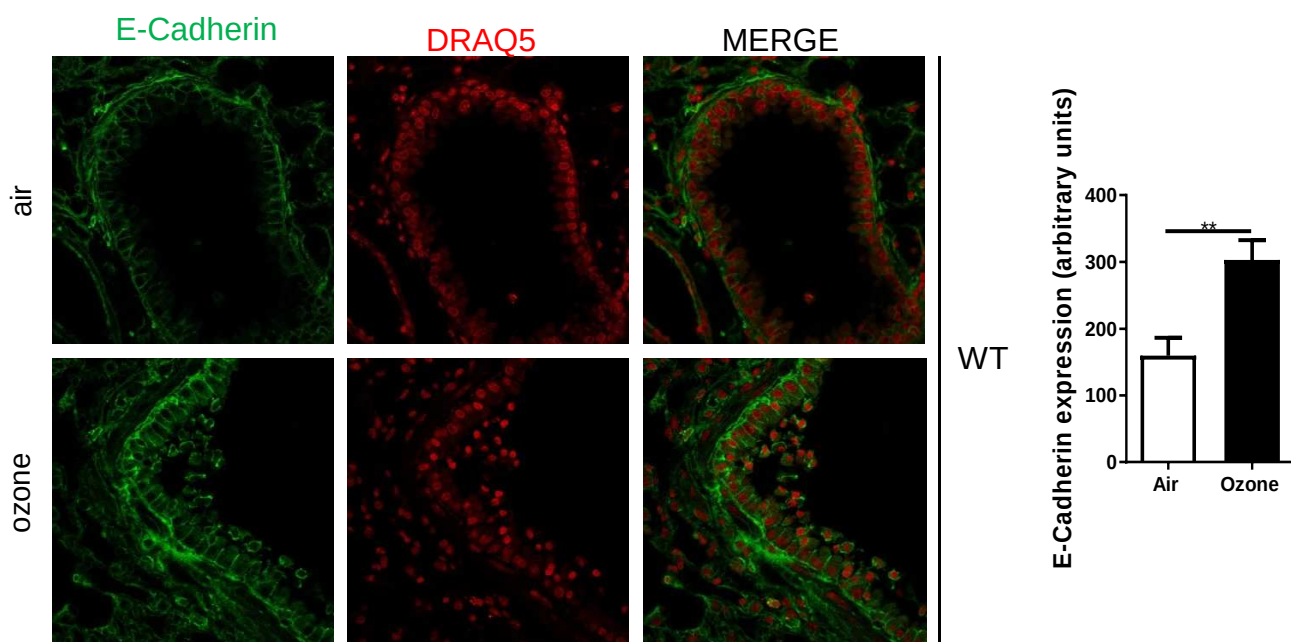
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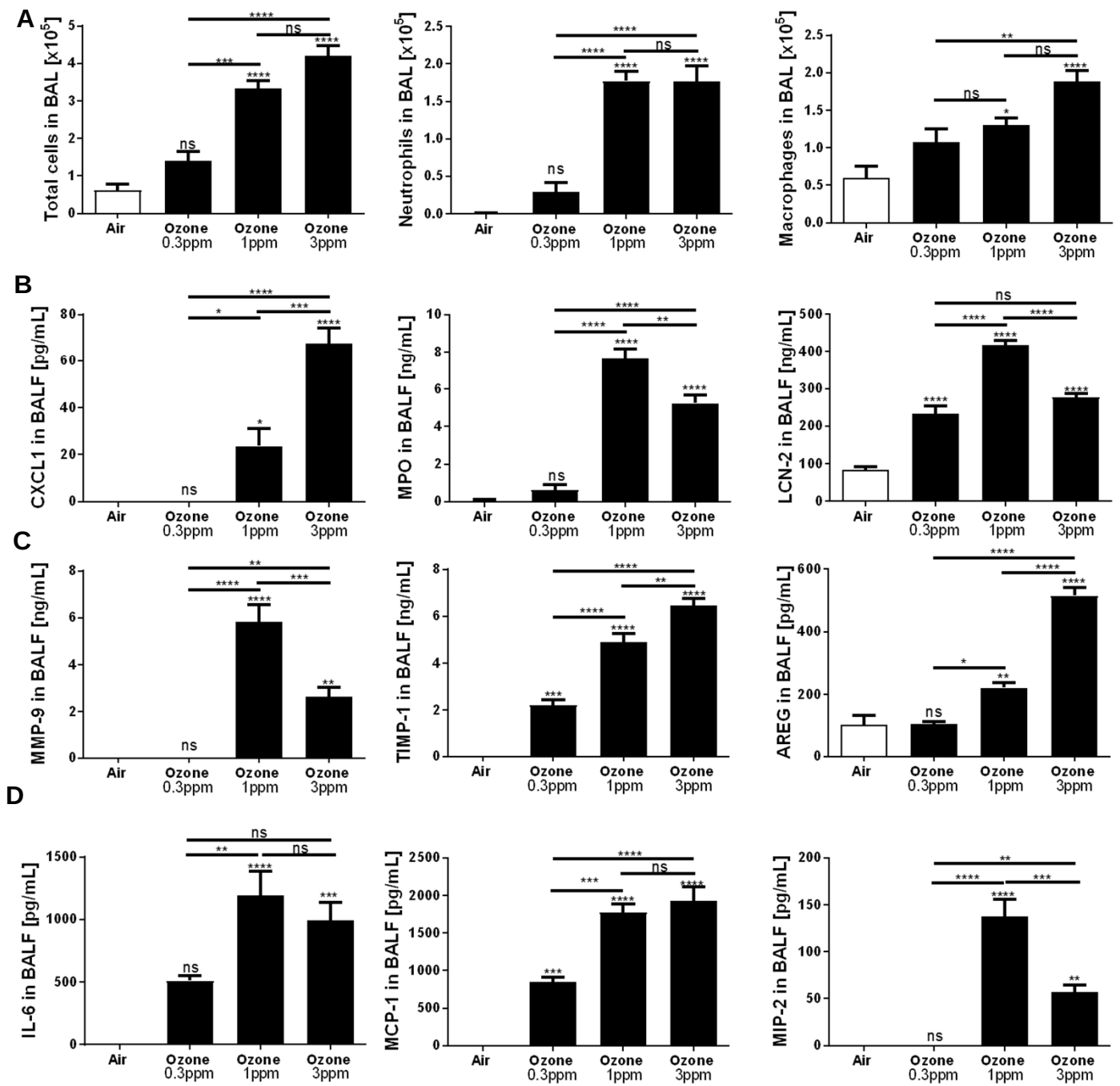
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F Lung- 4h

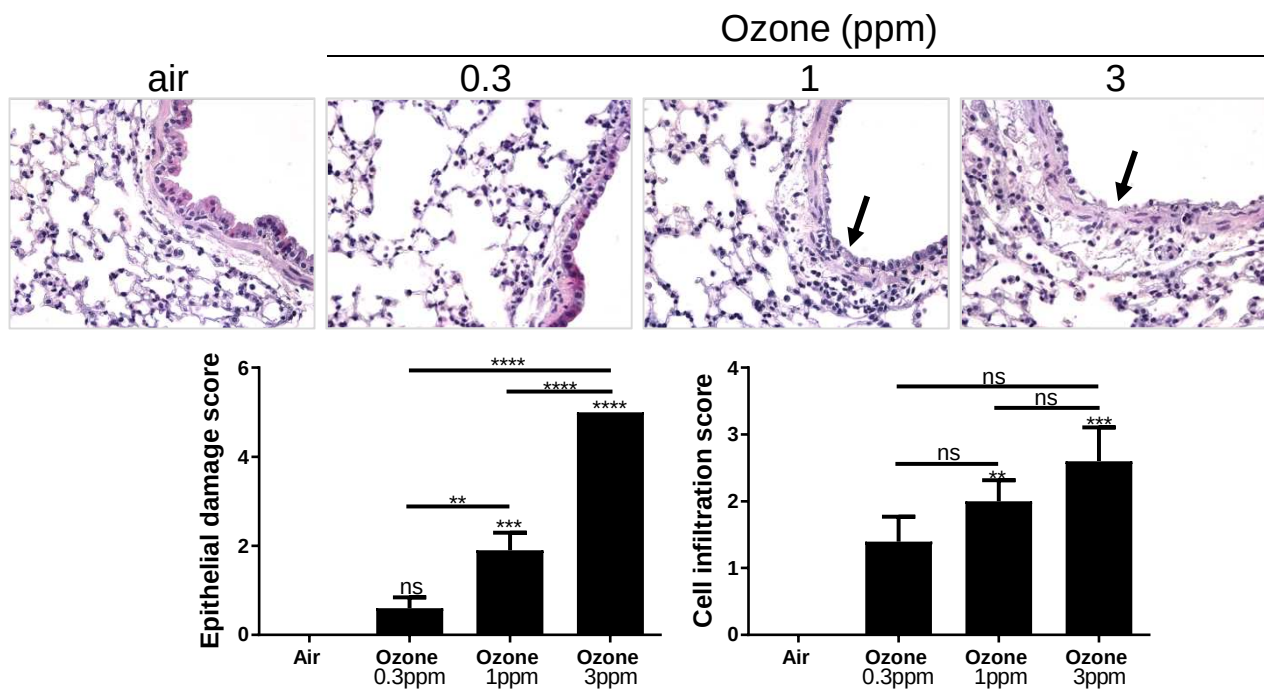


Supplementary Figure E2



Supplementary Figure E2

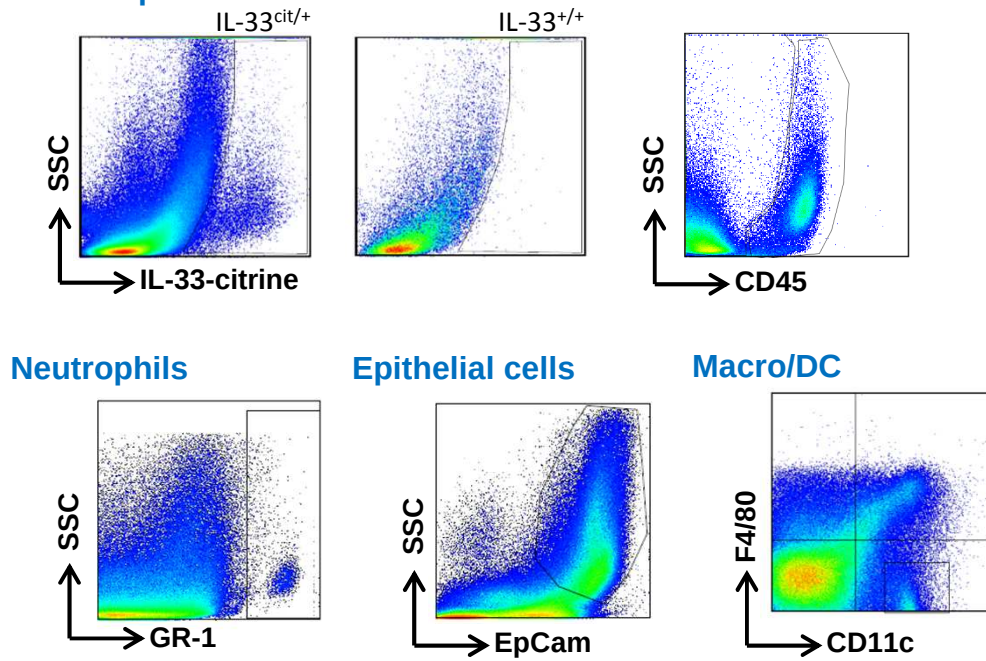
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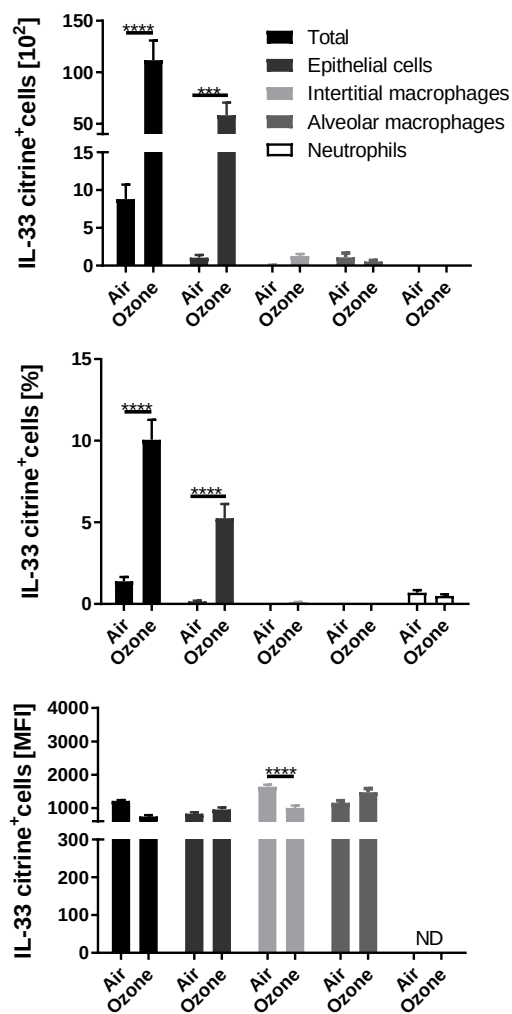
Supplementary Figure E3

A

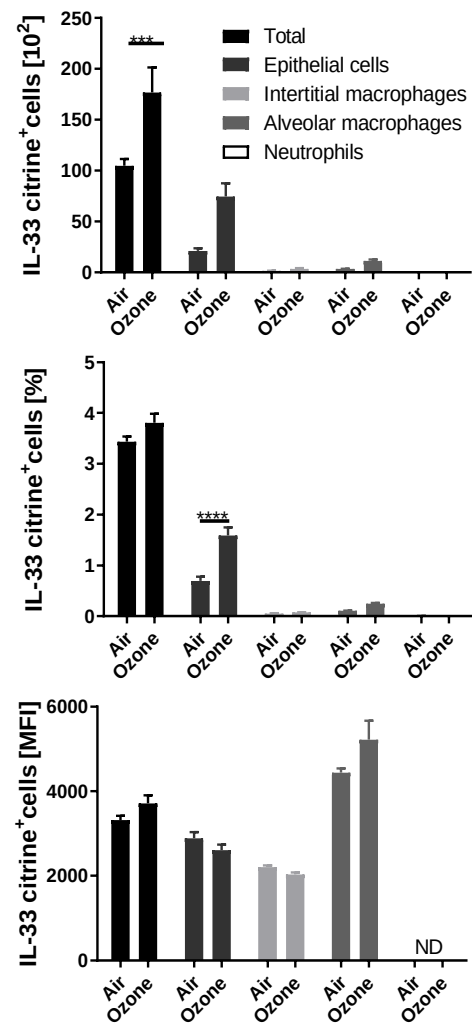
IL-33 expression



B BAL - 4h

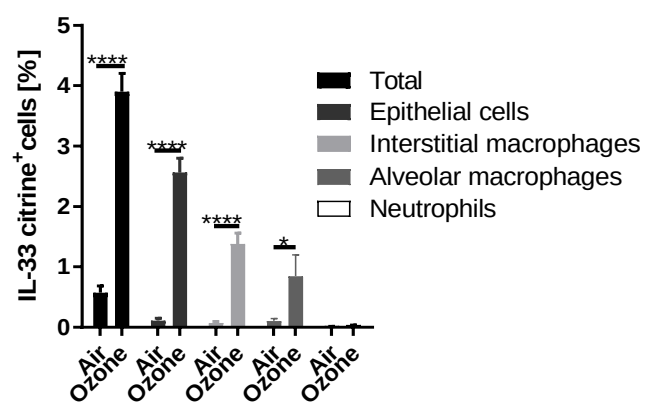


C Lung - 4h

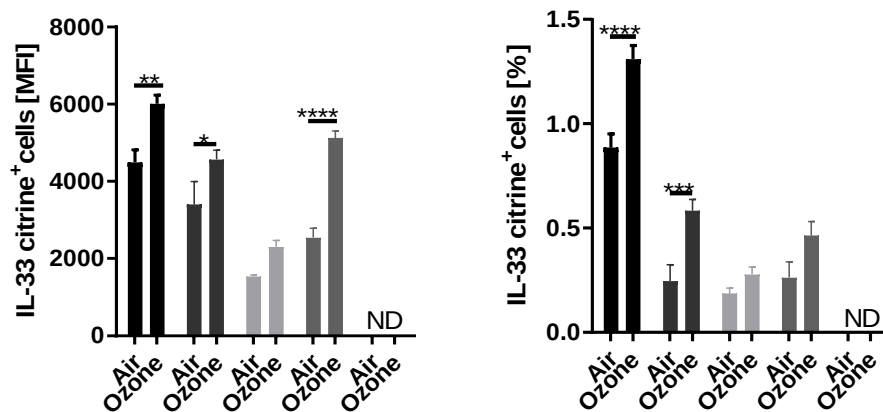


Supplementary Figure E3

D BAL - 24h

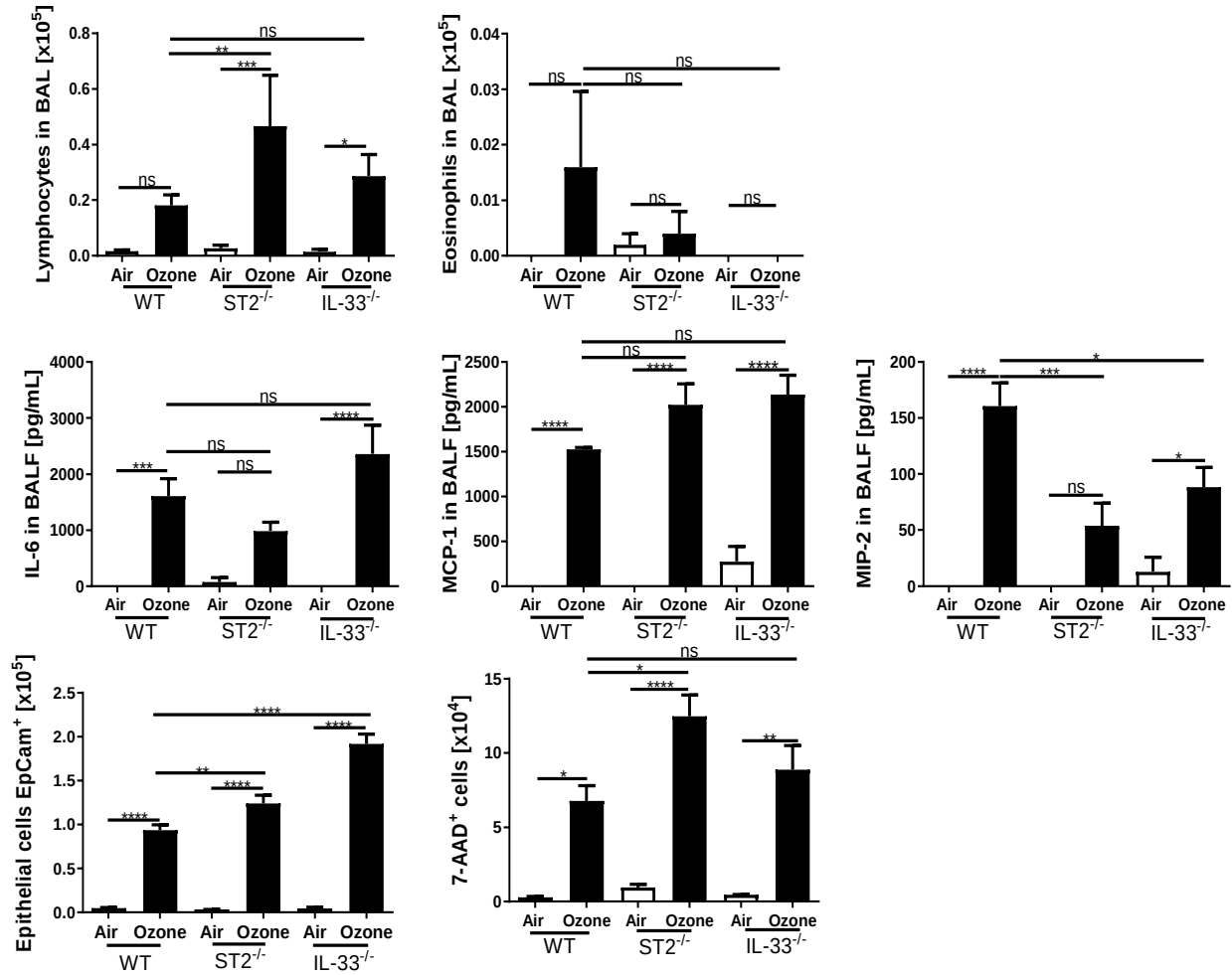


E Lung - 24h

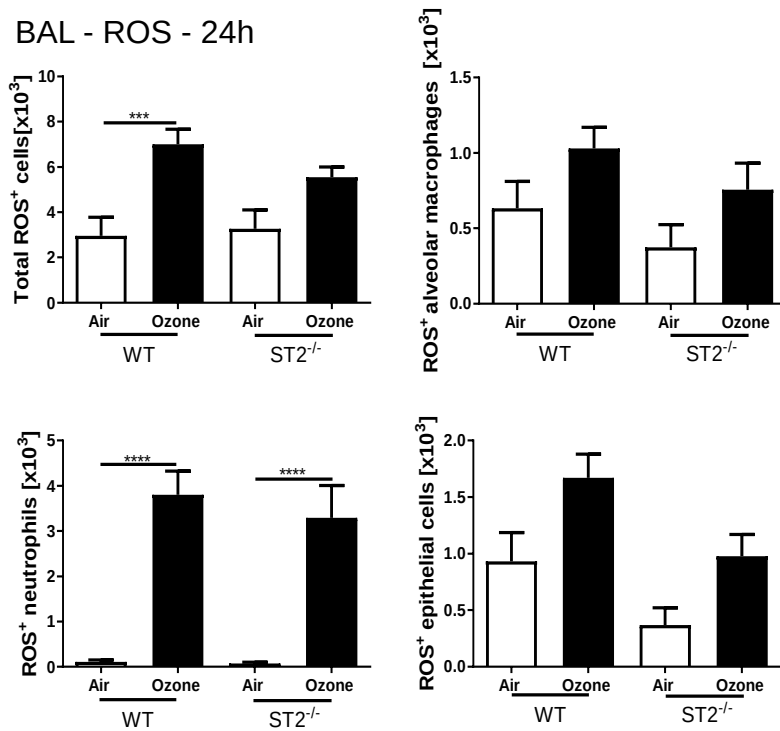


Supplementary Figure E4

A BAL - 24h

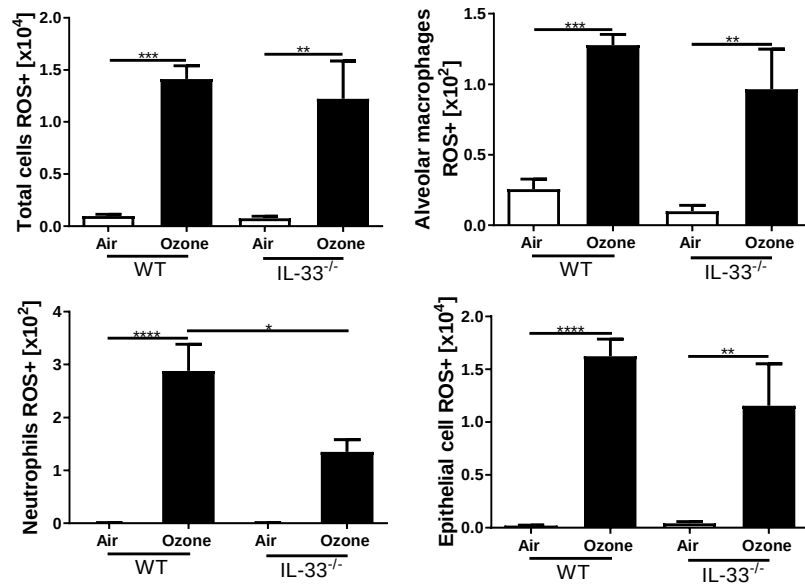


B BAL - ROS - 24h

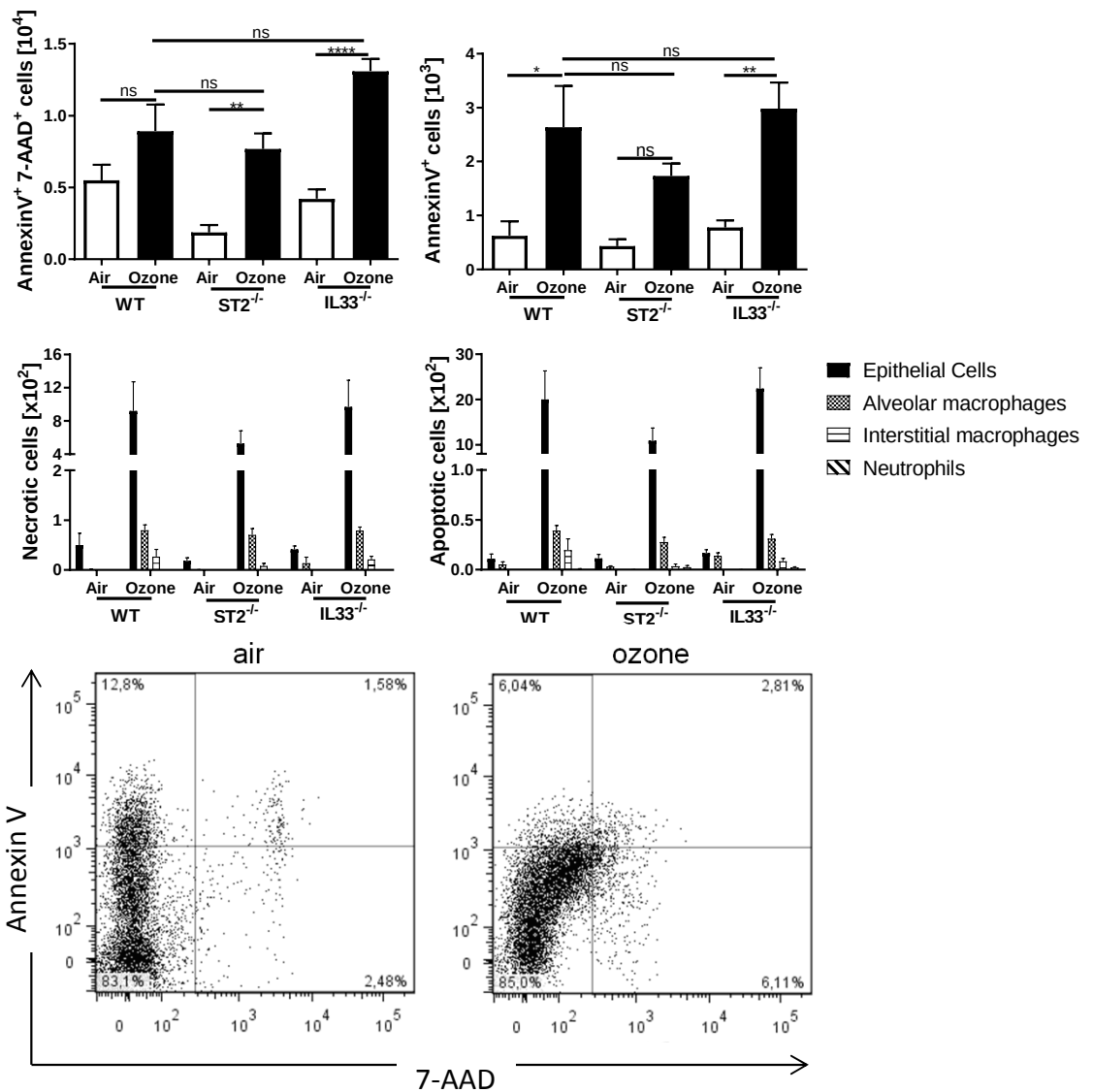


Supplementary Figure E4

C BAL - ROS - 24h

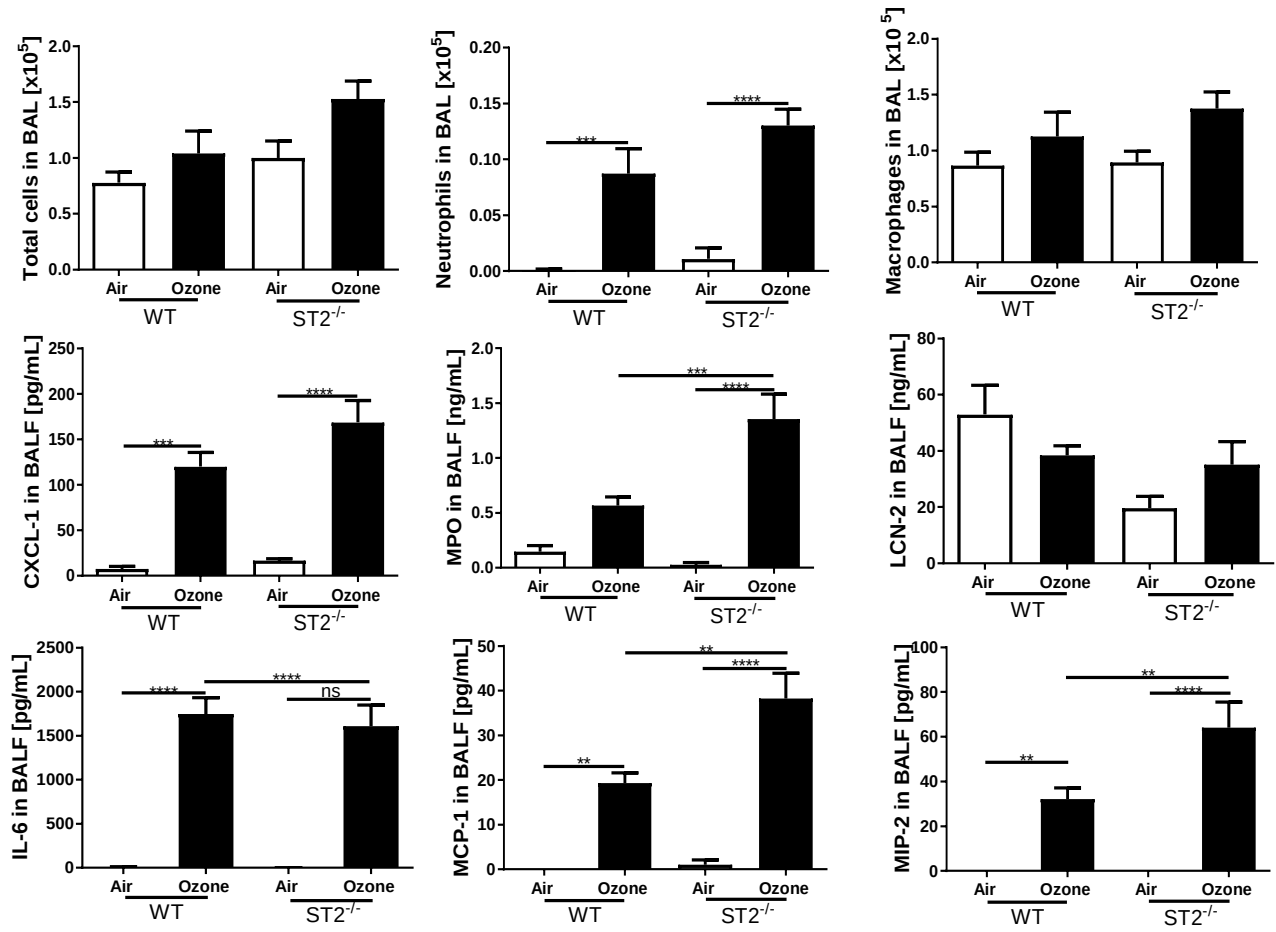


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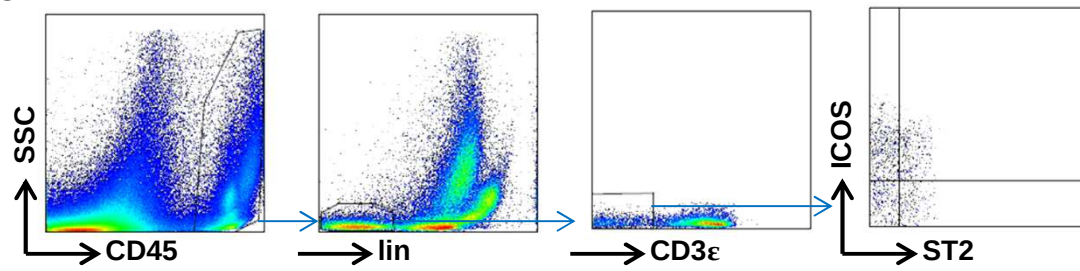
Supplementary Figure E4

E BAL - 4h

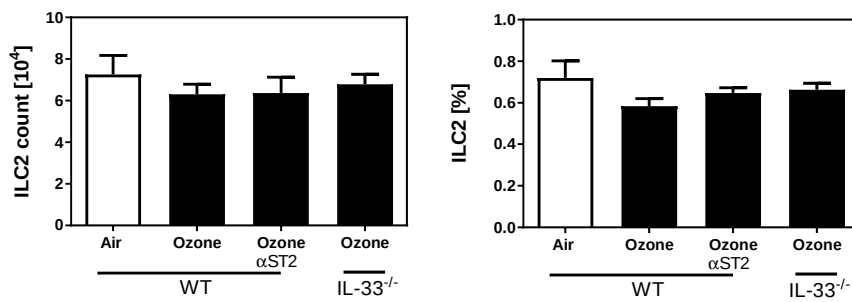


Supplementary Figure E5

A ILC2

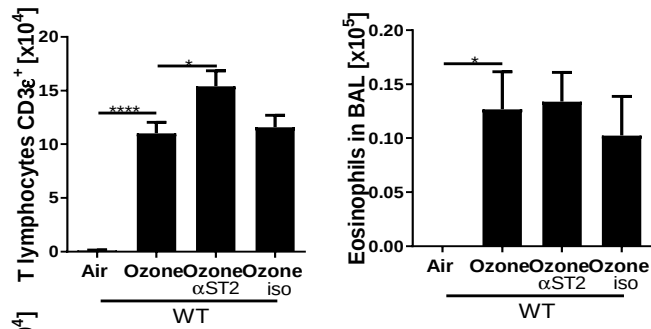


B Lung 24h

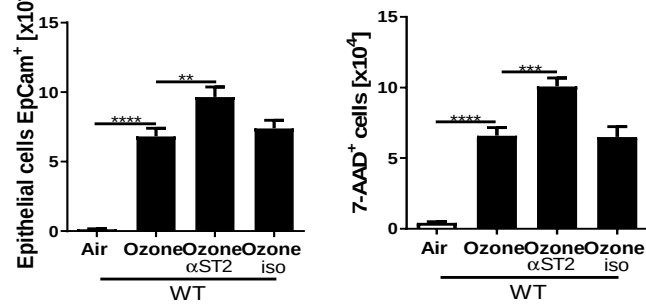


Supplementary Figure E6

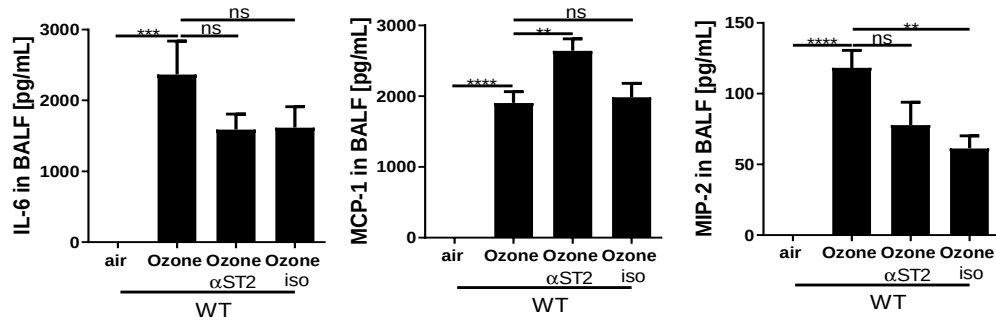
A BAL - 24h



B

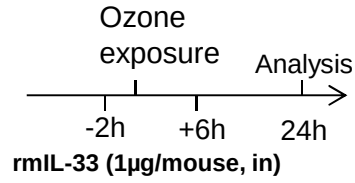


C

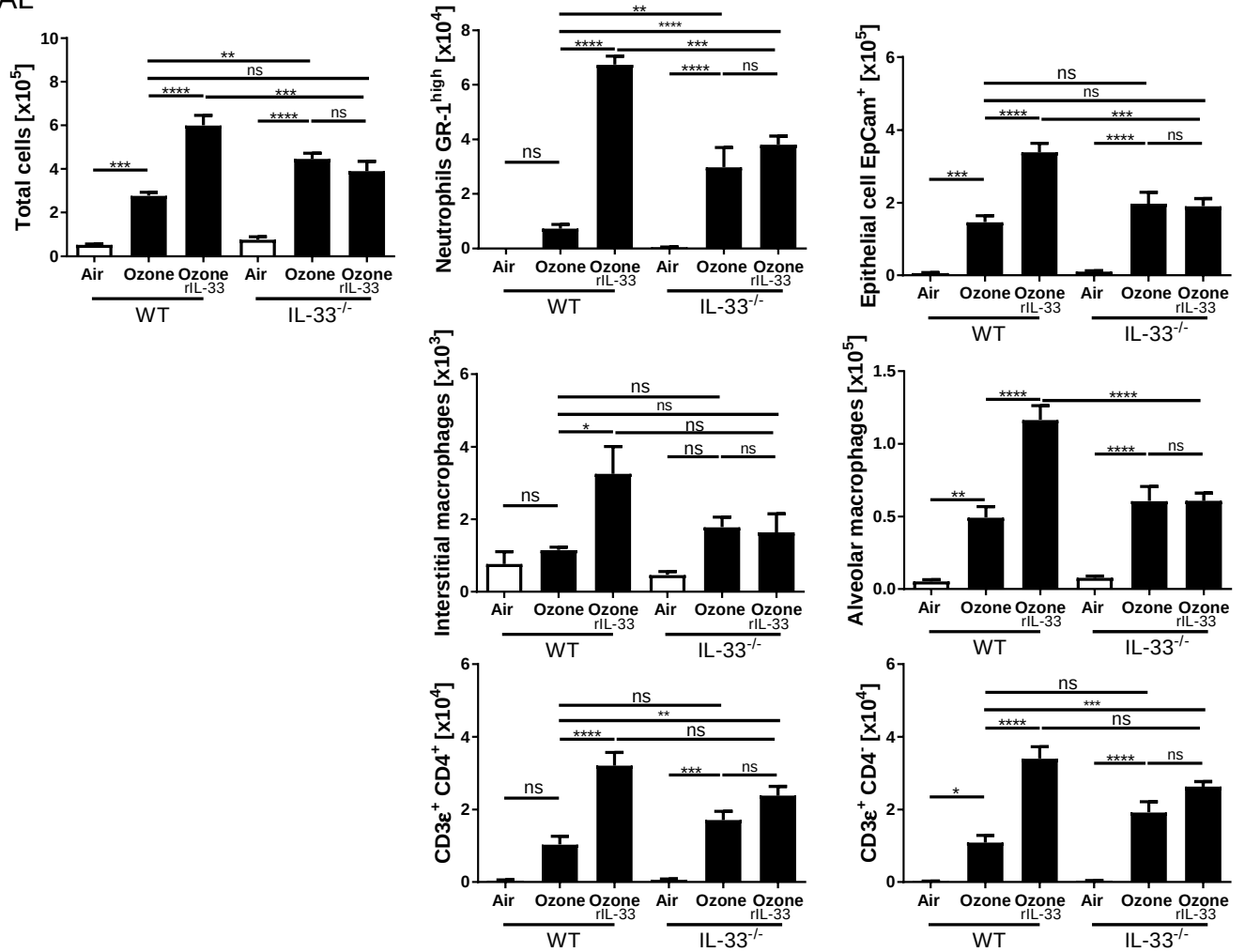


Supplementary Figure E7

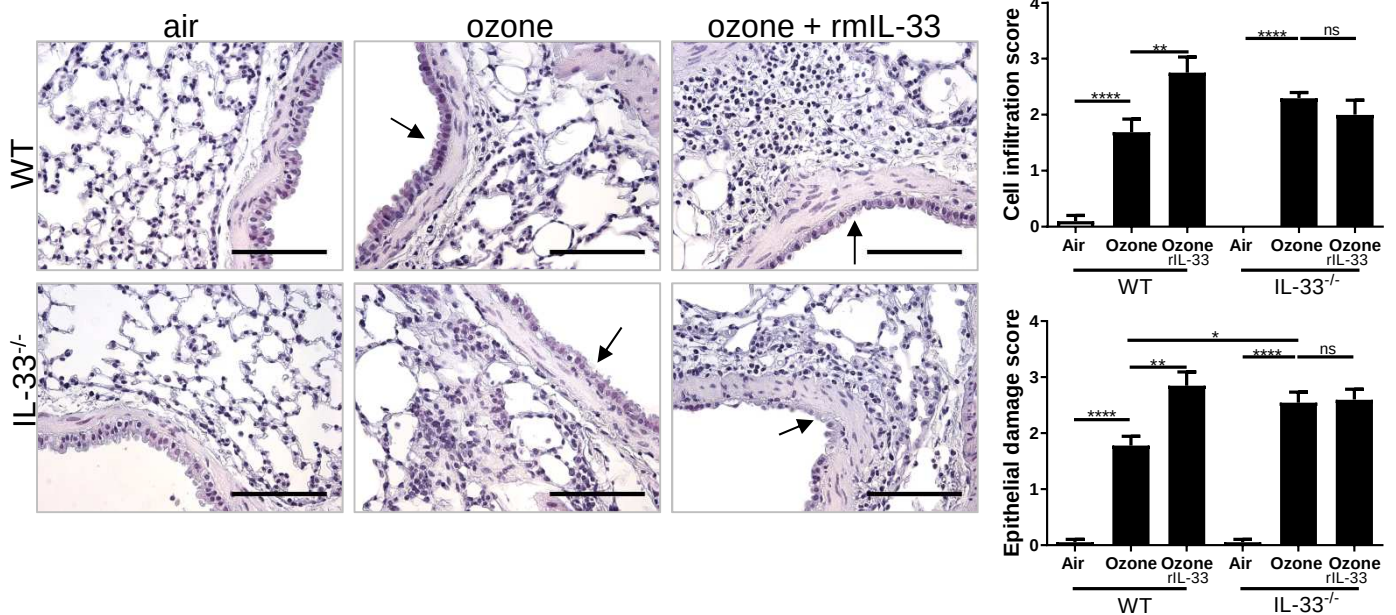
A



B BAL

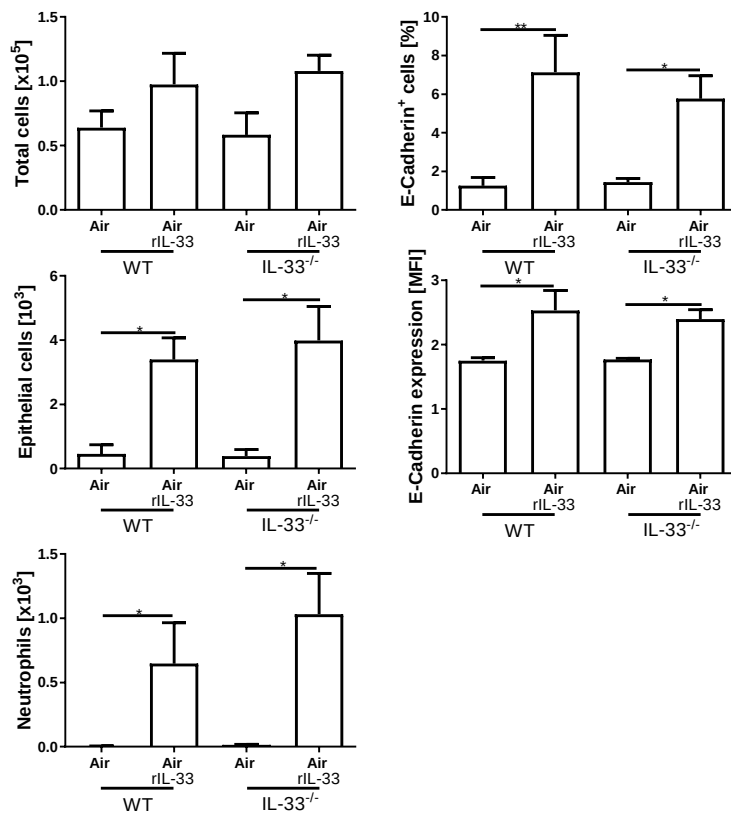


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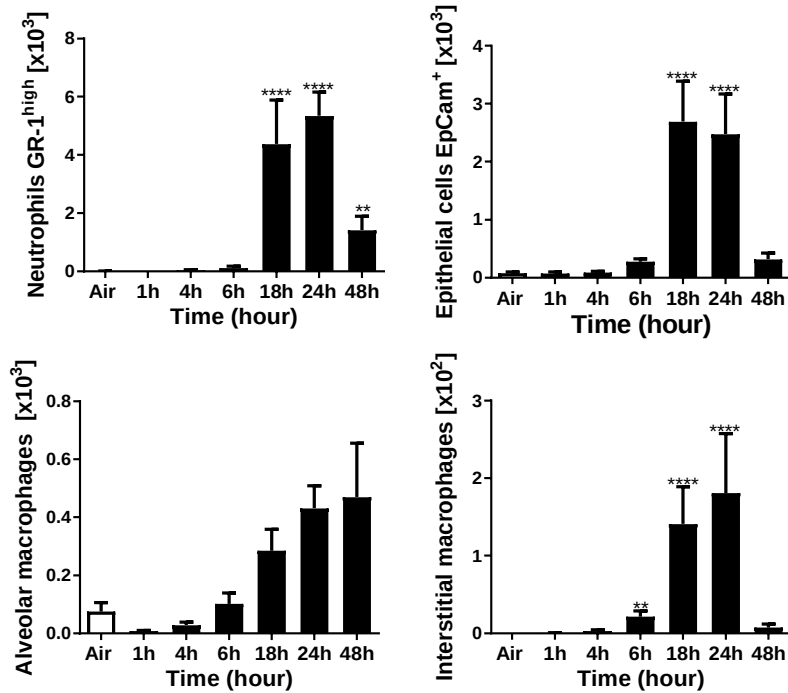
Supplementary Figure E7

D BAL 24h

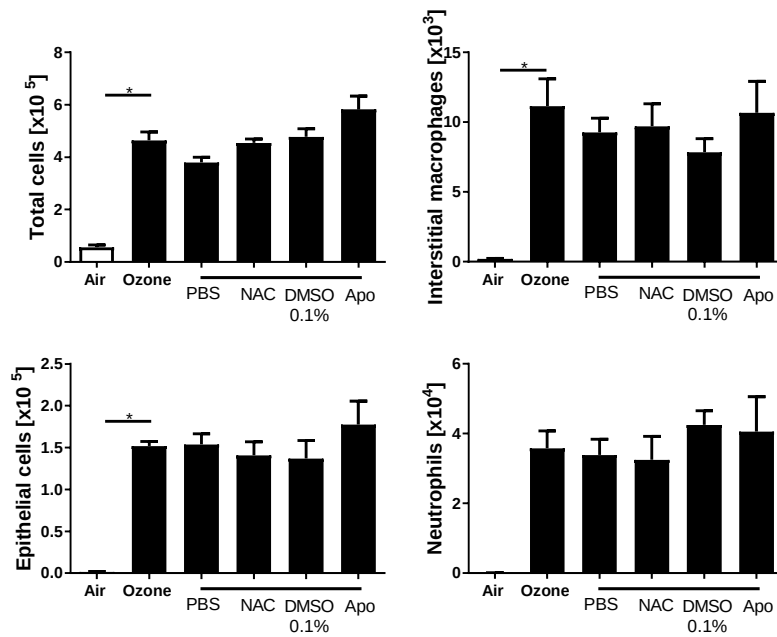


Supplementary Figure E8

A BAL – ROS⁺

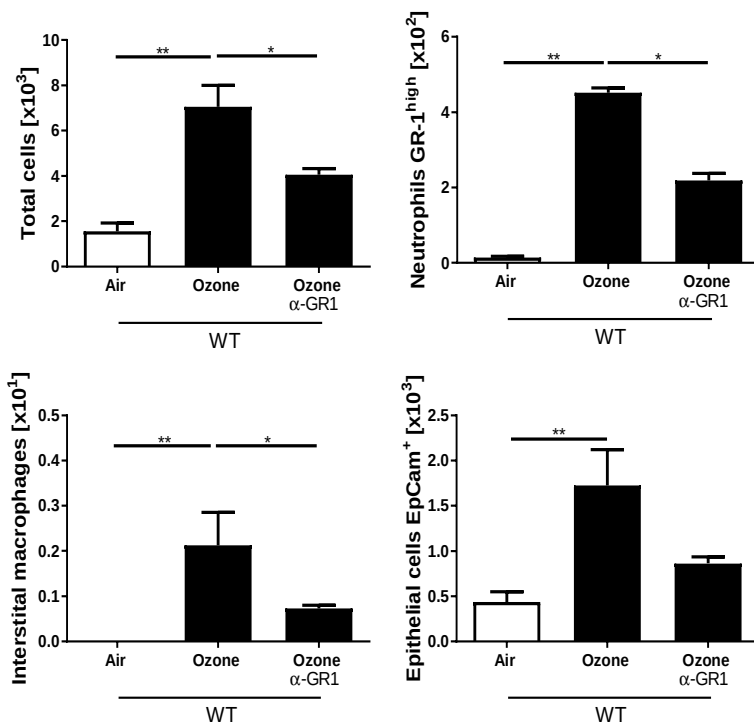


B BAL 24h

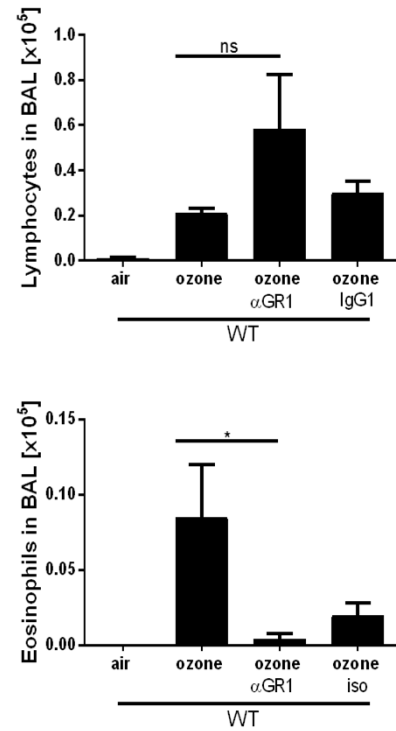


Supplementary Figure E8

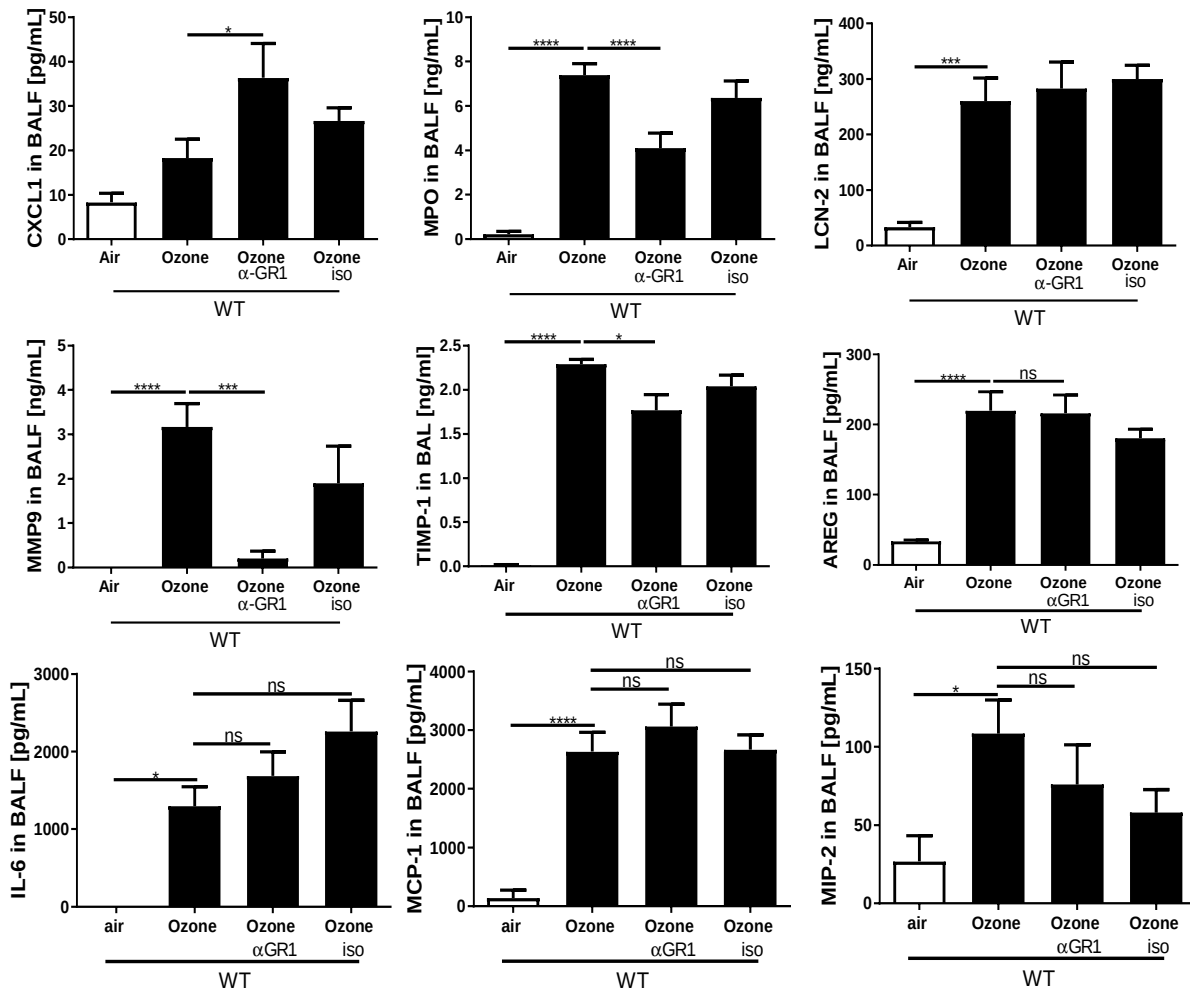
C BAL – ROS+



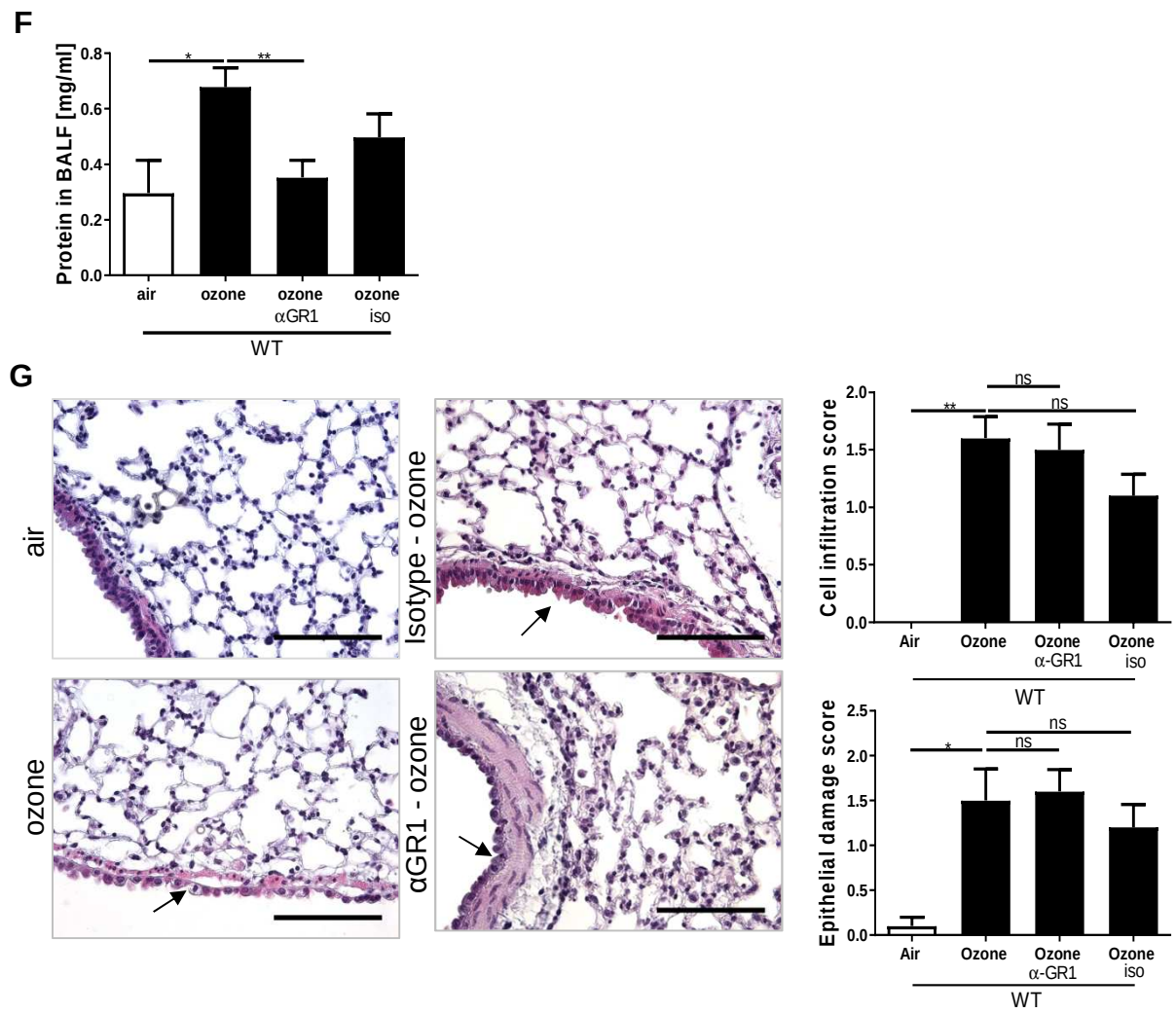
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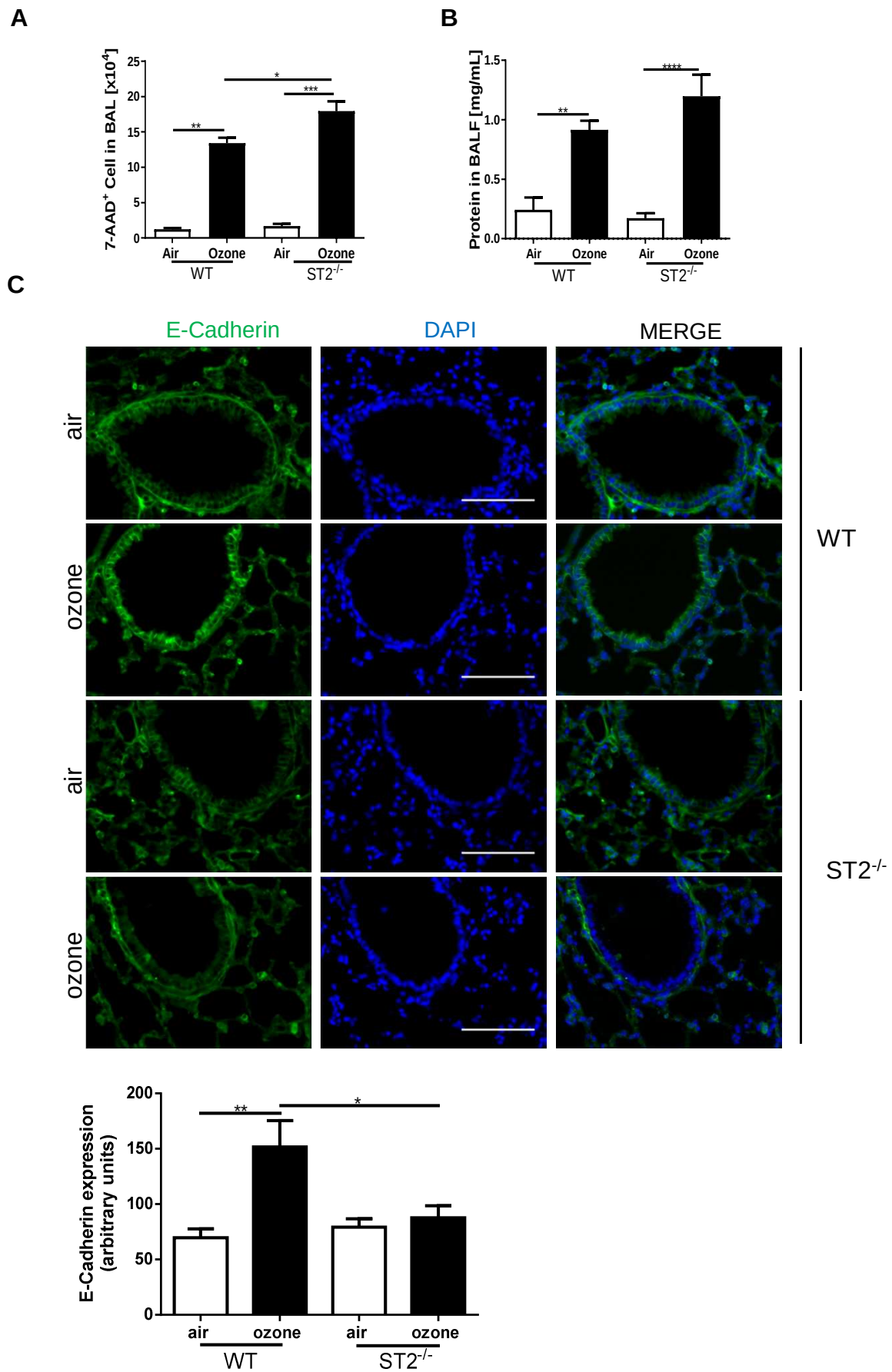
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Supplementary Figure E8

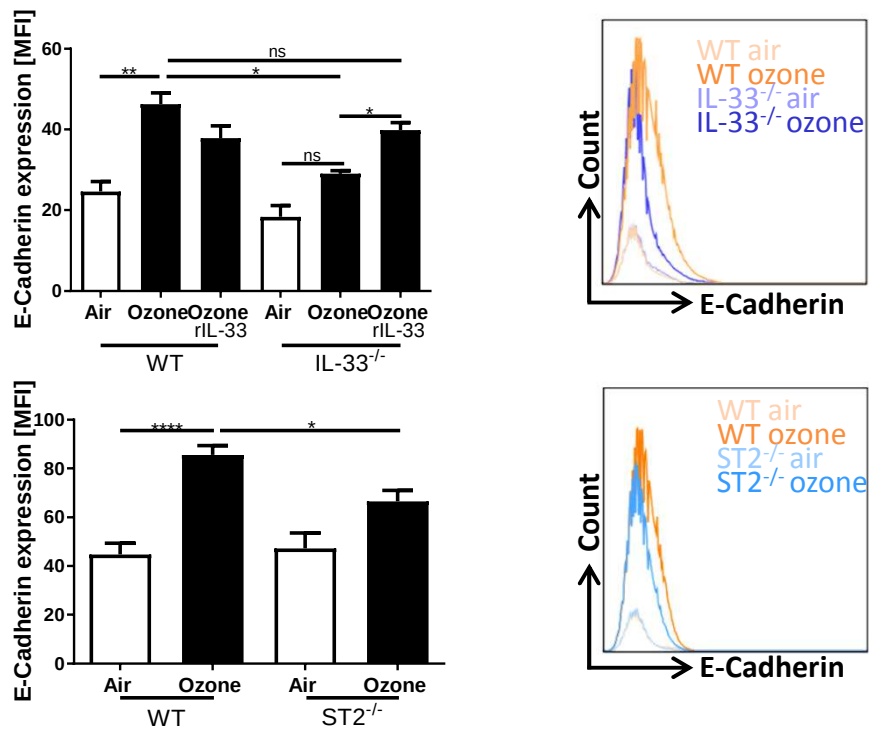


Supplementary Figure E9

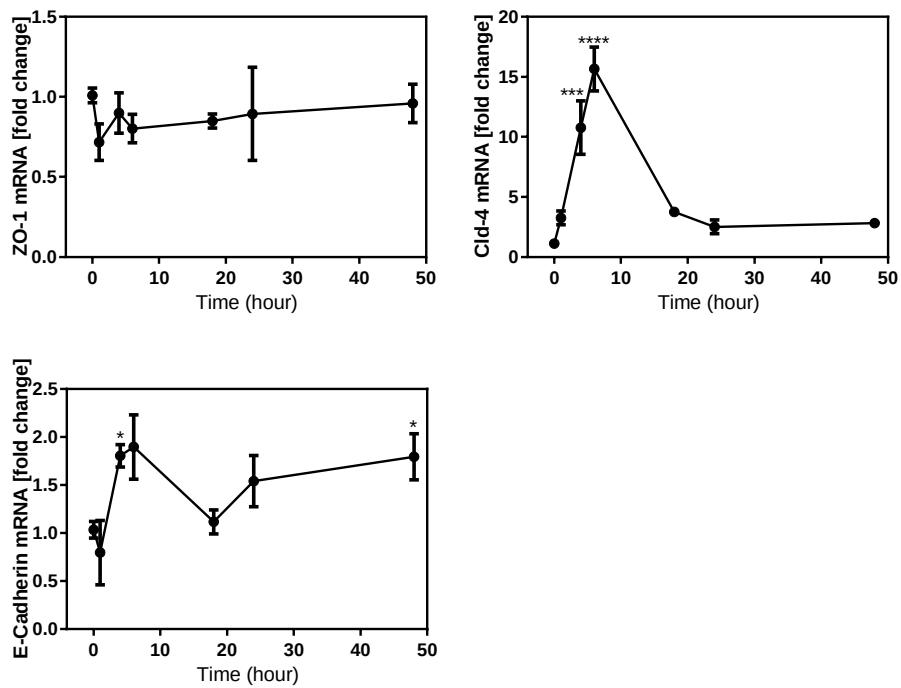


Supplementary Figure E9

D BAL

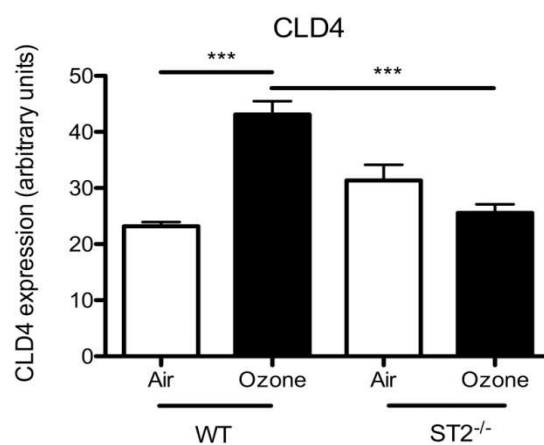
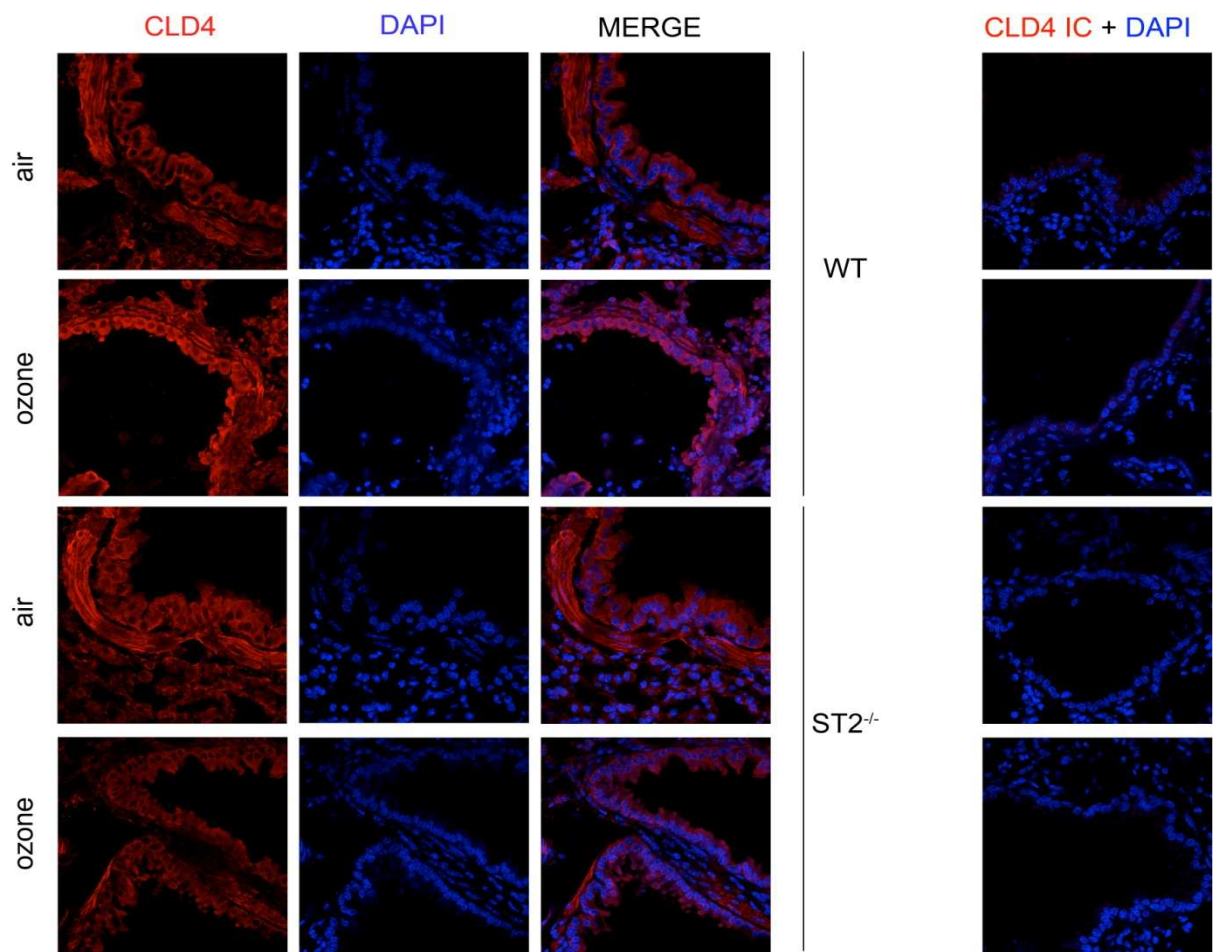


E



Supplementary Figure E9

F 24h



Supplementary Figure E9

G 4h

