DR. STEFANIE GILLES (Orcid ID : 0000-0002-5159-2558)

MRS. STEPHANIE MUSIOL (Orcid ID : 0000-0001-8356-4343)

PROF. FATIMA FERREIRA (Orcid ID : 0000-0003-0989-2335)

PROF. CARSTEN SCHMIDT-WEBER (Orcid ID : 0000-0002-3203-8084)

PROF. CLAUDIA TRAIDL-HOFFMANN (Orcid ID : 0000-0001-5085-5179)

DR. FRANCESCA ALESSANDRINI (Orcid ID : 0000-0002-9854-8968)

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Ragweed plants grown under elevated CO_2 levels produce pollen which elicit stronger allergic lung inflammation

Denise Rauer[1*], Stefanie Gilles[1*], Maria Wimmer[2], Ulrike Frank[3], Constanze Mueller[4], Stephanie Musiol[2], Behnam Vafadari[1], Lorenz Aglas[5], Fatima Ferreira[5], Philippe Schmitt-Kopplin[4], Jörg Durner[3], Jana Barbro Winkler[6], Dieter Ernst[3], Heidrun Behrendt[2], Carsten B. Schmidt-Weber[2], Claudia Traidl-Hoffmann[1,6*], Francesca Alessandrini[2*]

[1] Chair and Institute of Environmental Medicine, UNIKA-T Augsburg, Technical University Munich and Helmholtz Zentrum München, German Research Center for Environmental Health, Germany

[2] Center of Allergy & Environment (ZAUM), Technical University of Munich (TUM) and Helmholtz Zentrum München, German Research Center for Environmental Health, Germany, Members of the German Center of Lung Research (DZL), Munich, Germany.

[3] Institute of Biochemical Plant Pathology (BIOP), Helmholtz Zentrum München, Munich, Germany
[4] BGC, Research Unit Analytical BioGeoChemistry, Helmholtz Zentrum München, Munich, Germany
[5] Department of Biosciences, University of Salzburg, Salzburg, Austria
[6] Research Unit Environmental Simulation, Institute of Biochemical Plant Pathology, Helmholtz Zentrum München, Munich, Germany

[7] Christine-Kühne Center for Allergy Research and Education (CK-Care), Davos, Switzerland
[8] Outpatient clinic for environmental medicine, University clinic Augsburg, Germany

*equal contribution

Address correspondence to:
Prof. Dr. Francesca Alessandrini
Center of Allergy & Environment (ZAUM), Technical University of Munich and Helmholtz Zentrum Munich, German Research Center for Environmental Health
Ingolstaedter Landstr. 1
D-85764 Neuherberg, Germany
Tel: +49 (0)89 3187-2524
e-mail: franci@helmholtz-muenchen.de

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List of abbreviations

9-OTrE	9-oxo-10E,12Z,15Z-octadecatrienoic acid
ADO	Adenosine
BAL	Bronchoalveolar lavage

Cat	Catalposide
DC	Dendritic cell
MoDC	Monocyte-derived dendritic cell
HNEC	Human nasal epithelial cell
ICI	Inflammatory cell infiltration
IL	Interleukin
ILC2	Type 2 innate lymphoid cell
i.n.	Intranasal
IPCC	Intergovernmental Panel on Climate Change
Lumi	Lumichrome
Mal	Malvidin
LPS	Lipopolysaccharide
PALMs	Pollen-associated lipid mediators
pC4OG	*p*-Coumaryl alcohol 4-*O* glucoside
Pel	Pelargonidin
Q3OS	Quercetin-3-*O*-sophoroside
RWE	Aqueous ragweed pollen extract
Th2	T helper type 2 cell
Treg	Regulatory T cell
IPCC	Intergovernmental Panel on Climate Change
RCP	Representative concentration pathway

Abstract

Background: Common ragweed has been spreading as a neophyte in Europe. Elevated CO_2 levels, a hallmark of global climate change, have been shown to increase ragweed pollen production, but its effects on pollen allergenicity remains to be elucidated.

Methods: Ragweed was grown in climate-controlled chambers under normal (380 ppm, control) or elevated (700 ppm, based on RCP4.5 scenario) CO_2 levels. Aqueous pollen extracts (RWE) from control- or CO_2-pollen were administered *in vivo* in a mouse model for allergic disease (daily for 3-11 days, n=5) and employed in human *in vitro* systems of nasal epithelial cells (HNECs), monocyte-derived dendritic cells (DCs) and HNEC-DC co-cultures. Additionally, adjuvant factors and metabolites in control- and CO_2-RWE were investigated using ELISA and untargeted metabolomics.

Results: *In-vivo*, CO_2-RWE induced stronger allergic lung inflammation compared to control-RWE, as indicated by lung inflammatory cell infiltrate and mediators, mucus hypersecretion and serum total IgE. *In vitro,* HNECs stimulated with RWE increased indistinctively the production of pro-inflammatory cytokines (IL-8, IL-1β, IL-6). In contrast, supernatants from CO_2-RWE-stimulated HNECs, compared to control-RWE-stimulated HNECS, significantly increased TNF and decreased IL-10 production in DCs. Comparable results were obtained by stimulating DCs directly with RWEs. The metabolome analysis revealed differential expression of secondary plant metabolites in control- vs CO_2-RWE. Mixes of these metabolites elicited similar responses in DCs as compared to respective RWEs.

Conclusion: Our results indicate that elevated ambient CO_2 levels elicit a stronger RWE-induced allergic response *in vivo* and *in vitro* and that RWE increased allergenicity depends on the interplay of multiple metabolites.

Keywords: allergic lung inflammation, carbon dioxide, climate change, pollen metabolome, ragweed

Introduction

Common ragweed (*Ambrosia artemisiifolia* L.) is native to North America, where 26 % of the population is sensitized to its pollen,[1,2] causing hay fever, asthma and allergic rhinitis. In recent decades, this invasive neophyte has been spreading in Europe.[3,4] In 2016, around 33 million Europeans were sensitized to ragweed and these numbers are estimated to more than double by 2041-2060.[5] Because weed pollen are highly allergenic, even low exposure induces strong allergic reactions.[6]

This is important for understanding future health burdens, which will be heavily increased by climate change.[7] As a result of rising temperatures and favorable precipitation, we will experience a more widespread distribution of ragweed across Europe, expanding from Central towards Northern and Eastern European countries.[8-10] Rising temperatures lead to earlier pollen seasons of anemophilous plants in the Northern hemisphere, thereby increasing the abundance of airborne allergenic pollen.[11,12] Additionally, rising atmospheric CO_2 levels are driving forces of climate change, which resulted in higher ragweed biomass and pollen production in an experimental Intergovernmental Panel on Climate Change (IPCC) scenario.[13,14] Likewise, elevated CO_2 levels combined with drought stress increased the amount of ragweed allergens (Amb a 1, Amb a 8 and Amb a 9) at the protein and transcriptional level.[15,16]

The allergenic potential of pollen is also determined by pollen-associated lipid mediators (PALMs) and low molecular weight compounds.[17] PALMs, such as phytoprostanes, shift dendritic cell mediated T cell polarization towards a Th2 response.[18] Also, pollen-derived lipids of the linoleic acid pathway act as chemoattractants for granulocytes.[19] Additionally, low molecular weight compounds and lipid mediators such as PGE_2 and LTB_4 enhance cutaneous reactions and nasal allergic inflammation to common allergens.[20]

Research determining if and to what extent rising CO_2 levels influence the potential of ragweed pollen to induce pulmonary allergic disease is lacking. We used a combined approach of an *in vivo* mouse allergy model, human *in vitro* tests and untargeted metabolomics to investigate if elevated ambient CO_2 levels representative of climate change scenarios lead to enhanced allergenicity of ragweed pollen.

Methods

Growth of ragweed plants in climate chambers

In 2013, ragweed plants were cultivated as previously described.[21] Plants were grown under ambient (380 ppm) or enriched (700 ppm, based on IPCC scenario RCP4.5)[22] CO_2 levels for the whole vegetation period. Aqueous ragweed pollen extracts (control-RWE, CO_2-RWE) were prepared as previously described.[23] Here, concentrations of RWE correspond to the amount of pollen used for the extraction. For more information on plant cultivation and aqueous pollen extract preparation, see online supplement.

Murine sensitization model

Experiments were conducted according to federal guidelines for the use and care of laboratory animals and approved by the Government of the District of Upper Bavaria and the Animal Care and Use Committee of the Helmholtz Zentrum München (Approval # 55.2-1-54-2532-156-12).

An adjuvant-free ragweed sensitization protocol was performed as previously described.[24] In short, female, 6- to 10-week-old BALB/c mice received intranasal (i.n.) instillations of control-RWE (10 mg/ml, 10 µL/nostril), CO_2-RWE (10 mg/ml, 10 µL/nostril) or PBS (10 µL/nostril) on 3, 8, or 11 consecutive days. Mice were sacrificed 24 h after the last instillation (Fig. 1A). Blood samples were taken prior to the first instillation and at sacrifice. Measurements of airway hyperresponsiveness, performed after 11 RWE instillations and bronchoalveolar lavage (BAL) occurred as previously described.[24] Lung tissue was prepared for histology and FACS analysis.

Blood and nasal cell donors

Isolation, culture and stimulation of primary cells for this study was approved by the ethical committee of the Medical Faculty of the Technical University Munich (ethics statement code: 54/17 S) and the consultative commission of the Augsburg University Medical School (ethics statement code: 2016-7). Blood samples or human nasal epithelial cells from turbinoplastic surgery of healthy non-atopic donors were collected after written informed consent. Atopy status of blood or nasal cell donors was determined by measuring total serum IgE and allergen specific IgE by serum ImmunoCAP

(ThermoFisher, Massachusetts, USA). An overview of the donors, specifying gender, age, total IgE and RAST classes for the measured aeroallergens is available in Table 1.

Human nasal epithelial cells (HNEC) stimulations

HNEC isolation was performed as recently described[25]. For details see online supplement.

Submerged monolayer cultures of second passage HNECs were seeded in 48-well plates at a density of 2 x 10^4 cells/well in complete Airway Epithelial Cell Growth Medium (PromoCell, Heidelberg, Germany) and incubated at 37°C, 5 % CO_2 for five days. At 80 % confluence, the medium was changed to Airway Epithelial Cell Growth Medium without hydrocortisone (PromoCell) and cells were stimulated with control-RWE or CO2-RWE (0.3 mg/ml to 2.5 mg/ml). After 24 h, supernatants were collected and subjected to IL-8, IL-1β, TNF, CCL2, CCL22 (BDOptEIA, BDBioscience Pharmingen, San Diego, CA, USA), IL-33 (R&D Systems, Wiesbaden, Germany) and IL-6 (eBioscience, San Diego, CA, USA) ELISA.

Human monocyte-derived dendritic cells *(DC) stimulations*

DCs were isolated from PBMCs as previously described.[26] For details, see online supplement. A total of 10^5 day 5 immature DCs were stimulated with control- or CO_2-RWE (2.5 mg/ml), single pollen-derived compounds ($3x10^{-7}$M, Table 1) or corresponding compound mixes ($3x10^{-7}$M, Table 1). For DC stimulation with RWE-conditioned HNEC supernatants, HNEC supernatants of all donors were pooled and supernatants from cells stimulated with the two lowest (0.3 mg/ml and 0.6 mg/ml) or highest (1.25 mg/ml and 2.5 mg/ml) concentrations were combined resulting in 0.5 mg/ml (low) and 1.8 mg/ml (high) RWE stimulus concentrations, respectively. Unstimulated DCs correspond to DCs incubated with medium-stimulated HNEC. After 24 h, supernatants were analyzed by ELISA for IL-10, IL-1β, TNF (BDOptEIA), CCL17 (R&D Systems) and IL-6 (eBioscience) secretion and DC maturation markers were analyzed by flow-cytometry. For details, see online supplement.

Untargeted metabolome analysis

The metabolome of control-RWE and CO_2-RWE was analyzed using ultra high-resolution mass spectroscopy (ICR-FT/MS) as previously described.[21] For details, see online supplement.

Statistical analysis

In vivo and *in vitro* data are shown as boxplots indicating minimum, 25 % percentile, median, 75 % percentile, and maximum, or as mean ± SD. Statistical significance of the *in vivo* data was determined by Mann–Whitney U-test or by two-way ANOVA with post-hoc Bonferroni test for lung function analysis. *In vitro* data were normalized to unstimulated controls, mean ± SD of raw values are available in tables S2 and S3. Wilcoxon-signed rank test was used to compare two treatment groups of non-normally distributed data. Repeated measures one-way ANOVA with Sidak's post-hoc test, or Friedman using Dunn's correction was applied for multiple comparisons. Statistical analysis and graph design was performed using GraphPad Prism version 8.4.1. Spider plots for cytokine profiles were created in Excel (2013), using normalized data. Metabolomics data were analyzed using MetaboAnalyst 4.0 [27].

Results

Pollen of ragweed plants grown under elevated CO_2 levels elicit stronger allergic inflammation in vivo

The impact of elevated CO_2 exposure during plant growth on the allergenic potential of ragweed pollen was analyzed in an adjuvant-free mouse model of allergic lung inflammation.[24] To assess the kinetics of the allergic response on lung cell infiltration, mice were i.n. instilled on 3, 8 or 11 consecutive days with either PBS, control-RWE or CO_2-RWE (Fig. 1A). Increasing numbers of RWE instillations showed the typical shift from an early, neutrophil-based, to a later, eosinophil-based lung inflammation (Fig. 1B). Contrary to control-RWE, in the CO_2-RWE-treated group both neutrophil and eosinophil numbers were significantly elevated after 3 (neutrophils; $p < 0.05$) and 11 (eosinophils; $p < 0.05$) i.n. instillations (Fig. 1B). Total serum IgE measured 24h after 11x i.n. instillations was significantly increased in the CO_2-RWE treated group compared to the other groups ($p < 0.001$, Fig. 1D), including control-RWE. *Ex vivo* IgE production of mouse splenic B cells was also elevated upon CO_2-RWE stimulation compared to control-RWE ($p < 0.05$, Fig. 1E). Eleven i.n. instillations of RWE significantly increased airway resistance in both treatment groups compared to PBS control ($p < 0.01$ for CO_2-RWE and $p < 0.001$ for RWE, Fig. 1C), but no difference was detected between control-RWE and CO_2-RWE.

Flow cytometric analysis of lung tissue retrieved 24h after the last i.n. instillation revealed a significant increase in eosinophils after 8x and 11x instillations in the CO_2-RWE group, confirming the BAL data ($p < 0.05$, Fig. 2A, top). Furthermore, 8x instillations increased type 2 innate lymphoid cells (ILC2s) in lung tissues of mice treated with control-RWE and CO_2-RWE compared to 3x ($p < 0.05$) whereby the increase in CO_2-RWE was higher compared to control-RWE, but not significantly. 8x instillations increased Treg numbers in lung tissues of mice treated with control-RWE ($p < 0.001$ vs. 3x and $p < 0.05$ vs. PBS control, Figure 2A, middle). An increased percentage of $CD11b^+$DCs in lung tissue was detected in the CO_2-RWE group after 11x instillations compared to the other groups, although significantly only vs PBS ($p < 0.05$, Fig. 2A, bottom).

ILC2s were significantly increased in cervical lymph nodes of mice treated with CO_2-RWE vs control-RWE, although at a later time point compared to lung tissue (11x, $p < 0.01$ vs. PBS, $p < 0.05$ vs control-RWE, Supplement Fig. S2). Tregs in the same lymph nodes showed no significant differences

between the treatment groups, whereas higher percentage of DCs was detected after 8x instillations in the CO_2-RWE vs control-RWE group ($p < 0.05$, Supplement Fig. S2).

Histopathological analysis of H&E- and PAS-stained lungs after 11x instillations revealed increased perivascular and peribronchiolar inflammatory cell infiltration (ICI) and mucus hypersecretion in control-RWE and CO_2-RWE mice, with CO_2-RWE scoring highest (mucus hypersecretion: $p < 0.01$ and ICI: $p < 0.001$ for PBS vs. CO_2-RWE and $p < 0.05$ for PBS vs. control-RWE in both parameters, Fig. 2E-F). Analysis of Th1/Th2 and pro-inflammatory cytokines, as well as chemokines revealed significant increases of IL-17A ($p < 0.001$) and IL-17F ($p < 0.01$) after 11x instillations and CCL22 ($p < 0.05$) after 3x instillations in CO_2-RWE vs PBS. In control-RWE only IL-17A after 11x instillations and CCL17 after 3x instillations were significantly increased to PBS ($p < 0.05$; Fig. 3, Supplement Fig. S3). All other mediators were higher in CO_2-RWE, but did not reach statistical significance, apart from chemokines regulating neutrophil recruitment (CCL3, CCL4 and CXCL1), which were slightly higher in control-RWE (Fig. 3 and Supplement Fig. S3).

RWEs induce pro-inflammatory responses in human nasal epithelial cells

To analyze the allergenic potential of the different RWEs in a human *in vitro* system, we stimulated nasal epithelial cells as first port of entry for pollen into the body. Control- and CO_2-RWEs significantly increased IL-8 (control-RWE: $p < 0.01$ and $p < 0.001$; CO_2-RWE: $p < 0.01$), IL-1β ($p < 0.01$, $p < 0.001$) and IL-6 (control-RWE: $p < 0.01$; CO_2-RWE: $p < 0.05$) secretion compared to the unstimulated control (Supplement Fig. S4B, D-E). CCL2 and CCL22 secretion were unchanged (Supplement Fig. S4A, C). Only TNF release was differentially regulated by CO_2-RWE and control-RWE, being increased by low CO_2-RWE and high control-RWE concentrations ($p < 0.001$, $p < 0.01$ vs control-RWE and $p < 0.01$ vs CO_2-RWE, Supplement Fig. S4F). IL-33 could not be detected in the supernatants.

RWEs induce pro-inflammatory responses in human dendritic cells stimulated with RWE-conditioned epithelial cell supernatants

Because epithelial cells are important modulators of immune responses in the lung,[28] we investigated the effect of HNEC supernatants after RWE stimulation downstream of the nasal epithelium. Immature DCs were stimulated with the above characterized HNEC supernatants subsequently pooled in RWE-low and RWE-high, and the cytokine/chemokine profile and maturation markers were analyzed. IL-6 and CCL17 secretion was significantly higher than the baseline across all treatments (Fig. 4C, IL-6: $p < 0.001$; Fig. 4E, CCL17: $p < 0.001$ RWE-high, $p < 0.0001$ RWE-low, $p < 0.05$ unstimulated HNEC supernatants). CO_2-RWE-treated HNEC supernatants increased IL-6 ($p < 0.05$) and CCL17 ($p < 0.01$) secretion compared to unstimulated HNEC supernatants. IL-10 (Fig. 4A) was increased by unstimulated- and CO_2-RWE-treated HNEC supernatants ($p < 0.01$) and strongly increased by high control-RWE-treated HNEC supernatants ($p < 0.0001$). TNF (Fig. 4B) secretion was higher upon stimulation with CO_2-RWE-treated HNEC supernatants than control-RWE ($p < 0.01$). IL-1β was only increased by supernatants from unstimulated or CO_2-RWE-stimulated HNECs ($p < 0.05$, Fig. 4D). Overall, the cytokine profile induced by CO_2-RWE-treated HNEC supernatants was strongly pro-inflammatory (Fig. 4F). CD80 and CD86 were increased by supernatants from CO_2-RWE-stimulated HNECs ($p < 0.05$, $p < 0.0001$, Supplement Fig. S5B, D) whereas no difference in CD40 and HLA-DR was shown (Supplement Fig. S5A, E). CD83 (Supplement Fig. S5C) was elevated by unstimulated HNEC supernatants ($p < 0.01$) and reduced by CO_2-RWE-stimulated HNEC supernatants compared to unstimulated ($p < 0.01$).

CO_2-RWE induces a more pro-inflammatory response profile in human dendritic cells

Lastly, we analyzed the direct effect of RWEs on dendritic cell cytokine/chemokine secretion and surface marker expression. IL-10 was significantly less secreted by DCs stimulated with CO_2-RWE than control-RWE ($p < 0.05$, Fig. 5A). In contrast, CO_2-RWE significantly increased TNF levels ($p < 0.05$, Fig. 5B). No differences were detected for IL-1β, IL-6 and CCL17/TARC (Fig. 5C-E). Similar to the above described co-culture experiments, CO_2-RWE induced a pro-inflammatory cytokine profile (Fig. 5F). Both RWEs induced maturation profiles distinct from the unstimulated control, but similar between the treatments (Supplement Fig. S6, bottom). Expression of CD86 was increased by both

RWEs ($p < 0.05$), while CD80 was only higher in control-RWE-treated DCs ($p < 0.05$), (Supplement Fig. S6, top).

Extract analysis reveals candidate substances for enhanced allergenic potential of RWE

Pollen-derived substances act as immune-modulators or have pro-inflammatory properties.[18-20,24] As such, LTB_4, PGE_2, adenosine and LPS were slightly, although non-significantly higher in CO_2-RWE (Fig. 6A). The content of the major allergen Amb a 1 did not differ between control- and CO_2-RWE (Fig. 6A). To gain insight into secondary metabolites present in the RWEs we used untargeted mass spectroscopy. The metabolite profile of the extracts was distinctly different as revealed by principal component analysis (PCA) (Fig. 6C). We observed six candidate substances present only in control-RWE and 13 candidate substances present only in CO_2-RWE (Fig. 6D) and chose the ones commercially available or their analogues to stimulate moDCs (Table 2).

Pooled, but not single substances are responsible for the cytokine profiles of dendritic cells induced by control- and CO_2-RWE

We used the compounds either separately or in two combinations as present in control- or CO_2-RWE (Table 2) to stimulate DCs and compared the resulting cytokine response to whole RWEs. Pelargonidin and malvidin enhanced IL-10 secretion ($p < 0.0001$ and $p < 0.01$ vs. unstimulated), whereas pC4OG decreased IL-10 secretion ($p < 0.05$ vs. unstimulated) (Supplement Fig. S7A). Malvidin ($p < 0.001$) and 9-OTrE ($p < 0.05$) increased IL-1β secretion (Supplement Fig. S7D), and lumicrome decreased IL-6 secretion ($p < 0.05$ vs. unstimulated, Supplement Fig. S7C). Compared to a relatively low response to single substances, DCs stimulated with a compound pool mimicking CO_2-RWE secreted less IL-10 ($p < 0.05$, Fig. 6E) and more IL-1β ($p < 0.01$, Fig. 6H) than with the control-RWE compound mix. TNF and IL-6 secretion did not differ between the two compound mixes.

Discussion

Climate change poses a considerable threat to global health in the foreseeable future.[29] Elevated CO_2 levels are part of the driving forces behind our changing climate.[30] CO_2 naturally contributes to plant growth and doubling ambient CO_2 levels have led to increased pollen production of ragweed plants,[13,14] raising their impact on allergic patients.[31-33]

Here we investigated whether doubling ambient CO_2 levels to 700 ppm, a still rather conservative IPCC scenario, could also affect the allergenic potential of pollen.

We observed that pollen extracts from plants grown under 700 ppm CO_2 induced a stronger allergic phenotype in a mouse model, characterized by higher serum IgE levels, enhanced lung inflammatory cell recruitment and mucus hypersecretion, key hallmarks of allergic inflammation.[34,35] Moreover, we observed moderately increased inflammatory mediators in BAL fluid. In lung and cervical lymph nodes, numbers of dendritic and ILC2 cells, which play a critical role in mounting Th2 responses via IL-33/ST2 signaling under acute and chronic ragweed allergen exposure,[36] were increased. Airway hyperresponsiveness was increased by both RWEs compared to PBS control, but no difference was detected between them probably because of the overall moderately increased cytokine response in this study.

To translate our mouse-based results to humans we used different *in vitro* models to simulate the pollen passage through different immune checkpoints. As a first barrier, the nasal epithelium plays a key role in the allergic sensitization to airborne allergens, responding to pollen stimulation with inflammasome-related cytokines IL-18 and IL-1β.[25] RWEs also activate the inflammasome in keratinocytes by IL-1β secretion and caspase-1 activation.[37] In our study, RWEs induced IL-1β together with pro-inflammatory cytokines in HNECs, irrespectively of the plant growth conditions. In the absence of IL-12, IL-1 family cytokines have been shown to promote Th2[38,39] and, in the presence of TGF-β, Th9 differentiation[40] as well as proliferation of Th2 clones.[41,42] IL-1 has also been shown to be required for allergen-specific Th2 cell activation and airway inflammation in a mouse model of asthma.[43] Indeed, secretion of IL-1β in our RWE-stimulated HNECs potentially contributes to the Th2 promoting effect downstream of the nasal epithelium.

It is important to note that we used submerged HNEC monolayer cultures instead of air liquid interface. Although we did not measure tight junctions in our cultures, a characteristic of differentiated epithelia, they have been detected in confluent monolayer cultures of non-atopic donors, similarly to air liquid interface.[25,44]

Contrarily to the results obtained by stimulating directly HNECs with RWEs, we report stronger effects of plant treatments upon activation of DCs as downstream effector cells with HNEC supernatants. DCs incubated with supernatants from CO_2-RWE-stimulated HNECs produced more pro-inflammatory cytokines, especially Th2-cell attractant CCL17 and pro-inflammatory IL-6 and TNF, compared to DCs stimulated with control-RWE-treated HNEC supernatants.

Direct stimulation of DCs with pollen extracts clearly demonstrates that CO_2-RWE, which induced allergic airway inflammation *in vivo* more potently, induced less IL-10 in human DCs *in vitro* compared to control-RWE. IL-10 is the hallmark cytokine for DC-induced Treg differentiation.[45] This cytokine was reduced *in vitro* by CO_2-RWE and by the CO_2 compound mix, consistent with reduced pulmonary Treg numbers upon sensitization with CO_2-RWE *in vivo*. Our results are in line with a recent study indicating IL-10 signaling in DCs as essential for efficient tolerance induction.[46]

TNF is another critical factor in allergic sensitization,[47,48] acting as an adjuvant in house-dust mite allergic sensitization[49] and exacerbating allergic asthma.[50] CO_2-RWE induced TNF consistently in our *in vitro* experiments, but unfortunately we could not detect this cytokine in BAL fluid *in vivo*. IL-6 secretion, which was upregulated in DCs stimulated with CO_2-RWE conditioned HNEC supernatants, is also implicated in facilitating Th2 polarization and simultaneous Th1 inhibition by activating NFAC and up-regulating SOCS-1 expression in naïve $CD4^+$ T cells.[51]

Expression of CD80 and CD86 on antigen-presenting cells is important for Th2 differentiation.[52] Both markers were increased by CO_2-RWE-stimulated HNEC supernatants or by both RWEs by direct DCs stimulations. The role of CD83 on DCs is controversial,[53] but seems to be important for $CD4^+$ T cell activation.[54] CD83 was downregulated by CO_2-RWE-stimulated HNEC supernatants compared to unstimulated. Combined with the expression of CD80/CD86, our findings emphasize the importance of the mode of DC stimulation, either by RWE directly or indirectly via HNEC supernatants.

In addition to activating epithelial-DC cross-talk, RWE acts directly on B cells, increasing IgE secretion under Th2-mimicking conditions.[55] We demonstrate that CO_2-RWE increased the IgE response *ex vivo* as well as *in vivo* compared to control-RWE. Thus, RWEs appear to act on several levels of the immune response contributing to the clinical phenotype of ragweed allergy, i.e. DC-mediated sensitization and B cell-mediated IgE production, which are both enhanced under exposure to CO_2-RWE.

To identify one or more substances responsible for the observed CO_2-RWE-induced increased allergic response, we first analyzed PALMs, known pollen-derived immune modulators.[18,20] Pollen-derived adenosine appears to be protective during allergic sensitization by inducing regulatory responses in dendritic-primed T cells *in vitro,*[26] whereas it mediates exacerbation of allergic lung inflammation *in vivo.*[24] Slightly elevated PALMS and adenosine in CO_2-RWE can only partly explain the increased inflammatory response following CO_2-RWE exposure. Therefore, we broaden the analysis investigating the pollen metabolome. Here we found a plethora of secondary plant metabolites differentially regulated by growth conditions. Metabolites which were exclusively present in CO_2-RWE (malvidin, pelargonidin, catalposide, 9-oxo-OTrE) or in control-RWE (lumichrome, Q3OS and *p*-Coumaryl-alcohol-4-*O*-glucoside), exhibiting mostly anti-inflammatory/tolerogenic characteristics[56-63] were employed for *in vitro* stimulations of DCs. Pelargonidin and malvidin alone were anti-inflammatory, while the opposite was seen for *p*-Coumaryl-alcohol-4-*O*-glucoside, and the other substances had almost no effect. We showed synergistic effects of the compound mixes, which induced a cytokine profile comparable to whole pollen extracts. Indeed, substances with known anti-inflammatory properties exhibited pro-inflammatory properties when applied as a mix. Metabolomic screening was performed in a non-targeted, semi-quantitative manner, providing a global overview of the pollen metabolome without delivering absolute quantities of the significantly modulated compounds. The substances were annotated by their exact mass and elemental composition and chosen according to their immunological properties and commercial availability in case of multi-annotation. Nevertheless, we can conclude that more than a single adjuvant substance in the allergen matrix is needed to transmit

an integrated signal via DCs to downstream effectors of the adaptive immune response, i.e. T- and B-cells.

In summary, we showed that CO_2-RWE elicits a stronger allergic response compared to control-RWE and that allergenicity cannot be confined to a single factor, but rather stems from the interplay of different mediators. Given that IPCC reports predict a rise in atmospheric CO_2 from currently around 400 ppm to a range of 730 - 1,020 ppm expected by the year 2100, [30] it should be noted that the impact of most pessimistic IPCC scenarios (e.g. 1000 ppm CO_2) might further enhance not only pollen biomass, but also pollen allergenicity, which will most probably contribute to an increase of allergic responses to ragweed in the population. Together with our previous research on effects of climate change scenarios on pollen[16,64] we demonstrate that climate change affects plants and pollen allergenicity, emphasizing the importance of viewing climate change as an existential threat to our health.

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Figure legends

Fig. 1. *Pollen of ragweed plants grown under elevated CO_2 levels elicits stronger allergic inflammation in vivo*. (A) Experimental setup. (B) BAL cell analysis. (C) Airway hyperresponsiveness measured 24 h after 11x intranasal exposures. n = 5 mice/group; $^{**}p < 0.01$, $^{***}p < 0.001$ vs PBS at same methacholine concentrations. (D) Total IgE levels *in vivo* after 11 instillations and (E) *ex-vivo. In vivo*: n = 5 mice/group; $^{***}p \leq 0.001$. Representative data of two independent experiments; Mann-Whitney-U test, except AHR: ANOVA with post-hoc Bonferroni test. *Ex-vivo*: n = 7 mice/group; Wilcoxon-signed rank test; $^{*}p < 0.05$, dashed line represents unstimulated control.

Fig. 2. *Pollen of ragweed plants grown under elevated CO_2 levels elicits stronger allergic inflammation in vivo.* (A) Flow cytometric analysis of lung tissue. (B-D) Representative PAS-staining of lung sections from mice instilled 11x with pollen extract (**B**: PBS, **C**: control-RWE, **D**: CO_2-RWE). Arrows: inflammatory infiltrate; arrowheads: mucus hypersecretion; scale bar: 100 µm. (E-F) Histological scores after 11x instillations. n=5 mice/group; Mann-Whitney-U test; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs PBS, same number of instillations (if applicable). $^{\#}p < 0.05$ $^{\#\#\#}p < 0.001$ vs same experimental group, 3x instillations.

Fig. 3. *Inflammatory mediators in BAL fluid.* All mediators were measured 24 h after 3x, 8x or 11x i.n. instillations with pollen extract. n = 5 mice/group; Mann-Whitney-U test; **p* < 0.05; ***p* < 0.01; ****p* < 0.001 vs PBS, same number of instillations.

Fig. 4. *Co-culture of moDCs with supernatants of CO_2-RWE stimulated HNECs elicits pro-inflammatory cytokine profile.* (A-E) IL-10, TNF, IL-6, IL-1β, CCL17/TARC secretion and (F) cytokine profile of moDCs after 24 h stimulation with RWE-conditioned HNEC supernatants (corresponding to 0.5 mg/ml and 1.8 mg/ml RWE). Dashed line indicates baseline cytokine production of moDCs. n = 35 independent experiments using cells from different donors; A, C, D-E RM-one-way ANOVA with Sidak's correction for multiple comparisons, B Friedman's test with Dunn's correction; ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 vs. baseline unless indicated otherwise.

Fig. 5. *Pollen of ragweed plants grown under elevated CO_2 levels induce pro-inflammatory cytokine profile in moDCs.* (A-E) IL-10, TNF, IL-6, IL-1β, CCL17/TARC were measured in cell culture supernatants after 24 h stimulation with 2.5 mg/ml control- or CO_2-RWE and the results were summarized in a profile (F). Dashed line indicates unstimulated control. n = 24 independent experiments using cells from different donors; A) RM one-way ANOVA with Sidak's test for multiple comparisons, B-E Friedman's test with Dunn's correction for multiple comparisons;**p* < 0.05, ***p* < 0.01 comparison between treatment groups.

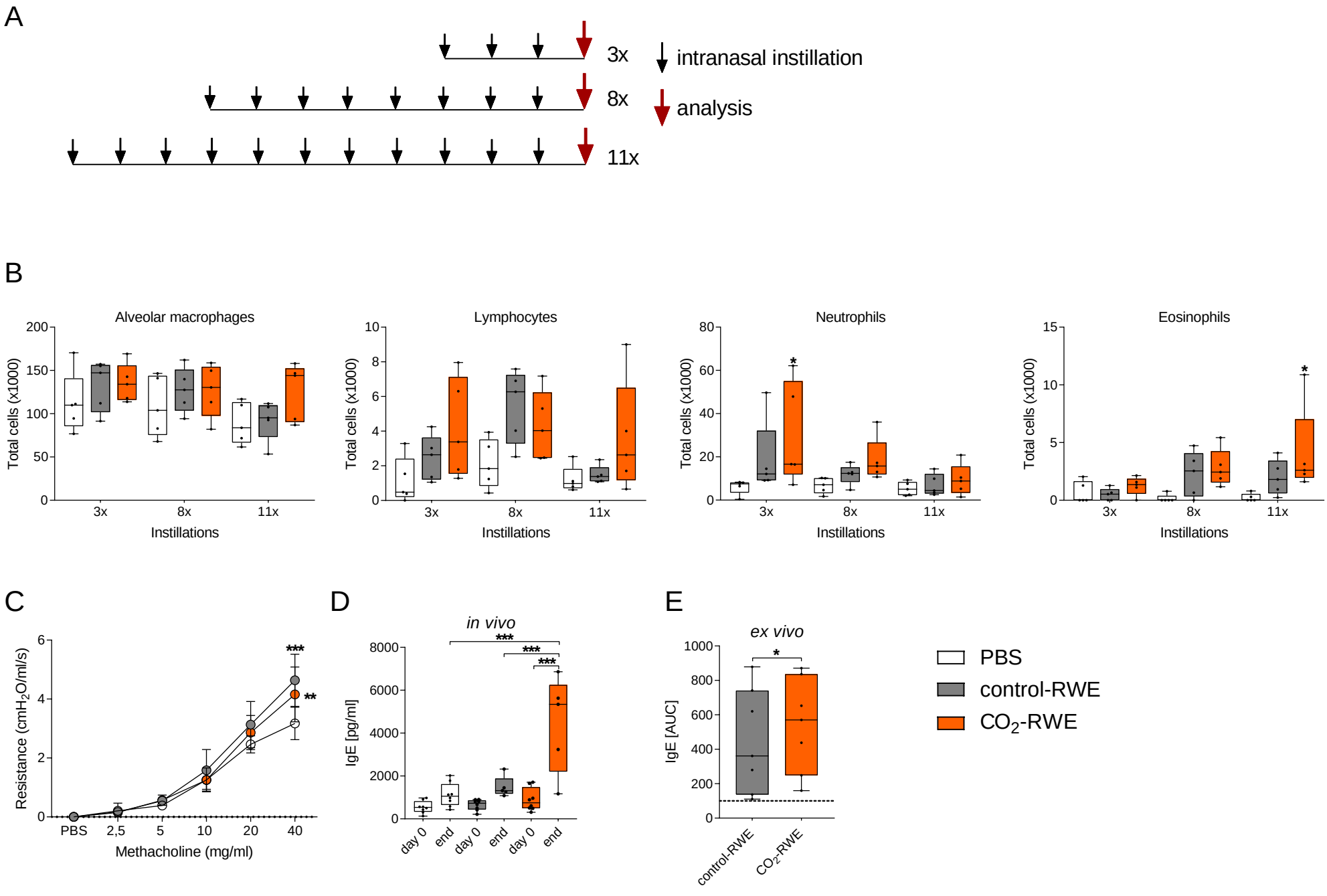
Fig. 6. *Metabolome analysis of RWEs reveals differentially expressed clusters of substances.* (A) PALMs, LPS, adenosine and Amb a 1 measured in extracts of single plants (n=10). (B) Heatmap of substances present in RWE are clustered using Euclidean distance measure and Ward's linkage-clustering algorithm. (C) Principal component analysis (PCA). (D) Univariate volcano plot analysis of all metabolites. n=3 control-RWEs and n=4 CO_2-RWEs for metabolome analysis. (E-H) Cytokines measured in DC supernatants 24 h after stimulation with compound mixes (concentration $3x10^{-7}$ M, table 1). n = 27 independent experiments using cells from different donors; Wilcoxon-signed rank test; **p* < 0.05, ***p* < 0.01 comparison between treatment groups.

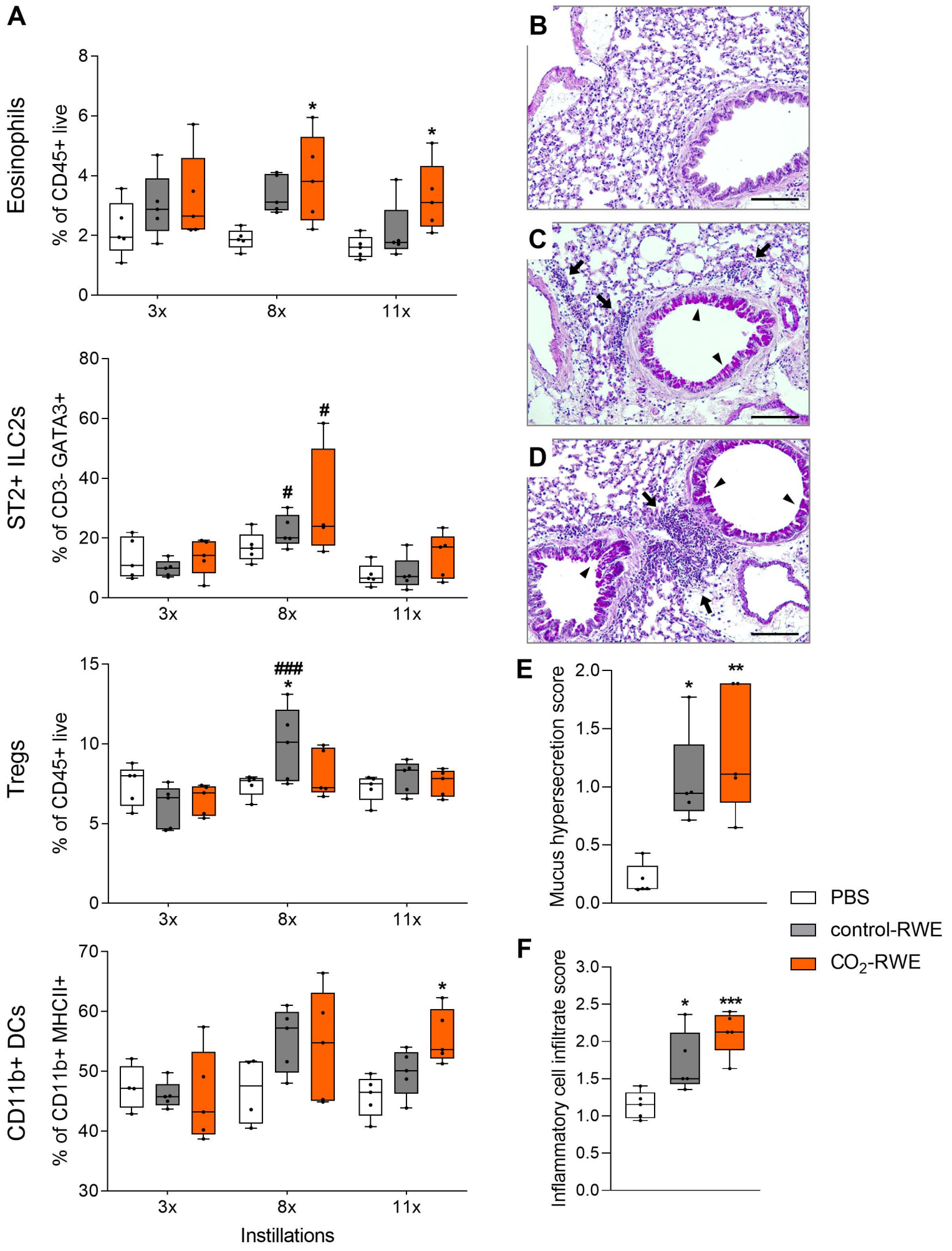
Table 1. Overview of cell donors for this study. A total IgE of <100 kU/ml and/or RAST class 0 for common airborne allergens was considered non-atopic.

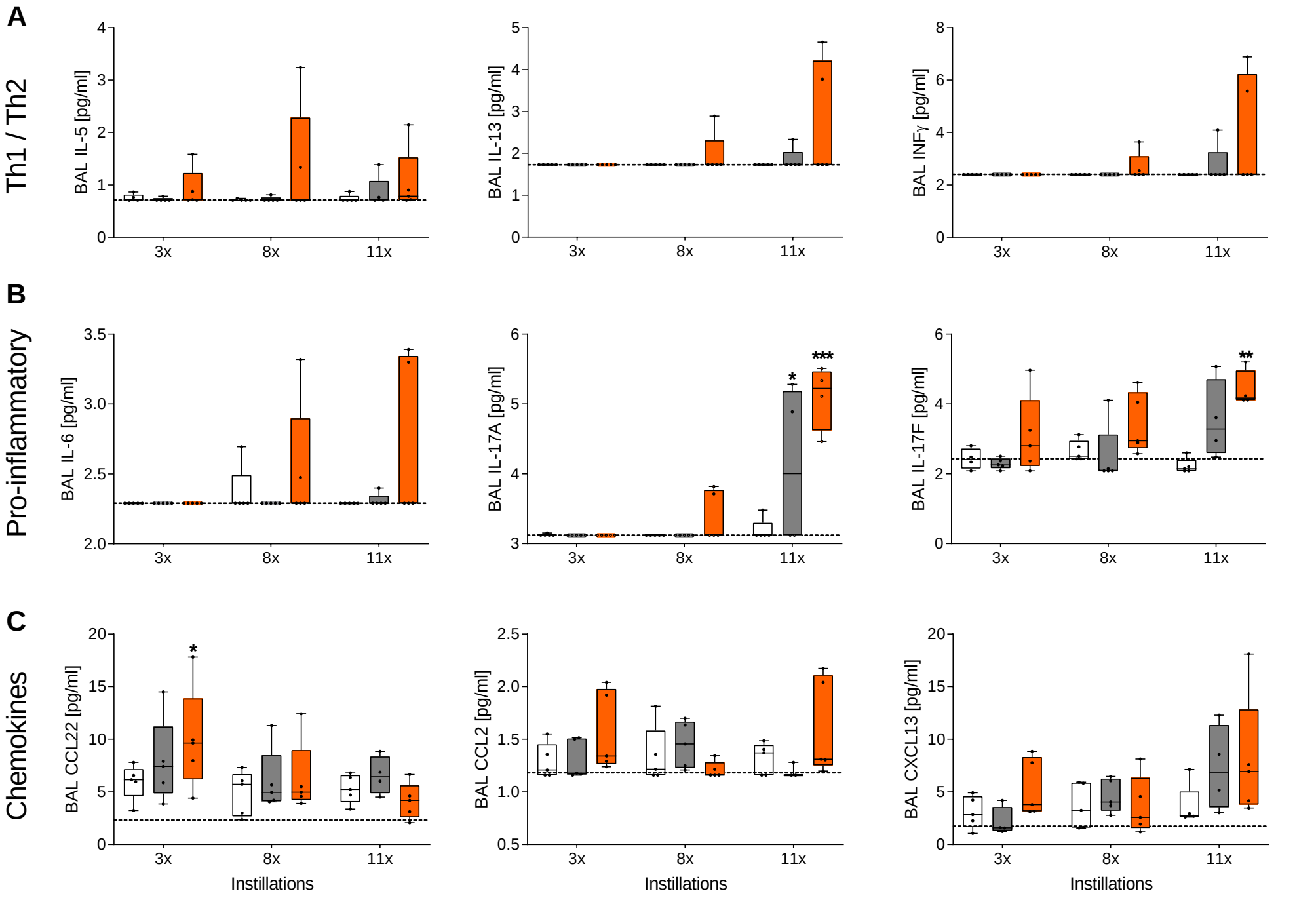
Gender	No. Donors	Age	Total IgE kU/ml (mean)	Aeroallergens (RAST class; HDM/Cat/Dog/Oat/Grasses/Rye/Penicillium/ Cladosporium/Aspergillus/Alternaria/Botrytis/Alder/ Birch/Hazel/Ash/ Mugwort/Buckhorn)
Female	23	20 - 61	37.00	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
Male	13	32 - 67	68.78	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0

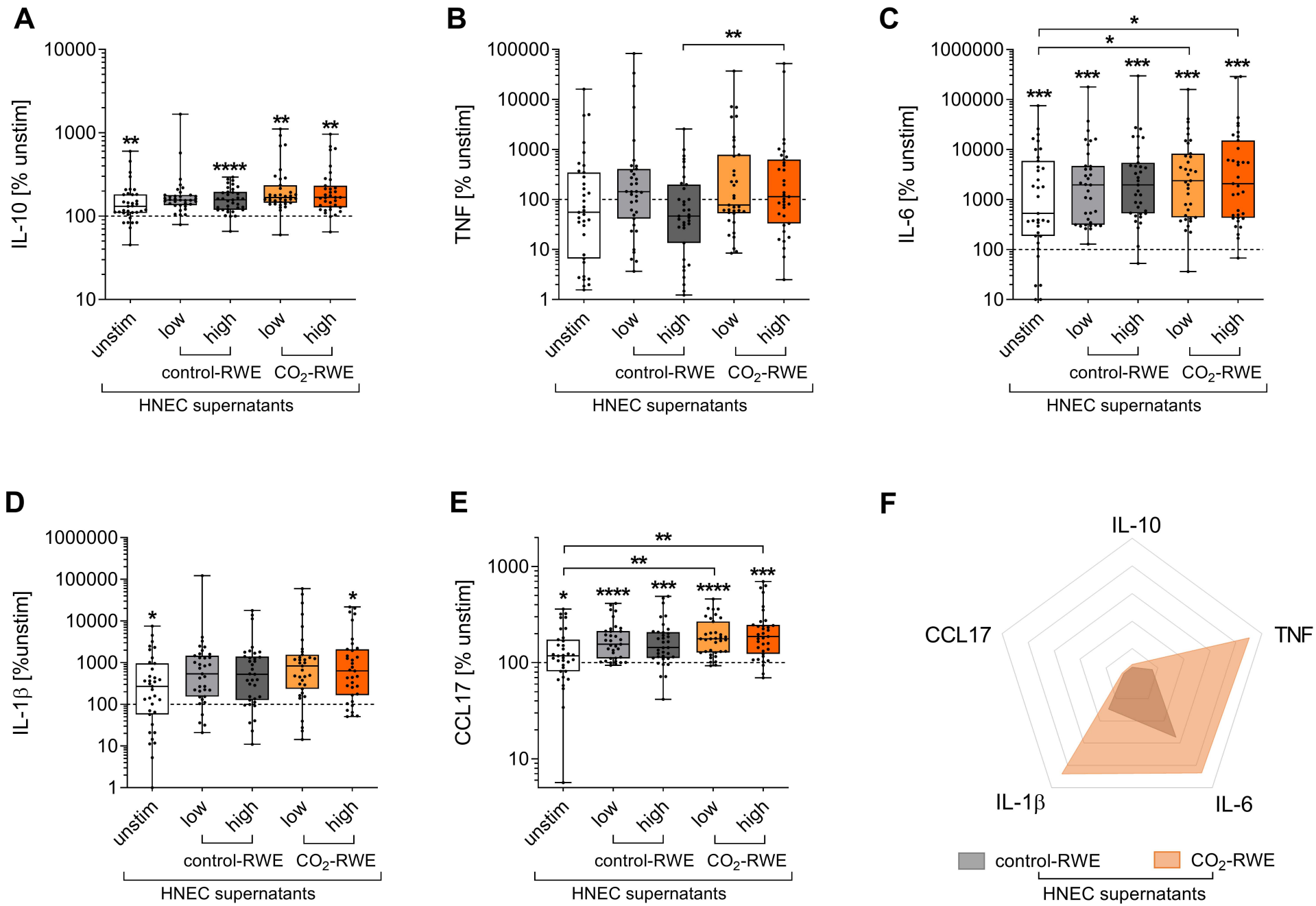
Table 2. Putative substances identified in CO_2-RWE and control-RWE, their compound class and corresponding compound mix.

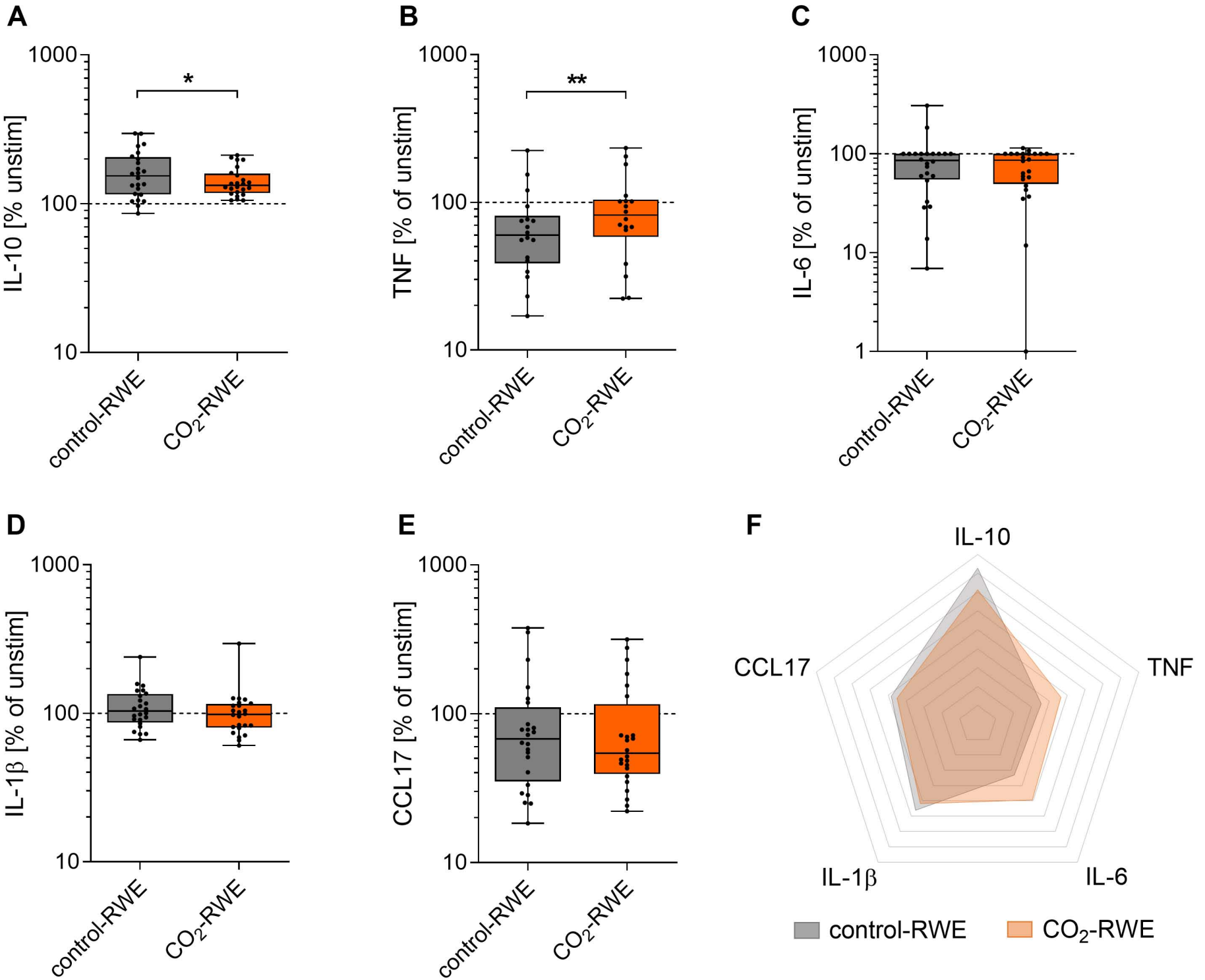
Compound	Compound class	Compound-Mix	Company
Pelargonidin (Pel)	Anthocyanidins	CO_2-Mix	Sigma Aldrich (Taufkirchen, Germany)
Malvidin (Mal)	Anthocyanidins		
Catalposide (Cat)	Terpenoids		
9-Oxo-OTrE (9-OTrE)	α-Linolenic acid metabolites		
p-Coumaryl alcohol 4-*O* glucoside (pC4OG)	Phenylpropanoids	Control-Mix	
Lumichrome (Lumi)	Riboflavins		
Quercetin-3-*O*-sophoroside (Q3OS)	Flavones and flavonols		F. Ferreira and L. Aglas, University of Salzburg, Austria

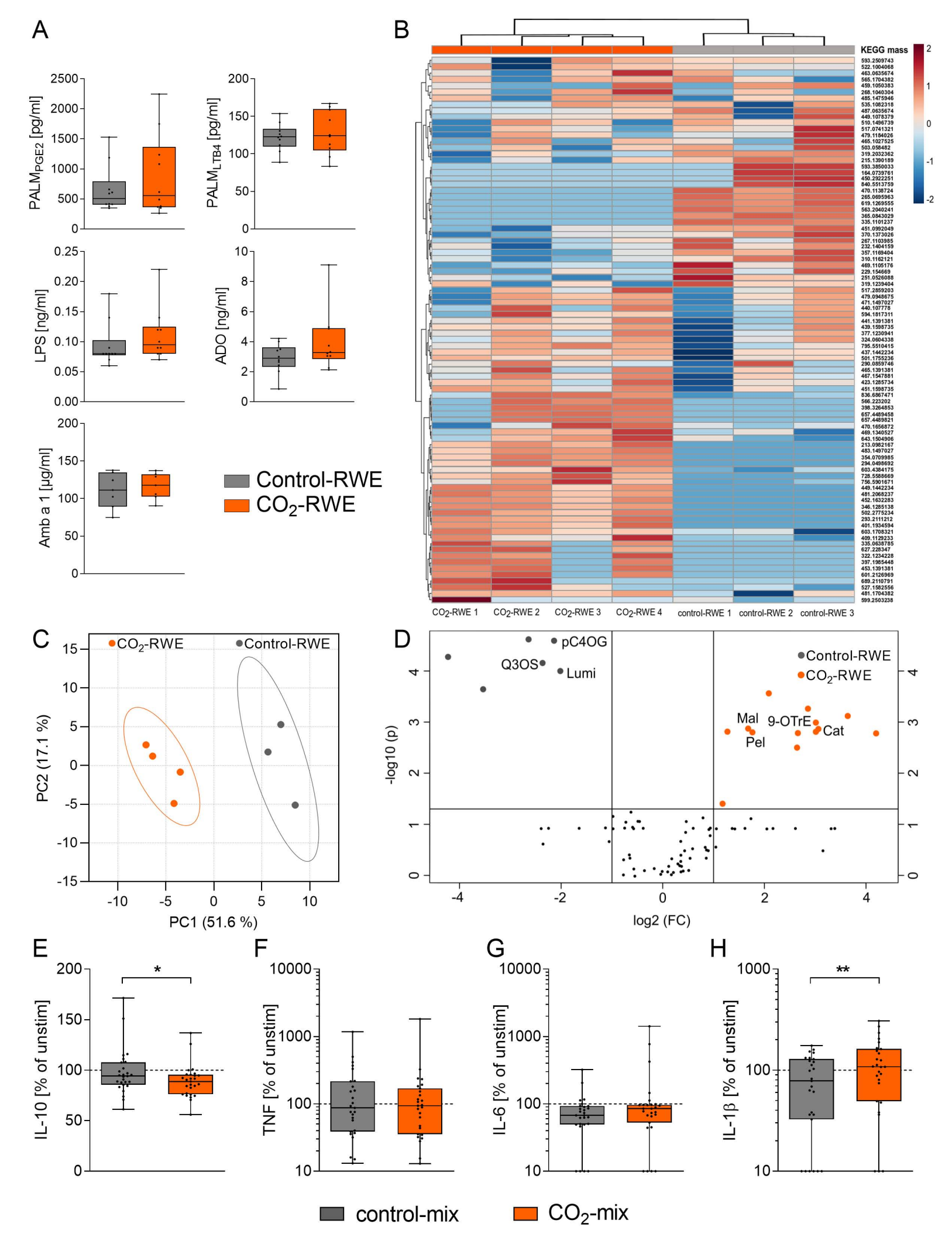

A
3x
8x
11x
intranasal instillation
analysis
B
Alveolar macrophages
Lymphocytes
Neutrophils
Eosinophils
Total cells (x1000)
Instillations
3x
8x
11x
C
Resistance (cmH2O/ml/s)
Methacholine (mg/ml)
PBS
2,5
5
10
20
40
D
in vivo
IgE [pg/ml]
day 0
end
E
ex vivo
IgE [AUC]
control-RWE
CO2-RWE
PBS
control-RWE
CO2-RWE


A
Eosinophils
% of CD45+ live
ST2+ ILC2s
% of CD3- GATA3+
Tregs
% of CD45+ live
CD11b+ DCs
% of CD11b+ MHCII+
3x
8x
11x
Instillations
B
C
D
E
Mucus hypersecretion score
F
Inflammatory cell infiltrate score
PBS
control-RWE
CO2-RWE


A
Th1 / Th2
BAL IL-5 [pg/ml]
BAL IL-13 [pg/ml]
BAL INFγ [pg/ml]
B
Pro-inflammatory
BAL IL-6 [pg/ml]
BAL IL-17A [pg/ml]
BAL IL-17F [pg/ml]
C
Chemokines
BAL CCL22 [pg/ml]
BAL CCL2 [pg/ml]
BAL CXCL13 [pg/ml]
3x
8x
11x
Instillations
*
***
**
PBS
control-RWE
CO2-RWE


A
IL-10 [% unstim]
10000
1000
100
10
unstim
low
high
low
high
control-RWE
CO2-RWE
HNEC supernatants
B
TNF [% unstim]
100000
10000
1000
100
10
1
C
IL-6 [% unstim]
1000000
100000
10000
1000
100
10
D
IL-1β [%unstim]
1000000
100000
10000
1000
100
10
1
E
CCL17 [% unstim]
1000
100
10
F
IL-10
TNF
IL-6
IL-1β
CCL17
control-RWE
CO2-RWE
HNEC supernatants


A
B
C
D
E
F
IL-10 [% unstim]
TNF [% of unstim]
IL-6 [% of unstim]
IL-1β [% of unstim]
CCL17 [% of unstim]
1000
100
10
1
*
**
control-RWE
CO2-RWE
IL-10
TNF
IL-6
IL-1β
CCL17


A
PALM$_{PGE2}$ [pg/ml]
PALM$_{LTB4}$ [pg/ml]
LPS [ng/ml]
ADO [ng/ml]
Amb a 1 [µg/ml]
Control-RWE
CO$_2$-RWE
B
KEGG mass
CO$_2$-RWE 1
CO$_2$-RWE 2
CO$_2$-RWE 3
CO$_2$-RWE 4
control-RWE 1
control-RWE 2
control-RWE 3
C
CO$_2$-RWE
Control-RWE
PC2 (17.1 %)
PC1 (51.6 %)
D
pC4OG
Q3OS
Lumi
Control-RWE
CO$_2$-RWE
Mal
9-OTrE
Cat
Pel
-log10 (p)
log2 (FC)
E
IL-10 [% of unstim]
F
TNF [% of unstim]
G
IL-6 [% of unstim]
H
IL-1β [% of unstim]
control-mix
CO$_2$-mix