UNIVERSITÀ DEGLI STUDI DI MILANO

Department of Pharmacological and Biomolecular Sciences

PhD in Pharmacological and Biomolecular Sciences,

Experimental and Clinical

XXXVI cycle

Assessing endocrine active substances' impact on the immune system through *in vitro* studies

SSD BIO/14

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R13025

Academic year 2022-2023

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Abstract

Endocrine active substances (EAS) are substances able to interact with the endocrine system and in case they lead to adverse effects on health they are called endocrine disruptors (EDs). They can be found mainly in the environment, food, cosmetics, pesticides, plastics, and industrial products. There is a close interplay between the endocrine and the immune systems and therefore EDs can putatively interfere with immunity. Over the past few decades, there has been a rise in the prevalence of specific diseases, notably immune-related conditions such as allergies and autoimmune disorders. Among the leading potential factors attributed to this increase, EDs have been suggested.

The aim of this thesis project was to investigate the ability of well-known EDs or suspected EAS to modulate the human immune system. To date, we have put forth various *in vitro* tests that utilize both cell cultures and human-derived primary cells. These tests could serve the dual purpose of eliminating the need for animal experimentation and enabling a more thorough evaluation of chemical hazards before they enter the market.

My thesis started with the use of glyphosate, a compound whose use is a subject of intense debate. Glyphosate is among the substances suspected of having endocrine active properties, although the European Food Safety Authority (EFSA) recently affirmed that glyphosate cannot be described as ED. It is one of the most used herbicides worldwide, which safety has been recently posed under discussion. Even if still under debate, several endocrine disturbances (i.e., reproductive and thyroid) of glyphosate have emerged and some evidence also suggests possible immunotoxic effects *in vivo* (i.e., lung inflammation, rhinitis, rheumatoid arthritis). Therefore, the ability of glyphosate to directly interfere with immune cells activation was investigated. To this end, peripheral blood mononuclear cells (PBMCs) obtained from healthy donors were used to assess the possible interference with the immune system, focusing mainly on T helper cells differentiation. A reduction of T helper 1 cells was the main effect observed following glyphosate exposure, which resulted in an unbalance in Th1/Th2 ratio, favoring Th2 which may support the *in vivo* data. This effect was mediated by the estrogen receptor-α and by the up-regulation of a miRNA (miR-500a-5p). Accordingly, glyphosate demonstrated the ability to directly act on T cells, with a mechanism suggesting the involvement of estrogen receptor-α.

Subsequently, my research activities were carried out within the PRIN project number 2017MLC3NF – EDONIS, focusing on the assessment of the immunotoxic potential of other

EAS. To this end, six well known EDs or EAS were selected, and a battery of *in vitro* test was applied to determine their ability to interfere with the immune system. Atrazine, cypermethrin, vinclozolin, ethynyl estradiol, diethylphthalate, and perfluorooctanesulfonic acid were tested. The first three are pesticides, ethynyl estradiol is a pharmaceutical and lastly two are industrial chemicals. Initially, the human monocytic cell line THP-1 was used to investigate the ability of the selected compounds to modulate RACK1 (receptor for activated C kinase 1) expression, a protein considered as a bridge between the immune and the endocrine system, as its expression is under steroid hormones control. All the compounds modulated RACK1 expression paralleled by changes of several inflammatory markers (namely IL-8, TNF-α, CD54, and CD86). These effects were then confirmed in human primary cells of both sexes (PBMCs). In addition, natural killer cells lytic activity and T cells differentiation were also investigated. Based on the results obtained, perfluorooctanesulfonic acid and diethylphthalate resulted to be immunosuppressive on most parameters, and by using pharmacological tools we could demonstrate that the immunotoxic effects were endocrine-mediated.

The studies on individual compounds were then followed by investigations on PFAS mixtures (perfluoroalkyl substances – PFAS). The effect of mixtures is quite often underestimated, but it is representative of real-life exposure. Three short-chain and three long-chain PFAS were evaluated and the mixture of all the six PFAS induced a general reduction of human basophil and lymphocyte activation. In particular, the most affected lymphocyte population was mucosal-associated invariant T (MAIT) cells, an innate-like T cells population. To better understand the effect of chemical mixtures on human immune system, four extracts of wastewater treatment plant effluents were also tested. Their exposure induced a reduced lymphocyte activation and basophil reaction to bacterial component, while increasing basophil allergy-like activation. Within the 339 detected chemicals in the extracts, 29 of them were considered prioritized chemicals for their strong correlation with most immune parameters. There are drugs, pesticides, industrial chemicals, and EDs, evidencing a contamination of water effluents that could perturb human health.

The battery of different *in vitro* tests applied were useful in the assessment of the immunotoxic potential of chemicals, including EAS and EDs. The data obtained reinforce the concept of immune-endocrine interplay and indicate that these tests can be integrated in the hazard assessment procedures in view of a more sustainable risk assessment.

Abstract - italiano

Le sostanze attive a livello endocrino (EAS) sono sostanze in grado in interagire con il sistema endocrino e in caso questa interazione induca effetti avversi, essi vengono identificati come interferenti endocrini (IE). Si trovano principalmente nell'ambiente, negli alimenti, nei cosmetici, nei pesticidi, nella plastica e nei prodotti industriali. Esiste una stretta interazione tra il sistema endocrino e quello immunitario e pertanto gli IE possono presumibilmente interferire con la risposta immunitaria. Recentemente, durante gli ultimi decenni, si è registrato un aumento di alcune patologie, tra cui condizioni che coinvolgono il sistema immunitario, come allergie e patologie di carattere autoimmune. Tra le principali cause attribuibili a questo aumento sono state proposti gli IE.

Lo scopo di questo progetto di tesi è stato quello di valutare se noti o presunti EAS o IE siano in grado di modulare la risposta immunitaria. Per far ciò, sono stati utilizzati diversi test *in vitro*, impiegando sia colture cellulari sia cellule primarie di origine umana. La batteria di test sviluppati potrebbe consentire in futuro di ridurre l'uso di animali e permettere una valutazione più approfondita durante la fase di caratterizzazione del pericolo di sostanze chimiche prima della commercializzazione.

Lo studio è iniziato con l'uso del glifosato; un composto il cui utilizzo è oggetto di intenso dibattito. Il glifosato rientra tra le sostanze sospettate per essere un EAS, sebbene recentemente l'Autorità Europea per la Sicurezza Alimentare (EFSA) abbia affermato che il glifosato non incontra i criteri per caratterizzarlo come IE. Il glifosato è uno degli erbicidi più utilizzati a livello mondiale, la cui sicurezza è stata recentemente oggetto di discussione. Anche se ancora dibattuto, diversi disturbi endocrini (es, riproduttivi e tiroidei) del glifosato sono stati riportati assieme ad alcune evidenze di effetti immunotossici *in vivo* (es, infiammazione polmonare, rinite, artrite reumatoide). Pertanto, la capacità del glifosato di interferire direttamente con l'attivazione delle cellule del sistema immunitario è stata valutata. In particolare, cellule mononucleate di sangue periferico (PBMC) ottenute da donatori sani sono state impiegate per valutare la possibile interferenza con il sistema immunitario, con un focus particolare sul differenziamento dei linfociti T helper. La riduzione delle cellule T helper 1 è stato il principale effetto osservato in seguito a esposizione a glifosato, che ha indotto uno sbilanciamento della ratio Th1/Th2, a favore dei linfociti Th2 che è in accordo con dati presenti in letteratura ottenuti *in vivo.* Questa azione è risultata essere mediata dal recettore degli estrogeni α e dall'aumento della regolazione di un miRNA (miR-500a-5p). Questi studi

dimostrano, quindi, che il glifosato è in grado di agire direttamente sui linfociti T, con un meccanismo che suggerisce il coinvolgimento del recettore degli estrogeni α.

Successivamente, la mia attività di ricerca si è sviluppata nell'ambito del progetto PRIN # 2017MLC3NF - EDONIS, focalizzandosi sulla valutazione degli effetti di certi o presunti EAS/IE sul sistema immunitario umano. A tal fine, sono stati selezionati sei composti appartenenti a diverse categorie chimiche e funzionali ed è stata utilizzata una batteria di test *in vitro* per valutare il loro impatto sul sistema immunitario. I composti selezionati sono stati atrazina, cipermetrina, vinclozolina, etinilestradiolo, dietilftalato, e acido perfluoroottansolfonico; i primi tre appartenenti a tre classi di pesticidi, etinilestradiolo è un farmaco e le ultime due sono sostanze chimiche ad uso industriale ampiamente utilizzate. Inizialmente è stata impiegata la linea cellulare monocitaria umana THP-1 per determinare la capacità dei composti selezionati di interferire con l'espressione di RACK1 (recettore per la chinasi C attivata 1), considerato come un ponte tra il sistema immunitario e quello endocrino, in quanto la sua espressione è sotto il controllo degli ormoni steroidei. Tutti i composti sono risultati in grado di modulare l'espressione di RACK1 e di diversi marcatori pro-infiammatori (IL-8, TNF-α, CD54 e CD86). Questi effetti sono stati successivamente confermati in cellule primarie umane di entrambi i sessi (PBMC). In aggiunta sono state valutate anche l'attività litica delle cellule *natural killer* e la differenziazione dei linfociti T. Sulla base dei risultati ottenuti, acido perfluorottansolfonico e dietilftalato sono risultati immunosoppressivi per la maggior parte dei parametri, e grazie all'utilizzo di modulatori farmacologici specifici è stato possibile dimostrare un effetto d'interferenza endocrina alla base degli effetti osservati.

Gli studi sui singoli composti sono stati seguiti da analisi sulle miscele di sostanze perfluoroalchiliche (PFAS). Il problema delle miscele è spesso sottovalutato, nonostante sia rappresentativo dell'effettiva esposizione umana. Sono stati valutati tre PFAS a catena corta e tre a catena lunga. La miscela di tutti e sei i PFAS ha determinato una riduzione generale dell'attivazione di basofili e dei linfociti umani. In particolare, la popolazione di linfociti più colpita è stata quella delle cellule T invarianti associate alla mucosa (MAIT), una popolazione di cellule T *innate-like*. Per comprendere meglio l'effetto delle miscele sul sistema immunitario umano, sono stati successivamente testati quattro estratti di effluenti di impianti di trattamento delle acque reflue. La loro esposizione ha indotto una riduzione dell'attivazione dei linfociti e della reazione dei basofili alla componente batterica, mentre ha aumentato l'attivazione dei basofili a stimolo allergico. Tra le 339 sostanze chimiche rilevate nei quattro estratti, 29 sono state considerate prioritarie per la loro forte correlazione con la maggior parte dei parametri

immunitari. Si tratta di farmaci, pesticidi, prodotti chimici industriali e IE, evidenziando quindi una contaminazione di questi effluenti con il potenziale di interferire con la salute umana.

La batteria di diversi test *in vitro* utilizzati si è rivelata utile per la valutazione del potenziale immunotossico delle sostanze chimiche, inclusi IE o EAS. I dati ottenuti rafforzano il concetto di interazione immuno-endocrina e indicano che questi test potrebbero essere integrati nelle procedure di valutazione del pericolo in vista di una valutazione del rischio più sostenibile.

Preface

There are more than 160 million known chemicals in the world, therefore, we are constantly exposed throughout life to different chemicals, both natural and man-made (Fernandes & Pestana, 2022; WHO, 2023). Chemicals represent an important part of human life, mainly due to their benefits (i.e., disease control, agricultural yield increase, industrial chemicals) (International Programme on Chemical Safety, 2000). The main routes of exposure of chemicals are ingestion, inhalation, or skin contact (Papadopoulou et al., 2019; Degrendele et al., 2022; Saravanan et al., 2022). The exposure to chemicals has been linked to several adverse health effects, some of which also irreversible (International Programme on Chemical Safety, 2000). Effects might include nervous and immune system disturbances, allergies, asthma, reproductive effects, and cancer (Government of Canada, 2023). In addition, subpopulations exist that can be more susceptible to their effects, e.g. children. Furthermore, occupational exposure may result in a major risk, due to a higher exposure. Indeed, more than 1 billion workers per year are exposed to hazardous chemicals, that can lead to illnesses, injuries or even deaths (ILO, 2021).

According to the Endocrine Society, within the 85'000 man-made chemicals in the world, more than 1'000 could be considered endocrine disruptors (EDs) (NIEHS, 2023). Endocrine active substances (EAS) are those substances able to interfere with the endocrine system (EFSA, 2023), and similarly EDs are considered natural or synthetic substances able to interfere with hormone synthesis, secretion, transport, binding, elimination or signaling of natural hormones, inducing adverse effects (Kavlock et al., 1996). EDs are mainly environmental pollutants, plastics, metals, by-products from industry, pesticides, food contaminants, personal care products, and pharmaceuticals (Benotti et al., 2008; Tijani et al., 2013). They can be found in many everyday products, like cosmetics, food and beverage packaging, and toys (NIEHS, 2023). Due to their widespread use, they cannot be completely avoided, that is why we are always exposed to their potential harm. The main EAS/ED categories are represented in Figure 1.

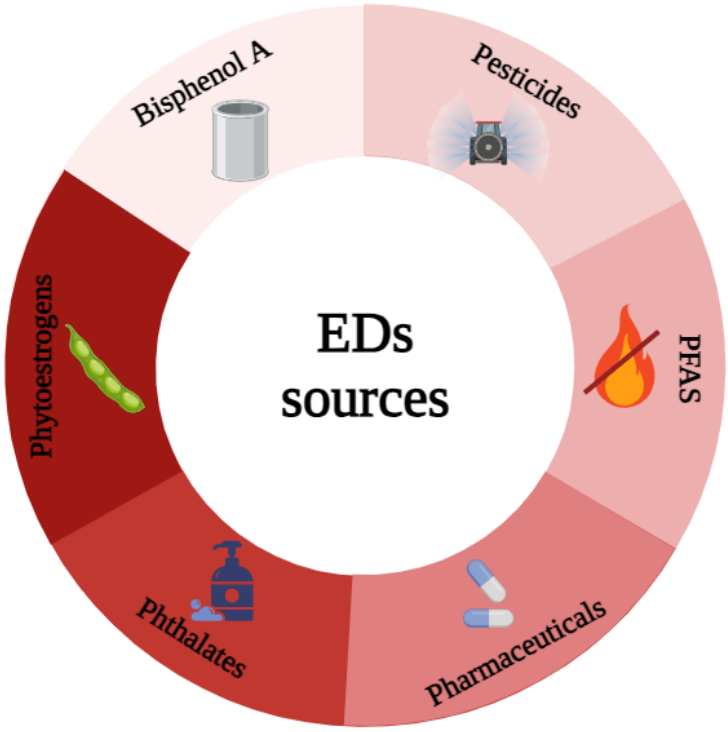

Figure 1. Principal sources of EDs (Biorender.com).

Examples of EAS/EDs are or can be found within specific listed categories (Diamanti-Kandarakis et al., 2009; Sabir et al., 2019; Endocrine Society, 2022; NIEHS, 2023):

- Bisphenol A: it is a component of polycarbonate plastics used mainly in food packaging and toys. Also its substitutes are suspected EAS and currently under evaluation;
- Pesticides, or in general, biocides: they prevent, destroy, or control harmful organisms or diseases, or protect plants (i.e., herbicides, fungicides, insecticides, repellents, or biocides). Examples of EAS are atrazine, glyphosate, cypermethrin, vinclozolin;
- PFAS (perfluoroalkyl substances): they are a group of chemicals widely applied in industry in firefighting foam, nonstick pans, and textile coatings;
- Pharmaceutical agents: some of them can be considered EDs due to primary or adverse effects. An example is ethynyl estradiol;
- Phthalates: they are a group of compounds used as liquid plasticizers. They are present in food packaging, cosmetics, fragrances, and toys. One example is diethylphthalate;
- Phytoestrogens: they are natural substances found in some plants (i.e., soy) with effects similar to estrogen.

There are also other sources of EAS/EDs, like cosmetics, flame retardants, and textiles.

In the last decades, an increased incidence of certain diseases (i.e., cancer, metabolic disorder, neurological disorder, allergy, autoimmunity) has been noticed, mainly in industrialized countries. Environmental factors, including the exposure to EDs, are considered the main culprit (Natha et al., 2015; Oliviero et al., 2022). Therefore, an increased interest was posed on them due to the emerging evidence on their negative impact on human health (Ho et al., 2022). In addition to the effects at the expense of the endocrine system, EDs can impact also metabolic, neurological, and immune systems (Monneret, 2017; Bansal et al., 2018; Kumar et al., 2020; D'Amico et al., 2021; Di Paola et al., 2022). The interconnection between the immune and the endocrine system is a relevant but underestimated topic. Immune system cells have receptors for hormones and can also produce hormones (Verburg-van Kemenade & Schreck, 2007; Carvalho et al., 2015). Therefore, hormones (i.e., androgens, estrogens, and glucocorticoids) can influence immune cell functions (Coutinho and Chapman, 2011; Islander et al., 2011; Racchi et al., 2017). And *vice versa* the immune system is able to influence and perturb the endocrine system (O'Connor et al., 2021). It is reasonable, therefore, to hypothesize that EDs exposure can perturb the immune system (Greives et al., 2017). In addition, the impact of EDs on the immune system is even more evident in a sex-specific perspective (Stelzer & Arck, 2016).

The aim of this PhD project was to evaluate if EDs or potential EAS were able to interfere with the immune system and to alter its functionality. In order to assess it, different *in vitro* methods were applied to evaluate different aspects of the human immune system. The project is part of a research supported by Ministero dell'Istruzione, dell'Università e della Ricerca (PRIN 2017, Project number 2017MLC3NF – EDONIS: Endocrine Disruptors: investigation of the effects On the Nervous and Immune Systems). The thesis is subdivided in three chapters composed of a collection of already published papers: starting from the case study on glyphosate (I), going through the evaluation of six known or suspected EAS/EDs (atrazine, cypermethrin, diethylphthalate, ethynyl estradiol, perfluorooctanesulfonic acid, and vinclozolin - II), ending with the effect of mixtures on the immune system (III). The following articles, in order, compose the three core chapters of my PhD thesis:

- **Maddalon, A.**, Iulini, M., Galbiati, V., Colosio, C., Mandić-Rajčević, S., & Corsini, E. (2022). Direct Effects of Glyphosate on In Vitro T Helper Cell Differentiation and Cytokine Production. Frontiers in immunology, 13, 854837. https://doi.org/10.3389/fimmu.2022.854837

- **Maddalon, A.***, Masi, M.*, Iulini, M., Linciano, P., Galbiati, V., Marinovich, M., Racchi, M., Buoso, E., & Corsini, E. (2022). Effects of endocrine active contaminating pesticides on RACK1 expression and immunological consequences in THP-1 cells. Environmental toxicology and pharmacology, 95, 103971. https://doi.org/10.1016/j.etap.2022.103971
- Masi, M.*, **Maddalon, A.***, Iulini, M., Linciano, P., Galbiati, V., Marinovich, M., Racchi, M., Corsini, E., & Buoso, E. (2022). Effects of endocrine disrupting chemicals on the expression of RACK1 and LPS-induced THP-1 cell activation. Toxicology, 480, 153321. https://doi.org/10.1016/j.tox.2022.153321
- **Maddalon, A.***, Cari, L.*, Iulini, M., Alhosseini, M. N., Galbiati, V., Marinovich, M., Nocentini, G., & Corsini, E. (2023). Impact of endocrine disruptors on peripheral blood mononuclear cells in vitro: role of gender. Archives of toxicology, 10.1007/s00204-023-03592-3. Advance online publication. https://doi.org/10.1007/s00204-023-03592-3
- **Maddalon, A.***, Pierzchalski, A.*, Kretschmer, T., Bauer, M., Zenclussen, A. C., Marinovich, M., Corsini, E., & Herberth, G. (2023). Mixtures of per- and poly-fluoroalkyl substances (PFAS) reduce the in vitro activation of human T cells and basophils. Chemosphere, 336, 139204. https://doi.org/10.1016/j.chemosphere.2023.139204
- **Maddalon, A.**, Pierzchalski, A., Krause, J. L., Bauer, M., Finckh, S., Brack, W., Zenclussen, A. C., Marinovich, M., Corsini, E., Krauss, M., & Herberth, G. (2023). Impact of chemical mixtures from wastewater treatment plant effluents on human immune cell activation: An effect-based analysis. The Science of the total environment, 906, 167495. Advance online publication. https://doi.org/10.1016/j.scitotenv.2023.167495

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Chapter I – Glyphosate

The first chapter focuses on the case study on the herbicide glyphosate. The use of glyphosate is currently under intense debate, since several studies highlighting its toxicity on non-target organisms are emerging. In particular, there are some evidence suggesting an action at the expense of the immune and endocrine systems. The endocrine disrupting potential targeting sex and thyroid hormones has been pointed out (de Souza et al., 2017; Guerrero Schimpf et al., 2017; Pham et al., 2019), although the European Food Safety Authority recently concluded that based on available data glyphosate cannot be considered an ED (EFSA et al., 2023). Evidence of immunotoxic effects is limited compared to those on endocrine system, but possible noxious effects, including lung inflammation and rhinitis have been associated to glyphosate exposure (Kumar et al., 2014; Hoppin et al., 2017). In addition, in the Agricultural Health Study, women with rheumatoid arthritis (RA) were somewhat more likely to have reported lifetime use of any specific pesticide versus no pesticides. Of the 15 pesticides examined, maneb/mancozeb and glyphosate women with RA were the ones showing an association (Parks et al., 2016). An attractive hypothesis to explain its potential immunotoxicity could be the one that connects microbiota dysbiosis with possible immune-endocrine outcomes (Maddalon et al., 2021). Indeed, some microorganisms that reside in the human intestine express the enzyme EPSPS (target of glyphosate), suggesting therefore a glyphosate-induced adverse health effects mediated by gut microbiota (Rueda-Ruzafa, 2019; Liu et al., 2022).

Considering the wide application and human exposure to glyphosate, mainly in occupational settings, and the paucity of studies concerning the immune system, we considered relevant to investigate if glyphosate could directly affect the human immune cells. Human peripheral blood mononuclear cells (PBMCs) exposed to glyphosate were used, the effects on T helper differentiation, and the underneath molecular mechanism investigated. The focus on T helper cells is based on an *in vivo* study showing a Th cells imbalance, with a predominant Th2 activation (Kumar et al., 2014), In particular, we evaluated the effect on T helper cells differentiation (Th1, Th2, and Th17) following stimulation with phorbol myristate acetate and ionomycin. The main effect observed was the dysregulation of the Th1/Th2 ratio, with a reduction of Th1 cells. Subsequently, to evaluate if the observed immune effect was mediated by an estrogenic effect, the estrogen receptor-α was inhibited and it resulted in a restore of IFN-γ basal levels. To further characterize the mechanism of action, the effect of glyphosate on miRNAs expression was also analyzed and hsa_miR-500a-5p resulted to be up-regulated, and this overexpression was responsible for the altered IFN-γ release.

Therefore, the initial hypothesis of an effect of glyphosate on the immune system with a link to its EAS activity was confirmed.

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frontiers in Immunology

ORIGINAL RESEARCH
published: 10 March 2022
doi: 10.3389/fimmu.2022.854837

Direct effects of glyphosate on *in vitro* T helper cell differentiation and cytokine production

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Frontiers in Immunology, 10 March 2022, doi: 10.3389/fimmu.2022.854837

Abstract

Glyphosate (G) is the active ingredient of the most used herbicides worldwide. Its use is currently very debated, as several studies indicating its hazard and toxicity are emerging. Among them, there is evidence of adverse effects on the immune system. The aim of this work was to investigate if G could directly affect immune cells. Peripheral blood mononuclear cells (PBMC) obtained from healthy donors were used as experimental model. PBMC were expose to G and stimulated with PMA/ionomycin, T helper (Th) cell differentiation and cytokine production were assessed by flow cytometry and enzyme linked immunosorbent assay, respectively. A reduction of Th1/Th2 ratio, mainly due to a decrease in Th1 cells, was observed following G exposure. Results show an enhancement of IL-4 and IL-17A production, and a reduction of IFN-γ. Based on literature evidence that suggest G being an endocrine disruptor, we investigated the role of nuclear estrogen receptors (ER). ERα/ERβ inhibition by ICI 182,780 abolished the effects of G on IFN-γ and IL-4 release, suggesting a role of ER in the observed effects. To further characterize the mechanism of action of G, miRNAs, both in exosome and intracellular, were investigated. A statistically significant increase in miR-500a-5p was observed following G treatment. The blockage of miR-500a-5p, using a specific antagomir, prevented G-induced reduction of IFN-γ production. Finally, a relationship between miR-500a-5p up-regulation and ER was observed. Overall, these results suggest that G can directly act on T cells, altering T cell differentiation and cytokines production.

Keywords

Glyphosate, immunotoxicity, T helper cells, estrogenic effect, miR-500a

Introduction

Glyphosate (N-(phosphonomethyl) glycine; G) is the active ingredient of the most used broad-spectrum herbicide worldwide (1, 2). It was commercialized starting from 1974 under the name of Roundup®, together with adjuvants, like polyoxyethylene tallowamine, that, although considered inert compounds, can increase G toxicity (3).

Its success further increased following the introduction of genetically modified G-resistant crops (4). The herbicidal activity is based on the competitive inhibition of the shikimate pathway, which leads to the blockage of the synthesis of aromatic amino acids in plants (5). Since animals do not have this pathway, G has always been considered safe for humans by regulatory agencies (6, 7).

However, evidence of G toxicity and its alleged threats to the ecosystem and human health are emerging (8, 9). Humans can be exposed to G through different routes; mainly orally, but also through dermal and inhalation exposures (10, 11). Although the majority of studies addressing G toxicity used high doses, there are also studies using environmental relevant doses showing noxious effects in both animals and humans (12–16).

As for general toxicity, there is also evidence of adverse effects against the immune system. Indeed, the latter can be the target of several pesticides (17–19). G-induced immunotoxicity could be the result of a direct action of the herbicide, or can be due to an indirect mechanism, secondary to endocrine, nervous system, or microbiota alterations (20–22). Regarding mammals, evidence indicates the ability of G and G-based herbicides to alter immune responses and to induce inflammation (22). More specifically, intranasal injection of G-rich air samples in mice enhanced eosinophils, neutrophils, and mast cells degranulation in lungs, suggesting an asthma-like pathology (23). In this study, an activation of T helper (Th) 2 response, as reported by the higher production of Th2 type cytokines in lungs, namely IL-4, IL-5, IL-10, IL-13 and IL-33, was observed. Roundup® was shown to induce pro-inflammatory cytokines (i.e., IL-1β and TNF-α) in liver of rats exposed via feeding at the doses of 100 and 250 mg/kg bw/day (24). Similarly, the exposure via gavage was able to increase the mRNA levels of IL-1β, IL-6, TNF-α, MAPK3, NF-κB, and caspase-3 in the jejunum of rats exposed to the doses of 50 and 500 mg/kg bw/day (25). Authors hypothesized a role of microbiota in G-induced immunotoxicity, as a significant decrease in the relative abundance of Firmicutes and Lactobacillus, while an enrichment in several potentially pathogenic bacteria were observed

(25). The same result was obtained by Qiu et al. (26), which treating piglets with Roundup® in diet, observed increased mRNAs of IL-6 and NFκB in the jejunum following the exposure to 10, 20 and 40 mg/kg bw/day.

Beside animal studies, there are also few epidemiological studies supporting G-based herbicides affecting human immune system. The Agricultural Health Study demonstrated an association between G occupational exposure and current rhinitis and increased rhinitis episodes (27, 28). This study revealed also an association between allergic and non-allergic wheezes among G sprayers (29).

As microbiota is known to influence the immune system and considering that the shikimate pathway is also found in some microorganisms, the interaction of G with commensal microorganisms represents a plausible explanation of G induced immunotoxicity. Indeed, recently G-based herbicide ability to inhibit the shikimate pathway in the microbiome was demonstrated (30). Furthermore, Roundup LP Plus® was demonstrated to alter the metabolism of *E. coli*, that in turn impacted on the inflammatory immune response, inducing a higher production of TNF-α (31). In addition, associations between G and immune-endocrine disturbances have been described, which also represent a plausible mechanism underlaying G immunotoxicity (reviewed in 22).

The aim of this study was, therefore, to investigate if G could directly affect T helper (Th) cell differentiation and functions. The study focused on Th cells as they have been identified in mice as a possible target of G (23). Investigations were also conducted to get insights on the underlying mechanism of action focusing on the role of the estrogen receptor (ER) and miRNAs in G-induced immunotoxicity. Results obtained demonstrated that G can directly act on T cells, altering T cell differentiation and cytokines production, with a role of estrogen receptor and miR-500-5p in the observed effects.

Materials and Methods

Chemicals

G (CAS #1071-83-6, purity ≥ 99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). It was dissolved in Dulbecco's Phosphate Buffered Saline. Phorbol 12-myristate 13-acetate (PMA – CAS #16561-29-8, purity ≥ 99%) and ionomycin from *Streptomyces conglobatus* (CAS #56092-81-0, purity ≥ 98%) were purchased from Sigma-Aldrich and used to stimulate immune cells, as well as phytohaemagglutinin (PHA, CAS #9008-97-3). 17b-estradiol (E2 – CAS #50-28-2, purity 98%) was used as reference compound for ER activity,

and it was obtained from Sigma-Aldrich. ICI 182,780 (CAS # 129453-61-8, purity ≥ 99%) was used as ER antagonist and it was obtained from Tocris Bioscience (Bristol, United Kingdom). All the substances, with the exception of G, were dissolved in dimethyl sulfoxide (DMSO, CAS # 67-68-5, purity ≥ 99.5%), with a final DMSO concentration in culture medium ≤ 0.2%. Cell culture medium and all supplements were also purchased from Sigma-Aldrich.

Cells

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll gradient centrifugation from anonymous buffy coats of 5 male healthy donors, purchased from the Niguarda Hospital in Milan (Italy). After centrifugation, PBMC layers were removed and washed 5 times with Dulbecco's Phosphate Buffered Saline. Isolated cells were diluted to 10_6 cells/mL in RPMI 1640 with phenol red containing 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, gentamycin 10 μg/mL, 50 μM 2-mercaptoethanol, supplemented with 10% heated-inactivated fetal bovine serum (culture media) and cultured at 37°C in 5% CO_2 incubator. Experiments with E2 and ICI 182,780 were performed using RPMI 1640 without phenol red containing 2 mM L-glutamine, 0.1mg/mL streptomycin, 100 IU/mL penicillin, gentamycin 10 μg/mL, 50 μM 2-mercaptoethanol, supplemented with 5% heat inactivated dialyzed fetal bovine serum. Preliminary experiments were conducted to identify non-cytotoxic concentrations (cell viability > 90%). Cytotoxicity was assessed by LDH Cytotoxicity Detection Kit (Takara Bio USA, Inc.) (data not shown).

Th Cells Analysis

Phenotyping of Th cells was conducted using the Human Th1/Th2/Th17 Phenotyping Kit (BD Pharmingen, Inc.). PBMC (10^6 cells/mL) were incubated overnight (o.n.) and then treated in the presence or absence of G at the concentrations of 0.01, 0.1, 1 and 10μg/mL. After 1hour, cells were stimulated with PMA (50 ng/mL), ionomycin (1 μg/mL) and GolgiStop™ Protein Transport Inhibitor for 5 hours, following manufacturer's instructions. After treatment, cells were centrifuged at 1300 rpm for 5 minutes and stained with anti-human CD4 PerCP-Cy5.5 conjugated, anti-IL-17A-PE, anti-IL-4-APC and anti-IFN-γ-FITC following manufacturer's instructions. Cells were analyzed using NovoCyte 3000 flow cytometer, and data were quantified using NovoExpress software (Acea Biosciences, Inc). Results are expressed as fold-change of G treated versus control cells.

Cytokine Production

PBMC (10^6 cells/mL) were incubated o.n. and then treated with G at the concentrations of 0.01, 0.1, 1 and 10 µg/mL. After 1 hour, cells were stimulated with PMA (10 ng/mL) and ionomycin (100 ng/mL) or PHA (1.2 mg/mL) for 72 hours. Following treatment, cells were centrifuged at 1300 rpm for 5 minutes and supernatants collected for cytokine measurement and stored at -20°C until measurement. Cytokine production was assessed in cell-free supernatants by specific sandwich enzyme-linked immunosorbent assays (ELISA) commercially available. ELISA for IL-4 and IFN-γ were purchased from ImmunoTools (Friesoythe, Germany), while IL-17A ELISA from BioLegend (San Diego, CA, USA). Antibodies dilutions were performed according to manufacturer's instructions. Limits of detection were 2.3 pg/mL for IL-4, 24 pg/mL for IFN-γ, and 3.9 pg/mL for IL-17A. Results are expressed as fold-change of released cytokines of G-treated versus control cells.

miRNAs Contained in Exosomes Analysis

PBMC (3×10^6) were incubated o.n. and then treated with G at the concentration of 0.1 µg/mL for 72 hours in culture medium without fetal bovine serum. Following treatment, PBMC were centrifuged at 260g at 25°C for 5 minutes and supernatants used for exosome extraction. Cell-free supernatants were centrifuged at 20'000g at 4°C for 30 minutes, and supernatants centrifuged again at 100'000g at 4°C for 90 minutes. The pellet obtained represented the exosomes released by PBMC. Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. Retro-transcription was performed according to manufacturer's instructions (miScript II RT Kit, Qiagen). Before Real-Time PCR, the selected miRNAs were pre-amplified using the miScript PreAMP PCR kit (Qiagen). For PCR analysis miScript SYBR® Green PCR Kit (Qiagen) was used. All the primers were purchased from Qiagen and used according to manufacturer's instructions. The quantification of the miRNAs was performed by the $2^{-\Delta\Delta Ct}$ method. The fold-changes of 7 miRNAs (hsa_miR-10b, hsa_miR-27a, hsa_miR-100, hsa_miR-136, hsa_miR-424, hsa_miR-500a, hsa_let-7f) were analyzed, and miRNA hsa_RNU6-2 was used for normalization.

Intracellular miR-500a Analysis

Following the above-mentioned treatment and centrifugation at 260g at 25°C for 5 minutes, total RNA was extracted from the pellet, using RNeasy Plus Mini Kit. RNA was retro-transcribed and the fold-change of hsa_miR-500a was analyzed using miScript SYBR® Green

PCR Kit. The quantification was performed by the $2^{-\Delta\Delta Ct}$ method, using miRNA hsa-RNU6-2 for normalization.

miRNA Silencing

Hsa_miR-500a-5p was silenced through the use of miRCURY LNA™ miRNA inhibitor (Qiagen). 400'000 cells, after o.n. incubation, were silenced with 25 pmol of miRNA inhibitor and 3 µl of HiPerFect Transfection Reagent (Qiagen) for 24 hours. Then, the medium was replaced, and cells treated in the presence or absence of G (0.1 µg/mL) for 1 hour, and then stimulated with PMA and ionomycin for 72 hours. Following treatment, cells were centrifuged at 1300 rpm for 5 minutes and supernatants collected for IFN-γ measurement.

Data Analysis

With exception of PHA-induced cytokine release (n=3), all experiments were conducted using 5 donors. Data are expressed as mean ± standard error (SEM). Statistical analysis was performed using GraphPad Prism version 9.1.1 (GraphPad Software, La Jolla, CA, USA). Data were analyzed by One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test or Tukey's multiple comparison test or by paired Student's t test. Differences were considered significant at $p \leq 0.05$.

Results

Effects of G on Th Differentiation

Epidemiological studies (27, 29) and mice study (23) suggest that G may be associated with increased risk of allergic reactions with increased Th2 responses. To assess if G was able to directly affect immune cells, its effects on Th differentiation were evaluated *in vitro*. PBMC were treated with G alone at increasing concentrations for 1 hour and stimulated with PMA and ionomycin for subsequent 5 hours. IFN-γ, IL-4 and IL-17A positive cells were analyzed by flow cytometry (Figure 1). Gating strategies for flow cytometry panels are reported in Supplementary Material (Supplementary Figure 1). A reduction of IFN-γ positive cells was observed following G treatment that reached statistically significance at 1 µg/mL (Figure 1A). No significant changes were observed in IL-4 positive cells (Figure 1B). When analyzing the ratio between IFN-γ and IL-4 positive cells (Figure 1C), a decrease was observed, reaching the statistical significance at the concentration of 10 µg/mL. No statistically significant effects were observed in IL-17A positive cells (Figure 1D). Overall, data indicate that G can directly affect

Th cell differentiation, resulting in an imbalance of Th1/Th2 subpopulations, supporting an increase in Th2 responses.

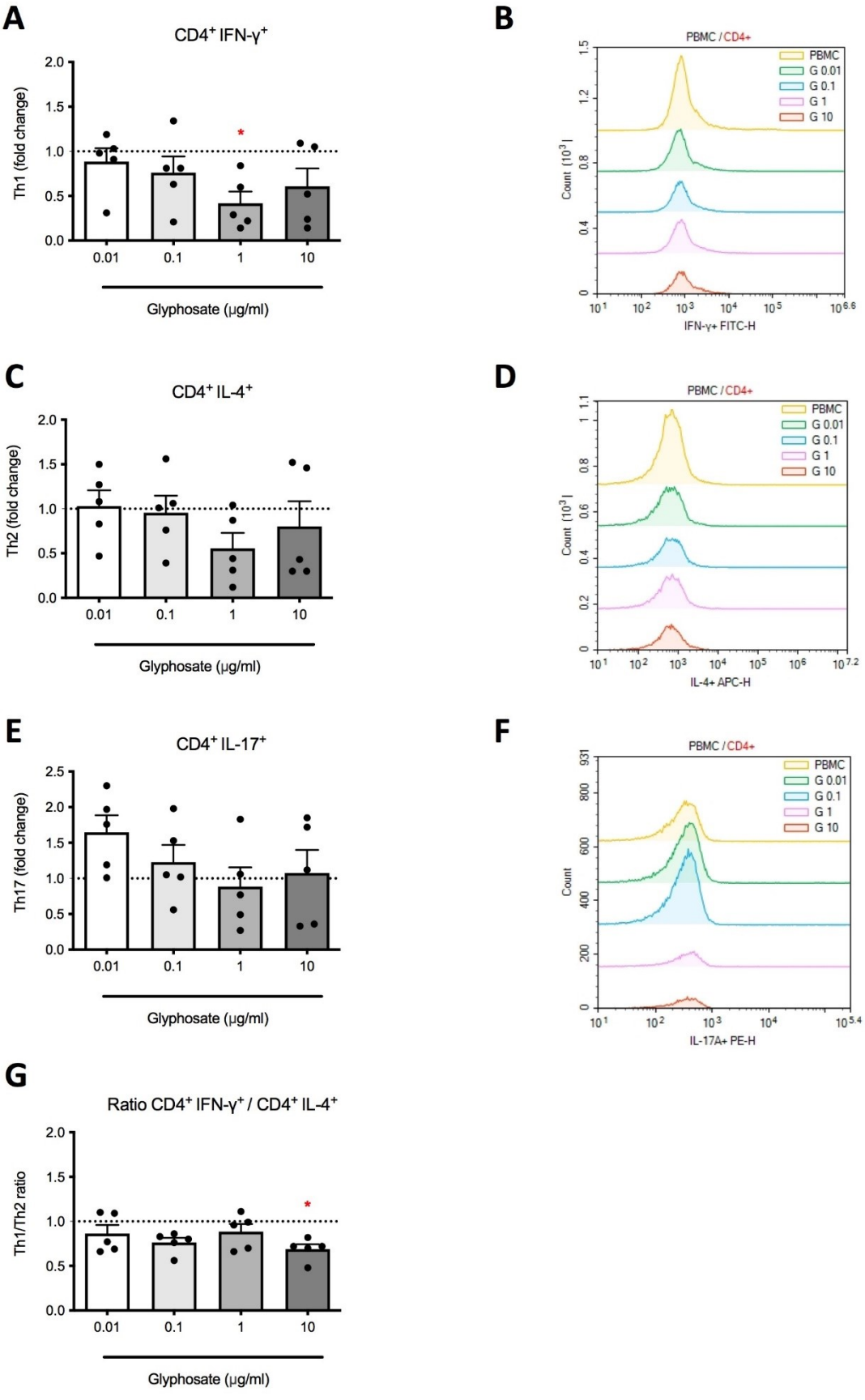

Figure 1. Effect of G on PMA plus ionomycin-induced $CD4^+$ cell differentiation. PBMC (10^6/mL) were treated for 1 h with increasing concentrations of G (0.01, 0.1, 1 and 10 μg/mL), and then stimulated with PMA, ionomycin and GolgiStop™ for 5 h, as described in the Materials and Methods section. (A) $CD4^+$ IFN-γ^+ cells. (C) $CD4^+$ IL-4^+ cells. (E) $CD4^+$ IL-$17A^+$ cells. (G) $CD4^+$ IFN-γ^+/$CD4^+$ IL-4^+ cell ratio. Results are expressed as fold-change of the number of positive cells in G treated cells compared to control cells. The dotted line reported is set at 1.0 (control). Each value represents the mean ± SEM, n = 5 donors. Each dot represents the value of the single individual. Statistical analysis was performed with Dunnett's multiple comparison test, with *$p < 0.05$ *vs* control cells. Flow cytometric analysis of representative histogram overlay were reported in (B, D, F).

Effects of G on Cytokine Production

Next, the effect of G on cytokine release, namely IFN-γ, IL-4, IL-17A, in response to PMA + ionomycin or PHA was investigated. PBMC were treated with increasing concentrations of G alone for 1 hour and then stimulated with PMA and ionomycin or PHA for 72 hours. Results were overall consistent with data shown in Figure 1. G reduced IFN-g release (Figure 2A) which resulted statistically significant at 0.1 and 1 μg/mL. The release of IL-4 (Figure 2B) was enhanced, although not linearly, reaching statistically significance at the concentrations of 0.01 and 0.1 μg/mL. This imbalance in IFN-γ and IL-4 release resulted in a reduction in the IFN-γ/IL-4 ratio (Figure 2C), indicating an action in favor ofTh2 cells and against Th1 cells. Regarding IL-17A production (Figure 2D), a statistically significant increase was observed at the concentrations of 0.1 and 10 μg/mL, with no clear dose-response. Similar results were also observed using PHA (Table 1), a lectin known to bind to T-cell membranes stimulating activation and proliferation (32), confirming the ability of G to unbalance Th1/Th2 responses, favoring Th2.

Table 1. Effect of G on PHA-induced cytokine production.

Treatment (μg/mL)	IFN-γ	IL-4	IFN-γ/IL-4 ratio
0.01	0.51 ± 0.22 *	0.76 ± 0.05	0.65 ± 0.24
0.1	0.38 ± 0.13 **	0.91 ± 0.16	0.38 ± 0.07 **
1.0	0.58 ± 0.28	0.90 ± 0.23	0.43 ± 0.15 **
10.0	0.67 ± 0.18	0.89 ± 0.17	0.58 ± 0.16 *

PBMC (10^6/mL) were treated for 1 h with increasing concentrations of G (0.01, 0.1, 1 and 10 μg/mL), and then stimulated with PHA (1.2 μg/mL) for 72 h. Cytokines were measured by ELISA in cell-free supernatants. Results are expressed as fold changes vs cells treated with PHA alone. Each value represents the mean ± SEM, n = 3 donors. Statistical analysis was performed with Dunnett's multiple comparison test, with *$p < 0.05$ and **$p < 0.01$ *vs* control cells.

Our findings demonstrated a non-monotonic response of G on some endpoints of Th differentiation and cytokine production. For instance, lower concentrations can exert higher effects, with a nonlinear trend.

Based on these results, subsequent experiments were conducted using G at the concentration of 0.1 µg/mL. This concentration is biologically relevant as serum level of 0.1891 mg/mL were detected in Thai women involved in agricultural activities (33).

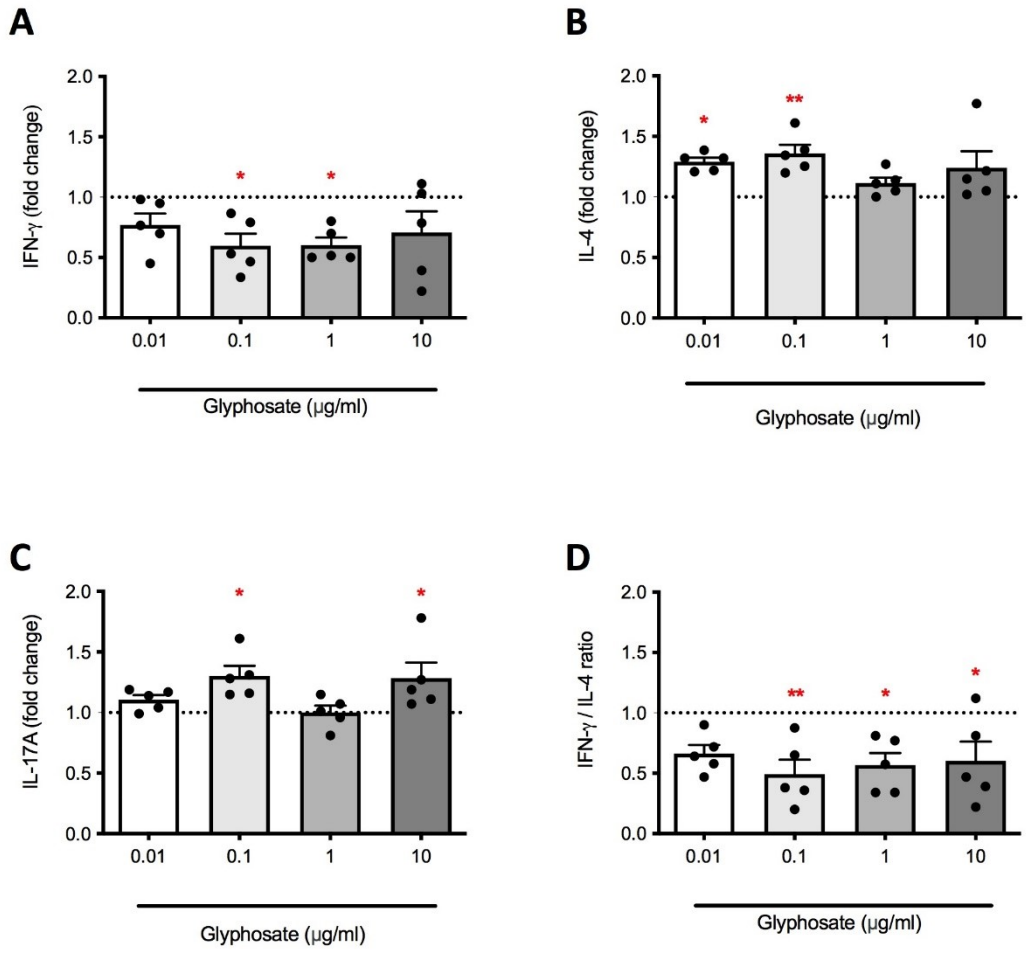

Figure 2. Effect of G on PMA plus ionomycin-induced IFN-γ, IL-4 and IL-17A release. PBMC (10^6/mL) were treated for 1 h with increasing concentrations of G (0.01, 0.1, 1 and 10 µg/mL), and then stimulated with PMA and ionomycin for 72 h. Cytokines were measured by ELISA in cell-free supernatants. (A) IFN-γ release. (B) IL-4 release. (C) IL-17A release. (D) IFN-γ/IL-4 ratio. Results are expressed as fold-change of the released cytokines in G treated cells compared to control cells. The dotted line reported is set at 1.0 (control). Each value represents the mean ± SEM, n = 5 donors. Each dot represents the value of the single individual. Statistical analysis was performed with Dunnett's multiple comparison test, with *$p < 0.05$ and **$p < 0.01$ *vs* control cells.

Role of ER in G-Induced Effects

Even if debated, some literature data suggest that G may act through the estrogen receptor (ER) (34). To investigate a possible role of ER in G-induced immunotoxicity the ER inhibitor ICI 182,780 was used (35). E2 was used as positive control. The release of IFN-γ, IL-

4 and their ratio was investigated, following treatment with G at the concentration of 0.1 μg/mL or E2 (10 ng/ml), with a pre-treatment of 15 minutes in the presence or absence of ICI (1 μM). G and E2 similarly reduced the production of PMA-ionomycin induced IFN-γ (Figure 3A). ICI pre-treatment combined with G or E2 was able to completely restore IFN-γ production. The same behavior was observed with the release of IL-4 (Figure 3B), which was enhanced by both G and E2 and restored by ICI. The decrease in IFN-γ/IL-4 ratio induced by both G and E2, was prevented by ICI, indicating a role of ER in G-induced Th1/Th2 imbalance.

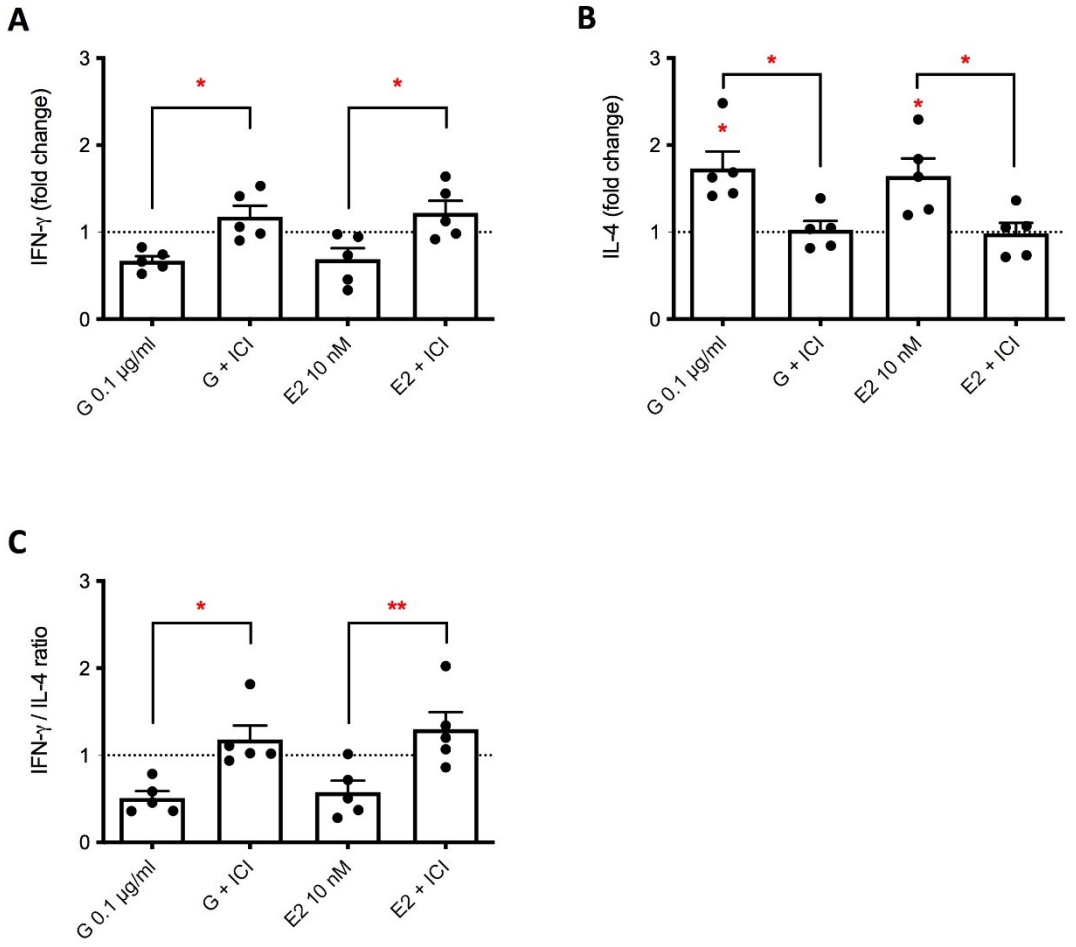

Figure 3. Role of ERα in G-induced cytokine release. PBMC (10^6/mL) were treated with ICI 182,780 (1 μM) for 15 minutes, and then with G (0.1 μg/mL) or with E2 (10 nM) for 1 h. After that, cells were stimulated with PMA plus ionomycin for 72 h. Cytokines were measured by ELISA in cell-free supernatants. (A) IFN-γ release. (B) IL-4 release. (C) IFN-γ/IL-4 ratio. Results are expressed as fold-change of the cytokine released in G treated cells versus relative control cells (untreated cells for G- or E2-treated cells and cells treated with ICI for G+ICI- or E2+ICI-treated cells). The dotted line reported is set at 1.0 (control). Each column represents the mean ± SEM, n = 5 donors. Each dot represents the value of the single donor. Statistical analysis was performed with Tukey's multiple comparison test, with *$p < 0.05$ and **$p < 0.01$ *vs* control untreated cells or the corresponding not ICI-treated cells.

Role of miR-500a in G-Induced Effects on Lymphocytes

To further investigate the mechanism underlying G immunotoxicity, starting from a miRNA panel conducted on exosomes obtained from plasma samples of farmers occupationally exposed to G-based herbicide (manuscript in preparation), seven de-regulated miRNAs were selected, namely miR-10b, miR-27a, miR-100, miR-136, miR-424, miR-500a, let-7f. PBMC were treated with G (0.1 µg/mL) for 72 hours, and the presence of the selected miRNAs in exosomes investigated (Figure 4A). Hsa-miR-100 appeared to be slightly down-regulated following G treatment, whereas the other 6 miRNAs investigated resulted to be up-regulated. Even if miR-424 resulted clearly up regulated, the only statistically significant change was observed for hsa-miR-500a-5p, which expression was over 3 times higher following G exposure (Figure 4B). The same over-expression was also observed for the intracellular miRNA (Figure 4C), indicating an effect of G both on intracellular and exosome-released miR-500a-5p.

To investigate if hsa-miR-500a-5p was involved in G-induced inhibition of IFN-γ release, miR-500a-5p was silenced by the use of an antagomir. The pre-incubation with hsa-miR-500a-5p inhibitor was able to restore the production of IFN-γ, in a statistically significant manner (Figure 4D), indicating a role of miR-500a-5p in the observed effects.

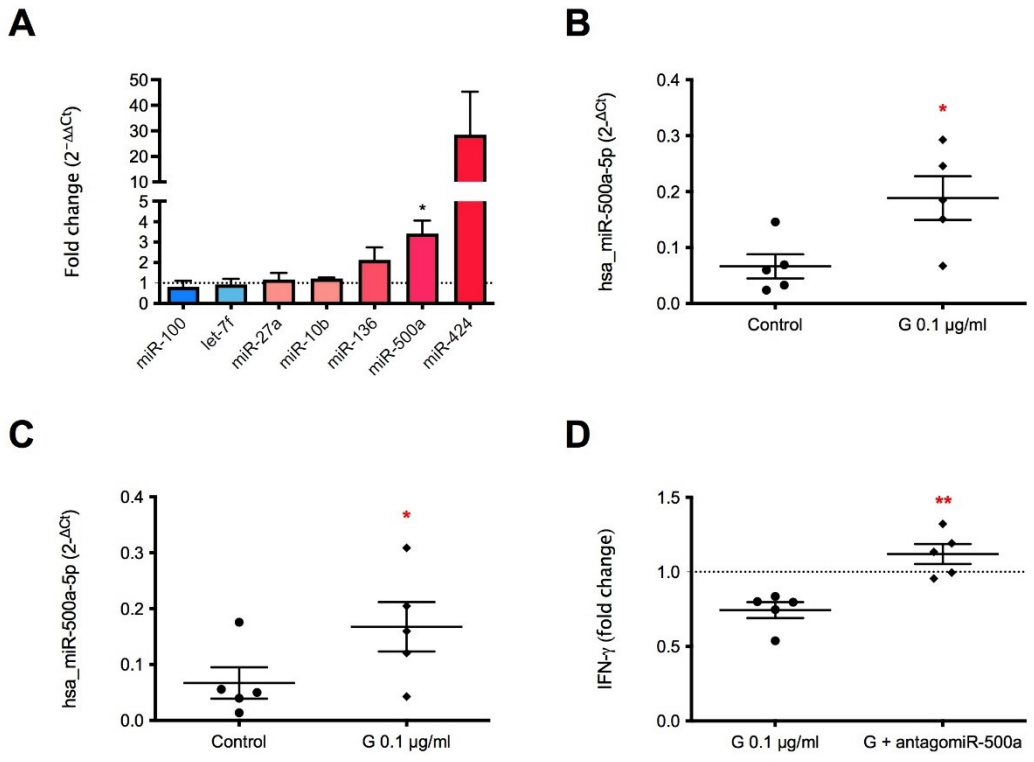

Figure 4. Effect of G on the exosome and intracellular expression of the selected miRNAs. PBMC (10^6/mL) were treated for 72 h with G (0.1 µg/mL). Exosomes were prepared as described in the Material and Methods section. Total RNA was extracted, retro-transcribed, and target miRNAs were pre-amplified and detected through Real-Time PCR. (A) Fold-changes ($2^{-\Delta\Delta Ct}$) of the selected miRNAs in exosomes (hsa_miR-10b, hsa_miR-27a, hsa_miR-100, hsa_miR-136, hsa-miR-424, hsa_miR-500a, hsa_let-7f) are reported. (B) Fold-change (2−DCt) of hsa_miR-500a contained in exosomes. (C) Fold-

change ($2^{-\Delta Ct}$) of intracellular hsa_miR-500a. hsa-RNU6-2 as negative control. (D) Role of miR-500a in IFN-γ production. PBMC (40'000 cells per well) were silenced with the inhibitor of hsa_miR-500a-5p for 24 h, after medium replacement cells were treated with G (0.1 μg/mL) for 1 h and then with PMA and ionomycin for 72 h. IFN-γ was measured by ELISA in cell-free supernatants. In (B, C) dots represent the individual values of control cells and rhombuses of G-treated cells for each donor. In (D) each dot represents the fold change of IFN-γ release induced by G of each individual, whereas rhombuses represent the fold change of IFN-γ release obtained from antagomir pre-treatment. Each value represents the mean ± SEM, n = 5 donors. Statistical analysis was performed with Paired t test, with $*p < 0.05$ and $**p < 0.01$ *vs* control cells (or G treated cells for (D).

Therefore, we highlighted an association between hsa-miR-500a-5p and IFN-γ, which release can be also affected by the pretreatment with ICI 182,780. To correlate these two phenomena, the expression of hsa-miR-500a-5p induced by G was assessed following ER inhibition. The inhibition of ER was able to statistically significantly reduce the expression of hsa-miR-500a-5p in the intracellular compartment (Figure 5), indicating the implication of ER in G-induced enhancement of hsa-miR-500a-5p expression. Despite the individual variability of miRNA expression, a reduction of hsa-miR-500a-5p expression, following ER inhibition, can be observed.

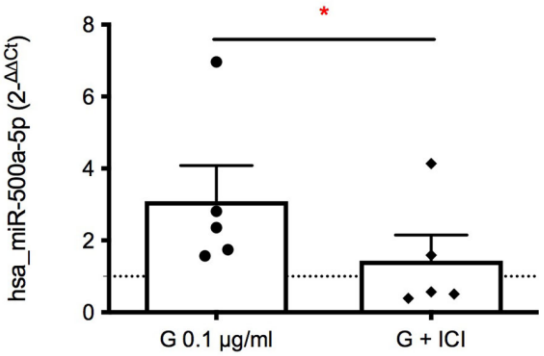

Figure 5. Role of ERα in G-induced intracellular expression of hsa-miR-500a-5p. PBMC (10^6/mL) were treated with ICI 182,780 (1 μM) for 15 minutes, and subsequently with G (0.1 μg/mL) for 72 h. Total RNA was extracted, retrotranscribed, and hsa-miR-500a-5p detected through Real-Time PCR. Foldchanges ($2^{-\Delta\Delta Ct}$) are reported, using hsa-RNU6-2 as negative control. Each dot represents the fold change of hsa-miR-500a-5p expression induced by G over control for each individual, whereas rhombuses represent the fold change of hsa-miR-500a-5p expression obtained from cells treated with ICI 182,780 and G over ICI-treated cells. Each value represents the mean ± SEM, n = 5 donors. Statistical analysis was performed with Paired t test, with $*p < 0.05$ *vs* G-treated cells.

Discussion

The purpose of this study was to investigate the ability of G to directly affect Th lymphocytes. Results demonstrated that G was able to decrease the Th1/Th2 ratio, inhibiting IFN-γ production. Effects could be reversed by the ER antagonist ICI 182,780, supporting a role of endocrine disturbance, and by miR-500a-5p antagomir. Results are in agreement with

the study conducted in mice by Kumar et al. (23) that demonstrated that G-rich air samples promoted Th2 type cytokines.

Even if still controversial, the ability of G to activate ER *in vitro* has been demonstrated (34, 36), and confirmations also came from experimental animals, showing ERα activation following herbicide exposure in male and female rats (37, 38). The possible role of ER in G-immunotoxicity is strengthened by the ability of estrogens to influence Th1 and Th2 responses, with a promotion toward Th2 activity (39), and to allergic airway inflammation (40). Estrogens can act on immune cells through both ERα and β (41, 42). ERs are involved in shaping the differentiation of Th cells, affecting transcriptional regulation, with possible consequences in inflammation (43). In this study, the role of ER was demonstrated, by the ability of ICI able to restore IFN-γ and IL-4 modulation, and results further support the endocrine disrupting activity of G (44).

G shown a non-monotonic dose-response relationship in its toxic effects against Th. Many endocrine disruptors, and mainly estrogenic substances, display this characteristic (45), strengthening the hypothesis of G considered as an endocrine disruptor. Some possible explanations to this phenomenon could be the cytotoxicity at high concentrations, the different receptor affinity, or receptor desensitization (45, 46).

To further investigate the mechanism underlying G immunotoxicity, a different expression of miRNAs in exosomes by PBMC following G exposure was investigated. MiRNAs are single-stranded RNAs of about 22 nucleotides, that function as post-transcriptional regulators, through the interference with mRNA processes (47). They can affect both mRNA stability and target mRNA for the subsequent degradation (48). The majority of miRNAs are localized in the intracellular compartment, however several miRNAs in various body fluids have been described (49). Exosomes are miRNAs carriers (50), of great relevance in pharmacology and toxicology (51). Notably, circulating miRNAs have been described as potential biomarkers of autoimmune and inflammatory disease (reviewed by 52). Considering the important role of miRNAs in the regulation of gene expression and their possible application as biomarkers and as cell-cell communication mechanism, we examined the differential expression of several miRNAs in farmers occupationally exposed to G (manuscript in preparation). Results presented in this work suggest miR-500a-5p as a possible target of G in immune cells. Only few studies investigated the effect of G on non-coding RNAs, mainly conducted in mice. Ji et al. (53) associated perinatal G exposure in mice with a differential miRNA expression in the prefrontal cortex (55 miRNAs up-regulated and 19 miRNAs down-regulated), mainly involved in neural development. Also, circular RNAs were affected, with

330 up-regulated and 333 down-regulated (54). Supporting this evidence, more recently, perinatal G exposure in mice was associated with aberrant expression of lncRNAs, highlighting a link with impaired neuronal development (55). Also changes in miRNA profile in female rat liver were assessed following G and G-based herbicide treatment, and interestingly these miRNAs were associated with carcinogenesis, like miR-17 and miR-22 (56). Furthermore, an *in vitro* study performed on carp lymphocytes shown that G induced lymphocyte apoptosis was mediated by the regulation of miR-203 and PI3K/AKT pathway (57). No studies examined the effect of G on miRNAs in human immune cells, in this regard this study represents the first evidence that G can affect human miRNAs expression linked to the immune system. In particular, a role of miR-500a could be demonstrated, linking miR-500a and lower IFN-γ production. MiR-500a-5p is not a well-characterized miRNA; to date the few available evidence address a role in oxidative stress in breast cancer (58) and in the promotion of breast cancer cell proliferation (59). It has also been described as oncogenic in colorectal cancer (60), as well as responsible for proliferation and metastasis of hepatocarcinoma (61). Based on TargetScan (v7.2; targetscan.org), a useful database to predict miRNA target sites (62), hsa-miR-500a-5p could hypothetically inhibit several mRNAs involved in the differentiation of Th cells towards Th1, preventing it. Among possible targets there are STAT1 (signal transducer and activator of transcription 1), TBX21 (T-box 21; or T-bet), SOCS5 (suppressor of cytokine signaling 5) and ANXA1 (annexin A1), which are all positive regulators of Th1. The up-regulation of miR-500a could be indeed in line with the decrease in Th1 as demonstrated in the current study. Furthermore, we speculate on the existence of a dependance between miR-500a expression and ER, since its inhibition reflected in a lower expression of hsa-miR-500a-5p. Preliminary results indicate indeed a reduced expression of STAT1 following G exposure, which could be reversed by ICI (data not shown), indicating STAT1 as a relevant target to explain G immunotoxicity.

Overall, data obtained indicate that G at biological relevant concentrations can directly affect Th cells, disturbing the balance between Th1 and Th2, through the ER pathway. We also demonstrated a role of miR-500a-5p in G-induced reduced IFN-γ production. Additional studies are required to identify the genes targeted by miR-500a-5p involved in Th1 differentiation and their link with ER.

Supplementary Material

Supplementary data to this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2022.854837/full#supplementary-material.

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