Glyphosate differentially affects the allergic immune response across generations in mice

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abstract

Exposure to environmental pollutants via food, particularly during the prenatal and early postnatal periods, has been linked to adverse effects on the immune system. Among these pollutants, the widely used pesticide glyphosate has been associated with endocrine disruption, autism, and cancer. Occupational high exposure to glyphosate has also been shown to influence immune function and exacerbate allergic asthma. However, there are no studies investigating the effect of a common low-dose glyphosate exposure on the allergic immune response – neither directly nor across generations. We therefore explored the impact of oral low-dose glyphosate exposure (0.5 and 50 mg/kg body weight/day) on airway inflammation in dams (F0) and the offspring (F1 and F2 generations) using a murine multi-generational asthma model.

While exposure to 50 mg/kg glyphosate induced a mild eosinophilic infiltration in the bronchoalveolar lavage and T_H2 cytokine production in the dams, the F1 offspring developed a reduced immune response after maternal exposure to 0.5 mg/kg glyphosate. In particular, decreased lung inflammation, HDM-specific IgE levels, and asthma-relevant cytokine production were primarily observed in the female F1 offspring. However, not only the T_H2 cytokines IL-13 and IL-5 but also the T_H17 cytokine IL-17 and T_H1 cytokine IFN-γ were reduced indicating a more general immunosuppressive function. Notably, the dampened immune response was no longer observed in the female F2 generation. Furthermore, female F1 offspring showed an increased abundance of bacteria in the gut, which have been associated with probiotic-mediated reduced allergic immune responses. Our results suggest a potential immunosuppressive effect of low-dose maternal glyphosate exposure in the F1 offspring that might be mediated by an altered microbiota composition. Further studies are needed to explore if this type of immune response modulation might also be

associated with impairments in immune defense upon infectious diseases or even cancer pathology.

1. Introduction

Humans are ubiquitously exposed to multiple environmental pollutants (Mitro et al., 2015). Next to well-investigated chemicals in plastic materials and commercial household products like phthalates, bisphenol A and perfluoroalkyl substances (PFASs), pesticide residues are the most common chemicals found in the natural environment (Kvalem et al., 2020; Martín et al., 2019; Ye et al., 2017). Pesticides are used to eliminate active organisms like insects (insecticides), fungi (fungicides), or weeds (herbicides) and are applied in agricultural contexts as well as in landscaping, public parks, or domestic homes (Dahiri et al., 2021; Kim et al., 2017). The general population is most commonly exposed to pesticides via the food chain, air, and water, however, dietary exposure seems to account for up to 90% of total pesticide exposure (Anderson et al., 2014; Yilmaz et al., 2020). While acute toxicity is well-established for a variety of pesticides, research is limited with respect to exposure scenarios seen in the general public with chronic low-dose concentrations (Requena-Mullor et al., 2021). These chronic low-dose exposures are suspected to trigger biochemical pathways in the highly susceptible in-utero development and the early postnatal phase potentially leading to adverse offspring health outcomes.

Among these pesticides, glyphosate is the most widely used pesticide worldwide. Even though its use in agriculture has been approved since the 1970s internationally, there is still a distinct lack of consensus between authorizing agencies like the European Food Safety Authority (EFSA), International Agency for Research on Cancer (IARC) or Food and Agriculture Organization (FAO), about the range of concentration at which glyphosate is to be considered safe (Agostini et al., 2020). Glyphosate and its residues have been readily detected in and on different food products (Louie et al., 2021), however, the quantities of glyphosate to which the general population is exposed daily

via food residues is still unclear (Connolly et al., 2020; Gillezeau et al., 2020). Recent human data extrapolated from glyphosate's urinary excretion rate showed that average glyphosate intake in westernized countries might be close to EFSA's acceptable daily intake (ADI) level (Connolly et al., 2020; Vandenberg et al., 2017), with exposures even higher in occupational settings or lower-income countries due to greater pesticide use and less restrictive pesticide regulations (Buralli et al., 2020). Indeed, it was shown that the general population exposure has continuously increased over the past decades (Gillezeau et al., 2020). So far, it is unclear whether low-dose glyphosate concentrations that are deemed acceptable for the common exposure by regulatory agencies, still have adverse effects in susceptible individuals like the unborn fetus exposed in utero or via breastmilk (Gillezeau et al., 2020; Mamane et al., 2015). There is increasing evidence that glyphosate may possess endocrine-disrupting properties, but research on a potential immune-modulatory impact with consequences for asthma development is still scarce (Maddalon et al., 2021). Epidemiological studies in an occupational context suggest a potential correlation between direct exposure to glyphosate-based herbicides (GBH) and wheeze and asthma in women (Hoppin et al., 2008; Hoppin et al., 2017). Experimental investigations using different animal models on whether glyphosate potentially affects innate and adaptive immune system remain inconclusive (Peillex et al., 2020). So far, experimental perinatal glyphosate exposure has been associated with autism, depression, or infertility (Milesi et al., 2021; Rueda-Ruzafa et al., 2019). To the best of our knowledge, there are no published experimental studies in mammals investigating the effects of maternal glyphosate exposure on the allergic immune response in the F0, F1, or F2 generation. To provide insights into this topic, we employed a well-established multi-generational mouse model (Jahreis et al., 2018; Junge et al., 2021; Junge et al., 2022) in which dams were chronically exposed to low doses of glyphosate during pregnancy, the lactational period and thereafter. In

this context, we studied the influence of glyphosate gavage on asthma development in the dams themselves but also in the next two generations.

2. Methods

1. Mice

Female Balb/cByJ mice (6-8 weeks of age) were purchased from the Elevage Janvier Laboratory (Le Genest St Isle, France) with a 7-day adaption period before the start of experiments. Animals were maintained in groups of 3-6 mice per cage in the animal facility at the University of Leipzig (Germany) under conventional conditions with 21.5 - 23°C room temperature, an average of 55% humidity, and a 12-hour day/night rhythm. Exposed and control dams as well as the offspring of exposed and control mice were housed separately. All mice were kept in multiple sealed cages with HEPA filters by Sealsafe® and bedded with LIGNOCEL® bedding material. Dams and pups received a phytoestrogen-free diet (C1077 from Altromin, Lage, Germany) and water *ad libitum*. All animal experiments were performed at least 3 times with at least 3 dams per group resulting in ≥ 8 pups per group and sex (with a maximum of 4 pups per sex per dam). The F2 generation included ≥ 4 female offspring. All animal experiments were conducted in accordance with institutional and state guidelines. Animal protocols used in this study were approved by the Committee on Animal Welfare of Saxony/Leipzig (Permit Number: TVV14/18).

2.2. Chronic exposure to glyphosate

Female Balb/c mice were exposed to the active substance glyphosate (N-(Phosphonomethyl)glycine; diluted in water) orally administered by gavage in 300 µl distilled water three times per week. The intervention lasted from one week before mating with unexposed BALB/c males until weaning of the pups at 3 weeks. After

143 weaning dams were further exposed to glyphosate until the end of the asthma protocol
144 (total exposure: 95 days; Supplementary Figure E1). Mice received a weekly
145 glyphosate concentration of either 8.75 mg or 87.5 µg, respectively equating to the no
146 observed adverse effect level (NOAEL; 50 mg/kg body weight/day, GLY_{NOAEL}) or
147 human acceptable daily intake (ADI; 0.5 mg/kg body weight/day, GLY_{ADI}) concentration
148 of glyphosate (EFSA, 2015). Control dams received distilled water. Respective
149 concentrations were chosen as they, per definition, should not induce direct adverse
150 effects on dams to allow an unbiased analysis of the progeny.
151
152 *2.3. HDM-induced asthma model*
153 To investigate the impact of glyphosate exposure on the development of allergic airway
154 inflammation in the dams and the offspring, mice were intranasally (i.n.) sensitized with
155 house dust mite extract (HDM, D. pteronyssinus 1, endotoxin: 1.273 EU/ml, Greer
156 Laboratories, USA) beginning at four weeks after weaning (dams) or the age of 10
157 weeks (offspring). Mice were first sensitized with an initial higher concentration of HDM
158 at 5 µg dissolved in 40 µl NaCl on day 1. This was followed by an allergen challenge
159 on days 8 - 12 and 15 to 17 applying half of the initial concentration (Supplementary
160 Figure E1). Control mice received normal saline i.n. The asthma-like phenotype was
161 characterized by airway responsiveness, bronchoalveolar lavage (BAL) infiltration,
162 lung inflammation, allergen-specific IgE levels, and the production of the T helper 1
163 and 2 (T_H1/ T_H2) cytokines (Junge et al., 2021; Junge et al., 2022).
164
165 *2.4. Measurement of airway responsiveness*
166 Lung resistance (R_L) was measured using a whole-body plethysmograph (EMKA
167 TECHNOLOGIES, Paris, France). Plethysmography was conducted 24 hours after the
168 last intranasal HDM administration as described earlier (Jahreis et al., 2018; Polte et

al., 2015). Briefly, to determine R_L mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine, Bayer, Leverkusen, Germany), intubated, and mechanically ventilated at a tidal volume of 0.2 ml and a frequency of 150 breath/min. Firstly, Baseline R_L and responses to aerosolized saline (0.9% NaCl) were assessed, followed by responses to increasing doses (2.5, 5, 10, 20, and 40 mg/ml) of aerosolized methacholine.

2.5. Bronchoalveolar lavage (BAL) & lung histology

The extent of inflammation in asthma development was characterized by the differential leukocyte count within the BAL fluid. BAL fluid was obtained after euthanasia by making a small incision in the trachea and then inserting a syringe to lavage the right lung three times with 400 µl with phosphate-buffered saline (PBS). All cells within the lavage fluid were counted using a hemocytometer. Diffquick® (Medion Diagnostics AG, Düdingen, CH) stained cytospins were differentiated into eosinophils, macrophages, lymphocytes and neutrophils according to conventional morphological criteria (Jahreis et al., 2018; Polte et al., 2015).

2.6. HDM-specific IgE

Blood was collected from the mice's hearts after they were sacrificed. The blood was centrifuged at 3500 rpm for 15 min. The serum was collected and stored at − 20 °C for subsequent analysis of serum HDM-specific IgE. Serum HDM-specific IgE was measured by sandwich ELISA according to a standard protocol. Briefly, 96-well microtiter plates (Nunc) were coated overnight with 50 µg/ml HDM. After washing and blocking of plates, samples were incubated for 2 hours. Subsequently, 96-well plates were washed, and Biotin-anti-mouse IgE antibody (BioLegend) was added.

Tetramethylbenzidine was used as substrate, and the optical density (OD) was determined at 450 nm.

2.7. Lung histology

Left lung was fixed in 10% formalin and stained with Haematoxylin & Eosin (H&E, MERCK, Darmstadt, Germany) to analyze inflammatory infiltrates in the airways (Junge et al., 2021; Petzold et al., 2014; Polte et al., 2015).

2.8. Cytokine production

One day after airway function test splenocytes (1 x 10^7 cells/ml per well) were isolated and re-stimulated in vitro with 100 µg/ml HDM in culture medium (RPMI medium supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin). After a 48-hour incubation period, the cultures were frozen for later cytokine expression analysis. Cytokines were measured in supernatants from re-stimulated spleen cells using DuoSet ELISA kits (R&D Systems, Wiesbaden-Nordenstadt, DE) according to the manufacturer's instructions.

2.9. Measurement of 8-isoprostane

To investigate lipid peroxidation/oxidative stress in the lung, tissues were homogenized using lysing matrix A (FastPrep®24 homogenizer, MP Biomedicals, LLC, Eschwege, Germany) and aliquots of the obtained supernatants were hydrolyzed (15% KOH) and deproteinized (ethanol containing 0.01% BHT). 8-isoprostane concentration was determined by specific immunoassay according to manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI, USA) as described previously (Junge et al., 2021).

2.10. Microbiota assessment

To assess microbiota community structure we used 16 S rRNA gene profiling of caecum samples of dams as well as three-week-old offspring upon sacrificing. DNA was extracted with QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) as previously described (Haange et al., 2020). V3-V4 variable regions of the 16 S rRNA genes were amplified by PCR and a library was constructed, followed by paired-end 2x250bp Illumina sequencing (StarSEQ GmbH, Mainz, Germany). Raw data were processed by Starseq using the QIIME 2 workflow (Bolyen et al., 2019). Here, data was de-multiplexed and quality checked, primers removed, paired-end reads were joined, and low-quality reads removed (de Sena Brandine et al., 2019). Amplicon Sequencing Variants (ASVs) were obtained, after read correction and chimera removal using the deblur workflow (Quast et al., 2013). The read counts per ASV with taxonomic annotation were normalized and relative abundances of each ASV and taxa were calculated using the R scripts Rhea (Lagkouvardos et al., 2017).

2.11. Statistical analysis

Experimental data sets were processed and analyzed in GraphPad PRISM 9.1.2 for macOS (GraphPad Software, Inc.). Data were expressed as mean ± SEM and *P* values of less than 0.05 were considered significant by t-test or Wilcoxon-Mann-Whitney test according to the individual data sets. Microbiome alpha-diversity (Richness and Shannon-Effective) and beta-diversity (Principal Component Analysis) analysis of taxa was done using an in-house written R-tools. For group statistics, Kruskal-Wallis test was used followed by a posthoc pairwise Dunn test. For global beta-diversity statistics, a PERMANOVA was performed to determine significant differences between treatment groups using the vegan R package (Dixon, 2003). Visualization of data was done in ggplot2 (Ginestet, 2011). For genus level, data with the highest relative

246 abundance/sample > 0.2 were included and analyzed using ANOVA with Dunnet
247 correction for multiple comparisons.
248
249 **3. Results**
250 *3.1. Asthma development in the F0 generation after direct glyphosate exposure*
251 Chronic exposure to GLY_{NOAEL} increased the eosinophilic infiltration in the BAL without
252 reaching significance (Figure 1A) in the dams while lung inflammation, IgE levels and
253 lung function were only slightly or not affected (Figure 1B-D, Figure 2A). However, the
254 production of the T_H2 cytokines IL-4 and IL-13 but also of IL-17 and IFN-γ was
255 significantly increased in mice directly exposed to GL_{NOAEL} (Figure 2B). Furthermore,
256 8-isoprostane levels as a marker for lipid peroxidation in lung homogenates were
257 slightly but not significantly increased after GLY_{NOAEL} exposure (Supplementary figure
258 E2A). Exposure to GLY_{ADI} had no effect on the asthma phenotype in the F0 generation
259 (Figure 1A-D, Figure 2A, B).
260
261 *3.2. Effects on asthma development in the F1 generation*
262 Next, we assessed the effect of maternal glyphosate exposure on the offspring's
263 asthma development in later life. In this context, maternal exposure to GLY_{ADI} led to a
264 significant decrease of eosinophils in BAL fluid (Figure 1A) as well as a diminished
265 lung inflammation in the female but not in the male offspring as demonstrated by HE-
266 stained lung sections (Figure 1B) and verified by an objective, investigator-
267 independent computer analysis (Figure 1C). Furthermore, HDM-specific IgE serum
268 levels were also significantly reduced in female mice from GLY_{ADI}-exposed dams while
269 no significant effects were observed in the offspring of GLY_{NOEL}-exposed dams (Fig.
270 1D). In contrast, glyphosate exposure during the pre- and postnatal period was not
271 associated with changes in lung resistance in the offspring at both concentrations

(Figure. 2A). Solely, maternal GLY_{ADI} exposure diminished T_H1 and T_H2 cytokine production in both males and females significantly (Figure 2B).

3.3. Effects on asthma development in the F2 generation

To investigate whether the diminished immune response in the female F1 generation of glyphosate-exposed dams persists up to the female F2 generation, female F1 mice from exposed dams were mated with non-exposed males and an asthma-like phenotype in the F2 generation was induced. In contrast to the F1 generation, there were no significant effects on eosinophils, lung inflammation, or lung resistance (Figure 3A-C). Also, neither HDM-specific IgE serum levels nor cytokine expression in splenocytes were affected in the female F2 offspring (Figure 3D, E).

3.4. Effects of glyphosate exposure on gut microbiota

Since the gut microbiome is assumed to be a crucial factor in asthma development in the first year of life (Depner et al., 2020) and glyphosate is supposed to induce alterations in microbiota (Rueda-Ruzafa et al., 2019), we have investigated the bacteria community structure using 16S rRNA gene profiling of caecum samples comparing the 3-weeks-old female offspring from glyphosate-exposed dams with the female offspring from control mice. While alpha-diversity of the gut microbiome was not affected in the F1 mice by maternal glyphosate exposure (Figure 4A) the variation of microbial communities between the groups was significantly different (Figure 4B). The distribution of microbial families did not indicate significant differences between groups (Figure 4C). To find mechanisms which may explain the immune-modulating effect of low-dose glyphosate (GLY_{ADI}) the relative abundance of the gut microbiota on genus level in female F1 offspring from glyphosate-exposed dams was investigated (Supplementary Table E1). Here, we observed a significantly increased relative

abundance of *Odoribacter* and *Lachnospiraceae NK4A136* at the genus level in the juvenile offspring after maternal GLY_{ADI} exposure compared to the offspring from GLY_{NOAEL}-exposed or control dams (Figure 4E).

To determine if the changes oberserved in the F1 generation constitue a mere transmission of the maternal microbiota, we also analyzed the dams' microbiota composition (Supplementary Table E2). Interestingly, increased relative abundance of Odoribacter and Lachnospiraceae NK4A136 that was seen in the F1 generation after maternal GLY_{ADI} exposure, was not observed in dams that were directly exposed to $GLY_{ADI.}$ However, GLY_{ADI} expsosure of dams led to alterations in the relative abudance of Akkermansia, Bacteroides, Clostridium sensu stricto 1, Lachnospiraceae UCG-006 and Lactobacillus at genus level compared to control dams without glyphosate exposure.

4. Discussion

Within this study, we investigated both the direct effect of chronic low-dose exposure to glyphosate on the allergic immune response in dams and the impact of glyphosate exposure during pregnancy and breastfeeding on the offspring's asthma development until the F2 generation. To our knowledge, cross-generational long-term effects of *in-utero* exposure to glyphosate have not yet been examined in the context of allergic diseases (Maddalon et al., 2021).

Our results show an increased eosinophilic infiltration in the BAL and increased lipid peroxidation in the lung of F0 mice directly exposed to GLY_{NOAEL} - although the effects did not reach significance level compared to unexposed control animals. However, a pro-inflammatory impact on the lung was also recently demonstrated in a mouse study after repeated exposure to LPS and low-dose glyphosate (Pandher et al., 2021). This

is in line with the significantly enhanced production of pro-inflammatory Th2 cytokines we found after GLY_{NOAEL} exposure compared to unexposed controls. There are several observational human studies on direct asthma effects in association with occupational exposure to glyphosate; however, there are discrepancies in the observed outcomes. While Hoppin et al. (2017) reported a positive association between occupational glyphosate exposure in farmers and wheeze or asthma severity, Henneberger et al. (2014) on the other hand found an inverse relationship between glyphosate exposure and asthma exacerbation in pesticide-using farmers with active asthma. Nonetheless, these studies' participants were exposed to exceedingly high concentrations of glyphosate via air in addition to common exposure via food, so these scenarios cannot be compared to the situation in the general population (Agostini et al., 2020; Peillex et al., 2020)

With respect to cross-generational effects of glyphosate exposure, it has been shown recently that low-dose maternal glyphosate concentrations can in fact lead to adverse health outcomes across several generations. For instance, low concentrations of glyphosate have been suggested to induce epigenetic changes in both sperm and ovaries in murine models leading to reproductive issues up to the F2 and even F3 generation (Guerrero Schimpf et al., 2021; Kubsad et al., 2019; Rossetti et al., 2021). We too observed fewer successful offspring deliveries in particular in the maternally exposed F1 generation leading to a limited number of mice we could characterize in the F2 generation in the glyphosate exposed groups. With respect to the asthma outcome, we found that maternal exposure to glyphosate during pregnancy and the lactational period was associated with a reduced immune response in the F1 but not in the F2 offspring. Interestingly, the asthma-reducing effects in our multi-generational mouse model were found only at the lower glyphosate concentration and primarily in

the female F1 offspring. With respect to concentration differences, this might suggest a U-shaped dose-response curve as described for other chemicals before (Calabrese et al., 2001; Vandenberg et al., 2012). The sex-specific effect could be an indicator of the known endocrine disruptive properties of glyphosate (Maddalon et al., 2021). Furthermore, it is also important to note that maternal glyphosate exposure not only alleviated the asthma-relevant T_H2 cytokines IL-13 and IL-5, but the T_H17 cytokine IL-17, and T_H1 cytokine IFN-γ as well, indicating a more general immunosuppressive function (Leung et al., 2010). The decrease in serum IgE level can likely be attributed to the observed reduction of T_H2-cytokines, as these cytokines in fact induce the conversion of B-cells into IgE-producing plasma cells. A general glyphosate-associated down-regulation of the T-cell immune response would also be of concern with respect to cancer risk – to which glyphosate exposure has been controversially linked for several years (Davoren et al., 2018; Peillex et al., 2020). Additionally, it is tempting to speculate that low-dose glyphosate can affect immune responses to bacterial, viral or parasitic infections, as recent data indicated an altered defense capacity to infections in glyphosate-exposed fish (Le Du-Carrée et al., 2022).

So far, the majority of experimental studies on glyphosate addressing its impact on the immune system have been conducted in fish models supporting the idea of a U-shaped dose-response relationship of glyphosate regarding immunomodulation. In summary, these studies indicated an immunosuppressive effect of glyphosate when low-doses were applied while an over activation of the immune system was observed in studies that applied excessive concentrations (Peillex et al., 2020). This is in line with our findings in the female F1 offspring showing immunosuppressive effects after exposure to low ADI concentration, while the 100 times higher NOAEL concentration did not exert significant effects. Further, Ma et al. (2015) also showed a reduction of cytokine

expression in splenocytes in carps due to low doses of glyphosate. Kreutz et al. (2010) demonstrated that low dosed glyphosate rendered fish more susceptible to infection - presumably due to reduced phagocytosis. Unfortunately, studies in mammals are scarce and merely investigated extremely high concentrations of glyphosate in air stating inflammatory immune responses with local inflammation and increased cytokine production (Kumar et al., 2014; Tang et al., 2020). However, it can be summarized that these studies are less comparable to occupational and common public scenarios as applied glyphosate concentrations are known to exert toxicity (Maddalon et al., 2021). Additionally, observational and experimental evidence on how low-dose exposures to pesticides during the perinatal phase affect the offspring's asthma development in a non-occupational context is still inconclusive – depending on how pesticide exposure was measured or which type of pesticide applied (Mamane et al., 2015; Rosa et al., 2018). A further limitation of most studies and also our's is the fact that while we study exposure to pure glyphosate, real-life exposure occurs to the commercially available solution that further contains additional substances, like surfactants. Thus, results obtained in mouse studies may not be easy to extrapolate to population studies as secondary effects of the herbicide may not be due to the main active substance only (Mesnage et al., 2019).

It has long been known that chronic exposure to low doses of pesticides like malathion or atrazine can impede immune functions, which might bear harmful effects for non-communicable diseases, like allergic diseases or even cancer, especially in the later life of unborn children (Corsini et al., 2008; Corsini et al., 2013; Dietert, 2014; Mansour, 2004). The *in-utero* environment, characterized by rapid cell division and organ growth in the developing fetus is particularly susceptible to certain low-dose chemical exposures that would otherwise not exert adverse effects in fully-grown children or

adults (Maddalon et al., 2021; Peillex et al., 2020). Furthermore, it has been shown that glyphosate passes the placental barrier, is present in amniotic fluid and placenta and is also found in breastmilk (Muñoz et al., 2021; Serra et al., 2021). Glyphosate has long been believed to be harmless in humans, as it targets the 5-enolpyruvylshikimate-3-phosphate synthase (ESPS) - an enzyme which exists in plants only and not in human cells. However, the human gut consists of a wealth of bacteria which are dependent on EPSP (Marques et al., 2007). Therefore, it has been proposed that, mechanistically, glyphosate may exert its adverse effects by causing dysbiosis in the gut microbiome and thus inducing changes in the gut-brain as well as gut-immune system crosstalk (Aitbali et al., 2018; Maddalon et al., 2021). It can also be speculated that glyphosate may disturb the interaction between the gut and certain immune cells affecting the onset, exacerbation or perpetuation of disease, or like in our experimental study, in the deviation of the immune response. The fact that the gut microbiome is of importance for childhood asthma development has been shown e.g. in relation to the well-accepted protective farm effect (Depner et al., 2020). In the current study, we observed an increased abundance of *Odoribacter* and *Lachnospiraceae NK4A136* in the gut of female F1 offspring of GLY_{ADI}-exposed dams, which showed a reduced allergic immune response. Both species have been shown to be enriched in the gut microbiota of mice treated with probiotic strains inducing tolerance in a cow milk allergy model (Esber et al., 2020). An increased abundance of *Odoribacter* was also found in human fecal samples after prebiotic intervention (Srivastava et al., 2021). The use of probiotics for prevention of atopic diseases like allergic asthma in infants has also been discussed for some time (Zuccotti et al., 2015). Therefore, the alteration in the gut microbiota in the offspring of low-dose GLY_{ADI}-exposed dams might at least in part contribute to the observed asthma-reducing effects. However, whether this observation is causally linked or whether there are other or additional mechanisms

leading to the detected rather general immune suppressive effect still has to be elucidated. Notably, a transmission of the dams' microbiota was not found since the increased relative abundance of Odoribacter and Lachnospiraceae NK4A136 seen in the F1 generation, was not observed in dams directly exposed to GLY_{ADI}. Instead, dams showed an altered abundances of Akkermansia and Bacteroides that is in line with earlier studies investigating glyphosate-induced effects on the microbiome (Liu et al., 2022; Mesnage et al., 2021). While we found an increased abundance of Lactobacillus after glyphosate exposure, other studies indicated the opposite (Blot et al., 2019; Tang et al., 2020). For the remaining altered taxa identified, Clostridium sensu stricto, Lachnospiraceae UCG 006, Turicibacter have not yet been identified as potential targets of glyphosate in murine models.

Considering the low number of studies on potential effects of glyphosate and the immune system, more studies in animals and humans are needed. In view of the immune suppressive findings within this study, further experiments should be conducted employing animal models in particular in models of other immune diseases like infections or cancer. In addition, the experimental results have to be validated in human mother-child cohorts may be also by using new methods to quantify glyphosate e.g. in in human hair (Alvarez et al., 2022). Moreover, immune system-related effects of formulated glyphosate products also needs to be addressed.

5. Conclusion

Low-dose glyphosate exposure in a multi-generational asthma mouse model was associated with concentration-dependent and sex-specific effects. In detail, GLY_{NOAEL} direct exposure of dams induced a slight eosinophilic infiltration in the BAL and a significantly increased T_H2 cytokine production. In contrast, maternal GLY_{ADI} exposure was associated with a reduced immune response primarily in the female F1 offspring.

Next to the observed phenotype of a mildered asthma outcome, changes in the gut microbial pattern were also identified. In particular, the multi-generational findings display initial evidence of a potential effect on the immune system of low-dose *in-utero* glyphosate exposure in mammals which has not been shown before. Further studies are required to deeper explore the link between perinatal glyphosate exposure and its immunomodulatory impact.

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712 **Figure legends**

713

714 **Figure 1: Effects of glyphosate exposure on airway inflammation and IgE levels**
715 **in HDM-sensitized F0 or F1 mice.** Cell number in BAL fluid (A), airway inflammation
716 examined by lung histology (B, H&E, x100), airway inflammation quantified by an
717 investigator-independent computer-based analysis (C) and HDM-specific IgE levels (D)
718 were examined in directly exposed mice (dams) or in the F1 generation from
719 glyphosate-exposed dams. Data are shown as means ± SEM, n ≥ 8 animals per group.
720 *P < .05.

721

722 **Figure 2: Effects of glyphosate exposure on lung function and splenocyte**
723 **cytokine production in HDM-sensitized F0 or F1 mice.** Lung resistance (A), and
724 respective cytokine levels (B) were examined in directly exposed mice (dams) or in the
725 F1 generation from glyphosate-exposed dams. Data are shown as means ± SEM. n ≥
726 8 animals per group. *P < .05.

727

728 **Figure 3: Effects of maternal glyphosate exposure on asthma development in**
729 **female HDM-sensitized F2 mice.** Cell number in BAL fluid (A), airway inflammation
730 (B, H&E, x100), HDM-specific IgE levels (C), lung resistance (D), and respective
731 cytokine levels (E) were examined in in the F2 generation from glyphosate-exposed
732 dams. Data are shown as means ± SEM. n ≥ 4 animals per group. *P < .05.

733

734 **Figure 4: Effects of maternal glyphosate exposure on the gut microbiota in**
735 **female F1 offspring.** Alpha diversity (A), Beta diversity (PERMANOVA) (B),
736 distribution of families (relative abundance per sample) (C), and relative abundance of
737 significantly affected bacteria on genus level in caecum samples of female offspring

738 from glyphosate-exposed dams are shown. Data are shown as means ± SEM (n ≥ 4
739 for A and D).
740
741

Figure 1

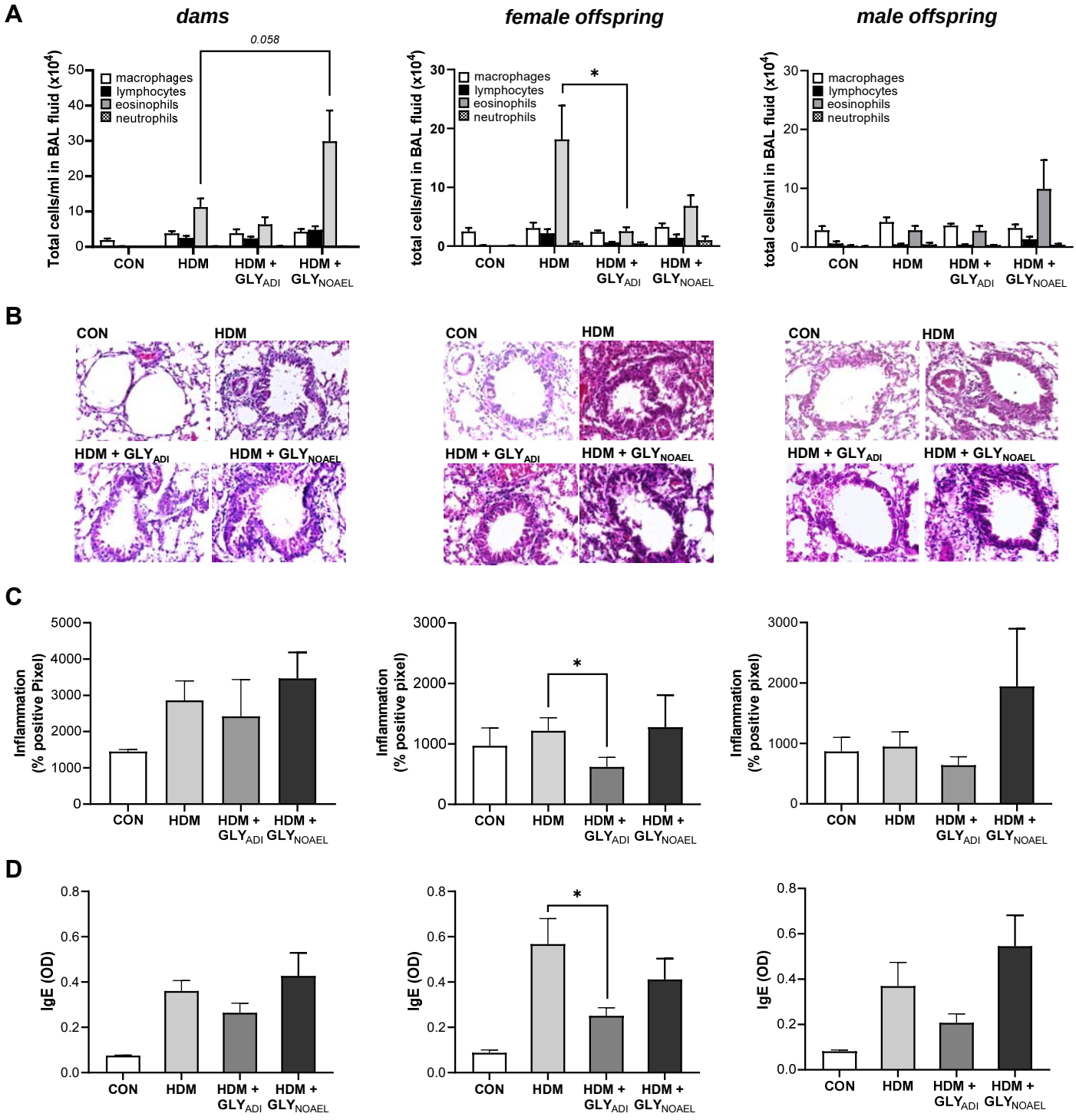

A
dams
female offspring
male offspring
Total cells/ml in BAL fluid (x10^4)
macrophages
lymphocytes
eosinophils
neutrophils
0.058
*
CON
HDM
HDM + GLY_ADI
HDM + GLY_NOAEL
B
CON
HDM
HDM + GLY_ADI
HDM + GLY_NOAEL
C
Inflammation (% positive Pixel)
Inflammation (% positive pixel)
D
IgE (OD)

Figure 2

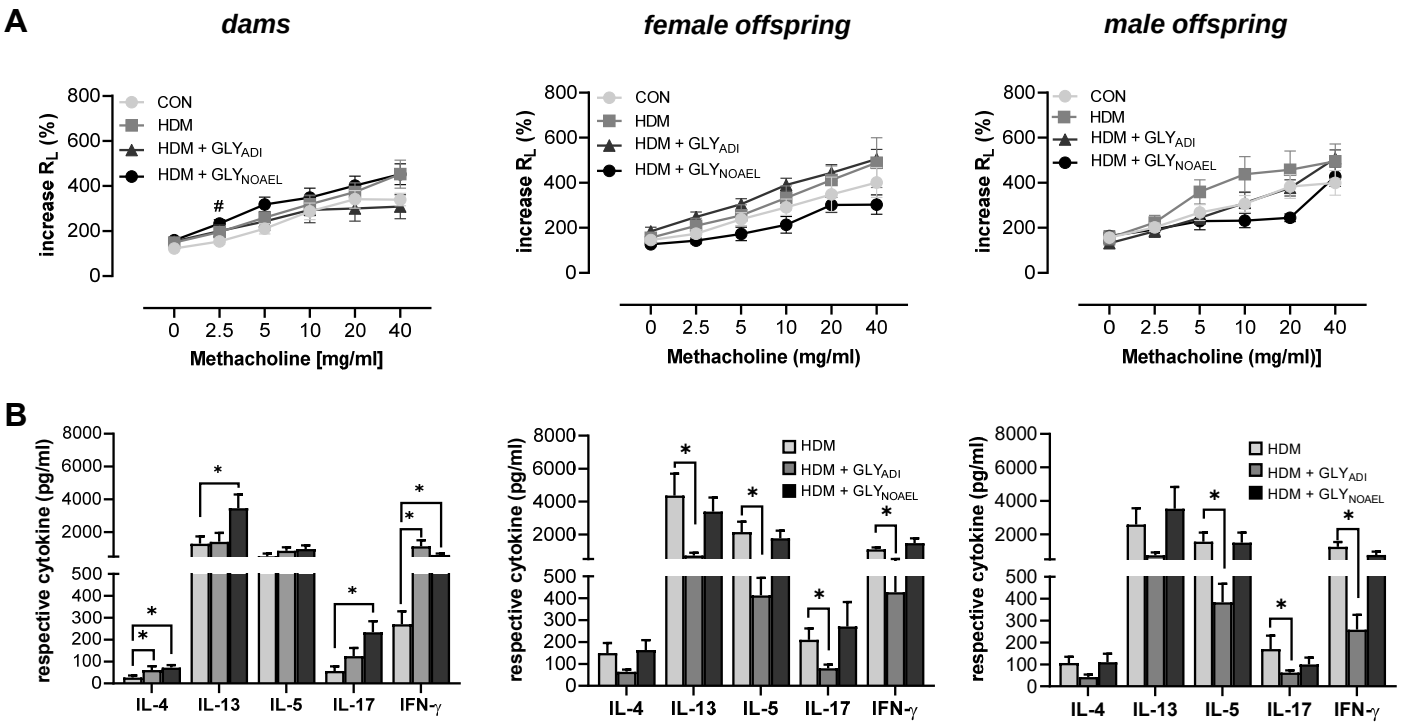

A
dams
female offspring
male offspring
CON
HDM
HDM + GLY$_{ADI}$
HDM + GLY$_{NOAEL}$
increase R$_L$ (%)
Methacholine [mg/ml]
Methacholine (mg/ml)
Methacholine (mg/ml)]
B
respective cytokine (pg/ml)
IL-4
IL-13
IL-5
IL-17
IFN-γ

Figure 3

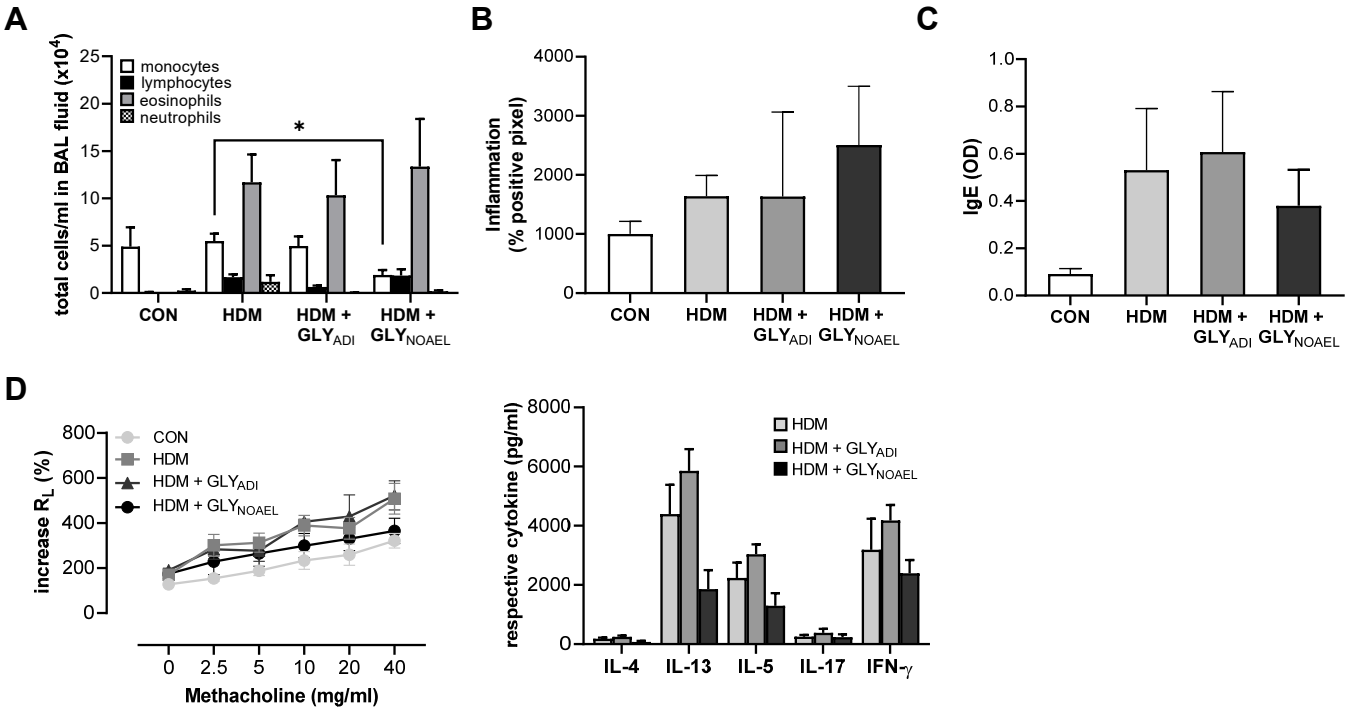
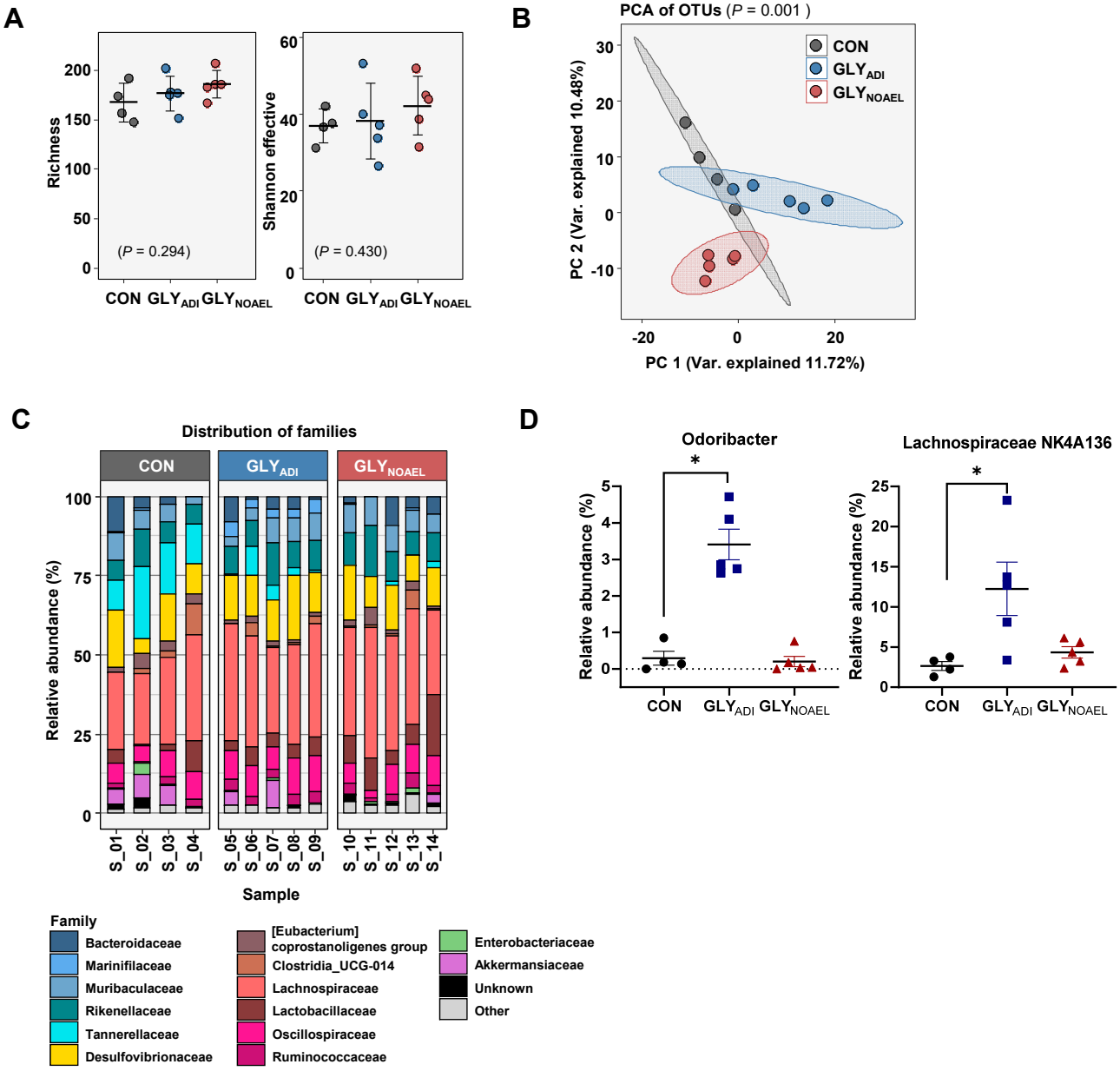

A
total cells/ml in BAL fluid (x10⁴)
monocytes
lymphocytes
eosinophils
neutrophils
*
CON
HDM
HDM + GLY_ADI
HDM + GLY_NOAEL
B
Inflammation (% positive pixel)
C
IgE (OD)
D
increase R_L (%)
CON
HDM
HDM + GLY_ADI
HDM + GLY_NOAEL
Methacholine (mg/ml)
respective cytokine (pg/ml)
IL-4
IL-13
IL-5
IL-17
IFN-γ

Figure 4


A
Richness
Shannon effective
(P = 0.294)
(P = 0.430)
CON GLY_ADI GLY_NOAEL
B
PCA of OTUs (P = 0.001)
CON
GLY_ADI
GLY_NOAEL
PC 2 (Var. explained 10.48%)
PC 1 (Var. explained 11.72%)
C
Distribution of families
Relative abundance (%)
Sample
Family
Bacteroidaceae
Marinifilaceae
Muribaculaceae
Rikenellaceae
Tannerellaceae
Desulfovibrionaceae
[Eubacterium] coprostanoligenes group
Clostridia_UCG-014
Lachnospiraceae
Lactobacillaceae
Oscillospiraceae
Ruminococcaceae
Enterobacteriaceae
Akkermansiaceae
Unknown
Other
D
Odoribacter
Lachnospiraceae NK4A136
Relative abundance (%)

Glyphosate differentially affects the allergic immune response across generations in mice

Lisa Buchenauer, Kristin M. Junge, Sven B. Haange, Jan C. Simon, Martin von Bergen, Anna-Lena Hoh, Gabriela Aust, Ana C. Zenclussen, Gabriele I. Stangl, Tobias Polte

Supplementary Information

Supplementary Table E1: Relative abundance of gut microbiota on genus level in female F1 offspring from glyphosate-exposed dams in alphabetical order. Significant p-values are printed in italic; significances only in the GLY_{ADI} group, related to the phenotype are printed in bold.

	CON (n=4)		**GLY_{ADI} (n=5)**			**GLY_{NOAEL} (n=5)**		
Genus	Mean	SD	Mean	SD	p-value[a]	Mean	SD	p-value[b]
A2	0,55	0,48	0,67	0,94	0,997	0,28	0,27	0,917
Acetatifactor	0,01	0,06	0,00	0,17	0,999	0,76	1,22	0,766
Aerococcus	0,00	0,01	0,00	0,00	>0,999	0,10	0,11	0,995
Akkermansia	5,39	3,18	0,02	3,88	0,178	0,60	1,16	*0,002*
Alistipes	5,33	1,46	7,96	2,11	*0,034*	9,78	3,85	*0,002*
Anaerotruncus	0,00	0,18	0,00	0,66	0,957	0,04	0,04	0,999
Bacteroides	2,99	4,87	4,05	2,92	0,824	4,09	3,38	0,970
Blautia	4,34	3,88	0,24	3,50	0,126	8,69	4,86	*0,001*
Butyricicoccus	0,17	0,29	0,04	0,05	0,977	0,06	0,05	0,975
Clostridia UCG-014	1,85	4,44	0,70	1,75	0,200	1,50	2,59	0,185
Clostridium sensu stricto 1	0,00	0,03	0,01	0,03	>0,999	0,07	0,15	0,998
Colidextribacter	1,40	0,49	4,25	1,15	0,051	2,45	1,42	0,583
Corynebacterium	0,00	0,02	0,00	0,00	>0,999	0,25	0,13	0,972
Enterococcus	0,16	0,16	0,00	0,03	0,977	0,80	0,63	0,845
Enterorhabdus	0,02	0,02	0,09	0,03	0,999	0,09	0,09	0,998
Erysipelatoclostridium	0,00	0,00	0,19	0,30	0,962	0,00	0,00	>0,999
Escherichia-Shigella	0,42	0,38	0,20	0,14	0,965	0,50	0,53	1,000
Eubacterium brachy	0,09	0,03	0,29	0,10	0,983	0,10	0,06	>0,999
Eubacterium coprostanoligenes	3,00	1,29	1,27	0,43	0,299	2,52	1,77	0,861
Gastranaerophilales	0,00	0,03	0,00	0,33	0,986	0,46	0,70	0,904
GCA-900066575	1,27	0,37	0,54	0,29	0,847	1,09	0,74	0,999
Harryflintia	0,15	0,07	0,27	0,13	0,996	0,11	0,06	0,999
Incertae_Sedis	0,55	0,53	0,95	0,64	0,919	1,61	0,88	0,616
Intestinimonas	0,40	0,19	0,27	0,12	0,993	0,25	0,18	0,995
Jeotgalicoccus	0,00	0,00	0,00	0,00	>0,999	0,08	0,09	0,997
Lachnoclostridium	1,60	0,63	0,13	0,51	0,444	3,51	3,16	0,218
Lachnospiraceae FCS020	0,26	0,18	0,18	0,17	0,995	0,14	0,08	0,988
Lachnospiraceae NK4A136	2,76	1,11	12,72	7,42	***<0,001***	4,34	1,58	0,274
Lachnospiraceae UCG-006	0,32	1,28	4,09	1,15	0,072	3,11	2,48	0,123
Lactobacillus	2,99	4,18	4,43	1,26	0,738	9,83	5,82	*<0,001*
Muribaculaceae	5,76	2,52	7,35	2,60	0,903	7,85	1,43	0,132
Odoribacter	0,16	0,38	2,85	0,94	***0,020***	0,20	0,32	0,996
Oscillibacter	0,78	0,73	2,28	0,87	0,355	1,09	0,55	0,983
Parabacteroides	14,40	5,70	2,07	3,51	*<0,001*	0,69	0,82	*<0,001*
Rikenellaceae RC9 gut	1,54	1,37	0,98	0,12	0,657	0,72	0,99	0,517
Roseburia	0,42	0,48	0,27	0,26	0,974	0,47	0,47	0,995
Staphylococcus	0,05	0,05	0,11	0,09	0,996	0,14	0,11	0,996
Turicibacter	0,00	0,00	0,00	0,02	>0,999	0,18	0,32	0,984
Tuzzerella	0,44	0,35	0,19	0,04	0,960	0,11	0,05	0,936

[a] 2way ANOVA with Dunnets; CON vs $GLY_{ADI;}$

[b] 2way ANOVA, with Dunnets; CON vs GLY_{NOAEL}

Supplementary Table E2: Relative abundance of gut microbiota on genus level in glyphosate-exposed dams in alphabetical order. Significant p-values are printed in italic; significances only in the GLY_{ADI} group, related to the F1 phenotype are printed in bold.

	CON (n=5)		**GLY_{ADI} (n=5)**			**GLY_{NOAEL} (n=5)**		
Genus	Mean	SD	Mean	SD	p-value[a]	Mean	SD	p-value[b]
A2	0,21	0,13	0,23	0,17	1,000	0,23	0,11	1,000
Acetatifactor	0,58	0,36	0,06	0,08	0,831	0,23	0,08	0,918
Acinetobacter	0,42	0,57	0,00	0,00	0,887	0,14	0,15	0,949
Akkermansia	1,01	1,50	6,84	4,97	***<0,001***	1,29	2,07	0,949
Alistipes	8,52	1,18	7,74	2,64	0,670	8,53	1,17	>0,999
Alloprevotella	0,34	0,32	0,66	1,42	0,934	0,10	0,10	0,960
Anaerotruncus	0,30	0,27	0,69	0,38	0,899	0,30	0,09	>0,999
Bacteroides	1,77	0,80	4,40	5,07	***0,022***	0,85	0,23	0,578
Blautia	2,01	1,79	4,16	2,97	0,070	4,49	2,06	*0,032*
Butyricicoccus	0,10	0,07	0,05	0,06	0,998	0,17	0,11	0,996
Clostridia UCG-014	0,61	0,81	0,36	0,62	0,959	0,50	0,31	0,992
Clostridia vadin BB60	0,08	0,09	0,15	0,16	0,997	0,28	0,19	0,974
Clostridium sensu stricto 1	3,86	1,74	0,59	0,59	***0,003***	3,55	1,81	0,939
Colidextribacter	2,07	0,51	1,98	0,80	0,994	2,29	0,44	0,968
Corynebacterium	0,64	0,54	0,64	0,78	>0,999	0,16	0,20	0,856
Dubosiella	0,30	0,19	0,38	0,52	0,996	0,34	0,75	0,999
Enterococcus	6,26	4,33	3,30	4,18	*0,008*	3,08	2,91	*0,004*
Erysipelatoclostridium	0,00	0,00	1,00	1,72	*0,525*	0,00	0,00	>0,999
Escherichia-Shigella	0,04	0,06	0,57	1,03	0,829	0,07	0,09	0,999
Eubacterium coprostanoligenes	0,94	0,15	0,51	0,73	0,886	0,49	0,18	0,874
Facklamia	0,06	0,11	0,10	0,11	0,999	0,00	0,00	0,998
Faecalibaculum	0,25	0,15	0,19	0,32	0,998	0,21	0,40	0,999
Gastranaerophilales	0,02	0,05	0,05	0,11	1,000	0,00	0,01	1,000
GCA-900066575	2,32	1,25	0,74	0,31	0,219	1,01	0,41	0,343
Incertae Sedis	1,78	0,66	1,88	1,06	0,993	1,71	0,53	0,997
Intestinimonas	0,29	0,24	0,04	0,04	0,960	0,15	0,08	0,986
Jeotgalicoccus	0,47	0,47	0,90	1,18	0,881	0,04	0,06	0,881
Lachnoclostridium	1,67	0,95	2,62	1,60	0,555	2,48	1,52	0,653
Lachnospiraceae FCS020	0,25	0,07	0,13	0,06	0,991	0,18	0,10	0,997
Lachnospiraceae NK4A136	2,73	1,60	4,69	1,64	0,107	4,05	1,15	0,338
Lachnospiraceae UCG-006	10,84	1,97	6,67	4,71	***<0,001***	10,12	4,96	0,715
Lactobacillus	3,62	1,83	9,74	6,54	***<0,001***	2,43	2,65	0,411
Muribaculaceae	4,36	1,40	6,05	3,63	0,180	5,33	1,14	0,544
Odoribacter	2,00	0,88	1,45	1,51	0,817	3,84	1,82	0,136
Oscillibacter	1,54	0,42	1,03	0,41	0,839	2,11	0,83	0,806
Parabacteroides	0,21	0,12	0,93	1,16	0,711	0,12	0,10	0,994
Rikenellaceae RC9 gut	0,32	0,14	0,35	0,34	1,000	0,23	0,04	0,994
Roseburia	0,35	0,29	0,11	0,15	0,963	0,21	0,17	0,987

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Solibacillus	0,00	0,01	0,00	0,00	>0,999	0,04	0,10	0,999
Sporosarcina	0,02	0,02	0,36	0,53	0,925	0,01	0,01	1,000
Staphylococcus	1,10	1,92	1,53	1,74	0,879	0,21	0,19	0,597
Turicibacter	4,16	1,35	0,58	0,72	***0,001***	4,35	2,12	0,974
Tuzzerella	0,16	0,08	0,08	0,07	0,996	0,28	0,22	0,991

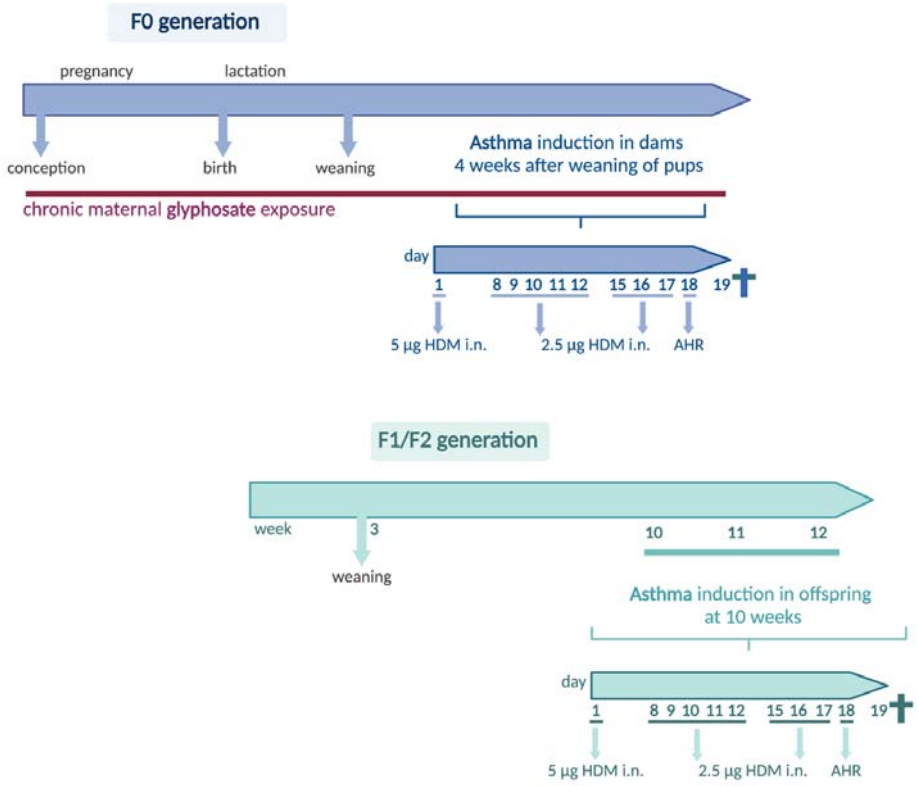
[a] 2way ANOVA with Dunnets; CON vs $GLY_{ADI;}$

[b] 2way ANOVA, with Dunnets; CON vs GLY_{NOAEL}

Supplementary Figure E1. Experimental procedure. Balb/c mice (dams) were exposed starting one week before mating until weaning of the pups at 3 weeks. After weaning dams were further exposed to glyphosate until the end of the asthma protocol. Dams and 10-weeks-old F1 generation were sensitized via the airways with house dust mite extract (HDM) on day 1 followed by HDM given intranasally (i.n.) on days 8 to 12 and 15-17. Control mice received normal saline i.n. Airway hyperreactivity (AHR) was measured on day 18 and mice were sacrificed on day 19. A subgroup of female F1 offspring was further mated with unexposed males with subsequent asthma induction within the F2 offspring.

Supplementary Figure E2. Effect of glyphosate exposure on isoprostane levels. Isoprostane concentrations in lung homogenates after GLY_{ADI} or GLY_{NOAEL} exposure in HDM-sensitized and control mice. Data are expressed as mean ± SEM, $n \geq 2$

Supplementary Figure E1



Supplementary Figure E2

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