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

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Chapter II – Endocrine disruptors

The second chapter reports the results obtained testing several known or suspected EAS/EDs in a battery of *in vitro* methods to address direct immunotoxicity. Six compounds, each with different applications and hormonal effects, were chosen:

- 17α-ethynyl estradiol (EE): a synthetic estrogen widely used in birth control pills in combination with progestins, considered an ED by EPA (US Environmental Protection Agency);
- atrazine (ATR): a widely used herbicide in some countries (banned in the EU) and a common contaminant of ground, surface and drinking water and under the assessment for being an ED by EPA;
- cypermethrin (CYP): a synthetic pyrethroid used as an insecticide on a large scale recently classified as non-ED but literature data suggest its endocrine active properties;
- diethylphthalate (DEP): a plasticizer under the assessment as ED by EPA and ECHA (European Chemical Agency);
- perfluorooctanesulfonic acid (PFOS): a fluorosurfactant widely used in industry, now regarded as a global pollutant and considered as ED by EFSA;
- vinclozolin (VIN): a common dicarboximide fungicide, toxic for reproduction (ECHA) and therefore with endocrine active properties.

These compounds have been selected based on their different or presumed endocrine targets (i.e., hormone receptors, enzymes, hormone synthesis). In detail, EE, being a derivative of estradiol, is known to interfere with estrogen receptors. ATR has been associated to reproductive dysfunctions (Chevrier et al., 2011; Griffiths et al., 2022; Owagboriaye et al., 2022), due to its ability to affect androgens and estrogen levels (Trentacoste et al., 2001; Eldridge et al., 2008). CYP endocrine effects are still debated, and several evidences indicate its ability to interfere with the endocrine system (Jin et al., 2011; Irani et al., 2022), but it has been classified as unlikely to be an ED (EC, 2019). DEP estrogen-mimetic action has been suggested, like other phthalates (Fiocchetti et al., 2021). PFOS is an ED contained in the European list of priority substances in the field of water policy (EC, 2012) and in the Stockholm Convention (UNEP, 2009). It has been linked to both thyroid and reproductive dysfunctions (Coperchini et al., 2017; Tarapore et al., 2021). VIN, instead, has been considered able to alter male reproduction (Anway and Skinner, 2008; Feijó et al., 2021).

The objective was to evaluate whether the chosen compounds could influence the immune system by conducting a series of *in vitro* and *in silico* tests. A protein that represents a possible link between the endocrine and the immune system is RACK1 (receptor of activated C kinase 1). RACK1 is involved in the activation of innate and acquired immunity and represents a relevant target of endocrine action due to the fact that its expression is under steroid hormone control (Buoso et al., 2017; 2020). Experiments started with THP-1 cells, a cell line representative of human monocytes, to evaluate if the selected compounds could modulate RACK1 expression and related molecules involved in the immune response, namely CD86, CD54, IL-8, and TNF-α. To better dissect the concept of endocrine disruption, *in silico* docking analysis was performed to evaluate the possible binding with hormone receptors. After having assessed the behavior of these chemicals on THP-1 cells, experiments were conducted in human PBMCs. Both male and female donors were used in order to investigate possible different effects related to sex. The ability of the selected compounds to modulate RACK1 expression, to interfere with NK cell activity, and lymphocyte differentiation, focusing on $CD4^+$ and $CD8^+$ cells, was investigated. The last experiments were done in collaboration with the Department of Medicine and Surgery (Section of Pharmacology) of the Università degli Studi di Perugia, under the supervision of Professor Giuseppe Nocentini, during a visiting scientist period at his laboratory. In particular, from the first two papers regarding the effects of the six EAS on THP-1 cell line, all of them resulted able to dysregulate RACK1 expression (at promoter, protein, and gene level) and of pro-inflammatory markers associated to RACK1, mainly with a down-regulation trend, with the exception of EE which induces an immunostimulation. Molecular mechanism experiments together with *in silico* analyses evidenced that the observed immune effects might be explained by an action on hormone receptors. In particular, EE exhibited estrogenic action, ATR, CYP, and VIN were able to interact/interfere with the androgen receptor with different mechanisms, and DEP and PFOS shown an activity on glucocorticoid receptor. Similar trends of RACK1 modulation were obtained also on primary cells of human origin. Furthermore, some differences between male and female donors were observed, with female donors resulting more susceptible than male ones. The six EAS resulted to be able to dysregulate also NK cells lytic activity and T cells differentiation. In particular, DEP and PFOS exposure were able to induce changes in most parameters evaluated and we were able to detect two populations highly reduced in women donors following EAS exposure, characterized by high expression of cytokines, FoxP3, and GITR, indicative of activated conventional T helper cells, with regulatory action, therefore the hypothesis of endocrine-immune disturbance ability of the six EAS was confirmed through the experimental work on different endpoints.

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Effects of endocrine active contaminating pesticides on RACK1 expression and immunological consequences in THP-1 cells

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Abstract

We have previously demonstrated that RACK1, which expression is under steroid hormone control, plays an important role in the activation of immune cells and its expression can be useful to evaluate the immunotoxic profile of endocrine disrupting chemicals (EDCs). Hence, we investigated the effects of three contaminating and persistent pesticides: the fungicide vinclozolin (VIN), the herbicide atrazine (ATR) and the insecticide cypermethrin (CYP) on RACK1 expression and on innate immune response. VIN resulted in modest alteration of RACK1 while ATR and CYP reduced in a dose dependent manner RACK1 expression, ultimately leading to the decrease in lipopolysaccharide-induced IL-8 and TNF-α release and CD86 and CD54 surface marker expression. Moreover, our data indicate that, after exposure to EDCs, alterations of RACK1 expression can also occur with mechanisms not directly mediated by an interaction with a nuclear or membrane steroid receptors. Therefore, RACK1 could represent a useful EDCs screening tool to evaluate their immunotoxic potential and to dissect their mechanisms of action.

Keywords

Endocrine disrupting chemicals, immunotoxicity, RACK1, vinclozolin, atrazine, cypermethrin

Introduction

Pesticides are used to destroy, repel, prevent, or control harmful organisms or plants (World Health Organization (WHO), 2012, 2016). Pesticides include different categories of substances, including herbicides, fungicides, and insecticides among others. Despite their beneficial use in agriculture and vector-mediated diseases, pesticides pose a risk for the environment and human health (Lee and Choi, 2020). Several pesticides are environmentally persistent, and their exposure has been reported to occur both occupationally and environmentally (Mokarizadeh et al., 2015). Among the adverse effects reported, pesticides can target the immune system and perturb its homeostasis, with possible severe implications (Corsini et al., 2008; Mostafalou and Abdollahi, 2013; Corsini et al., 2013; Gangemi et al., 2016; Lee and Choi, 2020). Noteworthy, a connection between the immune and endocrine systems has been reported and evidence of immune-endocrine alterations linked to various pesticides exposure in humans has been documented (Forawi et al., 2004; Maddalon et al., 2021; Liu et al., 2022). A growing list of widely used substances with industrial employment are classified as endocrine disrupting chemicals (EDCs), exogenous substances able to interfere with several aspects of the endocrine actions (Diamanti-Kandarakis et al., 2009). EDCs exposure can occur because of their presence in food, water, drugs, industrial and personal care products and can result in an altered ability of inter-organs hormonal communication and an impaired immune functionality (Nowak et al., 2019; Lee and Choi, 2020). Some pesticides are known to interact with the endocrine system and, therefore, can be classified as EDCs. Exposure to pesticides, and above all to persistent substances, may alter the immune system functionality, potentially resulting in a reduced ability to fight infectious diseases or in an enhanced susceptibility to allergies (Dietert et al., 2000; Puig et al., 2008; Gascon et al., 2013; Galbiati et al., 2021). Therefore, because of the observed EDC-induced immunotoxicity, the European Food Safety Authority (EFSA) and other regulatory authorities have prompted research and critical interpretation of EDCs effects on the immune system (EFSA, 2016). Since EDCs are perceived as a serious public health issue due to their potency, constant, and universal human exposure, research efforts are direct to identify molecular biomarkers and methods to rapidly predict and reveal immunotoxicity of EDC. In this regard, in the last two decades, we provided evidence for the existence of a complex hormonal balance, between glucocorticoids, androgens and estrogen, in the control of RACK1 expression and immune cells activation (Buoso et al., 2011, 2017a, 2017b; Racchi et al., 2017), indicating that RACK1 can be a target of EDC (Buoso et al., 2017c, 2020a, 2020b, 2021).

RACK1 is a scaffolding protein involved in essential cellular processes and important biological events, including cancer and immune response (Buoso et al., 2020c; Dan et al., 2020; Duan et al., 2020; Corsini et al., 2021). We demonstrated that RACK1 expression is tightly related to the activation of immune cells and the release of pro-inflammatory cytokines and surface markers upregulation, which production is dependent upon RACK1/PKCβ activation (reviewed in Corsini et al., 2021). Due to the tight connection between the endocrine and the immune system, the assessment of the effects of EDCs on immunity is highly relevant. The aim of this paper was to examine the modulation of RACK1 after the exposure to three different pesticides belonging to three different classes, namely the fungicide vinclozolin (VIN), the herbicide atrazine (ATR) and the insecticide cypermethrin (CYP), all showing evidence of endocrine-disrupting abilities (Lee and Choi, 2020; Hayes et al., 2011; Feijó et al., 2021; Zhang et al., 2021; Ge et al., 2021; Galbiati et al., 2021).

Materials and Methods

Chemicals

VIN (PubChem CID: 39676), ATR (PubChem CID: 2256), CYP (PubChem CID: 2912), lipopolysaccharide (LPS) from *Escherichia coli* serotype 0127:B8. All reagents were purchased at the highest purity available. Cell culture media and supplements were purchased from Sigma Aldrich (St Louis, MO, USA). The mouse anti-human RACK1 monoclonal antibody (sc-17754) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The mouse monoclonal anti-β-tubulin antibody (T0198) was from Sigma Aldrich (St Louis, MO, USA). The mouse anti-AR 441 (ab9474) was obtained from Abcam (Cambridge, UK). Electrophoresis reagents were from Bio-Rad (Richmond, CA, USA). Chemicals were dissolved in dimethyl sulfoxide (DMSO) at 50 mM stock solutions, and sequentially properly diluted.

Cell culture and treatments

The THP-1 cell line was maintained and treated as described in refs. Buoso et al., (2017, 2020, 2021). Preliminary experiments were conducted to identify non-cytotoxic concentrations (cell viability > 80 % (CV80)) as assessed by PI. For experiments, cells (10^6/ml) were treated with increasing concentrations of VIN, ATR and CYP (0.001–10 μM) or DMSO as vehicle control (final concentration of DMSO in culture medium < 0.1 %) for different times, as detailed in figure legends. For CD54 and CD86 expression and for the release of cytokines, cells were incubated for 24 h with increasing concentrations (0.1–10 μM) of the selected EDCs, or DMSO

as vehicle control. After 24 h, LPS was added at the concentration of 1 ng/ml (to assess CD54 expression) or 10 ng/ml (for the assessment of CD86 expression and for cytokines release) for further 24 h. LPS concentrations were selected based on previous dose response experiments as optimal for the selected markers.

Plasmid DNA preparation, transient transfections, and luciferase assays

Plasmids preparation, transient transfections and luciferase assays were performed as previously described (Buoso et al., 2019, 2020, 2021). The Δ1 reporter plasmid construct has been previously described (Del Vecchio et al., 2009). Plasmids for transfections were purified with the HiSpeed® Plasmid Midi Kit (Qiagen, Valencia, CA, USA) and DNA was quantified using QuantusTM fluorometer (Promega, Madison, WI, USA). After treatment, cells were lysed and analyzed following Dual-Luciferase Reporter Assay System specifications (Promega, Madison, WI, USA). Luminescence was measured with a 20/20 n Luminometer (Turner Bio-Systems, Sunnyvale, CA, USA), with 10 s integration time.

Reverse transcription quantitative PCR

Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer instructions. RNA was quantified using QuantusTM fluorometer (Promega, Madison, WI, USA). Quantitative PCR (qPCR) was performed as previously described (Buoso et al., 2017, 2020, 2021). Quantification of the transcripts was performed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Immunoblot analysis

RACK1 and β- tubulin expression in cell homogenates were assess by immunoblot analysis as previously described (Buoso et al., 2021; Masi et al., 2020). After immunoblot acquisition, bands optical analysis was performed with the ImageJ program (W. Rasband, Research Service Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, MD and Laboratory for Optical and Computational Instrumentation, University of Wisconsin). Bands relative densities were expressed as arbitrary units and normalized over control sample run under the same conditions.

Cytokine production

After treatment, the release of cytokine TNF-α, IL-6 and IL-8 was assessed in cell-free supernatants following centrifuge at 1200 rpm for 5 min. Cytokine release was assessed using

commercially available kits for human TNF-α (R&D Systems, Inc., Minneapolis, USA), for human IL-6 and IL-8 (ImmunoTools, Friesoythe, Germany). Limits of detection were 8 pg/ml for IL-6 and IL-8, and 7.8 pg/ml for TNF-α, respectively. Results are expressed in pg/ml.

Flow cytometric analysis of CD54 and CD86 expression

After treatment, THP-1 cells were centrifuged, washed once with cold PBS and suspended in 200 µL of PBS. Cells were stained in the dark for 30 min with specific PE-conjugated antibody against h-CD54 (BD Biosciences), FITC-conjugated antibody against CD86 (BD Biosciences) or with isotype control antibody (BD Biosciences) at 4°C, following supplier's instructions. Then, cells were centrifuged at 1200 rpm for 5 min and suspended in 0.5 ml of PBS. The intensity of fluorescence was analyzed using Novocyte 3000 flow cytometer and data were quantified using Novocyte software (Acea Bioscience Inc.). 10,000 viable cells were analyzed for mean fluorescence intensity (MFI). MFI of isotype control was subtracted to MFI of CD54/CD86 stained cells. Changes in CD54 or CD86 expression are reported as stimulation index (SI) calculated by the following equation:

SI = MFIt / MFIc

MFIt stands for chemical-treated cells, whereas MFIc for the untreated ones.

Molecular docking calculations

All molecular docking calculation were performed on the homology model of G-protein Estrogen Receptor (GPER), retrieved from the web server GPCR-ITASSER (Zhang et al., 2015; Zhang and Zhang, 2010), and already exploited in our previous work (Buoso et al., 2021). No structure of wild type human AR (hAR) in complex with an antagonist in the ligand binding domain (LBD) is available. For evaluating the binding mode of the antagonists at AR, molecular modeling approaches are frequently used starting from the available hAR-LBD-agonists complex (Duan and Sheu, 2017; Jin et al., 2019; Liu et al., 2018; Prekovic et al., 2016; Sakkiah et al., 2018; Wahl and Smieško, 2018; Zhou et al., 2018). In the present study, 3ZQT crystal structure of the hAR-LBD in complex with testosterone was used. Protein structures were prepared using the Protein Preparation Wizard tool, implemented in Maestro of the Schrödinger Suite (Schrödinger LLC New York (USA) 2014). Missing hydrogen atoms were added, and bond orders were assigned. The prediction of protonation states for the protein residues was accomplished by using Epik, with the pH set to 7.4. The chemical structures of VIN, ATR and CYP were designed in ChemDraw, imported into the Maestro software (Schrödinger, LLC,

New York, NY 2014b), and prepared using the LigPrep utility available within the Schrodinger suite (Schrödinger, LLC, New York 2014), and finally subjected to docking calculation. For GPER protein, the receptor grid was generated at the center of the putative binding site (Rosano et al., 2016), whereas for AR, the grid was defined using a 12 Å box centered on the cognate ligand. Docking studies were performed by using default setting of the Glide-SP protocol (Schrödinger, LLC, New York, NY 2014a), keeping the ligands flexible. For AR molecular docking calculation, the docking protocol was validated first by re-docking the cognate ligand into the parent receptor, thus estimating the capability of the software to reproduce the testosterone crystalized binding mode. The resulting ligand-protein complexes were ranked by docking score and visually inspected.

Statistical analysis

Data are expressed as means ± standard error (SEM) of at least three independent experiments. Statistical analysis was performed using the InStat software, version 9.0 (GraphPad Software, La Jolla, CA, USA). Significant differences were determined using analysis of variance (ANOVA), followed, when significant, by an appropriate post hoc test, as indicated in the Figure legends. In all the reported statistical analysis effects were designated as significant if the p value was ≤ 0.05.

Results

Effects of VIN on RACK1 expression and related pro-inflammatory markers

VIN is a dicarboximide fungicide widely used in agriculture to control different fungi-related diseases in vineyards, on fruits and vegetables (Anway and Skinner, 2006). As a pesticide, its use has been restricted in the US and banned in several European countries due to its classification as possible human carcinogen (U.S. EPA. 2000. Vinclozolin: Re-registration Eligibility Decision). VIN and its major metabolites are also considered EDCs with antiandrogenic effects, due to their structurally similarity to flutamide, and therefore able of competing for AR binding with endogenous androgen (Kelce et al., 1994; van Ravenzwaay et al., 2013). Based on our previous studies on the effects of EDCs on RACK1 expression and altered immune responses (Buoso et al., 2017c, 2020a, 2021), we investigated the effects of VIN on RACK1 expression by assessing the reporter luciferase activity using the human RACK1 gene promoter, mRNA expression by qPCR, and protein levels by western blot. THP-1 cells were treated for 6, 16, 18, 24 h with increasing concentrations of VIN (0.001–10 μM) or

DMSO as vehicle control. These time points were selected based on previous experiments as being optimal to investigate the effects of dehydroepiandrosterone (DHEA) and cortisol on RACK1 transcriptional activity, mRNA and protein expression (Buoso et al., 2011) as well as the effects of EDCs on RACK1 (Buoso et al., 2017, 2020, 2021). While 6 h of VIN treatment increased RACK1 gene promoter activity only at 1 μM concentration (Fig. 1A), 16 h VIN treatment modestly but statistically significant decreased RACK1 transcription with no clear dose response (Fig. 1B). This bidirectional effect on the transcriptional activity was not mirrored by changes in RACK1 mRNA (Fig. 1C) or protein (Fig. 1D) levels at 18 h and 24 h, respectively.

Regarding the assessment of the immunomodulatory effects on LPS induced stimulation, VIN induced a statistically significant reduction only at the highest tested concentration on CD86 (Fig. 1E), CD54 (Fig. 1F) and TNF-α (Fig. 1H). The same trend can be appreciated on IL-8 release (Fig. 1G).

The modest bidirectional effects of VIN on RACK1 promoter activity observed at 6 and 16 h are not sufficient to affect RACK1 mRNA and protein levels, most likely related to the turnover time of the protein in THP-1 cells (Del Vecchio et al., 2009; Corsini et al., 2002). The effect of VIN on CD86, CD54 and TNF-α cannot be recapitulated to an effect on RACK1 expression.

Results indicate a modest ability of VIN to induce an early RACK1 promoter activation and its late inactivation, which could not be captured in terms of alteration of RACK1 mRNA and protein expression. Nevertheless, the effect on the promoter appears to be sufficient to induce the downregulation of RACK1-related immune markers at high concentrations, in line with its acknowledged anti-androgenic effects reported as reported in the literature (Kelce et al., 1994; van Ravenzwaay et al., 2013). The levels of 3,5-dichloroaniline (3,5-DCA), used as biomarker of VIN exposure, detected in human urine samples indicative of both occupational and nutritional exposure (Will, 1995; Lindh et al., 2007), and are in line with the concentrations used in the current study.

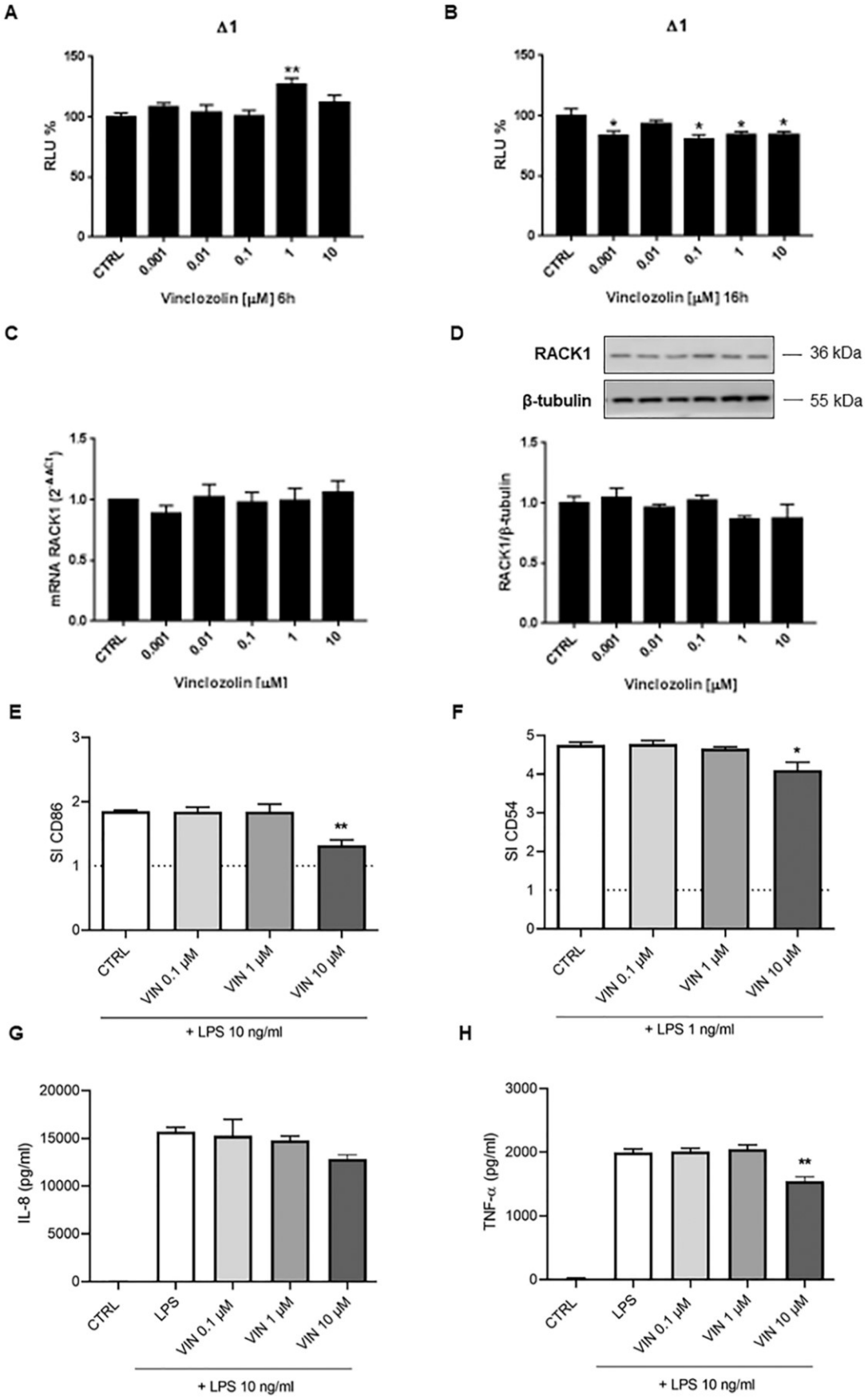

Figure 1. Effects of VIN on RACK1 expression and immune activation. A-B THP-1 cells transiently transfected with the Δ1 construct were treated for 6 h (A) or 16 h (B) with increasing concentrations of

VIN (0.001–10 μM) or DMSO as vehicle control (CTRL). Cells were lysed and luciferase activity was measured as described in Section 2. Luciferase activities are expressed as RLU % respected to non-treated construct (considered as 100 %). Results are expressed as mean ± SEM, n = 3 independent experiments performed in quadruplicate. Statistical analysis was performed with Dunnett's multiple comparison test with $*p < 0.05$ or $**p < 0.01$. C-D THP-1 cells treated for 18 h (C) or 24 h (D) with increasing concentrations of VIN (0.001–10 μM) or DMSO as vehicle control (CTRL). (C) mRNA levels evaluated by qPCR (endogenous reference, 18 S). (D) The image is a representative Western blot. RACK1 protein levels evaluated by Western blotting, normalized to β-tubulin expression. E-F THP-1 cells treated for 24 h with VIN (0.1–10 μM) or DMSO as vehicle control (CTRL). LPS (10 ng/ml for CD86 expression or 1 ng/ml for CD54 expression) was then added for further 24 h. CD86 and CD54 expression were assessed at flow cytometer and expressed as stimulation index (SI) calculated on DMSO-treated cells. G-H THP-1 cells treated for 24 h with VIN (0.1–10 μM) or DMSO as vehicle control. LPS (10 ng/ml) was added for further 24 h. IL-8 and TNF-α release were assessed with specific ELISA and expressed as pg/ml. Each value represents the mean ± SEM n = 3 independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with $*p < 0.05$, $**p < 0.01$ *vs* LPS-treated cells (CTRL).

ATR inhibits RACK1 expression and modulates LPS-induced proinflammatory pathway

ATR is an herbicide belonging to the triazine class and is the second most widely used herbicide after glyphosate. Although banned in Europe, ATR is still largely employed in the rest of the world for the control of broadleaf and grassy weeds (Atwood and Paisley-Jones, 2017). Human exposure to ATR still represents a public health problem due to groundwater and sediment contaminations. This is due to ATR and its metabolites (i.e., desethyl-ATR, deisopropyl-ATR and diaminochlorotriazine) long persistency in soil and water for years (Nödler et al., 2013). ATR and its metabolites do not bind to estrogen or androgen receptors (Eldridge et al., 2008; Galbiati et al., 2021), but display their endocrine disrupting action by enhancing aromatase activity (Gunderson et al., 2011; Martins-Santos et al., 2018; Sanderson et al., 2002). ATR has been classified as an evident EDC due to the observed reduction in androgen levels demonstrated in different vertebrate classes (Hayes et al., 2011; De Albuquerque et al., 2020). As RACK1 expression is positively regulated by androgens, we evaluated the effects of ATR on RACK1 by means of reporter luciferase activity using the human RACK1 gene promoter, mRNA expression using qPCR, and protein level by western blot analysis. THP-1 cells were treated for 6, 16, 18, 24 h with increasing concentrations of ATR (0.001–10 μM) or DMSO as vehicle control. As shown in Fig. 2, ATR treatment induced a statistically significant decrease of RACK1 transcriptional activity at 6 h (Fig. 2A) and 16 h (Fig. 2B), respectively. The effects on RACK1 gene promoter activation were mirrored by RACK1 mRNA at almost all concentrations here tested (Fig. 2C), while only higher concentrations (1 and 10 μM) were able to significantly decrease RACK1 protein levels (Fig. 3D), reflecting a different sensitivity of the different methods. In terms of surface markers expression and proinflammatory cytokines release following LPS exposure, ATR (1 and 10 μM)

up-regulated CD86 expression (Fig. 2E), whereas a reduction was observed for CD54 expression at 0.1 μM (Fig. 2F) and TNF-α release at 1 μM (Fig. 2H), with no changes in IL-8.

Overall, these results indicate the ability of ATR to induce the downregulation of RACK1 expression, which could be explained by its indirect anti-androgenic activity. The decreased RACK-1 expression could support the observed effects on CD54 and TNF-α, while a different mechanism is likely to be involved in the up-regulation of CD86. Plasma levels of ATR have been reported to be in line with treatment concentrations here used on THP-1 cells (Brzezicki et al., 2003), indicating that these effects were observed at *in vivo* relevant concentrations.

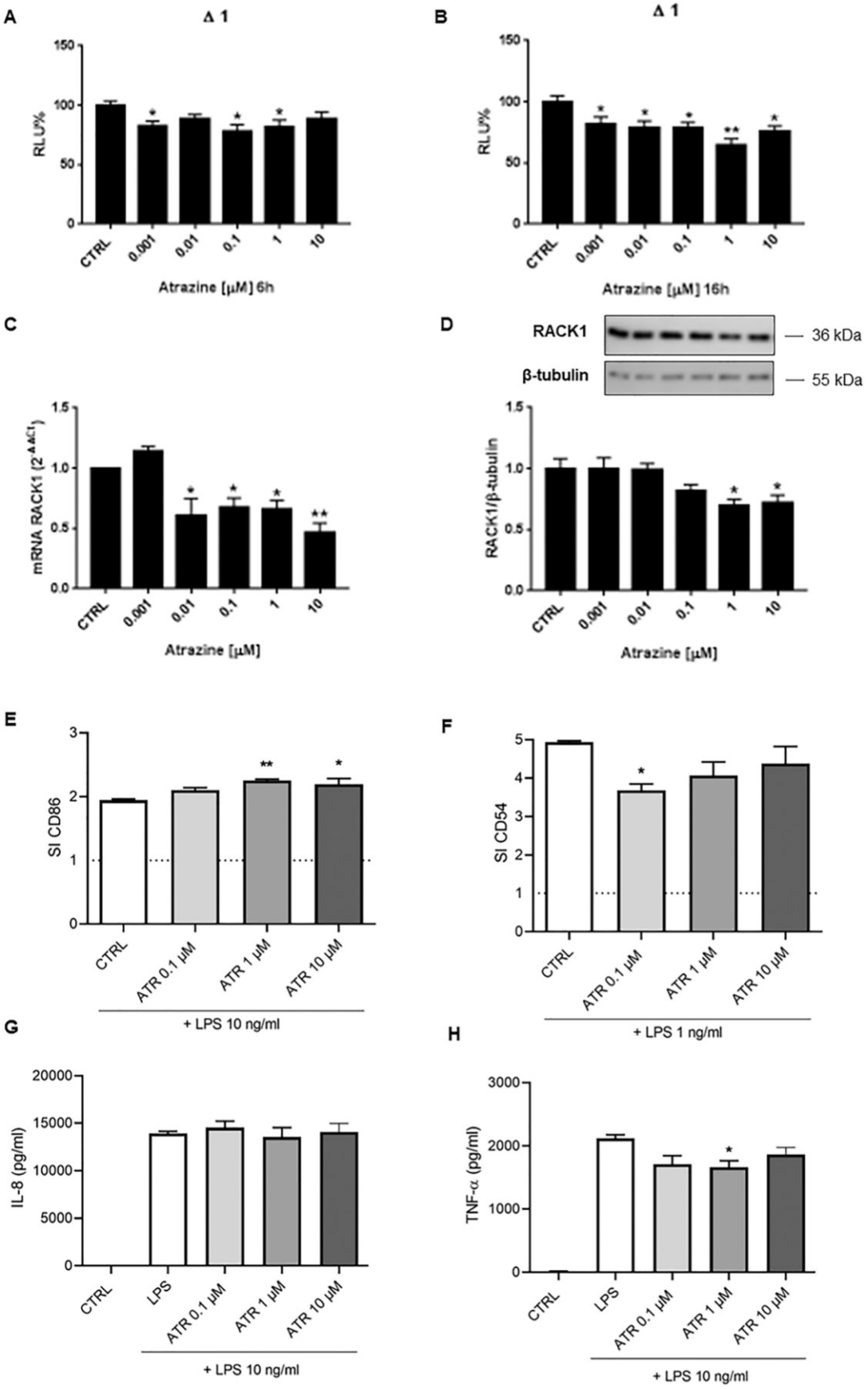

Figure 2. Effects of ATR on RACK1 expression and immune activation. A-B THP-1 cells transiently transfected with the Δ1 construct were treated for 6 h (A) or 16 h (B) with increasing concentrations of

ATR (0.001–10 μM) or DMSO as vehicle control (CTRL). Cells were lysed and luciferase activity was measured as described in Section 2. Luciferase activities are expressed as RLU % respected to non-treated construct (considered as 100 %). Results are expressed as mean ± SEM, n = 3 independent experiments performed in quadruplicate. Statistical analysis was performed with Dunnett's multiple comparison test with *$p < 0.05$ or **$p < 0.01$. C-D THP-1 cells treated for 18 h (C) or 24 h (D) with increasing concentrations of ATR (0.001–10 μM) or DMSO as vehicle control (CTRL). (C) mRNA levels evaluated by qPCR (endogenous reference, 18 S). (D) The image is a representative Western blot. RACK1 protein levels evaluated by Western blotting, normalized to β-tubulin expression. C-D Statistical analysis was performed with Dunnett's multiple comparison test with *$p < 0.05$ or **$p < 0.01$. E-F THP-1 cells treated for 24 h with ATR (0.1–10 μM) or DMSO as vehicle control (CTRL). LPS (10 ng/ml for CD86 expression or 1 ng/ml for CD54 expression) was then added for further 24 h. CD86 and CD54 expression were assessed at flow cytometer and expressed as stimulation index (SI) calculated on DMSO-treated cells. G-H THP-1 cells treated for 24 h with ATR (0.1–10 μM) or DMSO as vehicle control. LPS (10 ng/ml) was added for further 24 h. IL-8 and TNF-α release were assessed with specific ELISA and expressed as pg/ml. Each value represents the mean ± SEM n = 3 independent experiments. Significance was set at *$p < 0.05$ or **$p < 0.01$ *vs* LPS-treated cells (CTRL) by Dunnett's multiple comparison test.

CYP decreases RACK1 expression and related pro-inflammatory markers

CYP is a type II synthetic pyrethroid used as broad-spectrum insecticide to control insects in houses, restaurants, hospitals, schools, and industrial buildings. It is employed due to its behavior as fast-acting neurotoxin in insects (Pascual and Peris, 1992) and it is employed in large-scale commercial agricultural settings to control infesting ectoparasites (Xie and Zhou, 2008; Koureas et al., 2012). Due to its hydrophobic properties, CYP is strongly absorbed into the soil, where it is moderate persistent (Ostiz and Khan, 1994; Fenoll et al., 2011). Exposure to oxygen, sunlight and water accelerate CYP decomposition and, although being rapidly degraded on plants and soil, CYP can last and be effective for longer periods of time (i.e., days or even weeks) when applied on inert surfaces or indoor. Several studies reported that CYP displays anti-androgenic effects on the reproductive system of different exposed animals (Li et al., 2013; Ullah et al., 2018; Abdel-Razik et al., 2021) and multiple *in vitro* studies confirmed that CYP is able of reducing AR transcriptional activity (Sun et al., 2007; Xu et al., 2008; Hu et al., 2012; Pan et al., 2013; Christen et al., 2014) also at the same concentrations here tested (Du et al., 2010). Therefore, considering that RACK1 is positively regulated by androgens, THP-1 cells were treated with increasing concentrations of CYP (0.001–10 μM) or DMSO as vehicle control. As shown in Fig. 3A, 6 h CYP treatment did not alter RACK1 promoter activity at any of the concentrations tested, on the contrary 16 h treatment induced a statistically significant reduction at all concentrations tested (Fig. 3B). In agreement, a statistically significant decrease in RACK1 mRNA expression after 18 h of treatment (Fig. 3C), and at the higher concentrations (0.1, 1 and 10 μM) in RACK1 protein levels after 24 h treatment (Fig. 3D) was found. The decrease in RACK1 was parallel by a statistically significant down-

regulation of all LPS-induced parameters investigated at the highest concentration tested (Fig. 3E-H).

Altogether, these results indicate the ability of CYP to downregulate RACK1 expression, which could be explained by its anti-androgenic profile reported in literature data. Plasma levels of CYP have been reported to be in line with concentrations used (Appenzeller et al., 2017; Ferré et al., 2020), indicating that these effects were observed at *in vivo* relevant concentrations.

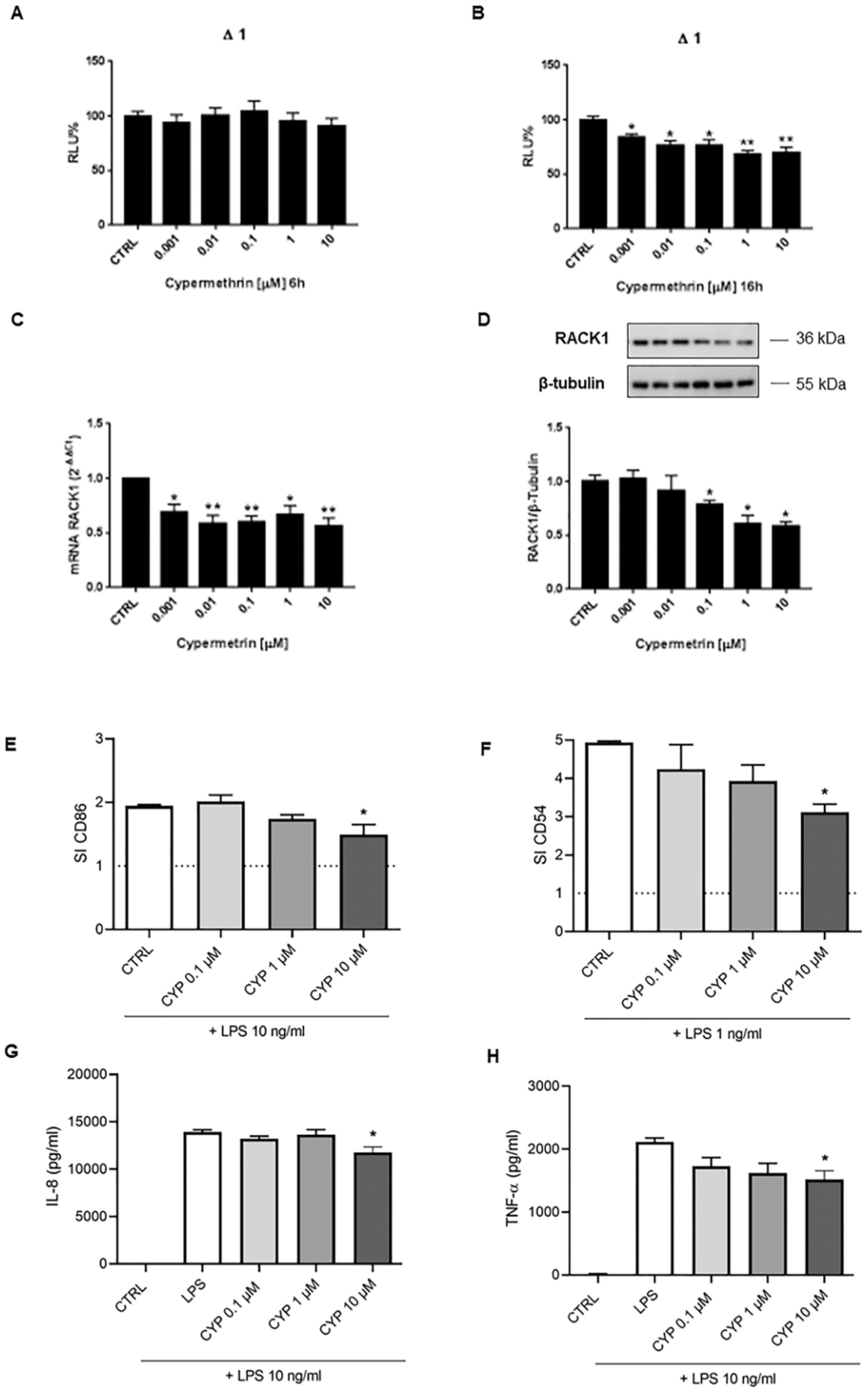

Figure 3. Effects of CYP on RACK1 expression and immune activation. A-B THP-1 cells transiently transfected with the Δ1 construct were treated for 6 h (A) or 16 h (B) with increasing concentrations of

CYP (0.001–10 μM) or DMSO as vehicle control (CTRL). Cells were lysed and luciferase activity was measured as described in Section 2. Luciferase activities are expressed as RLU % respected to non-treated construct (considered as 100 %). Results are expressed as mean ± SEM, n = 3 independent experiments performed in quadruplicate. Statistical analysis was performed with Dunnett's multiple comparison test with *$p < 0.05$ or **$p < 0.01$. C-D THP-1 cells were treated for 18 h (C) or 24 h (D) with increasing concentrations of EE (0.001–10 μM) or DMSO as vehicle control (CTRL). (C) mRNA levels evaluated by qPCR (endogenous reference, 18 S). (D) The image is a representative Western blot. RACK1 protein levels evaluated by Western blotting, normalized to β-tubulin expression. C-D Statistical analysis was performed with Dunnett's multiple comparison test with *$p < 0.05$ or **$p < 0.01$. E-F THP-1 cells treated for 24 h with CYP (0.1–10 μM) or DMSO as vehicle control (CTRL). LPS (10 ng/ml for CD86 expression or 1 ng/ml for CD54 expression) was then added for further 24 h. CD86 and CD54 expression were assessed at flow cytometer and expressed as stimulation index (SI) calculated on DMSO-treated cells. G-H THP-1 cells treated for 24 h with CYP (0.1–10 μM) or DMSO ad vehicle control. LPS (10 ng/ml) was added for further 24 h. IL-8, TNF-α and IL-6 release were assessed with specific ELISA and expressed as pg/ml. Each value represents the mean ± SEM n = 3 independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with *$p < 0.05$ vs LPS-treated cells (CTRL).

Molecular docking of VIN, ATR and CYP to GPER and AR

The lack of effects of VIN on RACK1 mRNA and protein could be explained by a possible balance between the early activation of its promoter and its late inactivation. VIN and its metabolites, in addition to their known anti-androgenic profile (Kelce et al., 1994), have been reported to display also estrogenic activity (Molina-Molina et al., 2006; Habib et al., 2020). In this regard, we previously demonstrated that estrogen-active compounds can induce RACK1 promoter activity through a GPER-mediated cascade (Buoso et al., 2020a, 2021). Therefore, we hypothesized that, due to its estrogen-like activity, VIN could exert an early (at 6 h treatment) transcriptional inducing effect on RACK1 expression which is then opposed and balanced by VIN and its metabolites anti-androgenic late effect (at 16 h treatment). To better understand this dual effect, molecular docking calculations were performed to gain insight into the putative binding of VIN at GPER. In absence of a crystallographic structure of GPER, the 3D structure of the protein was generated by homology modeling (HG ID: HG0714) (Zhang et al., 2015; Zhang and Zhang, 2010), and as reported in our previous study used for docking calculation of VIN at the putative ligand binding pocket of GPER (Rosano et al., 2016). As reported in Fig. 4, VIN binds in one of the superficial sub-pockets of the receptor site and its binding pose is highly conserved among the lowest docking score results (ranging from -6.715 to -5.963), thus ensuring the reliability of the predicted binding mode. Briefly, carbonyl moieties in position 2 and 4 of the oxazolidindione ring tightly anchor VIN to the receptor by accepting two hydrogen bonds from the amide nitrogen of Asn118 and from the hydroxyl group of Ser134, respectively. The di-chloro aromatic ring is instead projected toward a deepest and higher lipophilic cleft of the receptor binding site, partially protected from the solvent, and

delimited by Leu108, Leu134, Phe314, and Tyr65. Interestingly, an additional halogen bond is observed between one chlorine atom on the aromatic ring and the phenolic group of Tyr65, that contribute to stabilize the binding. Lastly both the vinyl and methyl moieties at the C-5 of the oxazolidindione ring are oriented in an additional hydrophobic cleft traced by Val196, Phe206, Phe 208 and Ala209. In particular, Phe206 and Phe208 are two key aminoacids involved in the binding of GPER agonists such as estradiol and G-1. The chirality at C-5 does not affect the binding since no differences in the binding mode of both (R)- and (S)-VIN are observed. Overall, the highly steric and electronic complementarity between VIN and the sub-pocket of GPER ligand binding site involved in the binding with GPER agonists supports our *in vitro* observation (Fig. 4A).

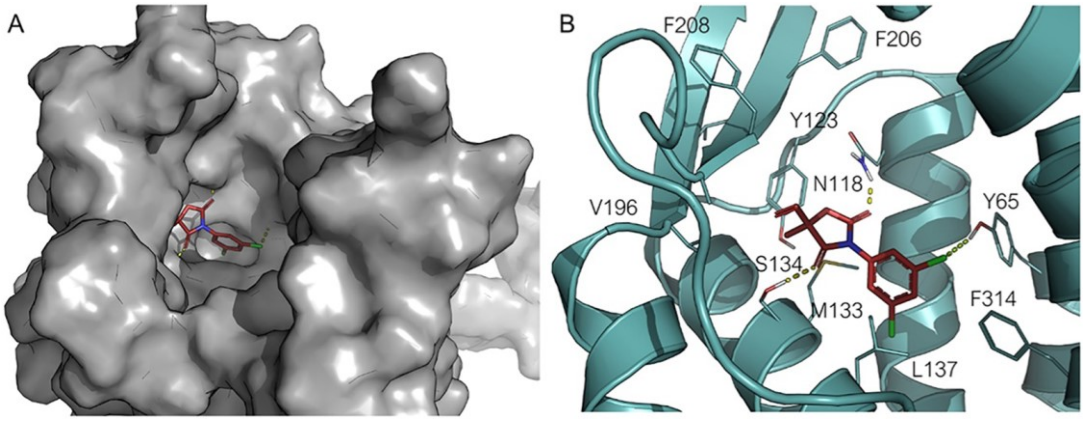

Figure 4. Predicted binding mode for VIN at GPER ligand binding site. A Close-up of the GPER binding site and B of the putative binding mode of VIN (in red stick carbons) predicted by docking calculation. The 3D structure of GPER is represented in teal cartoon, and the key amino acid residues interacting with the ligand are represented in lines. The heteroatoms are color-coded: oxygen in red, nitrogen in blue, sulfur in yellow and chlorine in green. The H-bonds and the halogen bond are represented in yellow, and green dotted lines, respectively.

Due to the known anti-androgenic properties of VIN, ATR and CYP, we investigated their potential to interact with AR. To this purpose, the X-ray protein structure of AR-LBD in binary complex with testosterone (PDB ID: 3ZQT) was used for the docking studies. Initially, the cognate ligand (testosterone) present in the crystal structures was docked to the parent protein in order to estimate whether the docking algorithm used is able to reproduce the bioactive conformation accurately. The top docked pose of DHT to AR is very similar to that of the crystallographic conformation, as measured by the RMSD (1.24 Å) between the docked pose and the crystallographic conformation, thus validating the docking protocol to predict a reliable binding mode for the new ligands under investigation. The AR-LBD is well-characterized as a hydrophobic cavity that forms strong hydrophobic interactions with a

steroidal core of testosterone and its congeners. Meanwhile, there are two polar patches, Arg752 and Gln711 at one end and Thr877 and Asn705 at the other end of the site, that are involved in a network of H-bonds with the carbonyl oxygen at position 3 and the hydroxyl group at position 17, respectively. The main difference between the agonist and antagonist ligand binding is the presence or absence of these H-bonds between the ligand and the receptor. Agonists form H-bonds that involves both the polar extremities of the AR-LBD and in particular Thr877 or Asn705. These interactions seem to provide a stabilization of the H12 helix in a suitable conformation for the recruitment and binding of transcriptional cofactors. Conversely, antagonists lack of these H-bonds (Azhagiya Singam et al., 2019), thus causing the destabilization of H12 or avoiding the stabilization of the receptor in the active conformation (Zhou et al., 2010).

CYP is the sole exception since the molecule possesses a huge molecular volume and it is too sterically bulky to effectively fit within the AR-LBD. Thus, we can exclude that CYP may act as a competitive antagonist for the AR. Conversely, VIN and ATR still demonstrated sufficient binding to the AR LBD (Fig. 5). To understand the essential features responsible for the binding of VIN and ATR, and to gain insight into their putative mechanism of action, the receptor-ligand interactions at the binding site were examined. In Fig. 5A is depicted the predicted binding mode for VIN. Briefly, the aromatic moiety of the ligand interacts with the AR-LBD portion usually involved in the binding of the ring-A and -B of the steroid-based AR agonists or antagonists. In particular, the phenyl ring is enclosed within the hydrophobic pocket lined by Leu707, Met745, Val746, Met749, Met787 and Leu873. Interestingly, in all the top ranked docking pose, the phenyl ring establishes a face-to-edge π- π interaction with the side chain of Phe764, whereas the chlorine atom is involved in a halogen bond with the guanidine group of Arg752. However, the small dimension of the molecule does not allow VIN to fulfil the entire AR-LBD, which eliminates the option for the isoxazolidindione moiety of anchoring the compound to Thr877 and Asn705 at the other end of the binding site. Likewise, to what has been observed for GPER, the binding of VIN to AR is not affected by chirality since both enantiomers maintain the same orientation within the LBD and establish the same key interactions. A comparable binding mode to VIN was observed for ATR (Fig. 5B). Due to the high lipophilicity of the molecule, ATR binds at the center of the AR-LBD and exclusively establishing hydrophobic interactions with the non-polar amino acids that delimit the core of the LBD. No contacts are detected with the polar amino acids located at the two ends of the pocket. Thus, the binding modes of VIN and ATR suggest that hydrophobic interactions may be essential for the ligand coordination, whereas lack of the hydrogen bonding network

necessary for the stabilization of AR in the active conformation may prove their antiandrogenic profile.

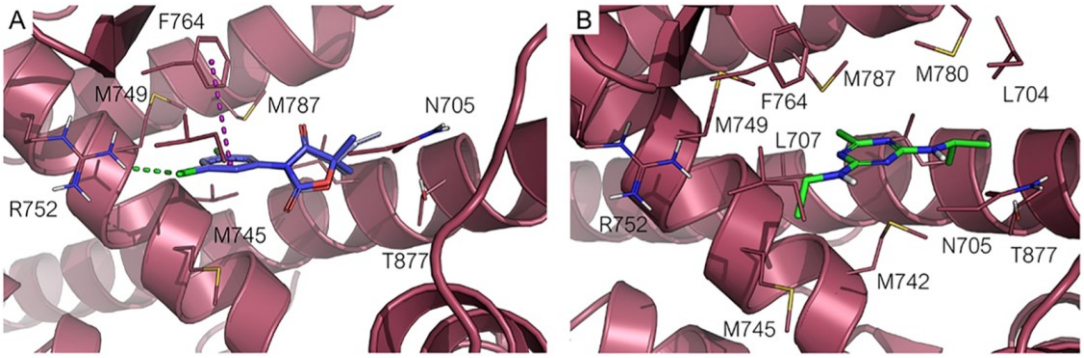

Figure 5. Predicted binding mode for VIN (A) and ATR (B) at AR ligand binding site. The 3D structure of AR is represented in raspberry cartoon, and the key amino acid residues interacting with the ligand are represented in lines. VIN and ATR are represented in sticks with carbons colored in blue and green, respectively. The heteroatoms are color-coded: oxygen in red, nitrogen in blue, sulfur in yellow and chlorine in green. The H bonds and the halogen bond are represented in yellow, and green dotted lines, respectively.

CYP exerts endocrine disrupting actions on RACK1 by mediating indirect anti-androgenic effects

Since both literature data and our molecular docking calculation excluded CYP as a competitive AR antagonist, an indirect endocrine disrupting, anti-androgenic effect – responsible for the CYP-induced RACK1 downregulation – was hypothesized. In this regard, *in vitro* studies demonstrated that CYP exposure induces the recruitment of AR corepressors Silencing Mediator for Thyroid Hormone Receptors (SMRT) and Nuclear Receptor Corepressor (NCoR) (Pan et al., 2013) and the inhibition of the interaction between AR and its coactivators ARA55 and ARA70 (Ding et al., 2020), resulting in a reduced AR transcriptional activity and an inappropriate AR conformation that leads to the increase of its degradation (Hu et al., 2012). This is in line with our previous data demonstrating that AR silencing in THP1 cells leads to a significant down-regulation of RACK1 expression (Corsini et al., 2016). Since the inhibition of AR-ARA70 interaction has been demonstrated to suppress AR transactivation and promote its 26 S proteasome-mediated degradation due to its arrest in the cytoplasm (Lin et al., 2001), we investigated AR protein levels after CYP exposure. As shown in Fig. 6A, CYP induced the reduction of AR levels in a concentration-dependent manner, that reached statistical significance for the higher concentrations here tested (0.1–10 μM). In parallel, based on literature data reporting the direct AR/mediated transcriptional control of IL-6 in monocytes and macrophages (Pang et al., 2020), and on *in vitro* evidence in other cellular contexts

highlighting that CYP can antagonize IL-6-induced ligand-independent AR activation (Wang et al., 2015, 2016; Zhou et al., 2017; Wang et al., 2016; Pan et al., 2012), we investigated a possible effect of CYP on IL-6 release. As shown in Fig. 6B, 10 μM CYP significantly downregulated LPS-induced IL-6 release, in line with its reported anti-androgenic profile. Hence, our data highlight that cypermethrin exerts its anti-androgenic activity on RACK1 expression not only reducing AR protein expression but, at higher concentration, interfering with IL-6 release as additional mechanism.

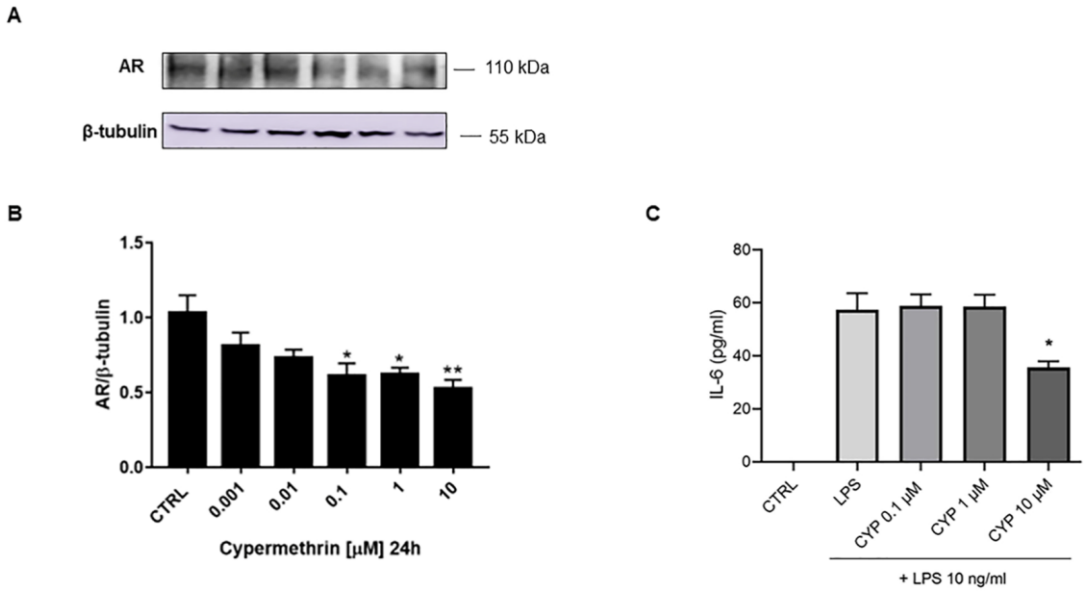

Figure 6. CYP treatment results in reduced AR expression and IL-6 production. A. representative Western blot, THP-1 cells were treated for 24 h with increasing concentrations of CYP (0.001–10 μM) or DMSO as vehicle control (CTRL). B. Densitometric analysis, AR protein levels evaluated by Western blotting were normalized to β-tubulin expression. Each value represents the mean ± SEM n = 3 independent experiments. Significance was set at $*p < 0.05$ or $**p < 0.01$ by Dunnett's multiple comparison test. C. THP-1 cells treated for 24 h with CYP (0.1–10 μM) or DMSO ad vehicle control (CTRL). LPS (10 ng/ml) was added for further 24 h. IL-6 release was assessed by ELISA, and expressed as pg/ml. Each value represents the mean ± SEM n = 3 independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with $*p < 0.05$ vs LPS-treated cells (CTRL).

Discussion

Our previous works showed that androgens, glucocorticoids and estrogen-active compounds can regulate RACK1 expression in immune cells (Buoso et al., 2017c; Racchi et al., 2017; Buoso et al., 2020a; Corsini et al., 2021). The canonical Androgen/Glucocorticoid Responsive Element (ARE/GRE) (Pihlajamaa et al., 2015) in the promoter region of human RACK1 is responsible for corticosteroids and androgens action at transcriptional level, resulting in the down- and upregulation of RACK1 expression respectively (Buoso et al., 2011; Corsini et al.,

2014a, 2014b). Hence, the modulation of AR transcriptional activity is a pivotal step in the mechanism supporting RACK1 expression in the immune system (Racchi et al., 2017; Corsini et al., 2021). In this context, because of its central role in immune cell activation (Corsini et al., 1999, 2005; Buoso et al., 2017b, 2017c, 2021; Racchi et al., 2017) and considering the complex hormonal balance that regulates its expression (Buoso et al., 2011, 2017b, 2017c; Racchi et al., 2017; Corsini et al., 2021), RACK1 could represent an important and useful tool to screen EDCs for their endocrine effects, and taking into account the emerging role of EDCs in immune system dysfunction (Corsini et al., 2018), for their immunotoxicity (Buoso et al., 2017a, 2020b; Racchi et al., 2017; Corsini et al., 2021). Therefore, this study aimed to assess if VIN, ATR and CYP, could affect immune function by regulating RACK1 expression and LPS-induced cytokine production and surface markers expression. These immune parameters were chosen since we previously demonstrated to be dependent upon RACK1/PKCβ activation (Corsini et al., 2005, 2014b; Buoso et al., 2011, 2017c, 2021).

Here we show that, to different extents, all three pesticides exerted an anti-androgen downregulating effect on RACK1This reduction was paralleled by a statistically significant decrease of cytokine release similarly to our previous data obtained using p'p'-DDT and p'p'-DDE (Buoso et al., 2017c). These results indicate RACK1 as a useful tool not only to unravel the interfering effects of EDCs on endocrine-regulated cellular processes but also to evaluate subsequent consequences on immune functions. Pesticides tested show an immunotoxic potential, possibly mediated by their effects on RACK1. Overall, the reduction of the release of pro-inflammatory cytokines and of the expression of surface markers supports an immunosuppressive effect of the three pesticides.

RACK1 expression following VIN exposure highlighted a complex molecular mechanism that involves both nuclear and membrane-bound receptors, AR and GPER respectively, showing a possible role of VIN and its estrogenic and anti-androgenic active metabolites in the observed effects on immune function. Hence, this result supports that RACK1 can be primarily exploited to unmask multiple molecular interactions of hormone-active substances to better dissect out their mechanisms of action as we also demonstrated in previous works (Buoso et al., 2020, 2021). VIN metabolism, however, has not been investigated, and additional studies are necessary to investigate the contribution of VIN and its metabolites in the observed effects.

Our data support a CYP anti-androgenic profile while molecular docking excluded CYP as a possible competitive anti-androgen, suggesting a different mechanism. Indeed, we demonstrated that CYP treatment decreased AR protein levels, which is in line with literature

data reporting CYP effects on proteasome-mediated AR degradation (Jaworski, 2006), resulting in anti-androgenic effect on RACK1. In addition, considering AR direct transcriptional control over IL-6 production due to an ARE sequence in its promoter (Pang et al., 2020) and that IL-6 exerts an autocrine action and a pro-inflammatory effect on monocytes and macrophages (Ogawa et al., 2021; Gidon et al., 2021; Gao et al., 2020), the reduction of IL-6 release here reported may further contribute to the observed anti-androgenic effect of CYP due to a reduced IL-6-mediated ligand-independent AR activation, remarkedly inhibited by CYP action as previously reported (Zhou et al., 2017; Wang et al., 2015, 2016; Pan et al., 2012, 2013; Hu et al., 2012).

Conversely, docking calculations performed indicate a possible ATR antagonist profile for AR, in line with our *in vitro* data and the antiandrogenic effect of ATR, even though we cannot exclude a role of ATR enhancing effect on aromatase activity as reported in literature data (Eldridge et al., 2008; Galbiati et al., 2021).

Overall, RACK1 expression and its correlate immune markers can capture both direct and indirect mechanisms of action which not directly involve a steroid hormone nuclear receptor activation. In both cases they lead to immunosuppressive effects.

In terms of hazard identification, our results indicate that exposure to the selected pesticides is mainly associated with immunosuppression. Similar results have been obtained on peripheral blood mononuclear cells obtained from healthy donors (manuscript in preparation). This may result in reduced immune response and inflammation dysregulation. In this regard, workers occupationally exposed to CYP (Costa et al., 2013; El Okda et al., 2017), *in vitro* and animal models to study ATR (Thueson et al., 2015; Ge et al., 2021) and VIN exposure (Anway et al., 2006) show different extents of immune activity impairment, demonstrating that immunological implications can occur due to exposure to high pesticide concentrations but suggesting that the outcome may strongly depend on the context and the immunological differences between exposed individuals, as also observed for bisphenols (Buoso et al., 2021). ATR adverse effects on the immune system have been previously reported in both animal studies and *in vitro* studies, indicating a reduction of immune functionality (Galbiati et al., 2021), mainly observed in young adult rodents previously exposed during the prenatal period (Rowe et al., 2008). ATR immunosuppression has been reported in animals, mainly in juvenile male mice (Rowe et al., 2008; Galoppo et al., 2020), and, interestingly, an *in vitro* study revealed effects on dendritic cells, showing inhibition of their maturation (Pinchuk et al., 2007). CYP effects on immune system have also been previously reported, in workers exposed to CYP exhibited lower levels of cytokines, including IL-8 (Costa et al., 2013), in line with our findings.

Furthermore, in support to our data, an *in vitro* study showed a reduction in LPS-induced cytokine production by murine macrophages, like TNF-α and IL-6, following β-CYP exposure (Wang et al., 2017). Regarding VIN and its immunotoxic potential, little information is present in literature. Only a multigenerational study on rats demonstrated VIN ability to modulate the immune response (White et al., 2004). In detail, adult female mice, following prolonged exposure to VIN, exhibited an increased number of B and T lymphocytes in a dose-dependent manner, and a decrease in NK cells. Instead, only in male offspring VIN induced an increase of antibody forming cells and an increase of NK cells, whereas female offspring were characterized by a decrease in NK cell activity, indicating a sexual dimorphism (White et al., 2004). Therefore, whereas ATR and CYP adverse effects on the immune system are well described, more studies investigating VIN immune effects are needed. Immunosuppression, induced by pesticide exposure, in particular during susceptible time windows, can lead to adverse outcomes not only strictly related to the immune system, like a reduced ability to fight against diseases or infections, but also on indirectly-related systems, like the central nervous system. Finally, considering the important role of the immune system in the tumor microenvironment and that exposure to different pesticides has been directly correlated to a higher hormone-sensitive cancers incidence (Weichenthal et al., 2010; Weichenthal et al., 2012; Ohlander et al., 2022), the endocrine-disrupting effect of multiple pesticides may interfere with the physiologic hormone signaling and, possibly contributing to cancer development and progression (Buoso et al., 2020b; Masi et al., 2021).

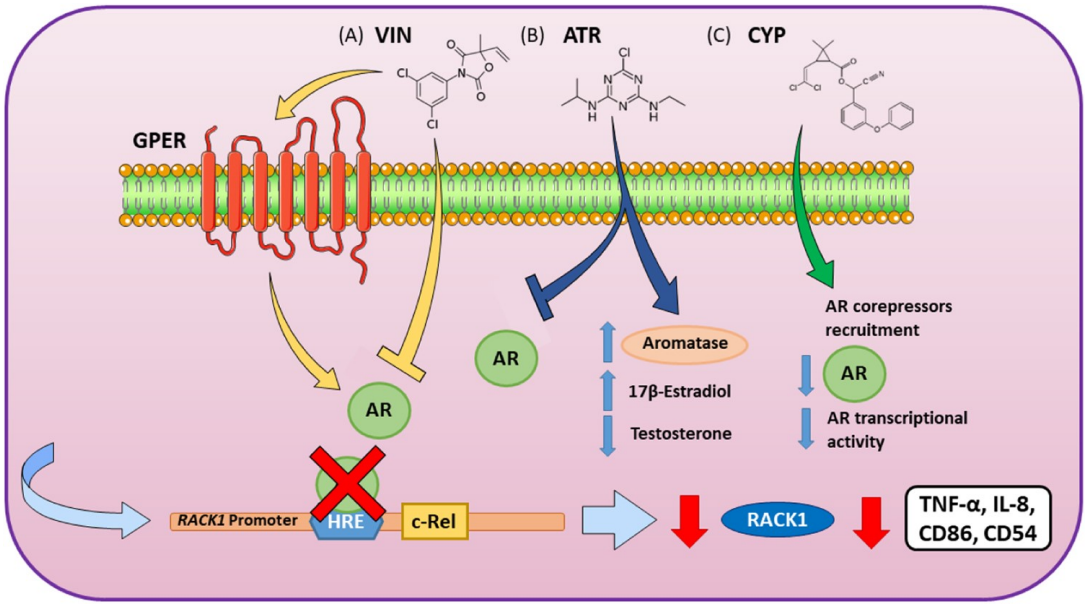

Figure 7. Proposed mechanisms of action of VIN, CYP and ATR on RACK1 expression and its related immune effects. (A) VIN modulates RACK1 expression by binding both nuclear and membrane-bound

receptors, AR and GPER respectively. *In vitro* and *in silico* data indicate the possible role of VIN and its estrogenic and anti-androgenic active metabolites in the observed effects on innate immune functions. (B) Docking calculations indicate a possible ATR antagonist profile for AR, in line with *in vitro* data and the anti-androgenic effect of ATR, however, we cannot exclude its enhancing effect on aromatase activity as reported in literature data. (C) CYP reduces RACK1 expression by a decrease of AR expression and consequently reduced transcriptional activity (see text for details).

Conclusions

The exposure to VIN, ATR and CYP, as a consequence of their wide use and/or their elevated environmental persistency, represents a critic public health issue of primary interest. Indeed, since exposure to VIN, ATR and CYP, as previously shown, and different hormone-active substances can have unintended effects on the immune system (Buoso et al., 2020), new and rapid predictive and screening methods, both *in silico* and *in vitro*, are now required for the increased interest in the immunotoxicity hazard identification of chemicals. Therefore, we propose an *in vitro* strategy composed of an initial screening based on molecular modelling and docking calculations to investigate the affinity of the known or putative EDC for different steroid hormones receptors, followed by the assessment of RACK1 promoter activity, RACK1 mRNA and protein expression to confirm the impact on RACK1 cellular level, and ultimately the evaluation of the immune functions after the observed modulation. The central point of using RACK1 as a screening tool is that monitoring the alterations of its expression can help to unravel the complex interplay of both transcriptional and non-transcriptional events associated with the exposure to hormone-active substances capable to interact with the hormonal system and the subsequent biological consequences (Buoso et al., 2011, 2017b, 2017c, 2020b). Importantly, present and previously results strongly warrant the analysis of further panels of EDCs due to the important concerns that these substances represent in terms of public health.

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Effects of endocrine disrupting chemicals on the expression of RACK1 and LPS-induced THP-1 cell activation

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Abstract

The existence of a complex hormonal balance among glucocorticoids, androgens and estrogens involved in the regulation of Receptor for Activated C Kinase 1 (RACK1) expression and its related immune cells activation, highlights the possibility to employ this protein as screening tool for the evaluation of the immunotoxic profile of endocrine disrupting chemicals (EDCs), hormone-active substances capable of interfering with the physiologic hormonal signaling. Hence, the aim of this work was to investigate the effect of the exposure of EDCS 17α-ethynylestradiol (EE), diethyl phthalate (DEP) and perfluorooctanesulfonic acid (PFOS) on RACK1 expression and on lipopolysaccharide (LPS)-induced activation of the human monocytic cell line THP-1, a validated model for this investigation. In line with our previous results with estrogen-active compounds, EE treatment significantly induced RACK1 promoter transcriptional activity, mRNA expression, and protein levels, which paralleled an increase in LPS-induced IL-8, TNF-α production and CD86 expression, previously demonstrated to be dependent on RACK1/PKCβ activation. EE mediates its effect on RACK1 expression through G-protein-coupled estrogen receptor (GPER) and androgen receptor (AR) ligand-independent

cascade, as also suggested by *in silico* molecular docking simulation. Conversely, DEP and PFOS induced a dose-dependent downregulation of RACK1 promoter transcriptional activity, mRNA expression, and protein levels, which was mirrored by a reduction of IL-8, TNF-α production and CD86 expression. Mifepristone pre-treatments abolish DEP and PFOS effects, confirming their GR agonist profile, also corroborated by molecular docking. Altogether, our data confirm that RACK1 represents an interesting target of steroid active compounds, which expression offers the opportunity to screen the immunotoxic potential of different hormone-active substances of concerns due to their human exposure and environmental persistence.

Keywords

Endocrine disrupting chemicals (EDCs), Ethynylestradiol (EE), Perfluorooctanesulfonic acid (PFOS), Diethyl phthalate (DEP), Immunotoxicity, RACK1

Introduction

Endocrine disrupting chemicals (EDCs) are hormone-active substances of natural or synthetic origin able to interfere with the synthesis, secretion, transport, binding, elimination or signaling of natural hormones (Kavlock et al., 1996). Synthetic EDCs can be subdivided mainly in industrial chemicals, plasticizers, pesticides, and pharmaceutical agents (Diamanti-Kandarakis et al., 2009) whose exposure can occur primarily via the oral route or through skin contact and inhalation (Kabir et al., 2015). In the last decades, an increased interest was posed on EDCs due to the emerging evidence on their negative impact on human health (Ho et al., 2022), including reproductive and hormonal diseases (Gore et al., 2015), but also metabolic, immune, and neurological disorders (Monneret, 2017; Bansal et al., 2018; Kumar et al., 2020; D'Amico et al., 2021; Di Paola et al., 2022). Indeed, exposure to different EDCs may display a role in the development of Parkinson's Disease (PD) (D'Amico et al., 2022a) and significantly increase the degree of inflammation and oxidative damage induced by arthritis thus also playing a deleterious role in the progression of rheumatoid arthritis (RA) (D'Amico et al., 2022b).

Hormones like androgens, glucocorticoids and estrogens, can influence immune cell functions (Coutinho and Chapman, 2011; Islander et al., 2011; Racchi et al., 2017) and, therefore, the relationship between the endocrine and the immune system can be affected by environmental conditions involving EDCs exposure (Greives et al., 2017). Here we describe the effects of three EDCs to which humans may come into contact with due to a possible high frequency exposure: 17α-ethynylestradiol (EE), perfluorooctanesulfonic acid (PFOS) and diethyl phthalate (DEP).

EE is a synthetic derivative of estradiol with improved bioavailability and resistance to metabolism, firstly employed for the treatment of diverse gynaecological disorders and menopausal symptoms. As an estrogen medication together with progestins is used in different birth control methods, from pills administered *per os* to vaginal rings and patches (Kuhl, 2005). EE presence in the urine of women taking contraceptives has been confirmed at increasing concentrations and contaminating rivers all over the world. A short-term exposure to EE during early pregnancy has severe consequences for fetal growth and survival depending on the dose. Exposition to synthetic estrogens also affects placenta growth and angiogenesis (Meyer et al., 2019). Hence, EE was added in the European list of priority substances in the field of water policy (European Commission, 2012; Loos, 2012) due to its wide use and low biodegradability that may induce adverse effects to the environment and wildlife (Kidd et al., 2007; Hu et al., 2017). EE levels in surface waters display high variations between the different continents, which ranged from non-detectable to high values (e.g., 17.112 ng/L in China) (Tang et al.,

2021). Concentrations reported in Europe were higher than 1 ng/L, exceeding 29–187 times the quality standards established by the Scientific Committee on Health and Environmental Risks (SCHER (Scientific Committee on Health and Environmental Risks), 2011).

Phthalate esters (PAEs) are widely employed as plasticizers to improve flexibility and hardiness in plastics, as solvents in personal care products (e.g. perfumes, cosmetics), insecticide materials and in pharmaceutical products. In recent decades, PAEs production has undergone a constant growth due to their widespread application and, consequently, increasing amounts of PAEs have been found extensively distributed in different environments (NICNAS, 2008; Giovanoulis et al., 2018). PAEs have been reported to have endocrine disrupting effects in different species of fish and mammals, including potential developmental toxicity (Yin et al., 2018), thus exerting potential impacts on ecosystem functioning and ultimately representing a public health issue (Net et al., 2015). Despite their biodegradability, humans are commonly and daily exposed to PAEs through food, drinking water, dust/soil, air inhalation and dermal exposure (Net et al., 2015). DEP represents the main PAE detected in surface waters, drinking water and sludge (Net et al., 2015) as well as in dust and air, both outdoor and indoor. Inhalation represents an important route of exposure to DEP (Tran and Kannan, 2015). In this regard, an agricultural area of western China reported high levels of DEP, in both outdoor (400–966 pg/m3) and indoor (297–549 pg/m3) air (He et al., 2020). Similarly, high concentrations of phthalates were detected in different private and public places in 2014 in Albany (New York, USA) (Tran and Kannan, 2015).

Literature data also report the highest levels of DEP in surface waters of Nigeria and South Africa, peaking at (538 ± 4) x 10^3 µg/L and 3.56 × 10^3 µg/L, respectively. DEP presence was also detected in some European countries, although at lower levels (from 2.9858 µg/L to < 0.01 µg/ L) (Net et al., 2015). A similar distribution was found in the Asan Lake of Korea (Lee et al., 2019). Moreover, DEP was also found at high level in wastewaters in France (3.29 µg/L) although in UK, DEP levels were higher than European countries (25 ± 16.7 µg/L). DEP in drinking water was also reported at high levels in Greece (300 ng/L) (Net et al., 2015). DEP was also revealed in the tissues of fish living in these contaminated waters, suggesting that it can easily enter the food chain, raising concerns for its possible harm to humans (Gao and Wen, 2016).

PFOS is another EDC present in the European list of priority substances in the field of water policy (European Commission, 2012; Loos, 2012) and in the Stockholm Convention (UNEP, 2009). PFOS is a fluorosurfactant widely employed in different industrial applications and consuming products (Jian et al., 2017), now regarded as a persistent and global pollutant

(OECD, 2002). It is known to be hazardous for both environment and human beings (EFSA, 2020) due to its long half-life (from four to five years) and its ability to significantly bioaccumulate not only in the wildlife but also in the human body (Olsen et al., 2007; Saikat et al., 2013; Tsuda, 2016; Sunderland et al., 2019; Glüge et al., 2020). Indeed, PFOS accumulation was reported to cause damage in particular areas of specific organs, like histopathological changes in the liver (Su et al., 2022) and in the marginal area of the heart (Li et al., 2021), and to cause neurotoxicity due to its ability to cross the blood-brain-barrier and enter the brain (Li et al., 2021). PFOS was detected in drinking waterin different areas, such as the Constance Lake in Germany (3 ng/L) (Lange et al., 2007) or in Osaka (13.7 ng/L) (Takagi et al., 2011) and levels of PFOS ranging from 0.39 to 0.87 ng/L were also reported in tap water in Spain (Ericson et al., 2008). A 345 ng/ml PFOS serum concentration was reported in a Swedish population exposed to contaminated PFOS drinking water (Li et al., 2018), with 307–458 ng/ml for PFOA and PFOS reported in people living in very contaminated areas or following occupational exposure, (Fromme et al., 2009). Indeed, PFOS serum concentrations in highly occupationally exposed workers have been reported to display a 145–3490 ng/ml range (Olsen et al., 2007).

EDCs represent a serious public issue due to their negative action on human body, including the immune system (Hansen et al., 2015; Chang et al., 2016) and exposure to immunotoxic compounds can have serious adverse health consequences (DeWitt et al., 2016). Therefore, research efforts are at identifying molecular biomarkers and methods to rapidly reveal immunotoxicity of EDCs. This includes the study of receptor for activated C kinase 1 (RACK1) as an EDC target (Buoso et al., 2017a; 2020a; 2021). Over the years, we provided evidence for the existence of a complex hormonal balance among glucocorticoids, androgens and estrogens, in the control of RACK1 expression and immune cells activation (Racchi et al., 2017; Buoso et al., 2011; 2017a; 2017b). Pro-inflammatory cytokines (e.g. IL-8 and TNF-α) release, CD86 expression, B and T cells proliferation were previously demonstrated to be dependent on RACK1/PKCβ activation (Corsini et al., 2002; 2005; 2014a; 2014b; 2016a; 2021; Doisne et al., 2008; Wu et al., 2013; Day et al., 2019). We demonstrated that glucocorticoids negatively regulate RACK1 expression whereas androgens and estrogen-active compounds promote RACK1 expression leading to the innate immune response activation (Buoso et al., 2011; 2020a; 2021; Corsini et al., 2016b; Racchi et al., 2017). Due to EDCs intrinsic features, RACK1 may represent an interesting target of EDCs, and its evaluation may offer the opportunity to screen the immunotoxic potential of hormone-active substances (Buoso et al., 2017a; 2020a; 2021). Therefore, in this study we investigated the effects of EE, PFOS and DEP on the modulation of RACK1 expression and the hormone receptors involved along with

molecular docking. As markers of immune functions, the response to lipopolysaccharide (LPS) was evaluated by assessing CD86 expression, and the release of IL-8 and TNF-α.

Materials and Methods

Chemicals

17α-ethynylestradiol (EE) (PubChem CID: 5991), perfluorooctanesulfonic acid (PFOS) (PubChem CID: 57652692), diethyl phthalate (DEP) (PubChem CID: 6781), Mifepristone (RU486) (PubChem CID: 55245), Flutamide (PubChem CID: 3397), Lipopolysaccharide (LPS) from *Escherichia coli* serotype 0127:B8, cell culture media and supplements were purchased from Sigma Aldrich (St Louis, MO, USA). G15 (PubChem CID: 1131380) was purchased from Tocris Bioscience (Bristol, United Kingdom). The mouse anti-human RACK1 monoclonal antibody (sc-17754) was from Santa Cruz Biotechnology (Dallas, TX, USA). The mouse monoclonal anti-β-tubulin antibody (T0198) was from Sigma Aldrich (St Louis, MO, USA). Electrophoresis reagents were from Bio-Rad (Richmond, CA, USA). All chemicals were purchased at the highest purity available. Chemicals were diluted in dimethyl sulfoxide (DMSO) for 50 mM stock solutions, and sequentially properly diluted whereas PFOS was an acid solution ~40% in H2O (T) (N◦ Cat. 77283-Sigma-Aldirch, PubChem CID: 57652692).

Cell culture

Human THP-1 cell line were purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). For experiments, THP-1 cells, were diluted to 106 cells/ml in RPMI 1640 without 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 5% dextran-coated charcoal-treated foetal bovine serum (DCC-FBS, Sigma Aldrich) and cultured at 37 ◦C in 5% CO2 incubator. The experiments were carried out on passages 5–15. Preliminary experiments were conducted to identify non-cytotoxic concentrations [cell viability > 80% (CV80)]. Cytotoxicity was assessed by propidium iodide staining and the CV80 determined for all compounds. For the different experiments, cells were then treated with increasing concentrations of EE (0.001–1 µM), DEP (0.001–10 µM) and PFOS (0.2–20 µM) or DMSO as vehicle control (final concentration of DMSO in culture medium < 0.1%) in different timings, as detailed in figure legends. For the analysis of the expression of CD86 and for the release of cytokines, cells were incubated for 24 h with increasing concentrations of the selected EDCs as following, EE (0.001–1 µM), DEP (0.1–10 µM) and

PFOS (0.2–20 μM), or vehicle control, then LPS was added at the concentration of 10 ng/ml for additional 24 h.

To investigate EDCs involvement with RACK1-related hormone receptors, cells were pre-treated with 10 μM mifepristone for 1 h (GR antagonist) (Corsini et al., 2014b), 50 μM flutamide for 1 h (AR antagonist) (Corsini et al., 2016b), or 0.5 μM G15 for 15 min (GPER antagonist) (Buoso et al., 2020a) as detailed in figure legends.

Plasmid DNA preparation, transient transfections, and luciferase assays

Plasmids preparation, transient transfections and luciferase assays were performed as previously described. The Δ1 reporter plasmid construct has been previously described (Del Vecchio et al., 2009). It was the longest construct available, 2105 nt long, which contains the RACK1 gene promoter region between nucleotides - 1744 and + 361 and includes the glucocorticoid responsive element (GRE) sequence. Plasmids for transfections were purified with the HiSpeed® Plasmid Midi Kit (Qiagen, Valencia, CA, USA). DNA was quantified and assayed for purity using QuantusTM fluorometer (Promega, Madison, WI, USA). Transient transfections were performed in 24-well plates; for each well, 5 × 105 cells were seeded in RPMI 1640 medium without phenol red, and with 5% DCC-FBS, 1% antibiotics and supplemented with 1% L-glutamine. Transfections were carried out using Lipofectamine ® 2000 (Invitrogen Carlsbad, CA, USA), following the manufacturer instructions. Each luciferase reporter construct plasmid DNA was co-transfected with the pRL-TK renilla luciferase expressing vector to measure the transfection efficiency (Promega, Madison, WI, USA). During transfection, THP-1 cells were incubated at 37 ◦C in 5% CO2, and then treated with the selected compounds in presence or absence of receptor antagonists for the times and at concentrations specified in figure legends. Cells were then lysed (Passive Lysis Buffer, provided by the Dual-Luciferase Reporter Assay System; Promega, Madison, WI, USA), following the manufacturer specifications. Luminescence was measured with a 20/20 n Luminometer (Turner Bio-Systems, Sunnyvale, CA, USA), with 10 s integration.

Reverse transcription quantitative PCR

Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer instructions and RNA quantification was performed with Quantus™ Fluorometer (Promega, Madison, WI). 2 μg total RNA was reverse transcribed using Qiagen QuantiTect reverse transcription kit following the manufacturer instructions. qPCR was performed with QuantiTect Sybr Green PCR kit and RACK1 and 18 S primers, provided by

Qiagen. 18 S was used as endogenous reference control (Buoso et al., 2020a; 2021) and transcripts quantification was performed with $2^{-\Delta\Delta Ct}$ method.

Immunoblot analysis

Immunoblot analysis was performed as previously described (Buoso et al., 2020a; 2021). RACK1 expression was determined at the protein level in cell lysates by immunoblot analysis. After the treatments, the cells were harvested, washed with phosphate-buffered saline (PBS) 1X, centrifuged, lysed in 100 µL homogenization buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, protease inhibitor mix) and sonicated for 10 s. Protein content was measured using Bradford assay. Samples for Western blotting were prepared by mixing cell lysates with sample buffer (125 mM Tris-HCl pH 6, 8.4% sodium dodecyl sulphate (SDS), 20% glycerol, 6% β-mercaptoethanol, 0.1% bromophenol) and denatured at 95 ◦C for 5 min. Equivalent amounts of extracted protein (10 µg) were electrophoresed into 10% SDS-PAGE under reducing conditions. Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Amersham, Little Chalfont, UK), blocked in 5% (w/v) bovine serum albumin (BSA), 1X TBS, 0.1% Tween-20 for 1 h with gentle shaking. Proteins were visualized using primary antibodies diluted in 5% (w/v) BSA, 1X TBS, 0.1% Tween-20 for RACK1 (1:1000) and β-tubulin (1:1000). In all the experiments, immunoreactivity was detected using host-specific secondary IgG peroxidase-conjugated antibodies (1:5000) and developed using enhanced chemiluminescence (Clarity Western ECL blotting substrates, Bio-Rad). Immunoblot images were acquired with Molecular Imager Gel Doc XR (BioRad), and the optical density of the bands was determined using the ImageJ software (W. Rasband, Research Service Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, MD, USA; and Laboratory for Optical and Computational Instrumentation, University of Wisconsin, WS, USA). The relative densities of the bands are expressed as arbitrary units and are normalized to the control samples run under the same conditions.

Cytokine production

After treatment, the release of cytokine TNF-α and IL-8 was assessed in cell-free supernatants following centrifuge at 1200 rpm for 5 min. Cytokine release was assessed using commercially available kits for human TNF-α (R&D Systems, Inc., Minneapolis, USA), and for human IL-8 (ImmunoTools, Friesoythe, Germany). Limits of detection were 8 pg/mL for IL-8, and 7.8 pg/mL for TNF-α, respectively. Results are expressed in pg/mL.

Flow cytometric analysis of CD86 expression

After treatment, THP-1 cells were centrifuged, washed once with cold PBS and suspended in 200 µL of PBS. Cells were stained in the dark for 30 min with specific FITC-conjugates antibodies against CD86 (BD Biosciences) or with isotype control antibody (BD Biosciences) at 4 °C, following supplier's instructions. Then, cells were centrifuged at 1200 rpm for 5 min and suspended in 0.5 ml of PBS. The intensity of fluorescence was analyzed using Novocyte 3000 flow cytometer and data were quantified using Novocyte software (Acea Bioscience Inc.). 10'000 viable cells were analyzed for mean fluorescence intensity (MFI). MFI of isotype control was subtracted to MFI of CD86 stained cells. Changes in CD86 expression are reported as stimulation index (SI) calculated by the following equation:

SI = MFIt / MFIc

MFIt stand for chemical-treated cells, whereas MFIc for the untreated ones.

Molecular docking

The 3D structure of GPER, generated by homology modeling, and the docking protocol for estimating the binding of small molecules at the GPER ligand binding domain (LBD) was already assessed in our previous study and therefore used as starting point for the present docking study. The crystal structure of the GR LBD domain in complex with the agonist dexamethasone (PDB entry: 4UDC) was retrieved from the Protein Data Bank (https://www.rcsb.org/) and properly prepared for docking by using the Protein Preparation Wizard utility of the Schrödinger suite 1,2. Missing amino acid side chains were rebuilt, and potential atom types and bond connectivity issues into the homology model structure were fixed. Moreover, ionization and tautomerization states potentially present at physiological pH were also calculated with the Epik sub-routine 3,4. Afterward, the pretreated structure was minimized according to the OPLS3e force field. The GPER receptor grid was generated at the center of the putative binding site 5, whereas the GR grid was centered on the cognate ligand present in the crystallographic structure. The chemical structures of 17alpha-Etinylestradiol, ethyl phthalate and PFOS were designed in ChemDraw, imported into the Maestro software 6, and prepared using the LigPrep utility available within the Schrodinger suite 7. All potential states of ionization and tautomerism at a physiological pH of 7.4 ± 0.2 were generated. The ligands thus prepared were finally docked to the protein. The Glide software was used for docking calculation, using the default settings of the Standard Precision (SP) protocol 8. The resulting ligand-protein complexes were ranked by docking score and visually inspected. For GPER, the top-ranking pose was further refined through an Induced-Fit Docking run.

Statistical analysis

Data are expressed as means ± standard error of mean (SEM) of at least three independent experiments. Statistical analysis was performed using the InStat software, version 7.0 (GraphPad Software, La Jolla, CA, USA). Significant differences were determined using analysis of variance (ANOVA), followed, when significant, by an appropriate post hoc test, as indicated in the figure legends. In all the reported statistical analysis effects were designated as significant if the *p* value was < 0.05.

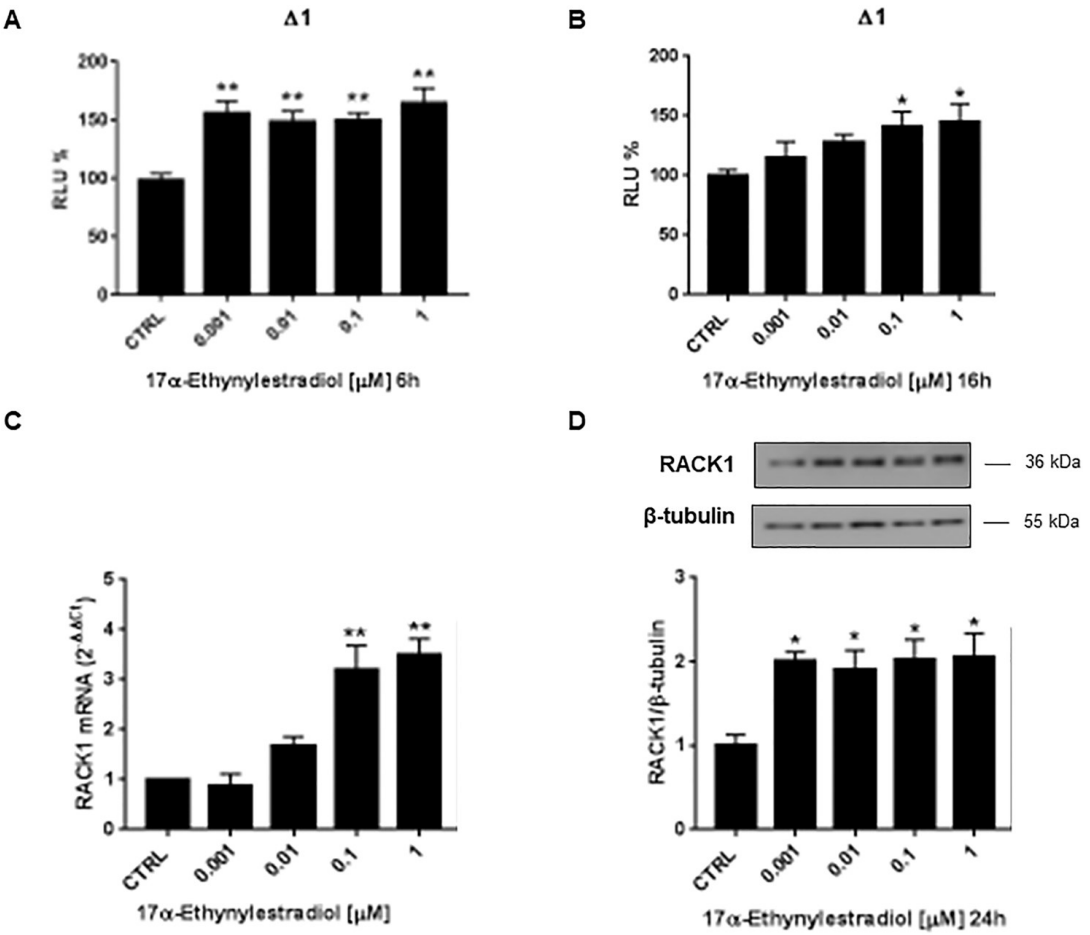
Results

17α-ethynylestradiol (EE) increases RACK1 expression and induces pro-inflammatory cytokines release

The effects of EE on RACK1 expression were evaluated by means of reporter luciferase activity using the human RACK1 gene promoter, mRNA expression using Real time-PCR, and protein level by western blot analysis. THP-1 cells were treated for 6–24 h with increasing concentrations of EE (0.001–1 μM) or vehicle control. These times were chosen from previous experiments as optimal for DHEA and cortisol induced transcriptional activity, mRNA and protein expression (Buoso et al., 2011). As shown in Fig. 1, EE induced a significant and concentration-related increase of RACK1 transcriptional activity at 6 h for almost all concentrations (Fig. 1A) and 16 h for high concentrations (Fig. 1B). The latter observation was mirrored by the increase of RACK1 mRNA expression at 18 h (Fig. 1C), while the increase in RACK1 protein evaluated at 24 h reached statistical significance at all concentrations tested (Fig. 1D).

Regarding the analysis of the immunomodulatory effects following LPS exposure, IL-8 was increased by EE at the concentrations of 0.001, 0.01 and 1 μM (Fig. 1E), and TNF-α by EE 0.01 μM (Fig. 1F). EE also induced a clearly up-regulation of CD86 expression at the lowest concentration tested (Fig. 1G).

Overall, these results indicate the ability of EE to induce the expression of RACK1 and the related immune markers. EE plasmatic levels of 158 pg/ml (0.5 nM) in normotensive women and of 328 pg/ml (1.1 nM) in hypertensive women with contraceptive drug therapy have been found (Ahluwalia et al., 1977). Therefore, plasma levels of EE are in line with treatment concentrations used in this study (Brenner et al., 1980; Kaufman et al., 1981; Kuhnz et al., 1991), thus indicating that the EE effects are observed at *in vivo* relevant concentrations.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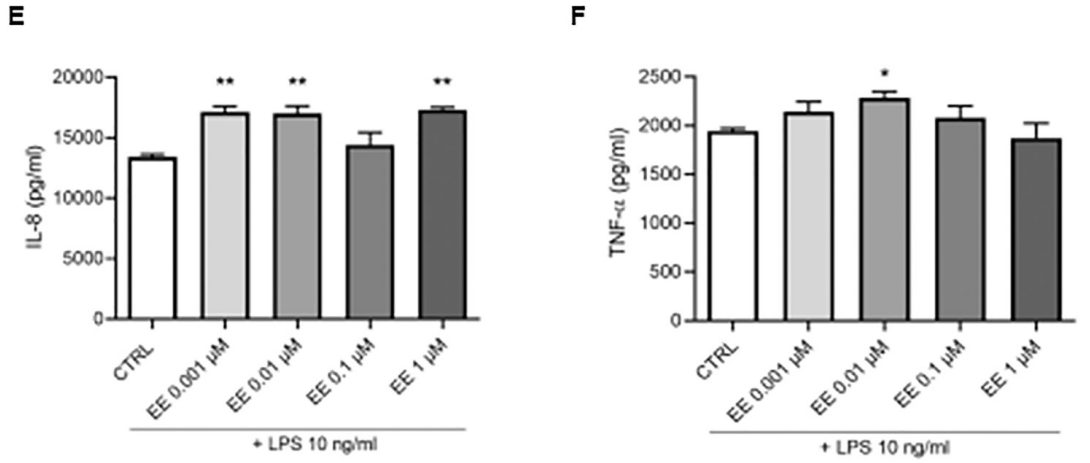
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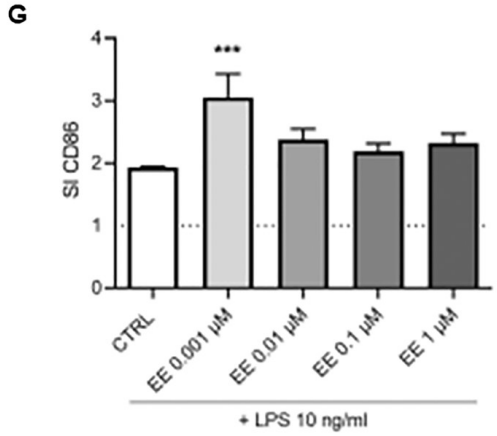
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EE 1 μM
+ LPS 10 ng/ml

Figure 1. Effects of EE on RACK1 expression and immune activation. A-B THP-1 cells transiently transfected with the Δ1 construct were treated for 6 h (A) or 16 h (B) with increasing concentrations of EE (0.001–1 μM) or vehicle control (CTRL). Cells were lysed and luciferase activity was measured as described in "Materials and Methods" section. Luciferase activities are expressed as RLU% respected to non-treated construct (considered as 100%). Results are expressed as mean ± SEM, n = 3 independent experiments performed in quadruplicate. Statistical analysis was performed with Dunnett's multiple comparison test with $*p < 0.05$ or $**p < 0.01$. C-D THP-1 cells treated for 18 h (C) or 24 h (D) with increasing concentrations of EE (0.001–1 μM) or vehicle control (CTRL). C. mRNA levels evaluated by qPCR (endogenous reference, 18 S). D. The image is a representative Western blot. RACK1 protein levels evaluated by Western blotting, normalized to β-tubulin expression. C-D Each value represents the mean ± SEM n = 3 independent experiments. Significance was set at $*p < 0.05$, $**p < 0.01$ by Dunnett's multiple comparisons test. E-G THP-1 cells were treated with EE (0.001–1 μM) or vehicle control (CTRL) for 24 h, then LPS 10 ng/ml was added for additional 24 h. IL-8 and TNF-α release were assessed using specific ELISA and results expressed as pg/ml. CD86 expression was assessed following flow cytometric analysis and results expressed as stimulation index (SI) calculated on DMSO-treated cells. Statistical analysis was performed with unpaired t-test with $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, vs LPS-treated cells.

Role of GPER and AR in EE-induced RACK1 expression

Molecular docking calculations were performed to gain insight into the putative binding of EE at GPER. In absence of a crystallographic structure of GPER, the 3D structure of the protein was generated by homology modeling (HG ID: HG0714) as reported in our previous study (Buoso et al., 2021). The binding mode of EE at GPER LBD is depicted in Fig. 2A, B. Briefly, EE binds in the same superficial sub-pockets of the receptor with a binding pose comparable to those predicted for estradiol, a well-known GPER agonist. Concurrently, a dense network of hydrogen bonds involving both hydroxyls' groups on A-ring and D-ring of the cyclopentanoperhydrophenanthrene core scaffold firmly anchors the molecule to the receptor. The hydroxyl group at position 17 simultaneously accepts and donate two hydrogen bonds involving the amide nitrogen of Asn118 and the carbonyl oxygen of Cys207, respectively. Besides, the phenolic hydroxyl in position 3 donate an additional H-bond to the carbonyl in the side chain of Gln138. The steroid scaffold resulted thus located into a hydrophobic cleft of the GPER LBD traced by Val196, Phe206, Phe208 and Ala209. In particular, Phe206 and Phe208 are two key aminoacids involved in the binding of GPER agonists (i.e. estradiol and G-1). Interestingly, from a structural standpoint, EE and estradiol just differ for the presence of an ethynyl moiety in position 17alpha of the semisynthetic estrogen. However, this ethynyl moiety has the proper steric and hydrophobic features to be well positioned within an additional small hydrophobic cleft lined by Ile114, Met133 and Cys130, thus suggesting a slight stronger binding at GPER for EE than estradiol, as proposed by the respective docking score (- 8.181 and - 6.163, respectively).

The role of GPER was confirmed in THP-1 cells treated with the lowest effective EE concentration (0.001 μM) in the presence or absence of the G15-GPER antagonist. As clearly shown in Fig. 2C, D, G15 completely blocked the effect of EE in LPS-induced CD86 expression (Fig. 2C) and IL-8 release (Fig. 2D), demonstrating the role of GPER in the observed effects. Since we previously investigated the mechanism through which GPER induces AR activation and consequently RACK1 expression (Buoso et al., 2020a; 2021), to demonstrate AR role, THP-1 cells were treated with the lowest EE effective concentration in the presence or absence of 50 μM flutamide, the AR irreversible antagonist, which completely blocked EE effect on RACK1 transcriptional activity. Similarly, flutamide completely prevented the increase of RACK1 expression even at the highest EE concentrations (0.01–1 μM) (Suppl. Fig. 1), confirming the role of AR in the observed effects.

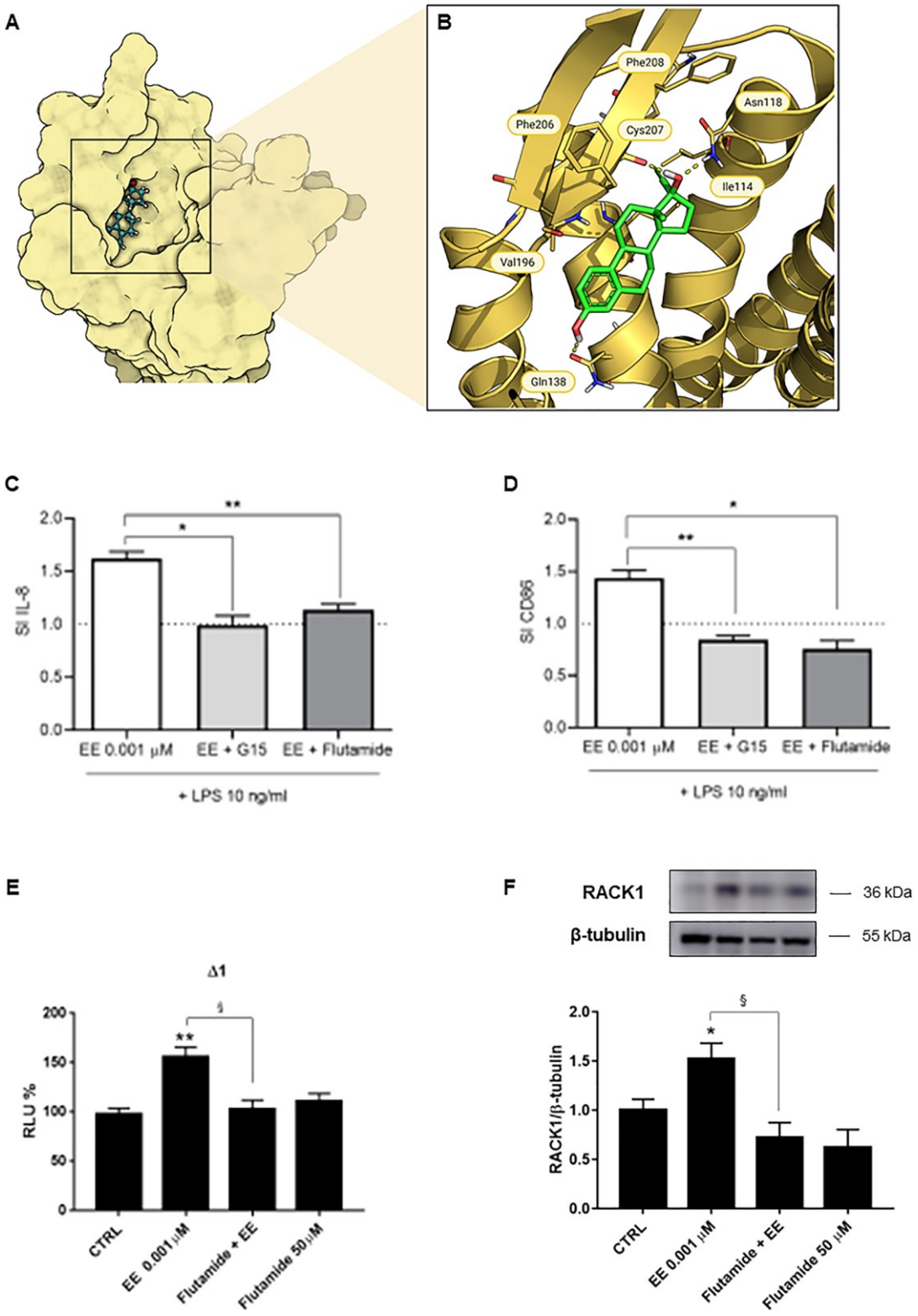

Figure 2. Role of GPER and AR in EE-induced RACK1 expression. A-B Predicted binding mode for 17alpha-etinylestradiol at GPER ligand binding site. (A) Close-up of the GPER binding site and (B) of the putative binding mode of 17alpha-etinylestradiol (in green stick carbons) predicted by docking calculation. The 3D structure of GPER is represented in yellow cartoon, and the key amino acid residues interacting with the ligand are represented in lines. The heteroatoms are color-coded: oxygen in red and nitrogen in blue. The H-bonds are depicted in yellow dotted lines. (Created with BioRender.com). C-D THP-1 cells were pre-treated with 0.5 µM G15 15 min or 50 µM flutamide 1 h, then treated with 0.001 µM EE or vehicle control (CTRL). After 24 h 10 ng/ml LPS was added for further 24 h. IL-8 release

was assessed using specific ELISA, CD86 expression was assessed following flow cytometric analysis and results expressed as stimulation index (SI) calculated on cells treated with DMSO, G15 or flutamide. Each value represents the mean ± SEM, n = 3 independent experiments. Statistical analysis was performed with Tukey's multiple comparison test with *$p < 0.05$, **$p < 0.01$ *vs* EE + G15; *$p < 0.05$, **$p < 0.01$ *vs* Flutamide. E THP-1 cells transiently transfected with the Δ1 construct were treated for 6 h with vehicle control (CTRL), 50 μM flutamide or pretreated for 1 h with 50 μM flutamide and subsequently 0.001 μM EE was added. Cells were lysed and luciferase activity was measured as described in "Materials and Methods" section. Luciferase activities are expressed as RLU% respected to non-treated construct (considered as 100%). Results are expressed as mean ± SEM, n = 3 independent experiments performed in triplicate. Statistical analysis was performed Tukey's multiple comparisons test with **$p < 0.01$ *vs* control (CTRL); §$p < 0.05$ *vs* Flutamide+EE. F THP-1 cells were treated for 6 h with vehicle control (CTRL), 50 μM flutamide or pretreated for 1 h with 50 μM flutamide and subsequently 0.001 μM EE was added. The image is a representative Western blot. RACK1 protein levels evaluated by Western blotting, normalized to β-tubulin expression. Each value represents the mean ± SEM, n = 3 independent experiments. Statistical analysis was performed with Tukey's multiple comparisons test with *$p < 0.05$ vs control (CTRL); §$p < 0.05$ vs Flutamide+EE.

DEP decreases RACK1 expression and hampers immune activation

DEP is recognized as a priority environmental pollutant with endocrine disrupting properties (Yu et al., 2021). Thus, we evaluated its effects on RACK1 expression and immune activation. THP-1 cells were treated for 6–24 h with increasing concentrations of DEP (0.001–10 μM) or vehicle control. As shown in Fig. 3, DEP induced a statistical significant decrease of RACK1 at 6 h starting from 0.01 μM (Fig. 3A), while only the highest concentrations (1 and 10 μM) were able to induce a decreased RACK1 transcriptional activity at 16 h (Fig. 3B). These effects on RACK1 gene promoter were reflected by both RACK1 mRNA (Fig. 3C) and protein levels (Fig. 3D), with concentrations 0.1–10 μM being statistically significant. Regarding the analysis of the immunomodulatory effects, no changes were observed in LPS-induced IL-8 release (Fig. 3E) whereas DEP induced a statistically significant down-regulation of TNF-α release (Fig. 3F) and CD86 expression (Fig. 3 G) at 10 μM. Collectively, these results indicate the capability of DEP to induce a decrease in RACK1 expression at plasma levels relevant concentrations (0.15–15 μM) after oral administration of 0.1–10 mg/kg DEP (Jeong et al., 2020; Lu et al., 2019). In rats and humans, the major metabolite following oral administration of DEP is the monoethyl phthalate (MEP), which has been used as a biomarker for estimating DEP exposure in humans (Api et al., 2001; Martino-Andrade et al., 2010). In this regard, a physiologically based pharmacokinetic model (PBPK) of DEP and its major metabolite MEP was developed in rats and extend this to risk assessment based on human exposure. Linearities for DEP and MEP at a dose of 0.1–10 mg/kg in human $AUC_{0-\infty}$ were excellent. The calculated amounts of MEP and DEP excreted in urine (as cumulative amounts of up to 24 h) after oral administration of 0.1–10 mg/kg DEP in humans also showed excellent linearity (Jeong et al., 2020). Therefore, DEP exposure is usually assessed with urine analysis, searching for its

metabolite MEP. In a study of 2000 in US, a concentration of 179 ng/ml was assessed (Silva et al., 2004) whereas in Asia a median of 391 µg/l was reported (Guo et al., 2011). In Europe lower concentrations have been reported in pregnant women in Norway (319 µg/l) (Villanger et al., 2020), in German adults (90.2 µg/l) and in children and adolescents (23.1 µg/l) (Koch et al., 2003; Schwedler et al., 2020), and in Spain (68.6 µg/l) (Herrero et al., 2015).

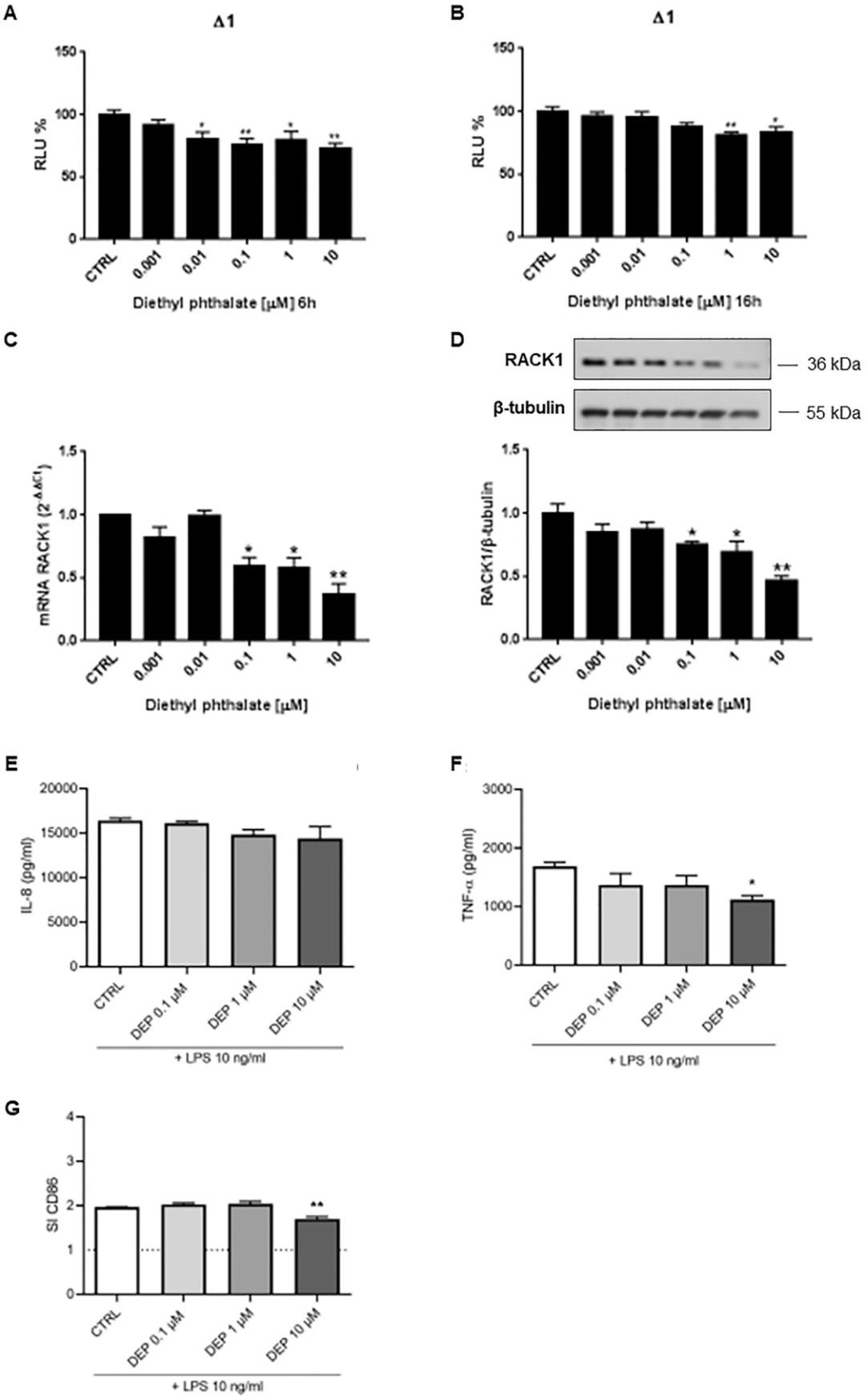

Figure 3. Effects of DEP on RACK1 expression and immune activation. A-B THP-1 cells transiently transfected with the Δ1 construct were treated for 6 h (A) or 16 h (B) with increasing concentrations of

DEP (0.001–10 μM) or vehicle control (CTRL). Cells were lysed and luciferase activity was measured as described in "Materials and Methods" section. Luciferase activities are expressed as RLU% respected to non-treated construct (considered as 100%). Results are expressed as mean ± SEM, n = 3 independent experiments performed in quadruplicate. Statistical analysis was performed with Dunnett's multiple comparison test with $*p < 0.05$, $**p < 0.01$ *vs* control (CTRL). C-D THP-1 cells treated for 18 h (C) or 24 h (D) with increasing concentrations of EE (0.001–10 μM) or vehicle control (CTRL). C. mRNA levels evaluated by qPCR (endogenous reference, 18 S). D. The image is a representative Western blot. RACK1 protein levels evaluated by Western blotting, normalized to β-tubulin expression. C-D Each value represents the mean ± SEM n = 3 independent experiments. Significance was set at $*p < 0.05$ or $**p < 0.01$ by Dunnett's multiple comparison. E-G THP-1 cells were treated with DEP (0.1–10 μM) or vehicle control (CTRL) for 24 h, after that LPS 10 ng/ml was added for further 24 h. IL-8 and TNF-α release were assessed using specific ELISA and results expressed as pg/ml. CD86 expression was assessed following flow cytometric analysis and results expressed as stimulation index (SI) calculated on DMSO-treated cells. Statistical analysis was performed comparing each condition to LPS-treated cells. Each value represents the mean ± SEM n = 3 independent experiments. Significance was set at $*p < 0.05$ or $**p < 0.01$ by Dunnett's multiple comparison.

PFOS inhibits RACK1 expression and decreases immune activation

Due to its endocrine disruptor profile, PFOS has been shown to affect the function of the immune system in several species, raising concerns towards the possible development of immunosuppression upon its exposition in animals and humans (DeWitt et al., 2019; Corsini et al., 2014c; Betts et al., 2008). Hence, we evaluated its effects on RACK1 expression and immune cell activation. THP-1 cells were treated for 6–24 h with increasing concentrations of PFOS (0.2–20 μM) or vehicle control. Concentrations were chosen accordingly to previous studies (Corsini et al., 2010; 2012).

PFOS induced a statistically significant decrease of RACK1 transcriptional activity at 6 h at all concentrations tested (Fig. 4A), but this reduction was not observed after 16 h treatment (Fig. 4B). These effects on RACK1 gene promoter were mirrored by both RACK1 mRNA (Fig. 4C) and protein levels (Fig. 4D), decreasing at all PFOS concentrations. The same trend can be also observed on the release of IL-8 (Fig. 4E), and TNF-α (Fig. 4F) in the analysis of PFOS immunomodulatory effects following LPS exposure. Moreover, PFOS shown a statistically significant down-regulation of CD86 expression at the highest concentration tested (Fig. 4G).

Altogether, these results indicate the ability of PFOS to decrease RACK1 expression and are in line with previous observations on the compromised function of the immune system in mice models and humans (DeWitt et al., 2019). As plasma levels of PFOS ranging between 3.7 and 12,000 ng/ml (0,0074 μM – 24 μM) have been found in occupationally exposed populations, the concentrations used in our *in vitro* studies reflect highly exposed humans (Olsen et al., 2007; Corsini et al., 2010; 2012; Eriksen et al., 2011; Lindh et al., 2012).

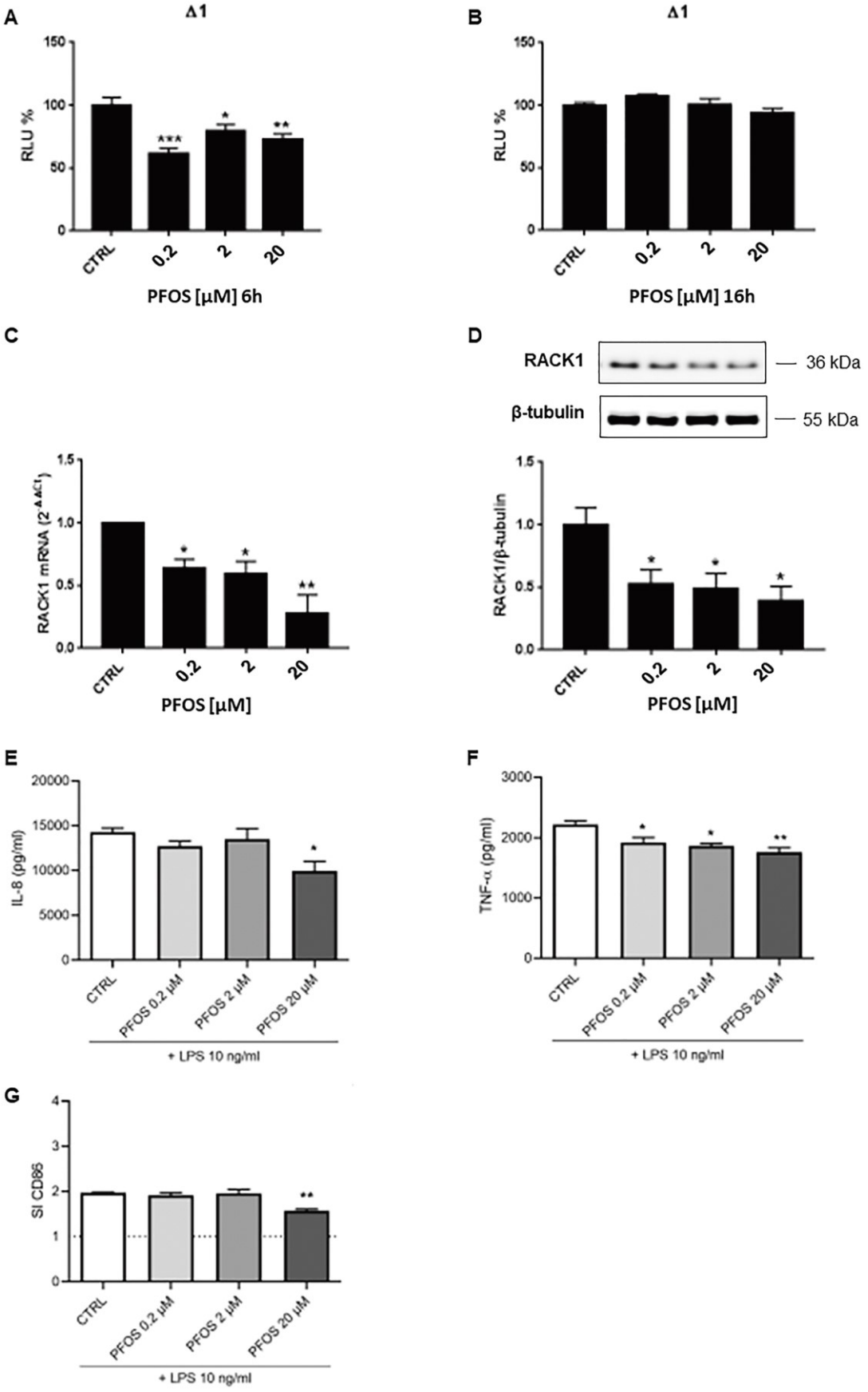

Figure 4. Effects of PFOS on RACK1 expression and immune activation. A-B THP-1 cells transiently transfected with the Δ1 construct were treated for 6 h (A) or 16 h (B) with increasing concentrations of

PFOS (0.2–20 μM) or vehicle control (CTRL). Cells were lysed and luciferase activity was measured as described in "Materials and Methods" section. Luciferase activities are expressed as RLU% respected to non-treated construct (considered as 100%). Results are expressed as mean ± SEM, n = 3 independent experiments performed in quadruplicate. Statistical analysis was performed with Dunnett's multiple comparison test with $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$ *vs* control (CTRL). C-D THP-1 cells treated for 18 h (C) or 24 h (D) with increasing concentrations of PFOS (0.2–20 μM) or vehicle control (CTRL). C.
mRNA levels evaluated by qPCR (endogenous reference, 18 S). D. The image is a representative Western blot. RACK1 protein levels evaluated by Western blotting, normalized to β-tubulin expression. C-D Each value represents the mean ± SEM n = 3 independent experiments. Significance was set at $*p < 0.05$ or $**p < 0.01$ by Dunnett's multiple comparison. E-G THP-1 cells were treated with PFOS (0.2–20 μM) or vehicle control (CTRL) for 24 h, after that LPS 10 ng/ml was added for further 24 h. IL-8 and TNF-α release were assessed using specific ELISA and results expressed as pg/ml. CD86 expression was assessed following flow cytometric analysis and results expressed as stimulation index (SI) calculated on DMSO-treated cells. Statistical analysis was performed comparing each condition to LPS-treated cells. Each value represents the mean ± SEM n = 3 independent experiments. Significance was set at $*p < 0.05$ or $**p < 0.01$ by Dunnett's multiple comparison.

Role of GR in PFOS- and DEP-induced RACK1 decrease

To further rationalize the inhibition activity of DEP and PFOS on protein expression, molecular docking calculation were carried out to investigate the binding conformation of the two compounds with the ligand binding domain of the glucocorticoid receptor (GR). The crystal structures of all GR ligand-binding domains obtained so far indicated the importance of the folding of 12 α-helices upon ligand binding for the activation or inhibition of the GR. The ligand alters how the α-helices compact around the ligand. The position of helix 12 in the final ligand bound conformation is different in the presence of an agonist compared with an antagonist. Agonists enable helix 12 to fold on the surface of the receptor thus allowing the stabilization of the co-regulators binding through a charge clamp. In this conformational state, GR can recruit the transcriptional factors triggering the gene expression. Conversely, the binding of the antagonists displaces the helix 12 thus avoiding GR to engage with co-regulators and transcriptional factors. Due to this conformational change of GR upon ligand binding and that allows to discriminate between agonists and antagonists, the docking of DEP and PFOS was carried out both on the crystal structure of the receptor antagonized by RU-486 (PDB ID: 1NHZ) and on the crystal structure of the GR bound to dexamethasone (an agonist, PDB ID: 4UCD). In the former the helix 12 is displaced and spread out far from the receptor; conversely, in the latter, GR is in its active conformation with the helix 12 properly folded on the surface of the receptor and bounded to a portion of the α-helix of the recruited co-regulator. The comparison of the docking poses obtained for both the active and inactive conformations of GR will give indications on the possible mechanism of action of DEP and PFOS. As shown in Fig. 5A, DEP can fit into the closed ligand binding cavity of GR and fasten to the binding site in a

similar orientation to that observed for dexamethasone. Briefly, the aromatic ring of DEP partially overlaps with the "pseudo" ring-A of dexamethasone and makes hydrophobic interactions with Leu563, Gln570, Met604 and Leu608; besides, it is involved in a face-to-edge π-π stacking with Phe623 which might help stabilizing the binding. The two ethyl groups point toward the entrance of the binding pocket and engage hydrophobic interactions with the amino acids of the helix 12. Whilst these hydrophobic interactions are weaker than the strong network of H-bond usually formed by more potent endogenous steroids or agonists, they might faintly contribute to stabilizing the helix 12 in its active conformation. Besides, DEP is too small to hamper the folding of helix 12 by steric hinderance like some GR antagonists (i.e., RU-486) do thus further suggesting that a DEP agonist profile.

Since our data reported in Fig. 4 showed that all the concentrations of DEP tested induced a significant RACK1 downregulation and considering our previous data demonstrated that RACK1 expression is negatively regulated by GR in immune context with the consequent reduction of cytokines release (Racchi et al., 2017; Buoso et al., 2011; Corsini et al., 2014b), GR involvement in mediating DEP effects was investigated. For this purpose, we pre-treated THP-1 cells with mifepristone, a well-known GR inhibitor (Bertagna et al., 1984) for 1 h before adding DEP (10 μM) or vehicle control. Mifepristone pre-treatment completely abolished DEP effects on RACK1 promoter activity (Fig. 5B), showing that these observed effects on RACK1 expression are correlated to DEP agonist profile for GR. The role of GR was also confirmed in LPS-induced pro-inflammatory pathways. Indeed, mifepristone was able to prevent the DEP-induced reduction of TNF-α and CD86 (Fig. 5 C, D).

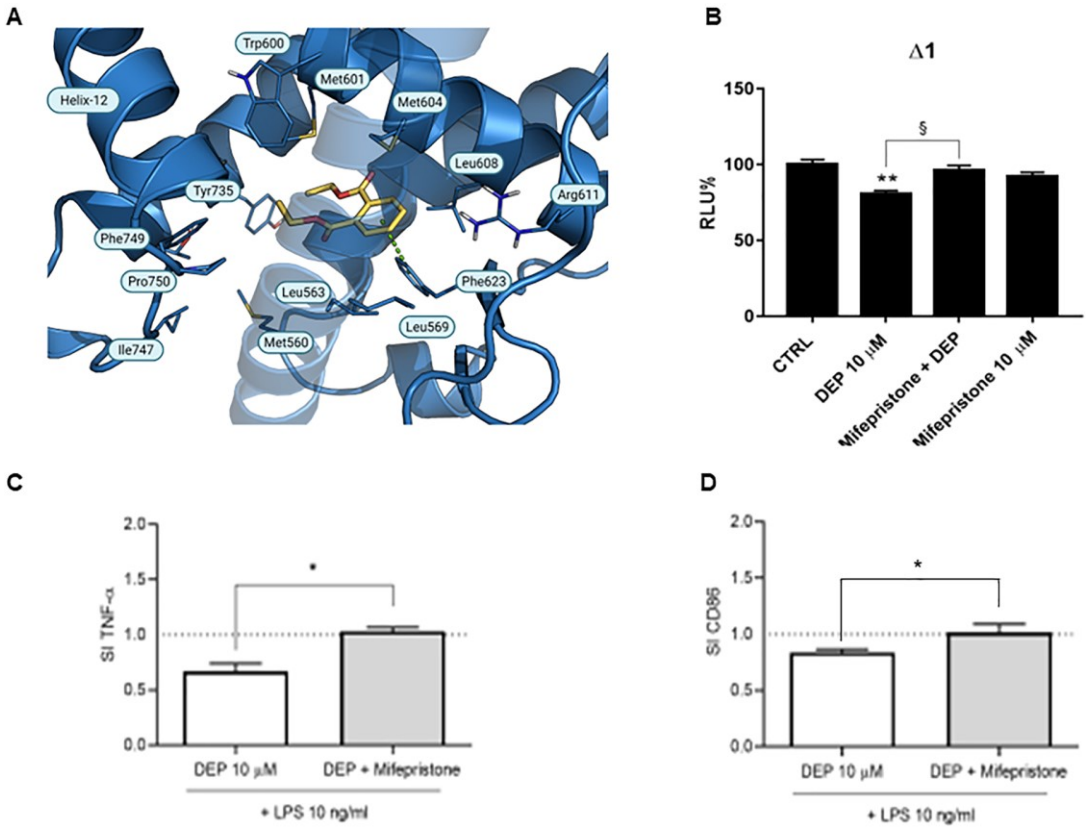

Figure 5. Role of GR in DEP-induced RACK1 expression. A. Predicted binding mode for DEP at GR ligand binding site. Putative binding mode of DEP (in yellow stick carbons) predicted by docking calculation. The 3D structure of GR is represented in blue cartoon, and the key amino acid residues interacting with the ligand are represented in lines. The heteroatoms are color-coded: oxygen in red, nitrogen in blue, sulfur in yellow and fluorine in cerulean. The face-to-edge π-π interaction and the salt bridge are depicted in green and magenta dotted lines, respectively (Created with BioRender.com). B. THP-1 cells transiently transfected with the Δ1 construct were pre-treated for 1 h with 10 μM mifepristone, then with 10 μM DEP or vehicle control (CTRL). Cells were lysed and luciferase activity was measured as described in "Materials and Methods" section. Luciferase activities are expressed as RLU% respected to non-treated construct (considered as 100%). Results are expressed as mean ± SEM, n = 3 independent experiments performed in triplicate. Statistical analysis was performed with Tukey's multiple comparison test with $^{**}p < 0.01$ vs control (CTRL) and $\S p < 0.05$ *vs* Mifepristone + DEP. C-D THP-1 cells were pre-treated with 10 μM mifepristone for 1 h then treated with 10 μM DEP or vehicle control (CTRL) for 24 h, and after that LPS 10 ng/ml was added for further 24 h. TNF-α release was assessed using specific ELISA, while CD86 expression was assessed following flow cytometric analysis and results expressed as stimulation index (SI) calculated on DMSO-treated cells or mifepristone-treated cells. Each value represents the mean ± SEM, n = 3 independent experiments. Statistical analysis was performed with Tukey's multiple comparison test with $^{*}p < 0.05$ *vs* Mifepristone + DEP.

Similar considerations can be performed for the binding mode of PFOS at GR. As depicted in Fig. 6A, the fluorochemical properly fits into the GR ligand-binding pocket engaging the sulfonic acid groups with a salt bridge to Arg611, whereas the fluorinated carbon tail adopts an extended conformation pointing toward the entrance of the binding site and helix 12. However, as observed for DEP, the polyfluorinated alkyl chain is not able to displace helix 12 from its folded conformation to prevent the inactivation of GR. These results indicated that

PFOS can efficiently bind to GR but without disturbing the active conformation of the receptor, thus suggesting an agonist activity.

To demonstrate GR involvement in mediating PFOS effects, similarly to DEP, cells were pre-treated with mifepristone before adding PFOS or DEP. Mifepristone pre-treatment completely abolished PFOS (Fig. 6B) effects on RACK1 promoter activity, showing that these observed effects on RACK1 expression are correlated to PFOS agonist profile for GR. Accordingly, mifepristone was able to prevent the PFOS-induced reduction of IL-8, TNF-α and CD86 (Fig. 6C-E).

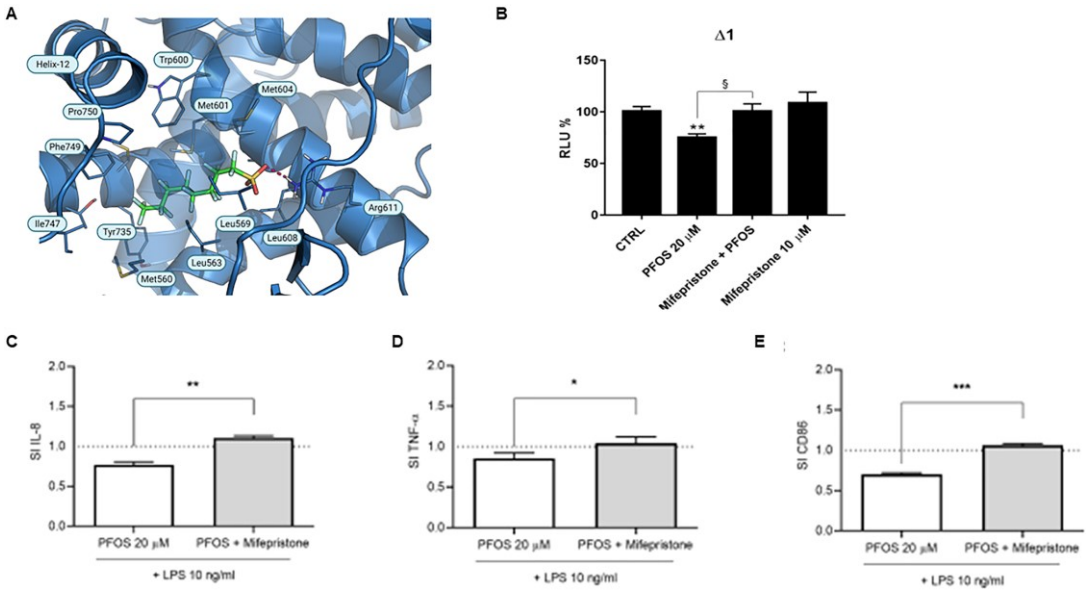

Figure 6. Role of GR in PFOS-induced RACK1 expression. A. Predicted binding mode for PFOS at GR ligand binding site. Putative binding mode of PFOS (in green carbon) predicted by docking calculation. The 3D structure of GR is represented in blue cartoon, and the key amino acid residues interacting with the ligand are represented in lines. The heteroatoms are color-coded: oxygen in red, nitrogen in blue, sulfur in yellow and fluorine in cerulean. The face-to-edge π-π interaction and the salt bridge are depicted in green and magenta dotted lines, respectively (Created with BioRender.com). B. THP-1 cells transiently transfected with the Δ1
construct were pre-treated for 1 h with 10 μM mifepristone, then with 20 μM PFOS or vehicle control (CTRL). Cells were lysed and luciferase activity was measured as described in "Materials and Methods" section. Luciferase activities are expressed as RLU% respected to non-treated construct (considered as 100%). Results are expressed as mean ± SEM, n = 3 independent experiments performed in triplicate. Statistical analysis was performed with Tukey's multiple comparison test with $^{**}p < 0.01$ *vs* control (CTRL) and $\S p < 0.05$ *vs* Mifepristone + PFOS. C-E THP-1 cells were pre-treated with 10 μM mifepristone for 1 h then treated with 20 μM PFOS or vehicle control (CTRL) for 24 h, and after that LPS 10 ng/ml was added for further 24 h. IL-8 and TNF-α release were assessed using specific ELISA, while CD86 expression was assessed following flow cytometric analysis and results expressed as stimulation index (SI) calculated on DMSO-treated cells or mifepristone-treated cells. Each value represents the mean ± SEM, n = 3 independent experiments. Statistical analysis was performed with Tukey's multiple comparison test with $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs* Mifepristone + PFOS.

Discussion

Altogether, our data confirm the opportunity to use RACK1 expression to screen the endocrine disrupting properties of chemicals and their immunotoxic potential. Chemicals targeting ER, GR and AR can modulate RACK1 expression, and the use of selective antagonists of the different steroid receptors and molecular docking allows the identification of the receptor involved.

We previously demonstrated that RACK1 expression can be regulated by the action of glucocorticoids, androgens, and estrogen-active compounds in immune context (Racchi et al., 2017; Buoso et al., 2017a; 2020a; Corsini et al., 2021). A bioinformatic analysis of RACK1 promoter region revealed the presence of several sites for transcriptional factors, including a GRE sequence similar to the consensus for a negative GRE (nGRE) (Del Vecchio et al., 2009), which is responsible for cortisol and corticosteroids effect at RACK1 transcriptional level, resulting in the downregulation of its expression and accounting for the immunosuppressive role of glucocorticoids (Buoso et al., 2011; Corsini et al., 2014a; 2014b). On the other hand, androgens, in particular dehydroepiandrosterone (DHEA) and its androgenic derivatives, have the opposite effect on RACK1 expression and on PKC activity regulation required for immune cell activation (Buoso et al., 2011; 2017b; Corsini et al., 2002; 2005) and AR modulation is at the basis of the mechanism controlling RACK1 expression in the immune context (Racchi et al., 2017; Corsini et al., 2021). Noteworthy, at transcriptional level, GR and AR can interact by recognising a similar palindromic sequence called canonical Glucocorticoid/Androgen Responsive Element (GRE/ARE) (Pihlajamaa et al., 2015) in RACK1 promoter region, that explains how these two nuclear receptors are capable of regulating RACK1 expression. Therefore, considering (1) the complex hormonal balance between corticosteroids and androgens involved in the regulation of RACK1 expression at transcriptional level (Racchi et al., 2017; Buoso et al., 2011; 2017a; 2017b; 2020a; 2021; Corsini et al., 2002; 2005; 2021), (2) RACK1 central role in immune cell function and activation (Racchi et al., 2017; Buoso et al., 2011; 2017a; 2017b; 2020a; 2021; Corsini et al., 2002; 2005; 2021) and (3) the emerging evidence that correlates EDCs effects with dysfunctions of the immune system (Bansal et al., 2018; Nowak et al., 2019), RACK1 could represent a solid and useful tool to screen EDCs effect on the immune function and their immunotoxic potential (Racchi et al., 2017; Buoso et al., 2011; 2017a; 2017b; 2020a; 2021; Corsini et al., 2002; 2005; 2021).

Due to EE, DEP and PFOS persistence in the environment and relevant human exposure, they are compounds of great concern. The aim of this study was to assess whether EE, PFOS and DEP could influence RACK1 expression and the production of its correlated cytokines IL-

8 and TNF-α and surface markers CD86 after LPS stimulation (Racchi et al., 2017; Buoso et al., 2011; 2017a; 2017b; 2020a; 2021; Corsini et al., 2002; 2005; 2021). Data obtained show that RACK1 was upregulated at promoter activity, mRNA and protein levels upon exposure to EE and this positive modulation was mirrored by an increase of TNF-α and IL-8 production and surface marker CD86 expression, indicating that the increased levels of RACK1 induced by this estrogen-active compound predisposes cells to an increased response to pro-inflammatory stimuli. In this regard, in genetically lupus-prone NZB/WF1 mice receiving EE treatment it was shown that EE impacted on splenic T cell composition, enhancing the activation of both CD4+ and CD8+ cells. Moreover, EE exposure enhanced LPS-induced IFN-γ, IL-1β and IL-10 production (Dai et al., 2019). However, high concentrations of EE have been reported to affect cell viability as shown in a macrophage cell line exposed to 20 μM EE (Kim et al., 2015). Our results were ultimately in line with EE estrogenic profile and were corroborated by our docking calculation and flutamide treatment, which confirmed the involvement of the same signalling pathway accounted for estrogen-active compounds on RACK1 expression previously demonstrated (Buoso et al., 2020a; 2021) (Fig. 7A).

On the other hand, DEP and PFOS both showed downregulating effects on RACK1 expression, and this reduction was paralleled by a decrease of the aforementioned cytokines production and surface marker expression, suggesting a significant decrease in immune cells functionality. Because of this observed reduction of RACK1 expression upon PFOS and DEP exposure and based on literature data on hormone mediated RACK1 transcriptional regulation, we used mifepristone to assess if PFOS and DEP either showed GR agonism or AR antagonism profile by means of luciferase reporter assay. In this regard, mifepristone allowed to reveal that both PFOS and DEP act as GR agonists, since mifepristone completely abolished their effect on RACK1 promoter activity and the correlated immune markers. Finally, our molecular docking calculation confirmed that both PFOS and DEP can act as GR agonists (Fig. 7B-C), further supporting their observed effects on RACK1 transcriptional regulation and immune functionality. In line with these observations, it has been reported the influence of phthalates on *in vitro* innate and adaptive immune responses (Hansen et al., 2015). Indeed, DEP treatment on peripheral blood mononuclear cells at high concentration (100 μM) was able to modulate the immune response induced by LPS thus enhancing the production of IL-6, IL-8 and IL-10 and reducing TNF-α stimulated by LPS (Hansen et al., 2015). Moreover, DEP can influence the immune response to pathogens by modulating the functions of macrophages (Kim et al., 2015). Similarly, in primary human leukocytes and the THP-1 cell line exposed to PFOS, reduced cytokine production has been observed. Moreover, monocytes and T lymphocytes, cells

involved in both innate and specific immune responses, were affected as LPS-induced cytokine production were decreased following PFOS exposure (Corsini et al., 2011; 2012). These results were also consistent with previous studies that reported immunomodulation in experimental animals exposed to PFOS, including altered inflammatory responses, cytokine production, reduced lymphoid organ weights and decreased antibody production (DeWitt et al., 2008; 2009). It has been shown that PFOS in mice, at levels similar to those found in humans, could suppress the immune system causing a reduced response to vaccination (T-cell dependent antibody response) (DeWitt et al., 2019; Peden-Adams et al., 2008). Epidemiological studies available since the publication of the previous EFSA CONTAM Panel opinion on PFOS provide support for the conclusion that PFOS is associated with reduced antibody response to vaccination (EFSA, 2020). Indeed, PFOS critical effect was identified in the decrease of the antibody in children (EFSA, 2018). In this regard, blood samples of children vaccinated against tetanus and diphtheria that were exposed to PFOS through diets, reported significant decrease in tetanus and diphtheria antibodies concentrations, below the protective level for these diseases (Grandjean et al., 2012; Timmermann et al., 2022). Moreover, by age of 3, children with higher exposure to PFOS had higher rates of bronchitis and pneumonia (Impinen et al., 2019). Some of the studies also suggest that serum levels of PFOS is associated with increased propensity for infection. Moreover, epidemiological studies also provide clear evidence for an association between exposure to PFOS and increased serum levels of cholesterol and the liver enzyme alanine transferase (ALT) although there is insufficient evidence to correlate PFOS exposure with cardiovascular disease, diabetes, obesity or metabolic syndrome (EFSA, 2018).

EDCs concentrations used in the current study were relevant to human exposure, thus indicating that EE, DEP and PFOS immunotoxic effects are observed at *in vivo* relevant concentrations. Altogether these results also demonstrate that the modulation of RACK1 expression and its downstream effects on the innate response can represent a useful tool for EDCs immunotoxic evaluation. In particular, this work shows that exists a strong correlation between EDCs-mediated RACK1 modulation and their immunotoxicity. Specifically, these data highlight that exposure to EE can predispose the cells to an increased response to proinflammatory stimuli, that can potentially lead, for example, to stronger allergic reactions, the onset of different autoimmune diseases and to a general misregulation of inflammation (Chen and Chai, 2022). In contrast, the decreased immunostimulation associated with GR agonists PFOS and DEP may also be correlated to misregulated inflammation but in the opposite direction, contributing to a decreased immune activation and general immunodepression with increased risk of infections and cancer (Buoso et al., 2020b).

Finally, our data show that monitoring the regulation of RACK1 expression can capture different mechanisms of endocrine disruption that involve either nuclear or membrane-bound receptors through the specific inhibition of renowned RACK1 transcriptional modulators. The present work perfectly fits in our research frame, for assessing estrogenic-active compounds, GR agonists/antagonists, and AR agonists/antagonists (Buoso et al., 2011; 2017a; 2017b; 2020a; 2021). In fact, while GR agonists can exert a direct transcriptional downregulation on RACK1 expression, the lack of an Estrogen Responsive Element in its promoter region indicates the membrane-bound receptor GPER as the mediator of estrogen-active substances effects on RACK1 expression via a non-genomic signalling, thus making RACK1 a potential screening tool also for compounds characterised by this profile.

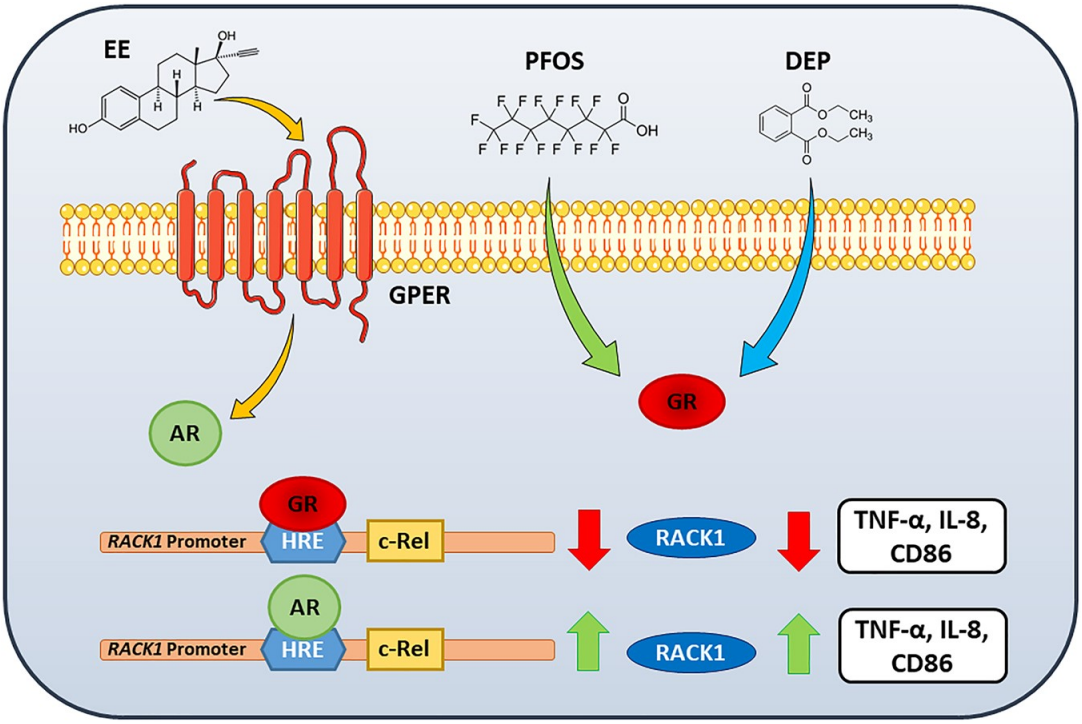

Figure 7. Proposed mechanism of action of EE, PFOS and DEP on RACK1 expression and its related immune effects. EE modulates the regulation of RACK1 expression through the binding of GPER which lead to AR activation and ultimately to RACK1 increase expression, CD86 and cytokines release. PFOS and DEP reduces RACK1 expression through their binding to GR thus determining the significant decrease of CD86 and cytokines release (see text for details).

Conclusions

The high exposure to EE, DEP and PFOS, as a consequence of their wide use and/or their elevated environmental persistency, represents a critic public health issue of primary interest. Indeed, since exposure to different hormone-active substances can have unintended effects on the immune system (Buoso et al., 2020b), new and rapid predictive and screening

methods, both *in silico* and *in vitro*, are now required for the increased interest in the immunotoxicity hazard identification of chemicals (Pappalardo et al., 2022). Therefore, our *in vitro* strategy, in parallel to *in silico* approaches (e.g., the mathematical model developed for Perfluorinated Alkylate Substances (PFAS) (Pappalardo et al., 2022) could be easily implemented in the evaluation of immunotoxicity hazard identification thanks to its rapid applicability and specific immune-related response.

Our *in vitro* strategy comprises three sequential steps: (1) initial screening step via molecular modelling, docking simulation and preliminary evaluation of the investigated EDC affinity for steroid hormones receptors relevant for RACK1 transcriptional regulation by means of promoter activity; (2) evaluation of RACK1 mRNA and protein levels to confirm the impact on RACK1 production of eventual promoter activity alterations; (3) assessment of the resulting biological consequences on the immune functions in terms of quantification of RACK1-correlated immune markers. We previously demonstrated that monitoring RACK1 expression upon EDC exposure can allow to unravel the multifaceted relationship between transcriptional and non-transcriptional events linked with the molecular action of hormonally active compounds (Buoso et al., 2011; 2017a; 2017b; 2020a; 2021).

Finally, considering the important role of the immune system in the tumor microenvironment and that exposure to different EDCs has been directly correlated to a higher hormone-sensitive cancers incidence (Weichenthal et al., 2010; 2012; Ohlander et al., 2022), the endocrine-disrupting effect of multiple EDCs may also interfere with the physiologic hormone signaling and, possibly contributing to cancer development and progression (Buoso et al., 2020b; Masi et al., 2020; 2021).

Supplementary Material

Supplementary data to this article can be found in the online version (doi:10.1016/j.tox.2022.153321), https://www.sciencedirect.com/science/article/pii/S0300483X22002335?via%3Dihub#sec0110.

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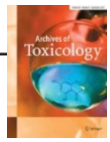
IN VITRO SYSTEMS



Impact of endocrine disruptors on peripheral blood mononuclear cells *in vitro*: role of gender

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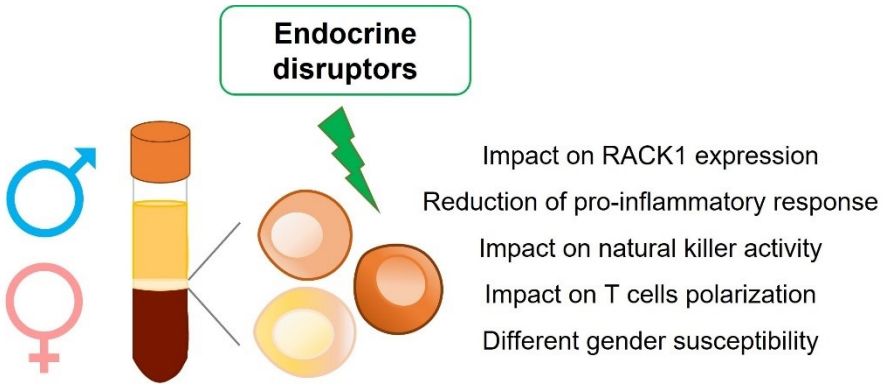
Abstract

Humans can be exposed to endocrine disruptors (EDs) in numerous ways. EDs can interfere with endogenous hormones at different levels, resulting in numerous adverse human health outcomes, including immunotoxicity. In this regard, this study aimed to investigate *in vitro* the possible effects of EDs on immune cells and possible gender differences. Peripheral blood mononuclear cells from healthy humans, both males and females, were exposed to 6 different EDs, namely atrazine (herbicide), cypermethrin (insecticide), diethyl phthalate (plasticizer), 17α-ethynylestradiol (contraceptive drug), perfluorooctanesulfonic acid (persistent organic pollutant), and vinclozolin (fungicide). We evaluated the effect of EDs on RACK1 (receptor for activated C kinase 1) expression, considering it as a bridge between the endocrine and the immune system, and putatively used as screening tool of immunotoxic effects of EDs. The exposure to EDs resulted at different extent in alteration in RACK1 (receptor for activated C kinase 1) expression, pro-inflammatory activity, natural killer lytic ability, and lymphocyte differentiation, with sex-related differences. In particular, diethyl phthalate and perfluorooctanesulfonic acid resulted the most active EDs tested, with gender differences in terms of effects and magnitude. The results from our study evidenced the ability of EDs to directly affect immune cells.

Graphical abstract



Keywords

Endocrine active compound, immunomodulation, immunotoxicity, peripheral blood mononuclear cells, T cells, NK cells, sex effects

Introduction

According to the definition by World Health Organization (WHO), 'an endocrine disruptor (ED) is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations (WHO, 2002)'. Compounds with possible endocrine activities can be found in consumer products, food-contact materials, plasticizers, pharmaceuticals, and pesticides (Kuo et al., 2012). Therefore, humans are daily exposed to EDs (Yilmaz et al., 2020). Hormones modulate the homeostasis of many systems, including the immune system. The link between the endocrine and the immune system is well established, and it is known that the immune function can be targeted by EDs (Greives et al., 2017). Both *in vivo* and *in vitro* evidence highlighted the interaction between EDs and the immune system with multiple targets and processes (Chalubinski and Kowalski, 2006; Rogers et al., 2013; Bansal et al., 2018; Nowak et al., 2019; Masi et al., 2021; Patisaul, 2021; D'Amico et al., 2022a). In this study, we selected six different EDs, covering a range of different uses: diethyl phthalate (DEP), 17α-ethynylestradiol (EE), perfluorooctanesulfonic acid (PFOS), atrazine (ATR), cypermethrin (CYP), and vinclozolin (VIN). DEP is a phthalate ester widely used in industry as plasticizer, fixative, and solvent in cosmetics and packaging materials (Kamrin and Mayor, 1991; Api, 2001). EE is a derivative of estradiol used in contraceptive pills. PFOS is a man-made fluorosurfactant and global pollutant (Liang et al., 2022; EU, 2019). ATR is an herbicide, which, although was banned in Europe, still represents a contamination issue due to its presence in waters and soils (Bhatti et al., 2022). CYP is a pyrethroid insecticide and VIN is a fungicide (Hrelia et al., 1996; Behnami et al., 2021; Kanyika-Mbewe et al., 2020). High levels of these EDs were found in drinking and surface waters, indicating their high exposure to humans (SCHER, 2011; Net et al., 2015; Domingo and Nadal, 2019; Tang et al., 2021; Li et al., 2022a). Adverse effects on the immune system were observed with several EDs, including bisphenols, phthalates, and several pesticides (Patisaul, 2021; Schjenken et al., 2021). These substances can interfere with the development and function of the immune system, acting on both innate and adaptive responses (Ahmed, 2000; Chalubinski and Kowalski, 2006; Rogers et al., 2013; Bansal et al., 2018; Buoso et al., 2021; Galbiati et al., 2021; Maddalon et al., 2022). Within the selected EDs, DEP is a phthalate compound, and in general thus family is considered to have endocrine disrupting properties (Hlisníková et al., 2020). The ability of DEP to mimic estrogen and activate the estrogen receptor has been assessed (Fiocchetti et al., 2021). Regarding the effects on the immune system, the few literature data available suggest a possible effect on it, like the induction of immune-related genes (Xu et al., 2013), but being a phthalate, a similar action is

suggested (Hansen et al., 2015). EE, being a drug used for birth control, has effects on the endocrine system, mainly regarding estrogen pathways. Its adverse effects on the immune system were observed in animal models, but studies in humans are sparse (Klinger et al., 2000; Cabas et al., 2012; Massart et al., 2014; Kernen et al., 2022). PFOS has been linked to both thyroid and reproductive dysfunctions (Coperchini et al., 2017; Tarapore et al., 2021), being able to affect hormone receptors and genes related to endocrine function (Du et al., 2013). Furthermore, its adverse action on immunity has been extensively investigated (Qazi et al., 2010; Guo et al., 2019; Torres et al., 2021; Liang et al., 2022), indicating the reduced antibody response following vaccination as the critical effects (EFSA Panel on Contaminants in the Food Chain, 2018). ATR, which was associated with reproductive dysfunctions (Chevrier et al., 2011; Hayes et al., 2011; Goodman et al., 2014; Namulanda et al., 2017; Almberg et al., 2018; Griffiths et al., 2022; Owagboriaye et al., 2022), due to its ability to affect androgens and estrogen levels (Trentacoste et al., 2001; Eldridge et al., 2008), is able also to affect immune functionality, mainly inducing immunosuppression and acting on T cells (Filipov et al., 2005; Pinchuk et al., 2007; Rowe et al., 2008; Zhao et al., 2013; Lee et al., 2016; Chang et al., 2021; Galbiati et al., 2021). CYP is considered able to alter immune functionality in rats (Liu et al., 2006) and exert myelotoxicity in human cells (Mandarapu and Mrakhya, 2015). The endocrine effects of CYP are debated, and several evidences indicate its ability to interfere with the endocrine system (Jin et al., 2011; Singh et al., 2020; Irani et al., 2022; Li et al., 2022b), but recently it has been classified as unlikely to cause endocrine disruption (EC, 2019). Finally, VIN effects on the endocrine system have been reported, evidencing altered male reproduction as the main effect, inducing a lower sperm quality and number, epididymal morpholohical changes, and prostate abnormalities (Anway and Skinner, 2008; Paoloni-Giacobino, 2014; Feijó et al., 2021). Regarding the action on the immune system, only few information are available, namely its ability to interact with NF-κB and with lymphocyte activity, increasing T and B cells percentage, while decreasing NK cells (White et al., 2004; D'Amico et al., 2022b).

The EDs have been selected based on their different endocrine targets (i.e., hormone receptors, enzymes, hormone synthesis). Indeed, EE is able to interfere with the estrogen pathway, DEP can act on estrogen and glucocorticoid pathway, while PFOS can impair the estrogen, glucocorticoid and thyroid signaling (Masi et al., 2022). Furthermore, ATR is able to interact mainly on the androgen pathway, but it can also interfere with estrogen and aromatase activity, whereas CYP acts indirectly on the androgen receptors, and VIN can act both on the androgen and estrogen pathways (Maddalon et al., 2022). The selected EDs were analyzed to assess their ability to interfere with a protein that represents a bridge between the endocrine and

the immune system. Recently, we demonstrated that these EDs were able to modulate monocytes' activation *in vitro*, through the modulation of RACK1 (receptor for activated C kinase 1) (Maddalon et al., 2022; Masi et al., 2022). This latter was identified as a target of EDs in the immune system and as a possible link between these two systems, since it is involved in the activation of innate immunity and represents a relevant target of endocrine action (Buoso et al., 2017; 2020). RACK1 expression, being under hormonal control, could be able to integrate the signals of different EDs and therefore influencing the immune response. This protein could serve as screening tool to evaluate the immunotoxic profile of EDs.

The purpose of this study was to evaluate the *in vitro* effects of the selected EDs on several immunological endpoints using primary cultures of human peripheral blood mononuclear cells (PBMC). Their ability to modulate RACK1 expression, to interfere with natural killer (NK) cell activity, and lymphocyte differentiation, focusing on $CD4^+$ and $CD8^+$ cells, was investigated.

Material and methods

Tested chemicals

The selected EDs are listed in Table 1, together with their acronym, CAS number, and the tested concentration.

Table 1
ED tested: name, acronym, CAS number, and concentration used.

Name	Acronym	CAS N°	Concentration (µM)
Diethyl phthalate	DEP	84-66-2	1
17α-ethynylestradiol	EE	57-63-6	0.001
Perfluorooctanesulfonic acid	PFOS	1763-23-1	0.2
Atrazine	ATR	1912-24-9	1
Cypermethrin	CYP	52315-07-8	1
Vinclozolin	VIN	50471-44-8	0.1

All the substances were purchased from Sigma-Aldrich (St. Louis, Missouri, US) at the highest purity available. They were dissolved in dimethyl sulfoxide (DMSO; CAS # 67-68-5, purity ≥ 99.5%) at 10 mM stocks that were stored at -20 °C. Working concentrations were then obtained diluting stock solutions for each treatment. The final DMSO concentration in culture

medium was ≤ 0.2%, and it was used as solvent control. Concentrations were selected based on previous studies conducted on THP-1 cell line (Maddalon et al., 2022; Masi et al., 2022), as the lowest concentration active on at least one immune parameter. Preliminary experiments were conducted to ensure that the concentrations used were not cytotoxic, as assessed by propidium iodide (PI) staining and flow cytometric analysis (data not shown).

PBMC treatment with EDs

PBMCs were obtained by Ficoll gradient centrifugation from buffy coats from anonymous healthy blood donors of both sexes, purchased from the Niguarda Hospital in Milan (Italy). Following centrifugation, PBMC layers were removed, and after washings with Dulbecco's Phosphate Buffered Saline (PBS), isolated cells were diluted to 10^6 cells/mL or $5x10^6$ cells/mL, based on the treatment, in RPMI-1640 without phenol red, containing 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, 10 µg/mL gentamycin, 50 µM 2-mercaptoethanol, supplemented with 5% heat-inactivated dialyzed fetal bovine serum (culture medium) and cultured at 37 °C in a 5% CO_2 incubator.

For the evaluation of RACK1 expression, PBMCs (10^6 cells/mL) were exposed to the different EDs or DMSO (vehicle control) for 24 hours at 37 °C in a 5% CO_2 incubator. RACK1 protein expression was evaluated by Western blot analysis and normalized to β-tubulin expression.

To evaluate the expression of CD86 and CD54, and the release of IL-8 and TNF-α, PBMCs (10^6 cells/mL), following 24 hours of exposure to EDs or DMSO, were stimulated with lipopolysaccharide (LPS) from *Escherichia coli* serotype 0127:B8 (Sigma-Aldrich) at the final concentration of 100 ng/mL for further 24 hours at 37 °C in a 5% CO_2 incubator.

To evaluate NK cell lytic activity, PBMCs ($5x10^6$ cells/mL) were exposed to the different EDs or DMSO for 24 hours. As target cells, K562 cells (AddexBio, US) stained with CellTrace™ CFSE (Invitrogen, Waltham, Massachusetts, US) were used. Briefly, 500 µL of K562 at the concentration of 10^6 cells/mL were centrifuged, the CellTrace™ CFSE was added to the cell pellet (1:1000 dilution) and incubated for 15 minutes at 37° C protected from light. After the incubation time, the reaction was stopped by adding culture medium containing 5% heat-inactivated dialyzed fetal bovine serum. CellTrace™ CFSE-stained K562 cells were then kept to the concentration of 10^5 cells/mL and co-cultured together with EDs/DMSO-exposed PBMCs. Three different ratios of effector (PBMC) and target (K562) cells were used: 50:1, 25:1, 12.5;1, maintaining fixed concentration of K562 cells. The cellular concentrations are reported in Table 2.

Table 2
Ratio between effector and target cells to assess NK cell's lytic activity.

Treatment ratio (effector:target)	Effector (PBMC)	Target (K562)
50:1	5 x 10^6 cells/mL	10^5 cells/mL
25:1	2.5 x 10^6 cells/mL	10^5 cells/mL
12.5:1	12.5 x 10^6 cells/mL	10^5 cells/mL

The cells are then co-cultured for 4 hours at 37 °C in a 5% CO_2 incubator.

For the assessment of T cell differentiation, 25 µL of Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation (ThermoFisher, Waltham, Massachusetts, US) were added to 10^6 PBMCs. Cells were then exposed to the EDs or DMSO and incubated for 4 days at 37 °C in a 5% CO_2 incubator.

Immunoblot analysis of RACK1 expression

After 24 hours of treatment, cells were harvested, washed and lysed in homogenization buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 and protease inhibitor). Protein content was assessed using the Bradford method. Cell lysates were mixed with sample buffer (125 mM Tris-HCl pH 6, 8.4% sodium dodecyl sulfate, 20% glycerol, 6% β-mercaptoethanol, 0.1% bromophenol) and denatured at 95° C for 10 minutes. 10 µg of extracted proteins were electrophoresed into 10% SDS-PAGE under reducing conditions and then transferred to PVDF membranes. The membranes were blocked with 1X TBS, 0.1% Tween-20, and BSA (5% w/v), and the expression of RACK1 and β-tubulin assessed following over-night incubation of the relative antibodies (dilution 1:1000) and following 1 hour incubation of secondary IgG peroxidase-conjugated antibodies (dilution 1:15000). Anti-human-RACK1 mouse antibody was purchased from Santa Cruz Biotechnology (B-3 clone; Dallas, Texas, US), anti-human-β-tubulin rabbit antibody was purchased from Novus Bio (R&D Systems, Minneapolis, Minnesota, US), goat anti-mouse IgG was purchased from Sigma-Aldrich, and goat anti-rabbit IgG was purchased from Bio-Rad. All the antibodies were diluted in 1X TBS, 0.1% Tween-20, and BSA 5% w/v. The band visualization was performed using Clarity western ECL blotting substrates (Bio-Rad, Hercules, California, US). Blot images were acquired with Image Lab Software version 4.0 (Bio-Rad) using the Molecular Imager Gel Doc

XR (Bio-Rad) and quantified normalizing on β-tubulin expression levels. The stimulation index (SI) was calculated on DMSO-treated PBMCs (vehicle control) set at 100.

Flow cytometric analysis of CD86 and CD54 expression

After 48 hours of treatment, PBMCs were centrifuged, and the supernatants were stored at -20° C for the assessment of cytokine release. Cell pellets were washed with PBS, suspended in 200 µl of PBS, and stained at 4° C in the dark for 30 minutes with specific PE-conjugated antibody against human CD54 or FITC-conjugated antibody against human CD86 or with isotype control antibodies, following supplier's instructions. All the antibodies were purchased from BD Biosciences (Franklin Lakes, New Jersey, US). After incubation, cells were centrifuged and suspended in 500 µL of PBS. The % of positive cells was analyzed using Novocyte 3000 flow cytometer (Acea Bioscience Inc., Agilent Technologies, Santa Clara, California, US) and data were quantified using Novocyte software (Acea Bioscience Inc.). 10'000 viable cells were analyzed for % of positivity to the respective marker. The % of isotype control was subtracted from the % of CD86/CD54 stained cells. Changes in CD86/CD54 expression are reported as SI calculated on DMSO-treated PBMC (vehicle control) set at 1. The gating strategy is reported in Supplementary Figure 1 and representative dot plots are reported in Supplementary Figure 2.

Cytokine production

From the same treatments in which surface markers expression was assessed, the cell-free supernatants were kept at -20° C for cytokine evaluation through commercially available ELISA kits. The ELISA kits to assess the release of IL-8 and TNF-α were purchased from ImmunoTools (Friesoythe, Germany) and R&D Systems, respectively. The limits of detection were 8 pg/mL for IL-8 and 7.8 pg/mL for TNF-α, respectively. Changes in IL-8/TNF-α release are reported as SI calculated on DMSO-treated PBMC (vehicle control) which was set at 1.

Assessment of the lytic activity of NK cells

After the treatment with EDs and the co-culture with CellTrace^{TM} CFSE-stained K562 cells, the plate content was transferred to flow cytometrical tubes and PI (5 nM) was added to each tube. The % of cells positive to PI, within CFSE-stained K562 cells was acquired using Novocyte 3000 flow cytometer. 1'000 CFSE-positive cells were analyzed for % of positivity to PI, indicative of dead K562 cells. The gating strategy is reported in Supplementary Figure 3 and representative dot plots are reported in Supplementary Figure 4.

Flow cytometric analysis of T cells differentiation

After 4 days of treatment, the Protein Transport Inhibitor Cocktail (Invitrogen) was added to each well, to stop cytokine secretion (1 µL every 500 µL of cell culture), for 5 hours at 37° C in a 5% CO_2 incubator. Following incubation, cells were harvested and washed, magnetic beads were removed using DynaMag 15 (Invitrogen) and washed again. The staining for surface markers was then performed, according to Table 3, for 30 minutes at room temperature avoiding the light.

Table 3
Antibodies used to stain PBMC, their dilution, the channel used to acquire them at the flow cytometer, and the supplier.

Antigen	Clone	Dilution	Channel	Supplier
		Surface antibodies		
GITR	DT5D3	1:200	BL2	Miltenyi
CD8	OKT8	1:200	VL2	ThermoFisher
CD4	OKT4	1:200	VL3	ThermoFisher
CD25	BC96	1:200	VL4	ThermoFisher
		Intracellular antibodies		
IL-4	8D4-8	1:200	VL1	ThermoFisher
IL-9	MH9D1	1:200	BL3	ThermoFisher
IL-10	JES3-9D7	1:200	BL1	ThermoFisher
IL-17A	eBio64DEC17	1:200	RL3	ThermoFisher
IL-22	IL22JOP	1:200	RL1	ThermoFisher
IFN-γ	4S.B3	1:200	RL2	ThermoFisher
FoxP3	236A/E7	1:200	BL4	ThermoFisher

After incubation, cells were washed and fixed using the fixation reagent for 45 minutes on ice avoiding the light. After washing, cells were permeabilized using permeabilization reagent for 5 minutes on ice avoiding the light and washed again, following supplier's instruction (eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set - Invitrogen). Then, cells were stained for intracellular proteins, according to Table 3, for 3 hours and 30 minutes at 4 °C avoiding the light. After that, 1 ml of PBS with 1% of fetal bovine serum was added, the cells were filtered using pre-separation filters (70 µM – Miltenyi Biotec, US) and the samples

acquired using the Attune NxT flow cytometer (ThermoFisher). Data were further analyzed through FlowJo V.10.8.1 (BD Biosciences). The % of positive cells to the different markers was retrieved. Data are presented as Log2 values calculated on DMSO-treated PBMC (vehicle control) which is set at 0. The gating strategy is reported in Supplementary Figure 5.

t-SNE analysis of T helper cell subpopulations

Following conventional analysis, through FlowJo, to deeply investigate T cell subpopulations, the t-distributed stochastic neighbor embedding (t-SNE) algorithm was applied. All the .FCS files were merged in two concatenated files as follows: a) DMSO, DEP, EE, and PFOS from 6 donors (3 males and 3 females) due to their high presence in the environment and high exposure levels to humans to and b) DMSO, ATR, CYP, and VIN from 6 donors due to their belonging to the pesticide class. Samples were down-sampled to 10'000 cells, and after the gating of the single populations within $CD4^+$ cells, as performed for the conventional analysis (Supplementary Figure 5), the t-SNE was run. Briefly, a Barnes-Hut t-SNE method, with a perplexity of 50 and 3000 iterations was chosen. The results were visualized in 2D t-SNE maps. The single treatment conditions were successively gated through the sample ID. Clusters of cells based on the expression level of the different analyzed markers were manually created on merged data, and the % of gated cells in each cluster was further analyzed for the single treatment conditions. Within $CD4^+$ cells, it was possible to recognize different subpopulations for IFN-γ, IL-4, IL-9, FoxP3, and GITR. For IL-10, IL-17A, IL-22, and CD25 it was not possible to define distinct sub-populations. Only the subpopulations common to all the 6 donors were further evaluated, and the % of cells present in the clusters was expressed as a Log2 value calculated on DMSO-treated PBMC, which is set at 0. The t-SNE density plots are reported in Supplementary Figures 6 and 7.

Statistical analysis

Statistical analysis was performed using Prism version 9.4.0 (GraphPad Software, San Diego, California, US). Data were reported as mean ± standard error (SEM) or as median of 3 (only for T cells differentiation) or 5 male and female donors, as reported in figure legends. To calculate differences between the treatment, t-test was applied, after the assessment of the normal distribution of the data through the Shapiro-Wilk test. Differences were considered statistically significant at $p \leq 0.05$.

Results

EDs modify RACK1 expression in human PBMC

First, to confirm previous results obtained in THP-1 cells (Maddalon et al., 2022; Masi et al., 2022), the ability of EDs to interfere with RACK1 expression was evaluated, being RACK1 a bridge between the immune and the endocrine systems, as previously explained.

Short-term exposure to different EDs resulted in changes in the expression of RACK1 in human PBMC, from both male and female healthy donors (Figure 1). In particular, DEP, PFOS, ATR, and CYP exposure was able to induce a statistically significant reduction of RACK1 expression in PBMCs from both males and females. Whereas EE and VIN were able to down-regulate RACK1 expression in females only. Furthermore, a sex bias in the response to EE exposure was found, highlighting a possible dimorphism in its action. While in female donors all the EDs induced a reduction in RACK1 activation, and possibly a reduced immune activation, EE and VIN had no statistically significant effect in male donors at the concentration tested. The effects obtained in primary PBMCs are in line with previous results obtained in THP-1 cells. THP-1 is a human monocytic cell line derived from acute monocytic leukemia of a male patient, and results obtained with male donors are closer to the one observed in THP-1 cells. In particular, DEP, PFOS, ATR, and CYP reduced RACK1 expression in both models, whereas an increase with EE and no effect with VIN were obtained in THP-1 cells (Maddalon et al., 2022; Masi et al., 2022). The modulation of RACK1 induced by the selected EDs is also in line with the previously performed molecular docking analysis. Indeed, DEP and PFOS revealed to activate glucocorticoid receptor, that in turn reduced RACK1 expression (Masi et al., 2022). Also ATR and CYP were able to decrease RACK1 expression, but the mechanism is linked to an anti-androgenic activity: ATR is able to competitively antagonize androgen receptor, whereas CYP acts in an indirect way, reducing androgen receptor expression and IL-6 release (Maddalon et al., 2022). Also VIN is an anti-androgenic compound, but it can also activate GPER (G-protein-coupled estrogen receptor) that in turn is able to activate androgen receptor (Maddalon et al., 2022). This dual mechanism could also explain the different effect observed in male and female PBMCs. Finally, EE is characterized by an estrogenic activity, linked to the action on both GPER and androgen receptor (Masi et al., 2022). Therefore, an increase RACK1 expression would be expected, as in the case of male donors, although not statistically significant. The involvement of GPER in the activity of EE and VIN could be an hypothesis of the different gender effect.

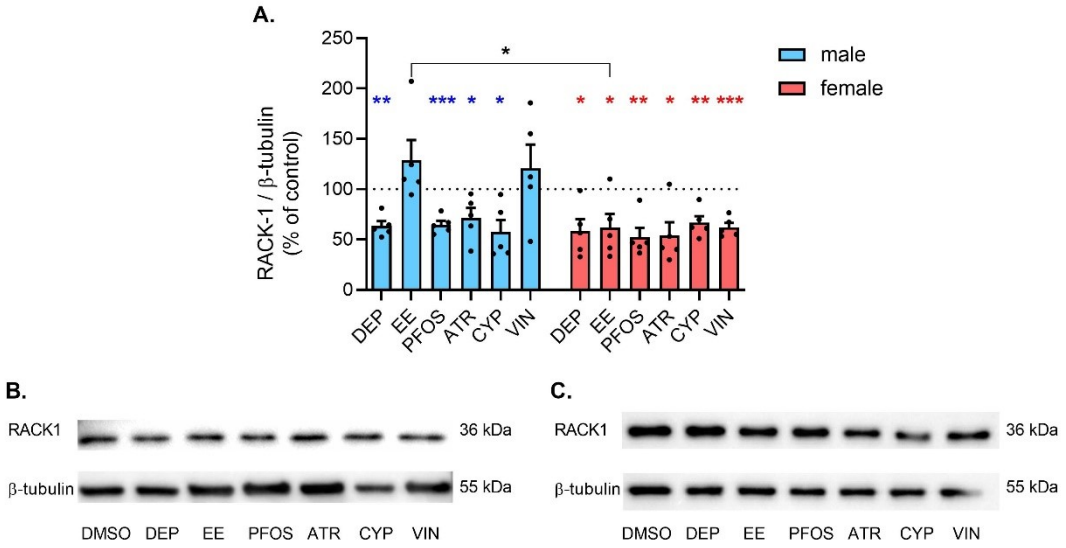


Figure 1. Effects of EDs on RACK1 expression. Male and female PBMC were exposed to the different EDs or DMSO (vehicle control) for 24 hours. RACK1 protein level was evaluated by Western blot analysis and normalized to β-tubulin expression (A). Data are referred to each sample DMSO-treated PBMC (vehicle control), which is set at 100 (dotted line). Results are expressed as mean ± SEM of 5 male donors (light blue) and 5 female donors (pink). Each dot represents the expression of the single donor. Statistical analysis was performed following unpaired t-test with Welch's correction, with **p*≤0.05, ***p*≤0.01, ****p*≤0.001 *vs* DMSO. Differences between males and females were assessed through unpaired t-test, with *p≤0.05 between males and females for EE exposure. (B and C) Representative Western blots for RACK1 and β-tubulin expression induced by EDs exposure in a representative male (B) and female (C) donor.

Effects of EDs on RACK1-related pro-inflammatory markers and cytokines production

Following the assessment of their ability to modulate RACK1 expression, the expression of CD86 and CD54 and the release of IL-8 and TNF-α were evaluated following LPS stimulation (Figure 2). CD86 and CD54 are two surface proteins important in the process of T cell activation, and their increase upon LPS stimulation in PBMC was observed (Fig. 2A, 2B).

EDs were able to modulate CD86 and CD54 expression in PBMC, with gender differences observed. The exposure to DEP was able to reduce LPS-induced CD86 and CD54 expression in male donors (Fig. 2A, 2B). PFOS decreased CD86 expression in both sexes (Fig. 2A). ATR and CYP exposure reduced, respectively, CD86 and CD54 expression, only in female donors (Fig. 2A, 2B). Similarly, the release of the pro-inflammatory cytokines IL-8 and TNF-α was increased by LPS treatment and it was modified by EDs pre-incubation (Fig. 2C, 2D). In detail, PFOS was able to reduce both IL-8 and TNF-α release in male donors only, and in case of TNF-α, a statistically significant difference between males and females was observed (Fig. 2C, 2D). Sex differences were obtained also following DEP stimulation, inducing a decrease in

IL-8 release in females only, and of TNF-α in both sexes, but with a higher susceptibility of female donors (Fig. 2C, 2D).

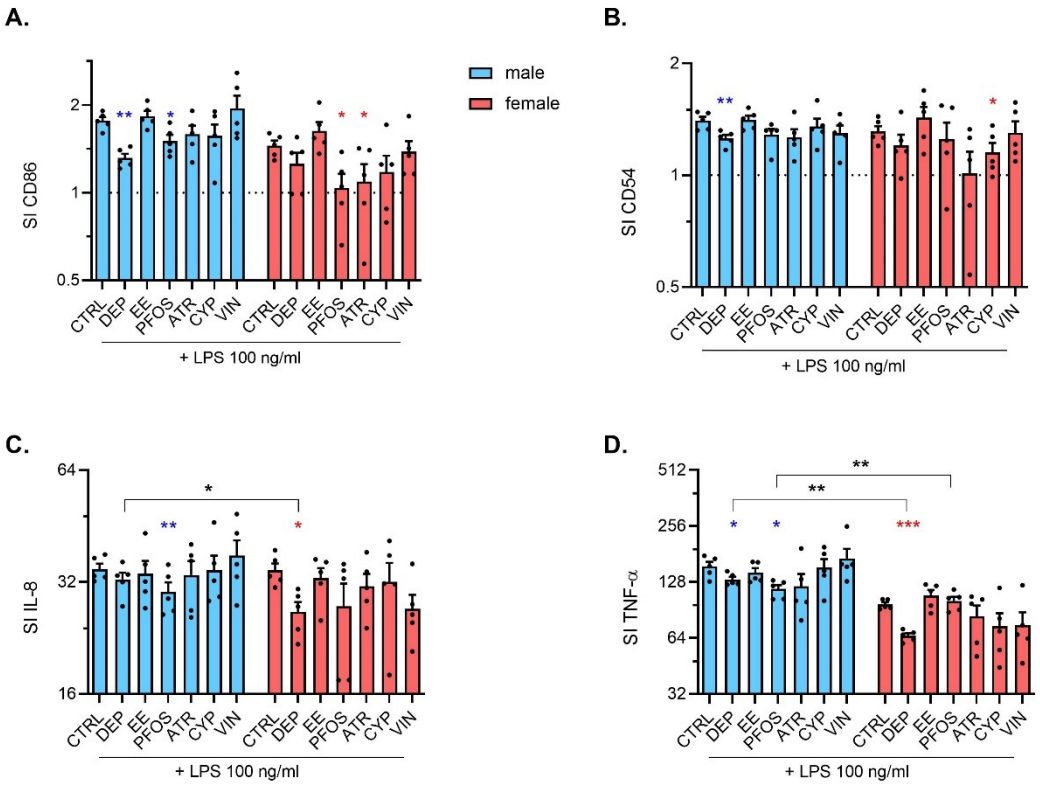


Figure 2. Effects of EDs on RACK1-related pro-inflammatory cytokines and activation markers. Male and female PBMC were exposed to the different EDs or DMSO (vehicle control) for 24 hours and then to LPS 100 ng/ml for further 24 hours. CD86 (A) and CD54 (B) expression were evaluated by flow cytometric analysis, whereas IL-8 (C) and TNF-α (D) release was assessed by ELISA. Results are expressed as SI calculated on DMSO-treated LPS-unstimulated PBMC set at 1 (dotted line) of mean ± SEM of 5 male donors (light blue) and 5 female donors (pink). Each dot represents the expression of the single donor. Note that a semi-log scale was used, to better show the results. Statistical analysis was performed following paired t-test, with *$p \leq 0.05$, **$p \leq 0.01$ *vs* DMSO-treated LPS-stimulated. Differences between males and females were assessed through unpaired t-test, with *$p \leq 0.05$, **$p \leq 0.01$.

Effect of EDs on NK cells' lytic activity

NK cells are cytotoxic cells able to kill target cells, such as tumoral or virus-infected cells. PBMC, in which cells with NK activity are present, were treated with EDs for 24 hours, and then co-cultured with CFSE-stained K562 cells (target cells) for 4 hours (Figure 3).

Different effector and target cell ratios were used: 50:1 (Fig. 3A), 25:1 (Fig. 3B), and 12.5:1 (Fig. 3C). Overall, only modest effects in NK cell activity following EDs exposure were observed, most of them in males. The most effective compound was EE, which was able to increase male donors' NK cells activity at all the tested effector:target ratios (Fig. 3A, 3B, 3C), with sex differences statistically significant at 12.5:1. Also PFOS exhibited sex-differences in its action, specifically it was able to increase NK cells activity in males (Fig. 3A), and to

decrease it in females (Fig. 3B). Minor effects were also observed in cells from healthy donors exposed to ATR, DEP, and VIN. DEP and VIN reduced NK cell activity in both sexes, males at the lowest ratio and females at the middle one, respectively (Fig. 3B, 3C). Instead, ATR was able to slightly increase target cell death in male donors only at the highest ratio (Fig. 3A). With the exception of CYP, every ED was able to affect NK cells activity at least at one effector and target cell ratios, with sex-differences observed for EE and PFOS.

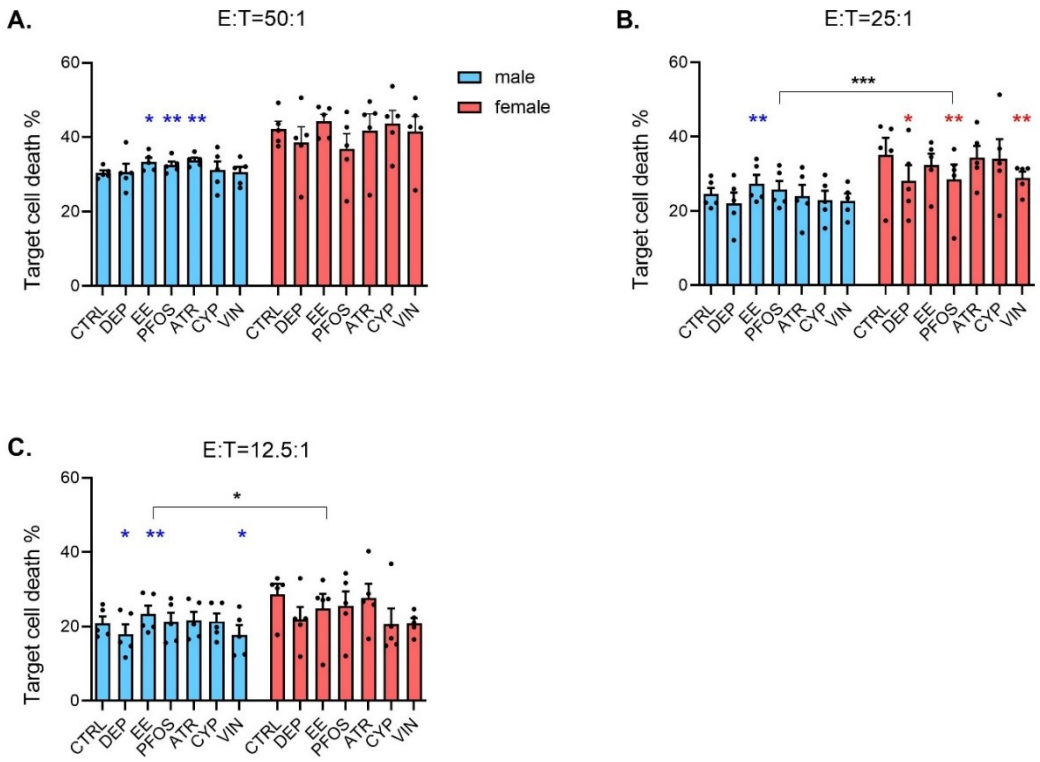


Figure 3. Effect of EDs on NK cell activity. Male and female PBMC were exposed to the different EDs or DMSO (vehicle control) for 24 hours and then co-cultured with K562 cells for 4 hours at three different ratio between effector and target cells: 50:1 (A), 25:1 (B), and 12.5:1 (C). Target cell death was assessed by flow cytometry. Results are expressed as mean ± SEM of 5 male donors (light blue) and 5 female donors (pink) of % of dead cells (% of cells positive to PI staining within CFSE-positive cells). Each dot represents the value of the single donor. Statistical analysis was performed following paired t-test, with *$p \leq 0.05$, **$p \leq 0.01$ *vs* CTRL (DMSO-treated PBMC co-cultured with K562 cells). Differences between males and females were assessed through unpaired t-test, with *$p \leq 0.05$, ***$p \leq 0.001$.

To better express the lytic activity, integrating the three different ratios used, the results are also expressed as lytic unit 35 (LU_{35}), meaning the ratio of effector cells necessary to kill the 35% of target cells. The LU_{35} are shown in Table 4. Both PFOS and ATR exposure in male donors resulted in a reduction in the lytic unit required to kill 35% of target cells, with respect to the control. Therefore, these EDs increased the lytic ability of male effector cells. Instead, in female donors DEP and VIN induced an increase of LU_{35}, reducing therefore the lytic ability

of female effector cells. Regarding sex differences, it can be noted that DEP, EE, PFOS, and VIN show different trend based on donors' sex, generally reducing LU_{35} in males and increasing them in females, meaning their ability to increase lytic activity in males, while reducing it in females.

Table 4
Lytic unit 35 (LU35) expressed as mean ± standard deviation. The regression linear fit method was used to calculate LU35 for each condition. Statistical analysis was performed following paired t-test, with *$p \leq 0.05$, **$p \leq 0.01$ vs CTRL, and differences between males and females were assessed through unpaired t-test, with #$p \leq 0.05$.

	Male donors	**Female donors**
CTRL	72.899 ± 12.661	25.408 ± 13.951
DEP	65.758 ± 17.242 #	44.322 ± 25.416 * #
EE	56.495 ± 13.897 #	29.888 ± 9.805 #
PFOS	63.871 ± 13.576 ** #	44.031 ± 28.870 #
ATR	55.485 ± 8.834 *	39.429 ± 36.992
CYP	94.498 ± 68.051	32.447 ± 19.383
VIN	71.162 ± 29.593 #	44.245 ± 24.615 * #

Effects of EDs on $CD4^+$ and $CD8^+$ cells differentiation

The main population of lymphocytes present in PBMCs are T lymphocytes. They can be divided into T helper (Th) cells and cytotoxic T cells, based on their expression of CD4 and CD8, respectively. Both populations can be further divided in subsets based on the expression of cytokines, transcription factors and surface markers. In particular, within circulating T helper cells, Th1, Th2, Th9, Th17, Th22, and regulatory T (Treg) cells can be found. Following activation, the first 5 populations may be recognized by the expression of IFN-γ, IL-4, IL-9, IL-17, and IL-22, respectively. Whereas Treg can be recognized by the expression of CD25, GITR, FoxP3, and IL-10. However, conventional activated $CD4^+$ cells are $GITR^+$ and can express IL-10. The four-day activation with anti-CD3/CD28 coated magnetic beads induced activation, proliferation and differentiation/polarization of $CD4^+$ and $CD8^+$ T cells present in PBMC and EDs exposure induced slight changes in $CD4^+$ (Figure 4) and $CD8^+$ lymphocyte differentiation (Figure 5).

Regarding $CD4^+$ lymphocytes polarization, EE and VIN induced a slight increase in the percentage of cells $CD4^+IL\text{-}17^+$ in PBMC from male donors only (Fig. 4E). DEP, EE, and PFOS

induced a decrease of the percentage of $CD4^+IL\text{-}22^+$ cells in PBMC from male donors, and a sex-difference could be observed for DEP and EE (Fig. 4F). Instead, no significant modulation of the percentage of $CD4^+IFN\text{-}\gamma^+$, $CD4^+IL\text{-}4^+$, $CD4^+IL\text{-}9^+$, and $CD4^+IL\text{-}10^+$ cells has been observed (Fig. 4A, B, C, D).

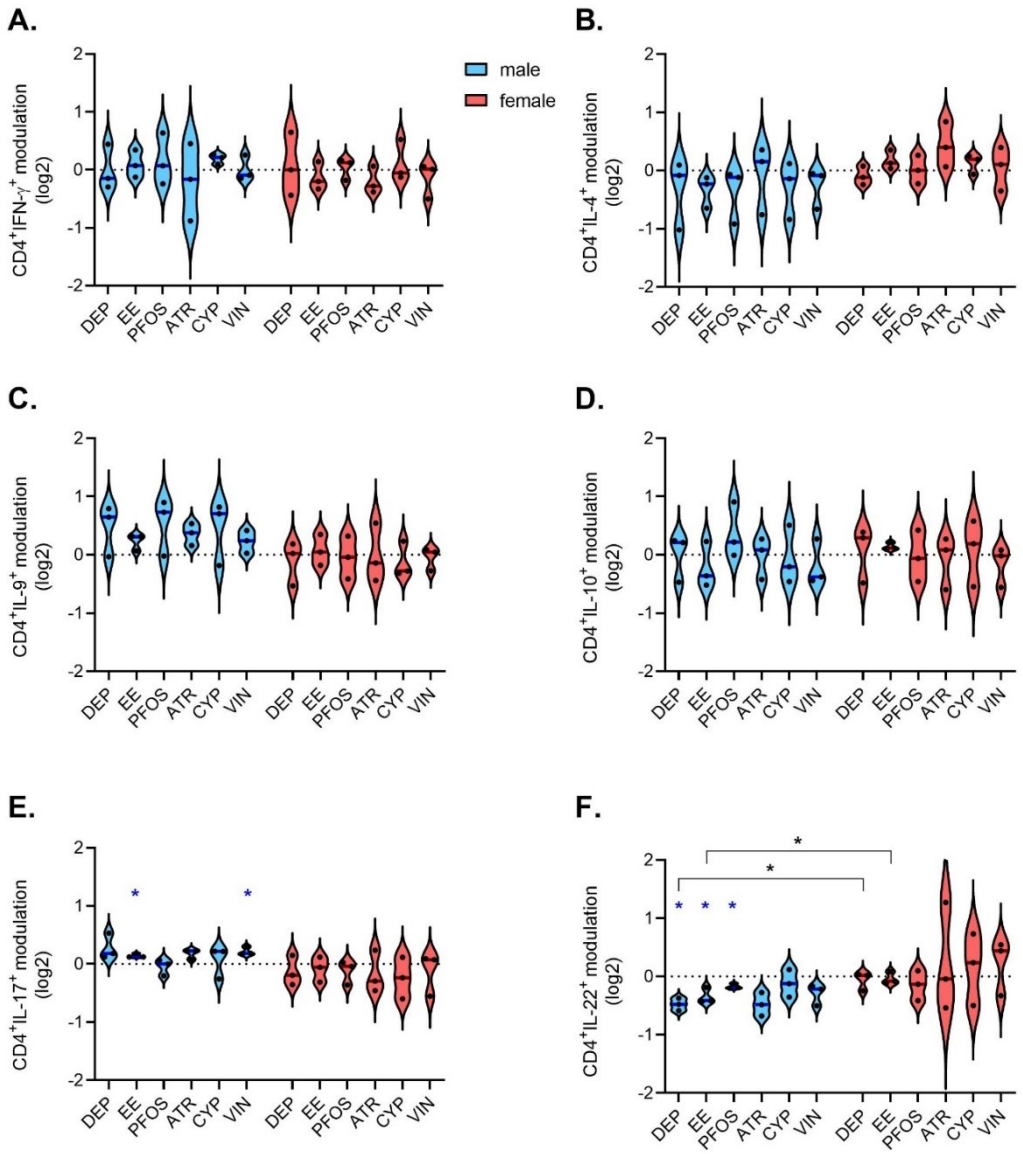


Figure 4. Modulation by EDs of cytokine-positive helper T cells. PBMC from males and females were exposed to the different EDs or DMSO (vehicle control) together with anti-CD3/anti-CD28 coated magnetic beads for 4 days. On gated $CD4^+$ lymphocytes, the cytokine-positive cells were identified by evaluating the expression of IFN-γ (A), IL-4 (B), IL-9 (C), IL-10 (D), IL-17 (E), and IL-22 (F) through flow cytometry. Results are shown as violin plots, with blue or red lines indicating the median fold modulation *vs* the respective DMSO-treated controls in 3 male (light blue) and 3 female (pink) donors, respectively. Each dot represents the cytokine modulation (log2 values) in a single donor. Statistical analysis was performed following unpaired t-test with Welch's correction, with *$p \leq 0.05$ *vs* DMSO. Differences between males and females were assessed through unpaired t-test (*$p \leq 0.05$).

While for T helper cells, the presence of different subpopulations is widely known and collectively accepted, for cytotoxic T cells only a few pieces of information are available, but they can be generally divided into Tc1, Tc2, Tc9, Tc17, and Tc22, similar to what occurs in T helper cells (Jiang et al., 2021; St Paul et al., 2021).

PBMC exposure to EDs induced some effects also regarding cytotoxic T-cell polarization. In particular, VIN increased the percentage of $CD8^+IL\text{-}9^+$ cells in PBMC from male donors only, with a statistical significance sex difference (Fig. 5C). Similarly to $CD4^+IL\text{-}22^+$ cells, DEP exposure resulted in a decreased percentage of $CD8^+IL\text{-}22^+$ cells in PBMC from male donors, with a sex-difference, that could be observed also following PFOS exposure (Fig. 5F). Instead, no statistically significant effects were observed on $CD8^+IFN\text{-}\gamma^+$, $CD8^+IL\text{-}4^+$, $CD8^+IL\text{-}10^+$, and $CD8^+IL\text{-}17^+$ cells (Fig. 5A, B, D, E).

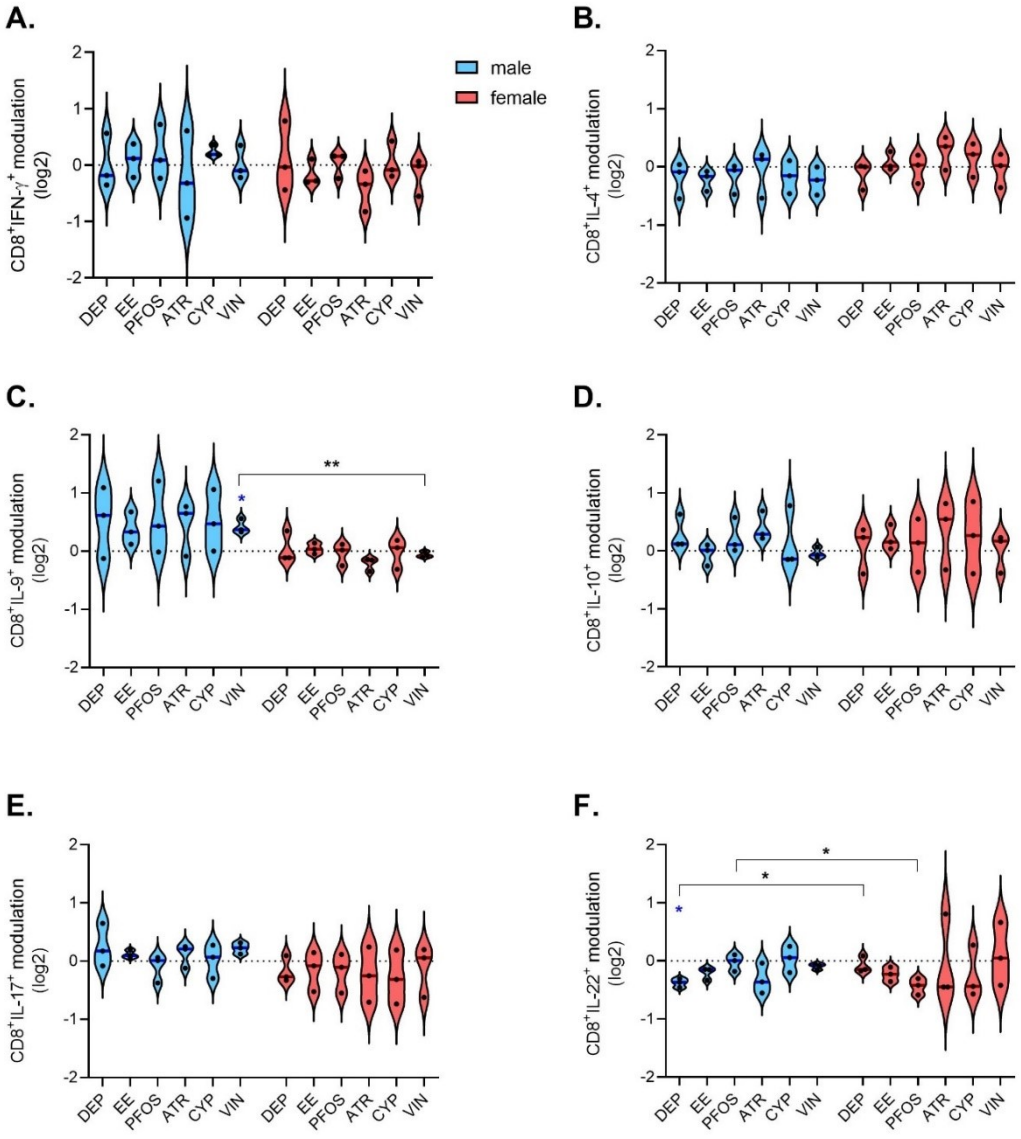


Figure 5. Modulation by EDs of cytokine-positive cytotoxic T cells. PBMC from males and females were exposed to the different EDs or DMSO (vehicle control) together with anti-CD3/anti-CD28 coated magnetic beads for 4 days. On gated $CD8^+$ lymphocytes, the cytokine-positive cells were identified by evaluating the expression of IFN-γ (A), IL-4 (B), IL-9 (C), IL-10 (D), IL-17 (E), and IL-22 (F) through flow cytometry. Results are shown as violin plots, with blue and red lines indicating the median fold modulation *vs* the respective DMSO-treated controls in 3 male (light blue) and 3 female (pink) donors, respectively. Each dot represents the cytokine modulation (log2 values) in a single donor. Statistical analysis was performed following unpaired t-test with Welch's correction, with *$p \leq 0.05$ *vs* DMSO. Differences between males and females were assessed through unpaired t-test (*$p \leq 0.05$ and **$p \leq 0.01$).

As previously mentioned, $CD4^+$ Treg cells express FoxP3 and co-express CD25 and GITR and FoxP3 is a regulatory marker even in $CD8^+$ cells. The role of CD25 and GITR in $CD8^+$ cells is not well understood, but the expression of these molecules in cytotoxic T cells has been documented, and their involvement in immune regulation and immune tolerance promotion has been proposed (Ronchetti et al., 2012; Churlaud et al., 2015; Niederlova et al., 2021).

Regarding $CD4^+$ Treg cells, no effects of the selected EDs have been observed (Fig. 6A, B). Instead, EE, PFOS, and ATR induced a modest decrease of $CD8^+FoxP3^+$ cells in female donors (Fig. 6C), and DEP induced a slight decrease of $CD8^+GITR^+CD25^+$ cells in female donors (Fig. 6D). Therefore, a modest decrease of $CD8^+$ Treg in female donors due to DEP, EE, PFOS, and ATR could be observed.

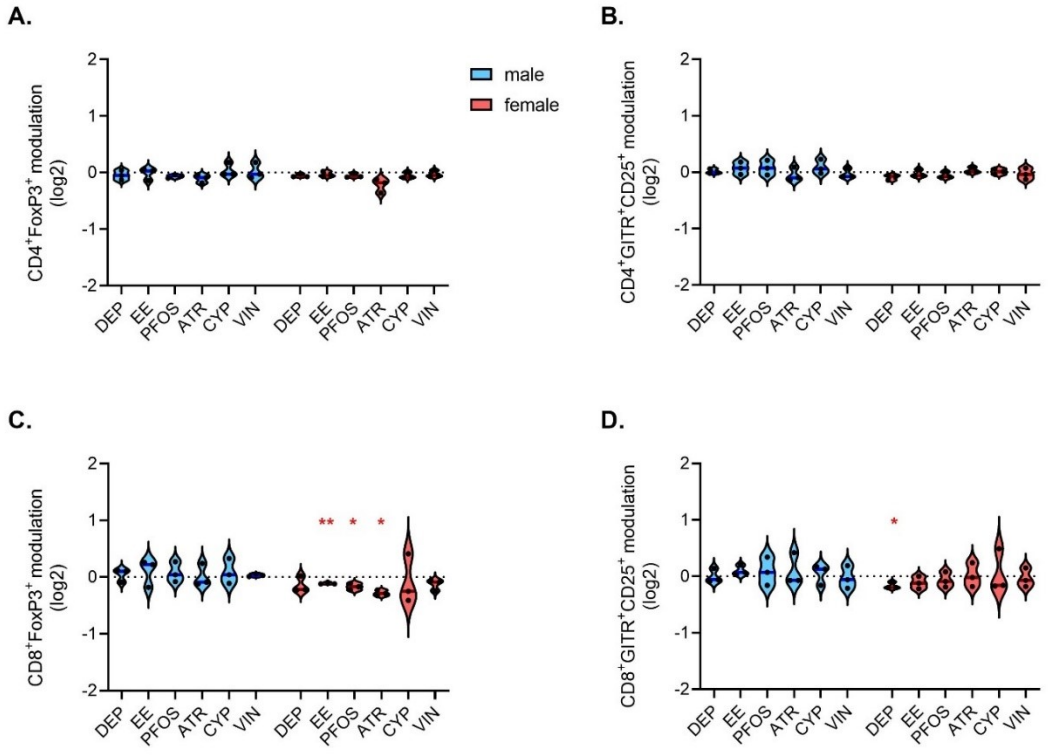


Figure 6. Modulation by EDs of cells expressing Treg-related markers. PBMC from males and females were exposed to the different EDs or DMSO (vehicle control) together with anti-CD3/anti-CD28 coated magnetic beads for 4 days. On gated $CD4^+$ (A, B) and $CD8^+$ (C, D) lymphocytes, FoxP3-positive (A, C) and GITR-CD25 double positive (B, D) cells were identified. Results are shown as violin plots, with blue and red lines indicating the median fold modulation *vs* the respective DMSO-treated controls in 3 male (light blue) and 3 female donors (pink), respectively. Each dot represents the marker modulation (log2 values) in a single donor. Statistical analysis was performed following unpaired t-test with Welch's correction, with $*p \leq 0.05$ and $**p \leq 0.01$ *vs* DMSO.

Sex differences in EDs' modulations of $CD4^+$ subpopulations

Using the canonical elaboration of flow cytometric, data suggested that EDs had few effects on T cell differentiation/polarization. Since it seemed unlikely, a more in-depth analysis was conducted using the t-SNE algorithm that groups cells in subsets, based on the different expression levels of the stained markers. To do this, we divided the EDs into two groups, highly contaminating EDs (DEP, EE, and PFOS) and pesticides (ATR, CYP, and VIN).

By considering data from the 6 donors following DEP, EE, PFOS exposure, the t-SNE analysis found 50 relevant subsets expressing high levels of one marker in $CD4^+$ cells: 8 IFN-γ^+ subsets, 3 IL-4^+ subsets, 14 IL-9^+ subsets, 14 $FoxP3^+$ subsets, and 11 $GITR^+$ subsets. Some of these subsets express more than one marker, as can be observed in Supplementary Figure 6. The mean percentage of cells in each subset after treatment is divided by that in the control solvent for males and females and the values are reported in Figure 7. The mean cell percentage of several subsets was modulated. In males, the percentage of cells present in subset #1 of IL-4^+ cells was decreased following EE exposure. Whereas, DEP exposure in general increased the percentage of IL-9^+ cells (mainly #9 and 10) and of $FoxP3^+$ cells (#5), while decreased subset #9 of $GITR^+$ cells. Also PFOS increased subset #9 of IL-9^+ cells. In females, instead, more modulations were observed. In detail, EE reduced the percentage of cells present in subset #7 of $FoxP3^+$ cells. DEP exposure reduced the percentage of cells present in subset #7 of IFN-γ^+ cells, reduced the percentage of IL-9^+ cells (#1, 3, and 5), of $FoxP3^+$ cells (#5, 11, and 14), and of $GITR^+$ cells (#2, 3, 5, and 6). PFOS also reduced the percentage of IL-9^+ cells (#3 and 4), #11 of $FoxP3^+$ cells, and #3 and 5 of $GITR^+$ cells. Even more interestingly, neither DEP, nor PFOS, nor EE had the same effects in females and males in each subset. In particular, looking at the subsets of cells positive to IL-9 and GITR (Fig. 7C and E) a general increase of the subsets in males and a decrease in females can be observed, and significant differences in the modulation in males *vs* females were observed. Subsets #5, 9, 10 and 12 of IL-9^+ cells were differently modulated by DEP based on the sex, and subset #5 also by EE exposure (Fig. 7C). Also several subsets of $FoxP3^+$ cells were altered differently based on the sex; #5 by DEP and PFOS exposure, and #13 by EE (Fig. 7D). Regarding $GITR^+$ cells, subsets #3 and 9 were

differently modulated based on the sex (Fig. 7E). Overall, a clear sex-bias in the effects induced by DEP, PFOS and EE exposure is observed. For those populations statistically significantly altered by EDs exposure, the log2 value of the modulation, together with the abundance of the population within $CD4^+$ cells is reported in Supplementary Table 1.

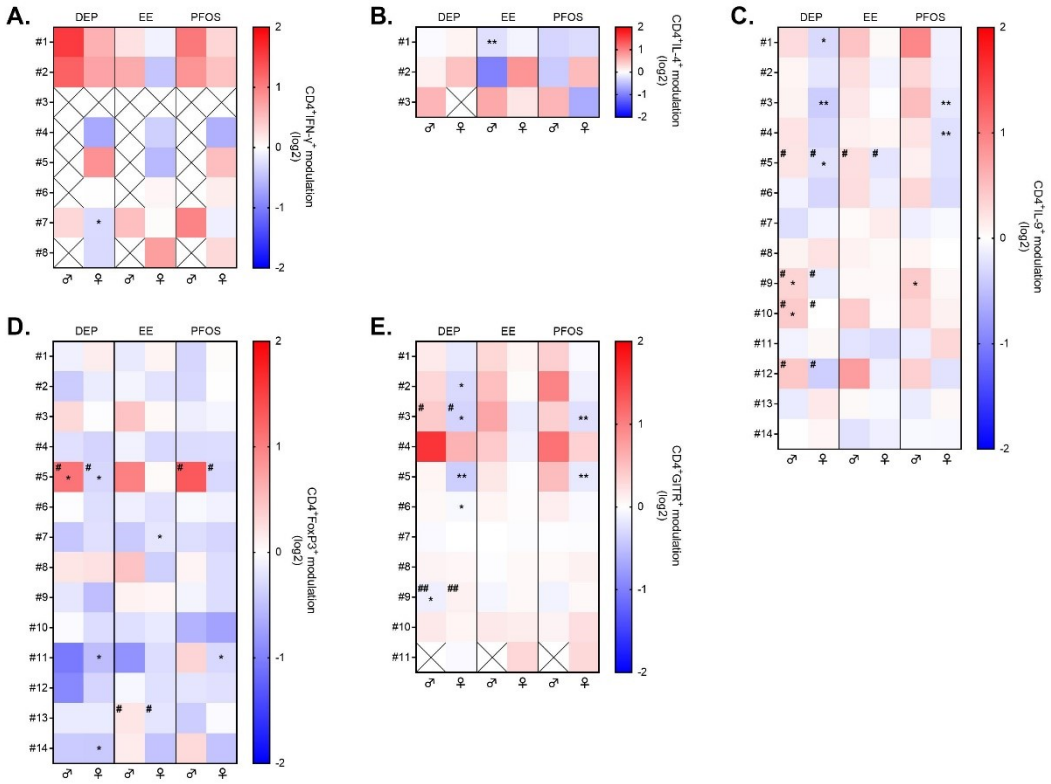


Figure 7. Modulation by DEP, EE, and PFOS of $CD4^+$ cell subsets, evaluated through t-SNE analysis. Male (♂) and female (♀) PBMC were exposed to the EDs or DMSO (vehicle control) together with anti-CD3/anti-CD28 coated magnetic beads for 4 days. After gating $CD4^+$ cells, subsets were identified based on staining with eleven antibodies as reported in the Material and Methods section. Cell subsets expressing high levels of IFN-γ (A), IL-4 (B), IL-9 (C), FoxP3 (D), and GITR (E) are numbered. The cell percentage of each treated donor was divided by the cell percentage of the same DMSO-treated donor (0, white) and expressed as log2 (modulation ratio). The mean modulation ratio of male and female donors is reported in the double gradient heatmap (red, increase; blue, decrease). White squares with the cross indicate that the subset of cells is absent in at least one DMSO-treated donor so the modulation ratio cannot be evaluated. Statistical analysis was performed following unpaired t-test with Welch's correction, with *$p \leq 0.05$ and **$p \leq 0.01$ *vs* DMSO-treated PBMCs. Differences between males and females were assessed through unpaired t-test (#$p \leq 0.05$ and ##$p \leq 0.01$).

Regarding the analysis including solvent control and treatment with ATR, CYP, and VIN in $CD4^+$ cells, the t-SNE analysis revealed 55 subsets expressing high levels of one marker: 8 IFN-γ^+ subsets, 2 IL-4^+ subsets, 16 IL-9^+ subsets, 16 $FoxP3^+$ subsets, and 13 $GITR^+$ subsets were retrieved. Some of these subsets express more than one marker, as can be observed in Supplementary Figure 7. The mean percentage of cells in each subset after treatment is divided by that in the control for males and females and the values are reported in Figure 8. The mean

cell percentage of some subsets was modulated in males or females. Also, the effects of ATR, CYP, and VIN showed sex-bias effects but the tendency was less evident. In males, the percentage of IFN-γ^+ cells (#5) was increased following CYP exposure and also subset #10 of FoxP3$^+$ cells. VIN exposure to male donors induced a slight increase of subset #10 of GITR$^+$ cells. In females, ATR was able to increase the percentage of cells positive to IFN-γ present in subset #6, and to decrease the percentage of cells present in subset #14 of FoxP3$^+$ cells. VIN exposure to female donors, instead, statistically significantly increased the percentage of IL-9$^+$ cells (#2) and of GITR$^+$ cells (#2). Differently, from the three highly persistent EDs, the three pesticides here analyzed do not show a strong difference between male and female donors. The only statistical differences were observed on subset #5 of IFN-γ^+ cells following CYP exposure, which increased the percentage of cells in males and decreased it in females (Fig. 8A), and on subset #14 of FoxP3$^+$ cells, where ATR decreased the percentage of cells in both sexes but with a greater effect on females (Fig. 8D). Similarly to the other EDs, the log2 values of the modulation relative to the populations statistically significantly altered by ATR, CYP, and VIN exposure are reported in Supplementary Table 2.

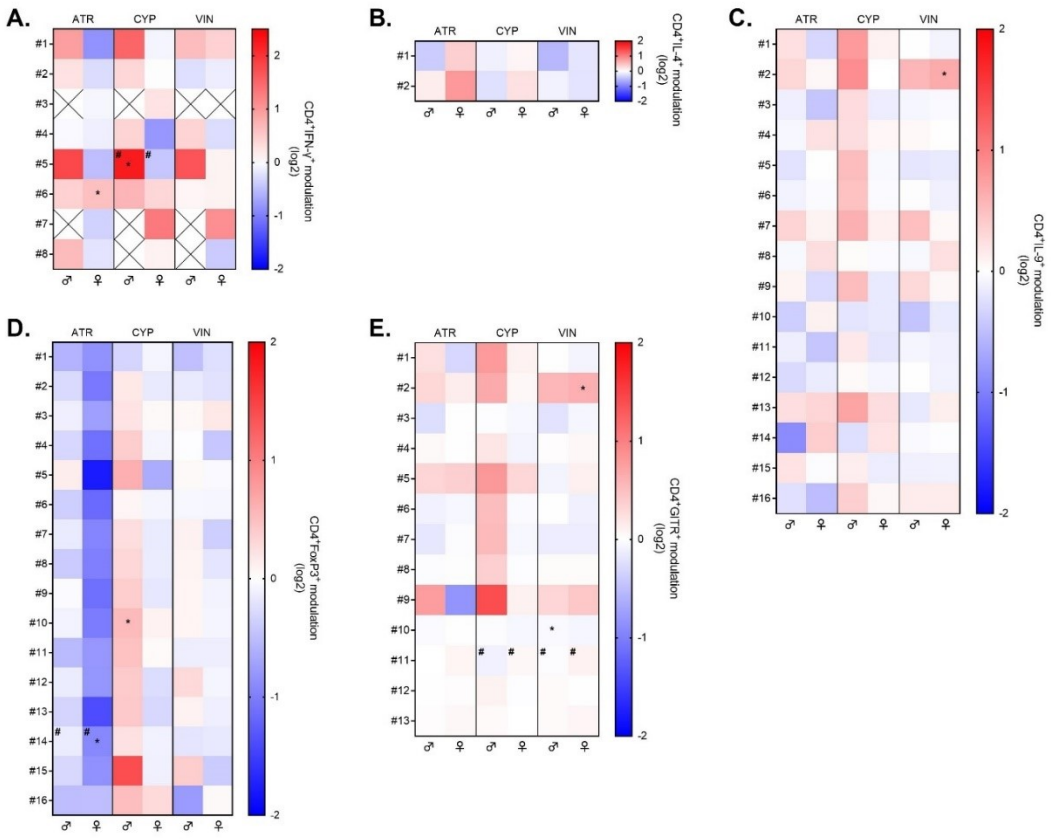


Figure 8. Modulation by ATR, CYP, and VIN of CD4$^+$ cell subsets, evaluated through t-SNE analysis. Male (♂) and female (♀) PBMC were exposed to the EDs or DMSO (vehicle control) together with

anti-CD3/anti-CD28 coated magnetic beads for 4 days. After gating $CD4^+$ cells, subsets were identified based on staining with eleven antibodies as reported in the Material and Methods section. Cell subsets expressing high levels of IFN-γ (A), IL-4 (B), IL-9 (C), FoxP3 (D), and GITR (E) are numbered. The cell percentage of each treated donor was divided by the cell percentage of the same DMSO-treated donor (0, white) and expressed as log2 (modulation ratio). The mean modulation ratio of male and female donors is reported in the double gradient heatmap (red, increase; blue, decrease). White squares with the cross indicate that the subset of cells is absent in at least one DMSO-treated donor so the modulation ratio cannot be evaluated. Statistical analysis was performed following unpaired t-test with Welch's correction, with $*p \leq 0.05$ *vs* DMSO-treated PBMCs. Differences between males and females were assessed through unpaired t-test ($\#p \leq 0.05$).

By overlapping the different 2D t-SNE projection maps obtained from the exposure to DEP, EE, and PFOS we realized that the cells in subset #7 of IFN-γ are the same as subset #1 of IL-9, #5 of FoxP3, and #2 of GITR (Fig. 7 and Supplementary Figure 6) and called the subset POP A (Fig. 9A). Moreover, the cells in the subset #8 of IFN-γ are present also in subset #3 of IL-9, #11 of FoxP3, and #5 of GITR (Fig. 7 and Supplementary Figure 6) and called the subset POP B (Fig. 9A). POP A and B are significantly decreased by DEP exposure in female donors only. POP B is significantly decreased also by PFOS exposure in female donors only. POP A and B are both $CD4^+FoxP3^+GITR^+IFN\text{-}\gamma^+IL\text{-}4^+IL\text{-}9^+$ with POP A expressing more GITR than POP B (Fig. 9B, Suppl Fig. 8A). The expression of GITR together with the cytokines may indicate that the cells of the subset belong to activated conventional $CD4^+$ T cells. However, the expression of FoxP3 may indicate that the cells of the subset act as regulatory T cells.
Lastly, analyzing the 2D t-SNE maps obtained from ATR, CYP, and VIN exposure, we discovered the subset POP C (Fig. 9C) which cells are present in subset #2 of IL-9 and #2 of GITR (Fig. 8 and Supplementary Figure 7). POP C is significantly increased by VIN exposure in both sexes, but the increase is significant only in woman donors. The phenotype of this population is $CD4^+IL\text{-}22^+IL\text{-}4^+IFN\text{-}\gamma^+IL\text{-}9^+GITR^+$, with a very high expression of GITR compared to the not affected population (Fig. 9D, Suppl Fig. 8B). Therefore, the main cytokines characterizing this population, which is increased by VIN exposure, mainly in women, are IFN-γ, IL-4, IL-9. The high level of expression of GITR may indicate that the cells of the subset belong to activated conventional $CD4^+$ T cells. The complete phenotypes can be retrieved in Supplementary Figure 8.

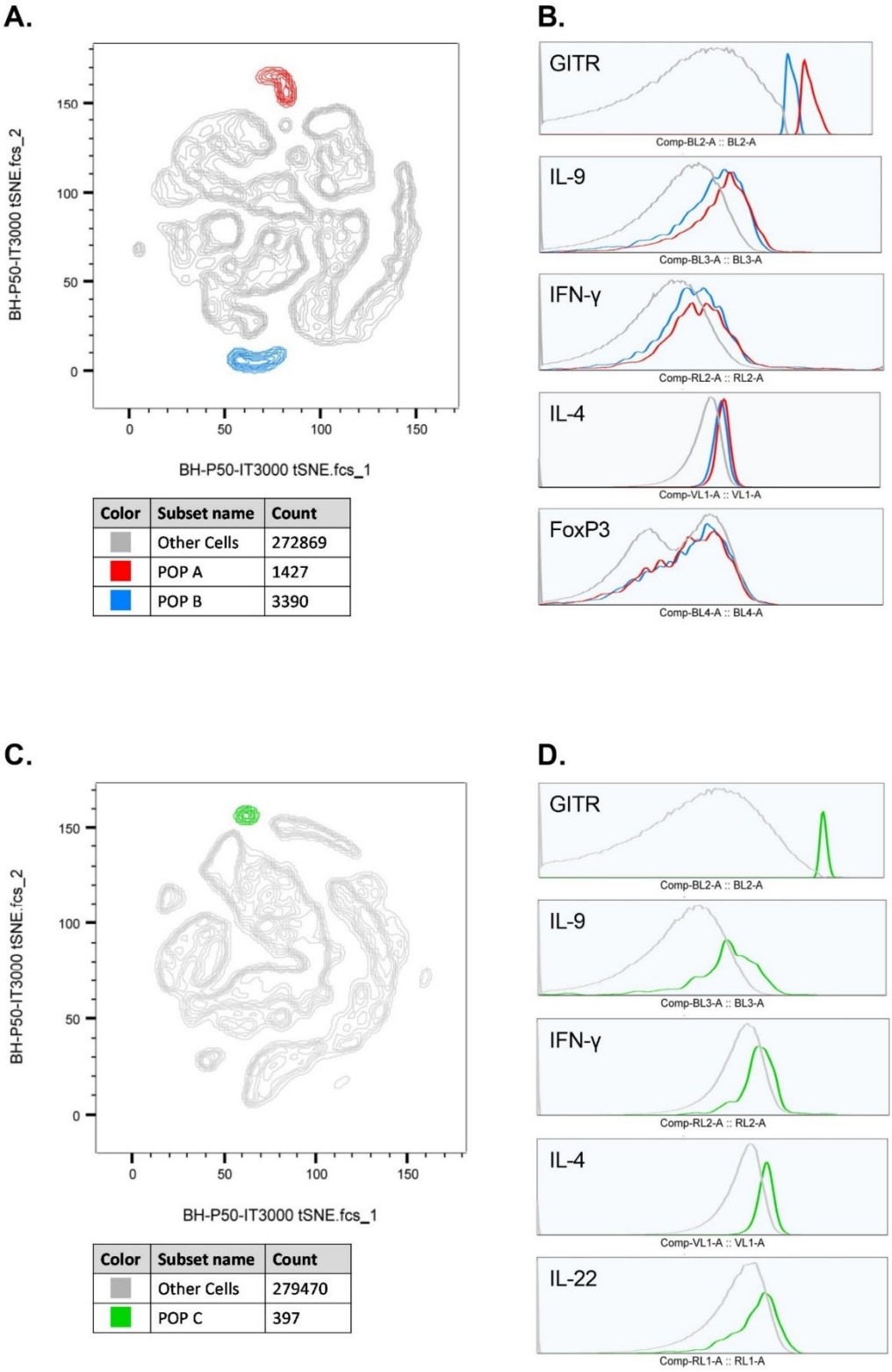


Figure 9. 2D t-SNE maps representing the most interesting subsets of population modulated by the selected EDs, which share multiple markers (A, C). Population A and B are referred to the t-SNE plot of DEP, EE, and PFOS (A), whereas Population C is referred to the t-SNE plot of ATR, CYP, and VIN (C). Population A (POP A – red) is represented by cells present in the subsets #7 of IFN-γ, #1 of IL-9, #5 of FoxP3, and #2 of GITR (Supplementary Figure 6) and they are reduced in a statically significant way by DEP exposure in female donors. Population B (POP B – blue) is represented by cells present in the subsets #8 of IFN-γ, #3 of IL-9, #11 of FoxP3, and #5 of GITR (Supplementary Figure 6) and they

are reduced in a statically significant way by both DEP and PFOS exposure in female donors. Population C is represented by cells contained in the subsets #2 of IL-9 and #2 of GITR (Supplementary Figure 7) and they are increased in a statically significant way by VIN exposure in female donors. Note that it was not possible to find POP C also in the other markers because these populations were not present in all the conditions of exposure and not in all donors. The phenotypes of POP A, B, and C are reported (B, D), where the grey line represents the phenotype of the other cells. The other phenotypes (all 11 markers) are shown in Supplementary Figure 8.

Discussion

In the last decades, an increase in several diseases, namely tumors, obesity, psychiatric disorders, and autoimmunity, has been observed, above all in developed countries. Within the main causes, environmental factors, including EDs, are considered the main responsible (Manley et al., 2018). Exposure to EDs has been related to diseases that involve, directly or indirectly, the immune system, such as inflammatory disorders, allergy, asthma, autoimmunity, obesity, type 2 diabetes, and cancer (Schug et al., 2011; Teitelbaum et al., 2012; Bekö et al., 2013; Bertelsen et al., 2013; Trasande et al., 2013; Buser et al., 2014; Schooling and Zhao, 2015; Benvenga et al., 2020; Predieri et al., 2020; Segovia-Mendoza et al., 2020; Schjenken et al., 2021). The majority of these disorders are characterized by a sex-dimorphism, meaning a different prevalence or susceptibility, different onset, progression, severity, survival, or response to therapy of the two sexes (Ortona et al., 2016; Selmi and Gershwin, 2019; Di Florio et al., 2020; Klein and Morgan, 2020; Massey et al., 2021). For example, autoimmune disorders usually affect more women than men (Quintero et al., 2012; Angum et al., 2020). Steroid hormones, such as sex hormones and corticosteroids, are known to interact with the immune system, leading to a sexual dimorphism that involves the endocrine, nervous, and immune systems (Gaillard and Spinedi, 1998; Bhatia et al., 2014; Taneja, 2018). Regarding the immune system, women's immune system is considered more reactive, and therefore less susceptible to infections but more prone to develop several immune disorders, like autoimmunity or exaggerated immune responses (Butterworth et al., 1967; Mangalam et al., 2013; Ngo et al., 2014). The interaction between steroid hormones and environmental factors can provoke different immune responses based on gender (Sugiyama et al., 2010; Ghosh and Klein, 2017). When considering the entire organisms, also the impact of microbiota must be mentioned due to the strict interconnection with the endocrine system (Mayer et al., 2015; Park and Choi, 2017; Qi et al., 2021). Therefore, due to the wide variety of factors that lead to a sex dimorphism in health and diseases, it is important to study the adverse effects of substances in both sexes, above all in the case of EDs.

In our study, we demonstrated that the selected EDs exerted adverse immune effects *in vitro* in both male and female PBMCs. Indeed, modulation of RACK1 was induced by all the tested EDs at least in one sex at the tested concentrations. Some of them also resulted in the modulation of the pro-inflammatory response, more specifically DEP, PFOS, ATR, and CYP induced a reduction of the pro-inflammatory response, which reflects the decreased RACK1 expression. These results confirm previous evidence of the modulation of immune parameters by EDs on cell lines (Maddalon et al., 2022; Masi et al., 2022). Regarding sex dimorphism, female donors seem to be more susceptible to DEP reduction of pro-inflammatory cytokines. Also EE induced different effects on RACK1 expression based on the sex, similar to what is observed on the impact on NK cells' lytic activity. On these cells also PFOS show a different effect, resulting in an increased lytic ability in males and a decreased one in females. Previous evidence showed the ability of PFOS to reduce NK activity in mice offspring following gestational exposure (Keil et al., 2008). All the other tested chemicals, with the exception of CYP, resulted in an impairment of NK activity, indicating their ability to modulate the immune system *in vitro*. NK activity decrease by VIN confirms what is already present in the literature (White et al., 2004). Regarding T cell differentiation, EE and VIN exposure resulted in a slightly increased percentage of IL-17-producing cells (likely Th17), while DEP, EE, and PFOS exposure decreased the percentage of IL-22-producing cells (likely Th22), with DEP and EE evidencing a higher activity in males *vs* females. Similarly, also Tc22 percentage resulted decreased in males upon DEP exposure, highlighting also in this case a higher men susceptibility. Finally, VIN increased Tc9 in male donors, differently from what was observed in women's cells. Regarding the effects observed in women, the percentage of $CD8^+FoxP3^+$ cells resulted to be reduced upon EE and mainly PFOS and ATR exposure, and DEP slightly reduced $CD8^+GITR^+CD25^+$ cells.

The deeper analysis of T helper cells subpopulations conducted by t-SNE evidenced other immunomodulation by EDs, with sex bias in several results, above all in the general modulation of Th1, Th2, Th9, and $GITR^+$ cells, mainly upon DEP and PFOS exposure. DEP's ability to reduce IL-4 and IFN-γ production was already demonstrated *in vitro* but without considering the possible sex bias (Hansen et al., 2015). Also for PFOS there are evidence of its ability to perturb the balance between Th1 and Th2 in mice (Dong et al., 2011; Zheng et al., 2011; Yang et al., 2021).

We observed the most relevant sex bias in DEP-treated samples with seven subpopulations demonstrating a significant sex bias and other subpopulation showing different DEP-dependent modulation in females and males, thought non-significant. In particular, DEP

determined decrease of 11 subpopulations in females and increase of 3 subpopulations in males. The relevant DEP-dependent decrease of POP A and B is of particular interest (Figure 7) because these subpopulations represent about 0.5% and 1.2% of $CD4^+$ T cells (Figure 9, panel A), respectively. They express FoxP3 at high levels, but, in our experimental setting, about half of $CD4^+$ cells express FoxP3 (Figure 9, panel B), suggesting that here, as in other experimental models, FoxP3 is expressed more by activated T cells than regulatory T cells (Wang et al., 2007). POP A and B are also characterized by high expression of GITR. GITR (TNFRSF18), originally described as induced by glucocorticoid in a T cell line, is mainly expressed in those active lymphocytes involved in immune tolerance and also in conventional T lymphocytes following activation (Placke et al., 2010; Ronchetti et al., 2015; Nocentini et al., 2017; Riccardi et al., 2018). Moreover, POP A and B express quite high levels of IFN-γ, IL-4 and IL-9, suggesting that they represent activated conventional T cells with a peculiar phenotype. Thus, the DEP-dependent decrease of POP A and B in females (Figure 7) would suggest an immunosuppressive effects of DEP in females in agreement with the DEP-dependent downmodulation of RACK-1, CD86, CD54, IL-8 and TNF-α expression (Figures 1 and 2) and the decrease of NK cell activity.

Interestingly, t-SNE analysis suggests that the activity of DEP and the other EDs is specific, having modulatory effects in some subpopulations of lymphocytes, whose functional meaning need to be investigated by dose-response *in vitro* and *in vivo* studies. For example, the PFOS-dependent decrease of POP B in males is somehow counteracted by the PFOS-dependent increase of POP A, suggesting a specific fine tuning of EDs on immune system of males and females. POP C (Figure 9, panels C-D), a subpopulation very similar to POP A and B, is another example of fine tuning. In fact, it is significantly increased by VIN in females but not in males (Figure 8). Finally, the relevant decrease of $FoxP3^+$ cells following ATR treatment is relevant in almost all subpopulations in females (significant in #14) and irrelevant or absent in males (Figure 8, panel D). Interestingly, we previously demonstrated the ability of DEP and PFOS to interact with the glucocorticoid receptor as agonists (Masi et al., 2022). Regarding VIN, its M2 metabolite (3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide) showed a weak antagonism toward the glucocorticoid receptor (Molina-Molina et al., 2006). Therefore, there could be a possible explanation of the relationship between DEP, PFOS, and VIN with GITR expression.
POP A, B and C are characterized also by high expression of IFN-γ, IL-4 and IL-9. A population of T helper cells co-expressing IL-9 and IL-4 was already reported in the literature, indicating that this population can activate eosinophils in colitis and is involved in the effector function of T helper cells in this disease (Moshkovits et al., 2017; Stanko et al., 2018). IFN-γ^+IL-4^+ double-

positive cells have been also described (Krawczyk et al., 2007). Furthermore, IL-9 can be also produced by Treg (Lu et al., 2006). Although Th1, Th2, and Th9 exhibit different T cell phenotypes, some gene clusters are similarly regulated (Xue et al., 2019). More in-depth investigation to discover the involvement of these cell subsets in EDs immunomodulatory effects must be performed.

The similar action between DEP and PFOS could be also explained by their common involvement in the estrogenic pathway (Du et al., 2013; Fiocchetti et al., 2021), and this could also represent a possible explanation for the sex bias. They both reduced RACK1 expression in both sexes, with a parallelism with immunological implications (reduced pro-inflammatory markers). They also reduced the percentage of $CD4^+IL\text{-}22^+$ cells and modulated $CD4^+IL\text{-}9^+$, $CD4^+FoxP3^+$, and $CD4^+GITR^+$ similarly.

Glucocorticoids are considered immunosuppressors, since they inhibit several immune cell activities (Van Laethem et al., 2001; Strehl et al., 2019). For example, they are able to suppress T cell activation and NK cell activity (Muscari et al., 2022), which is in line with the effects observed with PFOS and DEP exposure, both substances able to activate glucocorticoid receptor. Regarding T helper cells differentiation, glucocorticoids are able to suppress T helper cells, together with their effector functions (Liberman et al., 2018; Strehl et al., 2019; Taves and Ashwell, 2021). In general, all $CD4^+$ T cells are sensitive to glucocorticoid-induced inhibition, like Th1, Th2, Th9 and Th22 (Arya et al., 1984; Wu et al., 1991; Holz et al., 2005; Cao et al., 2012), with the only exception of Th17 and Treg (Banuelos and Lu, 2016; Cari et al., 2019). Also in this case, this is in line with the reduction of POP A and B exerted by DEP and PFOS in female donors, populations characterized by highly expression of cytokines representative of Th1, Th2, and Th9.

Due to the wide importance of the immune system and sex dimorphism in diseases, it is important to study the effect of EDs on the immune system, focusing on the possible sex difference. Our study provides an overview of the effects of EDs on the immune system, mainly focusing on PBMCs and lymphocytes. To confirm these data, more subjects should be tested, but since we already observed effects, it is presumable to find even more with more samples. We have also tested only one concentration of each ED. This allowed us to test and compare more substances at concentrations relevant to human exposure, but a wider range of concentrations should also be tested for a broader view. Therefore, with our *in vitro* study testing the effects of 6 EDs on primary immune cells of both sexes, we can affirm the ability of EDs to modulate the immune system, both innate and adaptive response. In detail, they modulated pro-inflammatory activity, natural killer lytic ability, and lymphocyte activation and differentiation

with different effects. In particular, DEP and PFOS appeared to be the two high concern EDs, within the ones selected in this study. Therefore, more studies focusing on them should be performed.

Supplementary Material

Supplementary data to this article can be found online at https://link.springer.com/article/10.1007/s00204-023-03592-3.

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