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Glyphosate-rich air samples induce IL-33, TSLP and generate IL-13 dependent airway inflammation

Sudhir Kumar¹, Marat Khodoun^{2,3}, Eric M. Kettleson¹, Christopher McKnight³, Tiina Reponen¹, Sergey A. Grinshpun¹, and Atin Adhikari^{1,4,*}

¹ Department of Environmental Health

² Department of Internal Medicine, College of Medicine, University of Cincinnati, Cincinnati, Ohio 45267, U.S.A.

³ Division of Cellular and Molecular Immunology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio 45229, U.S.A.

⁴Department of Environmental Health Sciences, Jiann-Ping Hsu College of Public Health, Georgia Southern University, Statesboro, Georgia 30460, U.S.A.

Abstract

Several low weight molecules have often been implicated in the induction of occupational asthma. Glyphosate, a small molecule herbicide, is widely used in the world. There is a controversy regarding a role of glyphosate in developing asthma and rhinitis among farmers, the mechanism of which is unexplored. The aim of this study was to explore the mechanisms of glyphosate induced pulmonary pathology by utilizing murine models and real environmental samples. C57BL/6, TLR4^{-/-}, and IL-13^{-/-} mice inhaled extracts of glyphosate-rich air samples collected on farms during spraying of herbicides or inhaled different doses of glyphosate and ovalbumin. The cellular response, humoral response, and lung function of exposed mice were evaluated. Exposure to glyphosate-rich air samples as well as glyphosate alone to the lungs increased: eosinophil and neutrophil counts, mast cell degranulation, and production of IL-33, TSLP, IL-13, and IL-5. In contrast, *in vivo* systemic IL-4 production was not increased. Co-administration of ovalbumin with glyphosate did not substantially change the inflammatory immune response. However, IL-13-deficiency resulted in diminished inflammatory response but did not have a significant effect on airway resistance upon methacholine challenge after 7 or 21 days of glyphosate exposure. Glyphosate-rich farm air samples as well as glyphosate alone were found to induce pulmonary IL-13-dependent inflammation and promote Th2 type cytokines, but not IL-4 for glyphosate alone.

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*Corresponding author: Atin Adhikari, Ph.D., Department of Environmental Health Sciences, Jiann-Ping Hsu College of Public Health, P.O. Box: 8015, Georgia Southern University, Statesboro, Georgia 30460, U.S.A..Tel.: +1- 912-478-2289; Fax: +1-912-478-0171. aadhikari@georgiasouthern.edu.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

This study, for the first time, provides evidence for the mechanism of glyphosate-induced occupational lung disease.

Keywords

Occupational health; lung disease; asthma; pesticide; innate immunity; immunotoxicology

1. Introduction

Many low-molecular weight chemicals including some pesticides and herbicides are capable of inducing occupational asthma (Henneberger et al., 2014). Glyphosate [(N-phosphonomethyl)glycine] is one of the most commonly used broad spectrum nonselective herbicides in the world. Approximately 83,000 tons of it was applied to agricultural fields and approximately 3000 tons was applied to the lawns and garden areas of homes in the United States (USA EPA, 2007).

Since glyphosate was brought to market in the 1970's as the active ingredient in the formulation of Roundup®, several animal studies have investigated the toxicity of glyphosate when administered by intravenous, oral, intraperitoneal, dermal, and ocular routes (Tai et al., 1990; Agriculture Canada, 1991; Cox, 1995). Its gastrointestinal toxicity to humans was also documented (Sawada et al., 1988; Talbot et al., 1991; Tominack et al., 1991; Menkes et al., 1991; Temple et al., 1992). According to the SAR (structure-activity relationships) model of Jarvis et al. (2005), the hazard index value of glyphosate is 0.6257, which evidently supports the hazardous nature of glyphosate and its possible role in inducing asthmatic symptoms. However, the inhalational effects of glyphosate particularly its effect on development of asthma was not entirely explored.

Because experimental asthma has been largely studied using various proteins as disease mediators, our understanding of asthma pathogenesis relies heavily on adaptive immune responses. The understanding of the induction of allergic pathology caused by small molecules like glyphosate is challenging due to a fundamentally distinct immune response that may not be obtainable like adaptive immune responses from environmental allergens of high molecular weights. Given that innate immune responses to stimuli are specific to the anatomic site involved, those animal studies that administered glyphosate to the airway would be best suited to provide insight into the pathogenesis of airway disease produced in agricultural workers. To our knowledge, the inhalational hazards of glyphosate have been studied experimentally by two groups (US EPA, 1982; Martinez et al., 1990). It was shown that glyphosate inhalation caused wheezing, reduced activity, and dark nasal discharge even at low exposure levels in rats. How these small molecules contribute to the development of these phenotypes remains a mystery. We hypothesized that exposure to air pollutant containing herbicides, endotoxin or other environmental contaminants induces airway inflammation by activation of the innate immune system through pattern recognizing innate pro-cytokines that contribute to the airway pathology. Here we report the exploration of the mechanism behind the airway inflammation caused by agricultural air samples containing

glyphosate, endotoxin, and other environmental contaminants as well as reagent-grade glyphosate delivered at low and high doses in the presence or absence of exogenous antigen.

2. Materials and methods

2.1. Mice

C57BL/6 female (6–9 weeks) mice were purchased from Jackson Laboratory (Sacramento, CA). TLR4^{−/−} mice (backcrossed 10 generations) were received from Cincinnati Children's Hospital Medical Center (CCHMC). Both strains were subsequently bred in house. Female mice of wild type and IL-13^{−/−} BALB/c background were received from the laboratory of Dr. Fred Finkelman, CCHMC. Mice were housed in individually ventilated cages in a pathogen free facility at the Department of Environmental Health, University of Cincinnati (UC) following the UC Institutional Animal Care and Use Committee (IACUC) guidelines and all experiments were conducted following a UC IACUC-approved protocol.

2.2. Antibodies and reagents

We purchased the following antibodies for flow cytometry: Ly-6G (Gr-1) eFluor® 450 (RB6-8C5; Isotype Rat IgG2b) from eBioscience (San Diego, CA). CD16/CD32 (2.4G2; Isotype Rat IgG2b) and SiglecF-PE (E50-2440; Isotype Rat IgG2a) were purchased from BD PharMingen (San Jose, CA). A kit for measuring serum levels of MMCP1 was purchased from eBioscience.

2.3. Collection of farm air samples during summer pesticide spray seasons

Air samples were collected by three sets of total inhalable aerosol samplers (Button Inhalable Aerosol Sampler, SKC Inc., Eighty Four, PA) operated in parallel on three farms in Butler County, Ohio during summer glyphosate spray seasons. Samplers were installed at 1.5 m height at the edge of the field downwind from the spraying locations (sizes: approx. 5000–10000 m²). The sampling period was approximately 24 hours starting from glyphosate spraying and air samples were collected at a flow rate of approximately 4 L/min on glass fiber filters. The filters from one set of samplers containing aerosolized glyphosate were eluted using PBS and the suspensions were filtered. A stock solution was prepared by pooling the samples collected from three farms (from now on referred as 'Real Env') and used for intranasal treatment of mice. The filters from the other two sets of samplers were analyzed for glyphosate and endotoxin to estimate the levels of glyphosate and endotoxin in 'Real Env' samples.

2.4. Analysis of glyphosate in filter extracts

Glyphosate residues from filters were extracted using KH₂PO₄ buffer /1M NaOH in an automatic shaker followed by freeze drying. The freeze dried samples were dissolved with deionized water and filtered through 0.45 µm Millipore filter. Glyphosate levels in the suspensions were determined by Abraxis ELISA Kit at 450 nm. The average amount of glyphosate per filter was 17.33 µg, which correspond to average airborne concentration of 22.59 ng/m³.

2.5. Analysis of endotoxin in filter extracts

Endotoxin in filter extracts were analyzed using the Limulus Amebocyte Lysate assay (Pyrochrome LAL; Associates of Cape Cod Inc, Falmouth, MA), as described previously (Adhikari et al., 2009; 2010). The samples were spiked with endotoxin standard of 0.50 EU/ml to assure that there was no inhibition or enhancement between the filter extracts and the reagents. The average amount of endotoxin per filter was 24.49 EU, which correspond to average airborne concentration of 4.87 EU/m³.

2.6. Treatment of mice with farm-derived air samples, glyphosate and sensitization with OVA

PBS suspended farm air sample ('Real Env'; estimated amount of glyphosate: 8.66 µg/ml) and reagent grade glyphosate (Sigma–Aldrich, St. Louis, MO) (100 ng, 1 µg or 100 µg) were delivered (in 30µl) to the nose of anesthetized mice which were witnessed to aspirate the solution. Treatments were administered either: daily for 7 days or 3 times a week for 3 weeks. Same exposure schedule was followed for OVA alone (100 µg) and for OVA (100 µg) plus different dose of farm air sample and glyphosate. Mice were sacrificed 24h after final airway treatment with sodium pentobarbital.

2.7. Histological analysis of lung

Formalin–fixed paraffin embedded lung sections (5 µm thick) were prepared for H&E and chloroacetate esterase (CAE) staining. The entire histological slide from each mouse was examined in blinded fashion and given a global categorical severity score based on infiltration of cells into parenchymal, peribronchial, and perivascular regions of lungs.

2.8. Immunohistochemical staining

To analyze IL–33 and TSLP expression in the lungs section, the following antibodies were used for immunostaining: mouse IL–33 (0.2 mg/ml, AF3626, R&D Systems, Minneapolis, MN); mouse TSLP biotinylated (0.2 mg/ml, BAF555, R&D Systems) and respective isotype controls (R&D Systems). IL–33 and TSLP antibody–antigen complex were detected using Cy3 donkey anti–goat IgG (1:10000) (Invitrogen/ Molecular probes, Grand Island, NY). Slides were counterstained with DAPI (Vector Labs, Burlingame, CA). Images were obtained using a Nikon A1R si microscope.

2.9. Isolation of lung inflammatory cells

Lungs were perfused with PBS, removed, manually minced into 1–2 mm fragments and then placed in Hank's Balanced Salt Solution (Sigma–Aldrich) containing Liberase TL (50µg/ml; Roche Diagnostics, Indianapolis, IN) and DNase I (0.5mg/ml; Sigma–Aldrich). Tissue was digested at 37°C in a CO₂ incubator for 30 min. The tissue suspension was then passed through a 40 µm cell strainer. ACK lysis buffer (Invitrogen) was used to clear red blood cells.

2.10. Flow cytometric analysis

Single cell suspensions from lungs (10⁶ per ml) were blocked with anti–mouse CD16/CD32 antibodies before cell–surface staining. Cells were stained with fluorescently– labeled

antibodies against SiglecF, Ly-6G/C (Gr-1), in different combinations according to the experiment. Analysis was performed using a FACSCanto II cytometer and FACSDIVA software (BD Biosciences). We defined eosinophils as being SiglecF⁺Gr-1⁺ and neutrophils as SiglecF⁻Gr-1⁺.

2.11. Cytokine measurement

IL-4, IL-10, IL-13, and IFN- γ production were measured by the *in vivo* cytokine capture assay (IVCCA) (Finkelman et al., 1999). Briefly, biotinylated cytokine-specific mAbs were injected via tail vein immediately before the last airway treatment, and blood was collected 24h later; sera or plasma were analyzed with microtiter plates wells coated with corresponding anti- cytokine mAbs. Cytokine levels were also assessed in bronchoalveolar lavage fluid (BALF) that was obtained 24h after the last airway treatment. A kit for measuring *in vivo* IL-4 production by IVCCA, R46A2 and XMG1.2 anti-IFN- γ mAbs was purchased from Becton-Dickinson (Franklin Lakes, NJ); eBio1316H and eBio13A anti-IL-13 mAbs, JES5-2A5 and JES5-16E3 anti-IL-10 mAbs, ELISA Ready-SET-Go analysis kits for measurement IL-33 and IL-5 were purchased from eBioscience. Assays were performed according to the kit's manufacturer protocols.

2.12. Statistical analyses

Data were analyzed with Sigma Plot 12.0 (Systat Software, Inc., San Jose, CA). Statistically significant differences in means were determined by one-way ANOVA followed by Bonferroni multiple comparison tests. Kruskal-Wallis tests were conducted if the data did not have a normal distribution. All the data are presented as means $\pm$ SD for each group. Probability values of <0.05 were considered significant.

3. Results

3.1. Exposure of air samples collected during glyphosate spray on farms stimulates airway inflammation

Wild type C57BL/6 (WT) and TLR4^{-/-} mice were intranasally exposed to 'Real Env' samples (PBS suspended farm air samples) daily for 7days. 'Real Env' exposure was found to substantially increase the cell count in both the lungs and BAL fluid of WT and TLR4^{-/-} mice. Additionally, the increase in pulmonary infiltrate in lungs was found to be higher in TLR4^{-/-} than in WT mice (Fig. 1A and B). Similarly, we also observed an increase in eosinophil and neutrophil levels in 'Real Env' treated mice (Fig. 1C-F). This inflammation was also confirmed by histological examinations (Fig. 1G) and elevated IgG1 and IgG2a levels (Supplementary Fig. S1C and D).

Additional experiments were conducted using reagent grade glyphosate of different doses. Administration of reagent grade glyphosate to the airway of mice produced substantial pulmonary inflammation whether the daily dose given was 100ng, 1 μ g or 100 μ g for 7days. In the BALF and lung digests, we found a significant increase in the total cell count when treated with glyphosate at 1 μ g or 100 μ g (Fig. 2A and D). Eosinophils (Fig. 2B and C), neutrophils, (Fig. 2E and F), and IgG1 and IgG2a levels (Supplementary Fig. S1A and B) were also increased in glyphosate-treated mice compared to controls. However, we did not

find any significant changes in the total cell count, eosinophils and neutrophils, IgG1 and IgG2a at glyphosate dose of 100 ng. Inflammation was confirmed by histological examination (Fig. 2M). Mice treated with both reagent grade glyphosate and OVA demonstrated significantly higher cell count (Fig. 2G and J), eosinophils (Fig. 2H and K), neutrophils (Fig. 2I and L), IgG1, and IgG2a (Supplementary Fig. S1A and B) compared to PBS treated mice.

Because pulmonary mastocytosis is typically observed in protein–allergen–induced experimental asthma, we assessed the pulmonary mast cell burden in our mice. We did not observe a significant increase in mast cell number in lungs treated with the substances isolated from the air on active farms ('Real Env') and reagent grade glyphosate (Fig. 3A and C; Supplementary Fig. S2). However, we did find the MCPT-1 levels to be substantially higher in both groups indicating increased mast cell degranulation in the treated mice (Fig. 3B and D).

3.2. Glyphosate-rich farm air samples induced airway inflammation and higher production of IL-10, IL-13, IL-5, IFN- γ and IL-4 but glyphosate alone failed to produce IL-4

To evaluate the glyphosate-induced inflammation, we measured the systemic cytokine profile (Fig. 4A-E) of 'Real Env' and glyphosate exposed mice using IVCCA (Finkelman et al., 1999). We found significantly higher levels of IL-5, IL-10, IL-13, and IL-4 upon treatment with 'Real Env' alone in WT and TLR4-/- mice (Fig. 4A-D) approaching the levels induced by treating with OVA alone. The production of IL-5, IL-13 and IL-10 following 'Real Env' exposures was higher in TLR4-/- than in WT mice. We did not find any significant difference in IL-4 production between TLR4-/- and WT mice (Fig. 4D). We then tested production of these cytokines in mice given two different doses of glyphosate and found significantly higher levels of IL-5, IL-10, IL-13 and IFN- γ (Fig. 4F) that approached those levels induced by treating with OVA alone. Notably, there was no additional or synergistic effect when OVA was co-administered with glyphosate (Fig. 4G). Another interesting finding is that glyphosate alone was unable to induce significant levels of IL-4 while airway treatment with glyphosate with OVA did so.

3.3. IL-33 and TSLP in lungs are increased upon exposure to glyphosate-rich air samples as well as reagent grade glyphosate alone

As the cytokine profile of mice treated with 'Real Env' and glyphosate approximated those treated with OVA, we looked at mediators known to promote type 2 pathology. IL-33 and TSLP appeared to be logical choices because of their well-recognized effector functions, and due to their source—the respiratory epithelium cells which would be the first cells to encounter inhaled glyphosate. We measured the IL-33 and TSLP content of BALF directly and found an abundance of both cytokines in 'Real Env'-treated WT and TLR4-/- mice (Fig. 5A and B). IL-33 production was observed to be significantly higher in TLR4-/- mice compared to WT mice. We also observed an abundance of both cytokines in glyphosate-treated mice (Fig. 5C and D). This finding was confirmed by immunohistochemical staining of IL-33 and TSLP in lung sections of glyphosate-treated mice (Fig. 5E) and 'Real Env'-treated WT and TLR4-/- mice (Supplementary Fig. S3A and B) which demonstrated

substantial production of both cytokines, which was limited to the respiratory epithelium after glyphosate exposure.

3.4. Glyphosate-induced pulmonary inflammation is attenuated in IL-13 $-/-$ mice

Glyphosate as a small molecule may not be efficiently presented to conventional T cells by antigen-presenting cells (Itano and Jenkins, 2003). The involvement of innate pathways upon glyphosate exposure, as we hypothesized, was supported by the absence of an increased production of IL-4. This absence would have been expected if type 2 innate lymphoid cells (ILC2s) were the primary source of the IL-5 and IL-13 detected.

IL-33 and TSLP have been well described to induce ILC2s, which in turn causes lung pathology particularly via IL-13-dependent mechanism. To test this hypothesis, we exposed IL-13 deficient mice to glyphosate for 7 and 21 days and assessed lung inflammation. While there was no change in IL-4 levels, we found that the inability to produce IL-13 prevented the rise in IL-5 production, but not the rise in IL-10 production, at both time points during glyphosate treatment. Deficiency in IL-13 also prevented a significant rise in IL-33 and TSLP levels at the early time point but not the latter one (Fig. 6A-D). Lack of IL-13 production was also associated with significantly less ($P < 0.05$) severe cellular infiltration noted on histology (Fig. 6E). Despite significant inflammation, we did not find airway hyperresponsiveness in glyphosate-treated wild type and IL-13 $-/-$ mice (Supplementary Fig. S4A and B).

4. Discussion

In the present study, we explored the ability and the mechanisms of airway asthma-like pathology caused by glyphosate-rich environmental samples collected on farms as well as glyphosate in a pure form. The data presented in this work for the first time demonstrate substantial airway inflammation upon exposure to farm air samples containing glyphosate as well as exposure to glyphosate alone. Notably, farm air samples and low dose of glyphosate provoked a mixed response with elevated pulmonary eosinophils and neutrophils.

Increasing the dose of glyphosate 100-fold up to 100 μ g did not substantially change the degree or character of inflammation; however, a longer exposure to glyphosate did significantly worsen histological pathology. No change of inflammatory response at higher dose is explainable because of the difference between inflammatory immune responses and toxic reactions. A toxic effect is unswervingly the result of the toxic chemical acting on cells. On the other hand, inflammatory responses are the result of a chemical stimulating the body to liberate natural chemicals (e.g., cytokines) which are in turn directly responsible for the effects observed. Thus, in an inflammatory reaction, the chemical can act simply as a trigger, but not as the bullet. In addition, the higher doses of glyphosate could exert some toxic effect on epithelial cells making them unresponsive to elicit allergic inflammatory responses.

In our study we observed minor or none exacerbation of immune response upon co-exposure of glyphosate and common allergen ovalbumin as well as upon glyphosate treatment alone. This was surprising due to a significant increase of IL-33 and TSLP expression upon

glyphosate treatment. Our results could be explained by a possibility that upon exposure to ovalbumin there is not enough damage of airway epithelial cells to release sufficient amount of IL-33 and TSLP. Other airborne allergens such as house dust mite that share the proteinase activity could be much more efficient in releasing of large amount of danger signals that could result in asthma exacerbation. There is also an unanswered question if glyphosate can exacerbate established asthma. The role of glyphosate is controversial due to the toxic properties of the herbicide that may result in immunosuppressive effects versus its ability to induce significant innate cytokine response. More studies are needed to elucidate these relevant questions.

Interestingly, there was no diminishment in the immune responses in TLR4^{-/-} mice upon exposure to farm air samples. However, these mice demonstrated a tendency to generate more IL-13, IL-4 and IL-10. The possible reason could be the decreased toleration ability in TLR4^{-/-} mice as has been shown in another allergy related study (Pochard et al., 2010). Taking into consideration the TLR4 polymorphism in the human population (Kerkhof et al., 2010; Kumar et al., 2012; Belforte et al., 2013; Vawda et al., 2014), it may be a significant risk factor for allergic symptoms in farmers.

Several previous studies have suggested cytotoxic and/or genotoxic effects of glyphosate on human cells (Richard et al., 2005; Monroy et al., 2005; Benachour et al., 2007; Benachour and Séralini, 2009). Reports also showed DNA damage and genotoxicity among individuals two months after their exposure to aerial spraying of glyphosate (Paz-y-Míno et al., 2007; Koller et al., 2014). However, the immunological consequences of glyphosate upon the airway have been rarely investigated experimentally. The findings from this study will help both researchers and clinicians to better understand the mechanisms of occupational asthma caused by one of the most extensively applied herbicides.

Since the asthma phenotype associated with occupational use of glyphosate is reported to be “atopic”, we assessed the cytokine profile produced in the murine lung and found canonical type 2 cytokines IL-5 and IL-13 were increased, but not IL-4 – both at early and later time points. We observed T-cell mediated antigen-specific antibody response in mostly IL-4 independent manner. Production of type 2 cytokines such as IL-5 and IL-13 in IL-4 independent manner has also been found to be associated with allergic disorders in a study conducted by Kurowska-Stolarska et al. (2008). As we also found that the condition induced by glyphosate exposure lacks the robust goblet cell metaplasia (data not shown) and lacks substantial AHR, the clinical implication would be that the inflammation produced by glyphosate in humans may be relatively asymptomatic—but could enhance symptoms of wheeze and cough in the allergic human lung.

To understand the mechanism of pulmonary inflammation and type 2 cytokine responses that we saw in our study, we wanted to look at IL-33 and TSLP in this experimental approach which had not been done before. The reasons for suspecting that IL-33 might be important in glyphosate-mediated airway disease were: 1) IL-33 is known to induce TNF- α , IFN- γ and IL-13 upon antigen challenge followed by activation and recruitment of inflammatory cells in the airways (Brightling et al., 2002), 2) IL-33 induced expression of IL-13 leads to severe pathological changes in mucosal organs (Schmitz et al., 2005), 3) IL-

33 enhances the eosinophil activation *in vitro* (Suzukawa et al., 2008), and increase airway inflammation *in vivo* (Kondo et al., 2008), 4) IL-33 is involved in the induction of allergic inflammatory responses by promoting pulmonary eosinophilia, IL-5, IL-13, and IgE (Smith, 2010; Liew et al., 2010), and 5) IL-33 is crucial for the induction of type 2 inflammation through innate pathways and is associated with tissue damage (Oboki et al., 2010). We observed increased IL-33 expression in both BALFs and lung sections, suggesting IL-33 may play an important role in innate immune response as well as in airway inflammation accompanied by significant accumulation of eosinophils caused by glyphosate exposure.

The involvement of TSLP has been similarly implicated in the mechanisms of pulmonary inflammation (Soumelis et al., 2002; Miyata et al., 2008). Furthermore, TSLP has also been found to be associated with the development and exacerbation of airway inflammation in mice (Zhou et al., 2005; Harada et al., 2009). When we examined TSLP and IL-13 levels in IL-13- deficient mice, their rise upon glyphosate treatment was delayed as compared to wild type mice – the rise being present at 21 days but not at 7 days. The reason for this is unclear, but suggests that: 1) IL-33/TSLP-mediated induction of type 2 cytokines is not necessarily a unidirectional process—at least in the absence of a classically activated adaptive immune response, 2) IL-13 appears to provide some degree of positive feedback for the propensity to release IL-33/TSLP from the epithelium, and 3) this deficit in IL-33/TSLP secretion associated with IL-13 deficiency can be overcome by further stimulation of glyphosate or inflammatory factors derived from the inflammatory milieu.

A second unexpected finding that recapitulates the central role of IL-13 in type 2 pathophysiology is that rise in IL-5 levels was not observed in mice deficient in IL-13 at both the early and late time points of glyphosate treatment. It is unclear how to interpret this finding, but this rise would presumably involve the overall decreased state of inflammation.

We observed a significant elevation of IL-10 production upon exposure to glyphosate. This is not an unexpected finding considering a significant ongoing inflammatory response. The elevated levels of IL-10 and possibly increased kinetics of its production were not sufficient to control the inflammation.

Collectively, our results showed that mice continuously exposed to glyphosate developed elevated levels of eosinophils, neutrophils, and asthma-related cytokines (IL-5, IL-10, IL-13, IL-33, TSLP) compared to control groups. Exposure to glyphosate results in airway barrier damage. This damage induces IL-33 and TSLP innate cytokines release that may have a sensitization role; co-exposure with an allergen leads to a profound inflammatory and antigen-specific innate and adaptive immune response, including release of IL-13, IL-5, IL-10, and antigen-specific antibodies. All these events could be a possible explanation of how glyphosate contributes to an induction and/or exacerbation of asthma-like airway pathology. Further studies are needed to explore the mechanistic role of glyphosate in triggering allergic inflammation eventually leading to asthmatic symptoms in agricultural workers and other populations exposed to higher levels of glyphosate, which is now widely used as a conventional herbicide around the world.

5. Conclusions

Our results demonstrate the capacity of glyphosate-rich air samples from farms as well as pure glyphosate to induce type 2 airway inflammation, over both short and longer time courses. Furthermore, glyphosate induced inflammation was found to be associated with induction of IL-33 and TSLP. This work also highlights the production of IL-13 as well as modulation of innate immune system by glyphosate, which may play an important role in exacerbation of airway inflammation by this low molecular weight chemical.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Adhikari A, Jung J, Reponen T, Lewis JS, DeGrasse EC, Grimsley LF, Chew GL, Grinshpun SA. Aerosolization of fungi, (1→3)-β-D glucan, and endotoxin from flood-affected materials collected in New Orleans homes. *Environ. Res.* 2009; 109:215–224. [PubMed: 19201399]
- Adhikari A, Lewis JS, Reponen T, Degrasse EC, Grimsley LF, Chew GL, Iossifova Y, Grinshpun SA. Exposure matrices of endotoxin, (1→3)-β-D-glucan, fungi, and dust mite allergens in flood-affected homes of New Orleans. *Sci. Total Environ.* 2010; 408:5489–5498. [PubMed: 20800874]
- Agriculture Canada. Pesticides Directorate. Discussion document: Pre-harvest use of glyphosate. Ottawa, Ontario, Canada: 1991. Food Production and Inspection Branch.
- Belforte FS, Coluccio LF, Poskus E, Penas SA. Toll-like receptor 4 D299G polymorphism in metabolic disorders: a meta-analysis. *Mol. Biol. Rep.* 2013; 40:3015–3020. [PubMed: 23275193]
- Benachour N, Séralini GE. Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. *Chem. Res. Toxicol.* 2009; 22:97–105. [PubMed: 19105591]
- Benachour N, Sipahutar H, Moslemi S, Gasnier C, Travert C, Séralini GE. Time- and dose-dependent effects of Roundup on human embryonic and placental cells. *Environ. Contam. Toxicol.* 2007; 53:126–133.
- Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *N. Engl. J. Med.* 2002; 346:1699–1705. [PubMed: 12037149]
- Cox C. Glyphosate. *Toxico. J. Pest. Ref.* 1995; 15:14–20. part 1.
- Finkelman FD, Morris SC. Development of an assay to measure in vivo cytokine production in the mouse. *Int. Immunol.* 1999; 11:1811–1818. [PubMed: 10545485]
- Harada M, Hirota T, Jodo AI, Doi S, Kameda M, Fujita K, Miyatake A, Enomoto T, Noguchi E, Yoshihara S, Ebisawa M, Saito H, Matsumoto K, Nakamura Y, Ziegler SF, Tamari M. Functional analysis of the thymic stromal lymphopoietin variants in human bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 2009; 40:368–374. [PubMed: 18787178]

- Henneberger PK, Liang X, London SJ, Umbach DM, Sandler DP, Hoppin JA. Exacerbation of symptoms in agricultural pesticide applicators with asthma. *Int. Arch. Occup. Environ. Health.* 2014; 87:423–432. [PubMed: 23670403]
- Itano AA, Jenkins MK. Antigen presentation to naive CD4 T cells in the lymph node. *Nat. Immunol.* 2003; 4:733–739. [PubMed: 12888794]
- Jarvis J, Seed MJ, Elton R, Sawyer L, Agius R. Relationship between chemical structure and the occupational asthma hazard of low molecular weight organic compounds. *Occup Environ Med.* 2005; 62:243–250. [PubMed: 15778257]
- Kerkhof M, Postma DS, Brunekreef B, Reijmerink NE, Wijga AH, de Jongste JC, Gehring U, Koppelman GH. Toll-like receptor 2 and 4 genes influence susceptibility to adverse effects of traffic-related air pollution on childhood asthma. *Thorax.* 2010; 65:690–697. [PubMed: 20685742]
- Koller VJ, Fürhacker M, Nersesyan A, Mišík M, Eisenbauer M, Knasmueller S. Cytotoxic and DNA-damaging properties of glyphosate and Roundup in human-derived buccal epithelial cells. *Arch. Toxicol.* 2012; 86:805–813. [PubMed: 22331240]
- Kondo Y, Yoshimoto T, Yasuda K, Futatsugi-Yumikura S, Morimoto M, Hayashi N, Hoshino T, Fujimoto J, Nakanishi K. Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. *Int. Immunol.* 2008; 20:791–800. [PubMed: 18448455]
- Kumar S, Khandpur S, Rao DN, Wahaab S, Khanna N. Immunological response to Parthenium hysterophorus in Indian patients with parthenium sensitive atopic dermatitis. *Immunol. Invest.* 2012; 41:75–86. [PubMed: 22091625]
- Kurowska-Stolarska M, Kewin P, Murphy G, Russo RC, Stolarski B, Garcia CC, Komai-Koma M, Pitman N, Li Y, Niedbala W, McKenzie AN, Teixeira MM, Liew FY, Xu D. IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *J. Immunol.* 2008; 181:4780–4790. [PubMed: 18802081]
- Liew FY, Pitman NI, McInnes IB. Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nat. Rev. Immunol.* 2010; 10:103–110. [PubMed: 20081870]
- Martinez TT, Long WC, Hiller R. Comparison of the toxicology of the herbicide roundup by oral and pulmonary routes of exposure. *Proc. West Pharmacol. Soc.* 1990; 33:193–197. [PubMed: 1703306]
- Menkes DB, Temple WA, Edwards IR. Intentional Self-poisoning with Glyphosate-Containing Herbicides. *Hum. Exp. Toxicol.* 1991; 10:103–107. [PubMed: 1675099]
- Miyata M, Hatsushika K, Ando T, Shimokawa N, Ohnuma Y, Katoh R, Suto H, Ogawa H, Masuyama K, Nakao A. Mast cell regulation of epithelial TSLP expression plays an important role in the development of allergic rhinitis. *Eur. J. Immunol.* 2008; 38:1487–1492. [PubMed: 18461563]
- Monroy CM, Cortes AC, Sicard DM, de Restrepo HG. Cytotoxicity and genotoxicity of human cells exposed in vitro to glyphosate. *Biomedica.* 2005; 25:335–345. [PubMed: 16276681]
- Oboki K, Ohno T, Kajiura N, Arae K, Morita H, Ishii A, Nambu A, Abe T, Kiyonari H, Matsumoto K, Sudo K, Okumura K, Saito H, Nakae S. IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proc. Natl. Acad. Sci.* 2010; 107:18581–18586. [PubMed: 20937871]
- Paz-y-Miño C, Sánchez ME, Arévalo M, Muñoz MJ, Witte T, De-la-Carrera GO, Leone PE. Evaluation of DNA damage in an Ecuadorian population exposed to glyphosate. *Genetics Mol. Biol.* 2007; 30:456–460.
- Pochard P, Vickery B, Berin MC, Grishin A, Sampson HA, Caplan M, Bottomly K. Targeting Toll-like receptors on dendritic cells modifies the T(H)2 response to peanut allergens in vitro. *J. Allergy Clin. Immunol.* 2010; 126:92–97. [PubMed: 20538332]
- Richard S, Moslemi S, Sipahutar H, Benachour N, Séralini GE. Differential effects of glyphosate and roundup on human placental cells and aromatase. *Environ. Health Perspect.* 2005; 113:716–720. [PubMed: 15929894]
- Sawada Y, Nagai Y, Ueyama M, Yamamoto I. Probable Toxicity of Surface-active Agent in Commercial Herbicide Containing Glyphosate. *Lancet.* 1988; I:299. [PubMed: 2893109]
- Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, Zurawski G, Moshrefi M, Qin J, Li X, Gorman DM, Bazan JF, Kastelein RA. IL-33, an interleukin-1-like cytokine that signals

- via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity*. 2005; 23:479–490. [PubMed: 16286016]
- Smith DE. IL-33: a tissue derived cytokine pathway involved in allergic inflammation and asthma. *Clin. Exp. Allergy*. 2010; 40:200–208. [PubMed: 19906013]
- Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, Gilliet M, Ho S, Antonenko S, Lauerma A, Smith K, Gorman D, Zurawski S, Abrams J, Menon S, McClanahan T, de Waal-Malefyt Rd R, Bazan F, Kastelein RA, Liu YJ. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat. Immunol*. 2002; 3:673–680. [PubMed: 12055625]
- Suzukawa M, Koketsu R, Iikura M, Nakae S, Matsumoto K, Nagase H, Saito H, Matsushima K, Ohta K, Yamamoto K, Yamaguchi M. Interleukin-33 enhances adhesion, CD11b expression and survival in human eosinophils. *Lab Invest*. 2008; 88:1245–1253. [PubMed: 18762778]
- Tai T, Yamashita M, Wakimori H. Hemodynamic effects of roundup, glyphosate and surfactant in dogs. *Jpn. J. Toxicol*. 1990; 3:63–68.
- Talbot AR, Shiaw MH, Huang JS, Yang SF, Goo TS, Wang SH, Chen CL, Sanford TR. Acute poisoning with a glyphosate–surfactant herbicide ('Round-up'): a review of 93 cases. *Hum. Exp. Toxicol*. 1991; 10:1–8. [PubMed: 1673618]
- Temple WA, Smith NA. Glyphosate Herbicide Poisoning Experience In New Zealand. *NZ Med. J*. 1992; 105:173–174.
- Tominack RL, Yang GY, Tsai WJ, Chung HM, Deng JF. Taiwan National Poison Center Survey of Glyphosate–Surfactant Herbicide Ingestions. *J. Toxicol. Clin. Toxicol*. 1991; 29:91–109. [PubMed: 2005670]
- United States EPA. Pesticide Market Estimates Agriculture, Home and Garden. 2007
- US EPA. Office of Pesticides and Toxic Substances. Memo from William Dykstra, Toxicology Branch, to Robert Taylor, Registration Division. 1982
- Vawda S, Mansour R, Takeda A, Funnell P, Kerry S, Mudway I, Jamaludin J, Shaheen S, Griffiths C, Walton R. Associations Between Inflammatory and Immune Response Genes and Adverse Respiratory Outcomes Following Exposure to Outdoor Air Pollution: A HuGE Systematic Review. *Am. J. Epidemiol*. 2014; 179:432–442. [PubMed: 24243740]
- Zhou B, Comeau MR, De Smedt T, Liggitt HD, Dahl ME, Lewis DB, Gyarmati D, Aye T, Campbell DJ, Ziegler SF. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nat. Immunol*. 2005; 6:1047–1053. [PubMed: 16142237]

Highlights

- Glyphosate-rich air samples induce antigen-independent airway inflammation.
- Glyphosate causes high expression of IL-33 and TSLP during the airway inflammation.
- Glyphosate exposure in airways produces canonical Th2 cytokines.
- Glyphosate-associated airway inflammation is partially dependent on IL-13.
- IL-13, TSLP, IL-33 can be potential targets to control glyphosate-induced inflammation.

Fig. 1.

Increase in total number of cells, eosinophils, and neutrophils in lung and BAL fluids upon airway exposure to farm air samples ('Real Env') and OVA for seven consecutive days (mean $\pm$ SD; n = 8). Increase in total number of cells in (A) lung and (B) BAL fluids. Increase in percentage (C) and total number (D) of eosinophils and neutrophils (E, F) per lung upon exposure to farm air samples ('Real Env'). (G) Representative lung sections (H&E staining) and its pathology score from mice treated with PBS, farm air samples ('Real Env') and OVA intranasally for seven consecutive days (mean $\pm$ SD; n = 8); magnification 200X.

* indicates statistically significant differences ($p < 0.05$) with respect to PBS treated control and in between WT and TLR4-/- mice group.

Fig. 2.

Increase in total number of cells, eosinophils, and neutrophils in lung and BAL fluids of WT mice upon airway exposure to glyphosate and combinations of glyphosate and OVA for seven consecutive days (mean $\pm$ SD; n = 8). Increase in total number of cells in (A) lungs and (D) BAL fluids upon exposure to different doses of glyphosate (100 ng, 1, or 100 μ g). Increase in percentage (B) and total number (C) of eosinophils and neutrophils (E, F) per lung upon exposure to two doses of glyphosate. Increase in total number of cells in (G) lungs and (J) BAL fluids upon exposure to combination of glyphosate (1 or 100 μ g) with OVA (100 μ g). Increase in percentage and total number of (H, K) eosinophils and (I, L) neutrophils per lung upon exposure to OVA and combination of glyphosate, respectively. (M) Representative lung sections (H&E staining) and its pathology score from WT mice treated with PBS, glyphosate (1 μ g) and OVA (100 μ g) intranasally for seven consecutive days (mean $\pm$ SD; n = 8); magnification 200X.

* indicates statistically significant differences (p < 0.05) with respect to PBS and treated WT mice group.

Fig. 3.

Farm air samples containing glyphosate as well as pure glyphosate alone induce increased mast cell degranulation but no increase in lung mast cell numbers upon airway exposure. (A) Mast cells number in CAE stained lung section and (B) serum MCPT-1 concentration in blood 4h after last exposure of PBS, farm air samples ('Real Env'), and ovalbumin (OVA). (C) Mast cells number in CAE stained lung section and (D) serum MCPT-1 concentration from mice treated with PBS, ovalbumin and 1 μ g of glyphosate delivered to intranasally for seven consecutive days (mean $\pm$ SD; n = 8).

* indicates statistically significant differences ($p < 0.05$) with respect to PBS and treated mice group.

Fig. 4.

(A–E) Higher production of IL-5, IL-13, IL-10, IL-4 and no change in the IFN-γ levels upon exposure to farm air samples in WT and TLR4^{-/-} mice. (F) The increased level of IL-5, IL-10, IL-13, IFN-γ and no change in the IL-4 levels upon glyphosate (1 or 100 μg) exposure to WT mice. (G) The increased level of IL-4, IL-5, IL-10, IL-13, and no change in IFN-γ levels upon combination of glyphosate (1 or 100 μg) and ovalbumin (100 μg) exposure to WT mice (mean ± SD; n = 8). Levels of cytokines were evaluated by IVCCA in serum of mice upon 7 consecutive days of intranasal treatment with farm air samples ('Real Env') and glyphosate. Blood samples were collected 24h after the last exposure. IL-5 was measured in the BAL fluids.

* indicates statistically significant differences (p < 0.05) with respect to PBS treated control and in between WT and TLR4^{-/-} mice group.

Fig. 5.

IL-33 and TSLP productions increased in the lung upon exposure to farm air samples and glyphosate. (A, B) ELISA based measurement of IL-33 and TSLP in BAL fluids of PBS, farm air samples and ovalbumin (100 µg) treated WT and TLR4^{-/-} mice, respectively (mean ± SD; n = 8). (C, D) ELISA based measurement of IL-33 and TSLP in BAL fluids of PBS, OVA and pure glyphosate (1 µg) treated WT mice, respectively (mean ± SD; n = 8). (E) Immunofluorescence staining of IL-33 and TSLP in the lung sections of the glyphosate treated WT mice, magnification 200X.

* indicates statistically significant differences (p < 0.05) with respect to PBS treated control and in between WT and TLR4^{-/-} mice group.

Fig. 6.

IL-13-deficient mice demonstrated diminished inflammatory response upon glyphosate exposure. (A, C) Diminished production of IL-5 but no change in IL-4 level, and (B, D) diminished production of TSLP, IL-33, IL-10 levels, between IL-13-deficient mice and WT mice upon glyphosate exposure (1 μ g) for 7 or 21 days, respectively (mean $\pm$ SD; n = 8). (E) Representative lung sections (H&E staining) from mice treated with PBS and glyphosate (1 μ g) intranasally three times a week for 21 days; magnification 200X (left panel). Arbitrary scores were based on inflammatory cells infiltration in lungs parenchyma, peribronchial, and perivascular regions. Analysis was performed in a double blinded manner (right panel).

* indicates statistically significant differences ($p < 0.05$) with respect to PBS treated control group.