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

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Chapter III – Mixtures

Previous data obtained (Maddalon et al., 2023) identified DEP and PFOS as EAS affecting the immune system. Therefore, a more detailed investigation into the effects of PFOS was conducted, primarily because of its extensive use in industrial applications, which resulted in elevated human exposure. PFOS falls within the chemical category of perfluoroalkyl substances (PFAS), which encompasses over 15,000 different chemicals (NIEHS, 2023). Since the exposure to PFAS usually occurs as mixtures and not as single entities, the effects of PFAS mixtures on human immune cells activation were investigated.

The concept of mixtures is often understated. We are daily exposed to mixtures of chemicals and the effect of chemicals, sometimes, can be potentiated by the co-presence of other chemicals, leading to potential greater effects (Groten, 2000). We are exposed to chemical mixtures through air, water, food and skin contact. Regarding water chemical exposure, several chemicals (i.e., urban runoff chemicals, pesticides, personal care products, and drugs) can pass through wastewater treatment plants (WWTP) and enter the water cycle (Robles-Molina et al., 2014; Kümmerer, 2009). Micropollutants coming from WWTP represent one of the main contributors to aquatic pollution (Loos et al., 2013). WWTP effluents represent a harmful source of micropollutants for both aquatic organisms and humans, mainly workers in the field of treatment plants, agriculture, and farm (Brack et al., 2016; Lu et al., 2020; Wu, 2020).

Therefore, two sources of mixtures were selected: one artificial represented by PFAS mixtures (I) and one representative of a real environment: WWTP effluent extract (II). Three *in vitro* protocols were used, using human peripheral blood or PBMCs, namely lymphocytes activation, MAIT cells activation, and basophils activation assays. Immune cells were exposed to PFAS mixtures or extracts from WWTP effluents and then cells were differentially stimulated to evaluate the possible interference of mixtures in their activation profile and transcript expression. Finally, the chemical profile of WWTP effluent extracts was correlated with the immune endpoints to obtain a list of high immune-concern chemicals.

These experiments were done in collaboration with the Department of Environmental Immunology of the Helmholtz Centre for Environmental Research (UFZ) located in Leipzig (Germany) under the supervision of Doctor Herberth, during a six-month period as visiting scientist. In particular, from the first paper, regarding the effect of PFAS mixture on human immune cells activation, we observed their ability to reduce the activation of CD4$^+$, CD8$^+$, natural killer T, and mucosal activated invariant T (MAIT) cells. This immunosuppression is induced by the mixture of the three short-chain PFAS and mainly by all the six PFAS (three

short-chain plus three long-chain). We noticed that MAIT cells were the most vulnerable lymphocytes to PFAS exposure, and 18 genes (within the 39 analyzed) resulted to be down-regulated by PFAS exposure (both short-chain and short/long-chain PFAS), such as pro-inflammatory cytokines, chemokine receptors, several genes typical of MAIT cells and NFKB2. Furthermore, also the activation of basophils was reduced following PFAS exposure, in this case mainly by long-chain and the mixture of short/long-chain PFAS. The article focusing on WWTP exposome also revealed a general trend of immunosuppression. In particular, the activation of CD4$^+$, CD8$^+$, natural killer T, and MAIT cells was highly reduced by all the four WWTP extracts tested. MAIT cells resulted to be the most susceptible lymphocyte population, and the transcript analysis revealed 12 genes down-regulated and 11 up-regulated within the 40 analyzed. Several cytokines and chemokine receptors were down-regulated together with typical lymphocyte transcripts, whereas several receptors like *AHR*, *PPARA*, and *PPARG* resulted to be up-regulated. The different WWTP extracts were also able to induce alterations in basophils activation. The correlation analysis between the 339 chemicals identified in the WWTP and the immune endpoints revealed that 2/3 of the chemicals correlated with the reduced lymphocyte activation. We pointed out 50 prioritized chemicals for their immunomodulatory profile, and 29 of them highly correlated with all the immune endpoints analyzed, comprehending mainly pharmaceuticals, pesticides, industrial chemicals, and EDs.

The battery of tests confirmed the ability of PFOS and PFAS mixture to interfere with the immune system, and also the hypothesis of real environmental mixtures (WWTP) adverse activity on the immune system was confirmed.

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Mixtures of per- and poly-fluoroalkyl substances (PFAS) reduce the in vitro activation of human T cells and basophils

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Abstract

In the last decades, per- and poly-fluoroalkyl substances (PFAS), widely used industrial chemicals, have been in the center of attention because of their omnipotent presence in water and soils worldwide. Although efforts have been made to substitute long-chain PFAS towards safer alternatives, their persistence in humans still leads to exposure to these compounds. PFAS immunotoxicity is poorly understood as no comprehensive analyses on certain immune cell subtypes exist. Furthermore, mainly single entities and not PFAS mixtures have been assessed. In the present study we aimed to investigate the effect of PFAS (short-chain, long-chain and a mixture of both) on the *in vitro* activation of primary human immune cells.

Our results show the ability of PFAS to reduce T cells activation. In particular, exposure to PFAS affected T helper cells, cytotoxic T cells, Natural Killer T cells, and Mucosal associated invariant T (MAIT) cells, as assessed by multi-parameter flow cytometry. Furthermore, the exposure to PFAS reduced the expression of several genes involved in MAIT cells activation, including chemokine receptors, and typical proteins of MAIT cells, such as GZMB, IFNG and TNFSF15 and transcription factors. These changes were mainly induced by the mixture of both short- and long-chain PFAS. In addition, PFAS were able to reduce basophil activation induced by anti-FcεR1α, as assessed by the decreased expression of CD63.

Our data clearly show that the exposure of immune cells to a mixture of PFAS at concentrations mimicking real-life human exposure resulted in reduced cell activation and functional changes of primary innate and adaptive human immune cells.

Graphical abstract
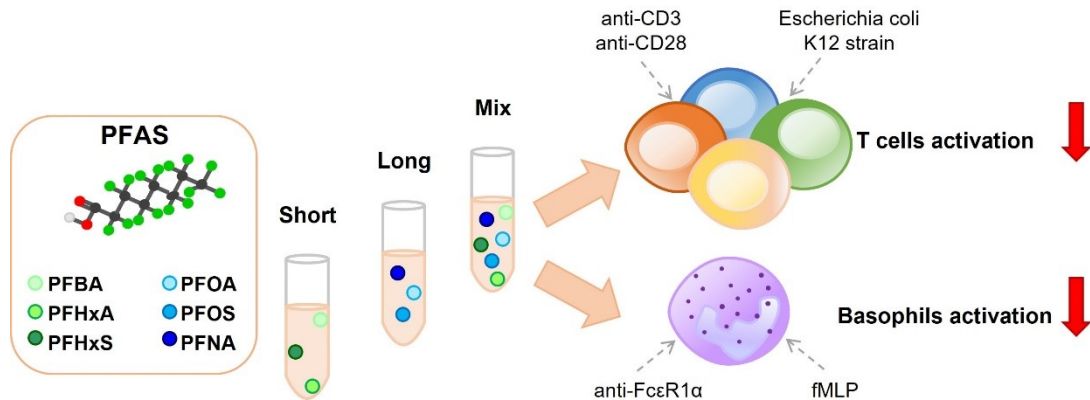
Introduction

Per- and poly-fluoroalkyl substances (PFAS) are a group of anthropogenic contaminants characterized by fluorinated aliphatic chain (that ranges from 2 to 16 carbons). The stable link between carbon and fluorine renders these molecules resistant to degradation and accounts for their environmental persistence and accumulation, leading to their reference as 'forever chemicals' (Kempisty and Racz, 2021). PFAS are widely used in industry, mainly in textiles, household products, fire-fighting, automotive, food processing, construction, and electronics (Glüge et al., 2020). They can be found in the environment and in food (EFSA Panel on Contaminants in the Food Chain et al., 2020). In human samples, PFAS are detectable in serum, urine, breast milk (Barbarossa et al., 2013; Worley et al., 2017; Zheng et al., 2021) but also in hair and nails (Worley et al., 2017), pointing out at their bioaccumulation. Due to their wide spread, emerging evidence addresses associations between PFAS exposure and pathologic health outcomes (Fenton et al., 2021). The majority of toxicity data have been obtained for perfluorooctanoic acid (PFOA), perfluorooctanesulfonic acid (PFOS), perfluorononanoic acid (PFNA), perfluorobutanesulfonic acid (PFBS), and perfluorohexanesulfonic acid (PFHxS) (Hu et al., 2016). Indeed, PFOA has been classified as a substance of very high concern (REACH, 2017), and PFAS are present in the list of persistent organic pollutants (EU, 2019, 2020). With the aim of limiting and replacing these two chemicals, a switch toward shorter chain PFAS has been recently implemented. However, their toxicity is still unclear. Usually, PFAS are classified as long-chain (C > 7), short-chain ($4 \leq C \leq 7$), and ultra-short-chain ($2 \leq C \leq 3$) (Ateia et al., 2019). Due to the higher elimination rate of short-chain PFAS, they are considered less toxic, but only few studies actually assessed their toxicological potential. Within these substitutes there are perfluorobutanoic acid (PFBA), perfluorobutane sulfonic acid (PFBS), perfluorohexanoic acid (PFHxA), and PFHxS (Mudumbi et al., 2017).

Possible noxious effects of PFAS in humans are associated with thyroid disease, liver damage, kidney and testicular cancer, increased cholesterol levels and alteration in development (reviewed in Fenton et al., 2021). Regarding the immune system, a reduced response to vaccines in childhood have been associated to PFAS exposure, and particularly to PFOS and PFOA (Grandjean et al., 2012, 2017; Granum et al., 2013; Looker et al., 2014; Abraham et al., 2020). Reduced response to vaccination has been identified by EFSA experts as most critical human health effect when determining the tolerable weekly intake (EFSA Panel on Contaminants in the Food Chain et al., 2020). Children's exposure to PFAS has been also associated to atopic dermatitis and respiratory tract infections (Kvalem et al., 2020), suggesting pregnancy and childhood as critical time points for PFAS immunosuppressive effects (Szilagyi et al., 2020;

von Holst et al., 2021). Moreover, prenatal exposure to PFOS and PFHxS has been associated to increased childhood infections (Goudarzi et al., 2017). Regarding other immune system-based outcomes, such as asthma and allergies, data are more inconsistent, deeming a higher number of investigations. However, most of these findings results from epidemiological studies and would benefit from validation for the underlying mechanisms using *in vitro* systems that employ primary cells.

Within more than 4700 PFAS (OECD, 2018), we decided to test three short-chain PFAS (PFHxA, PFHxS, PFBA) and three long-chain (PFOA, PFOS and PFNA) in concentrations found in human serum, to assess their possible impact on human immune cells activation. The decision to test these six PFAS derived from evidence of human exposure levels through different sources, mainly water (Wilhelm et al., 2010; Boiteux et al., 2012; Llorca et al., 2012). Furthermore, PFOA, PFOS, PFNA, and PFHxS were identified as the PFAS that mainly accumulate into the human body by EFSA, considering the immune system as critical effect (EFSA Panel on Contaminants in the Food Chain et al., 2020). We decided to include other two short-chain PFAS, namely PFHxA and PFBA, since the first is under evaluation to be restricted, following ECHA's opinion (ECHA, 2021), whereas regarding the latter few information are available, but in a study analyzing different PFAS in human urine samples, PFBA has been found in all the samples, at high levels (Perez et al., 2012). The six mentioned PFAS were tested in combination, since real-life exposure involves PFAS mixtures and not just single entities (Centers for Disease Control and Prevention, 2017). The concentrations of 0.02, 0.2, 2 ng/mL have been selected based on human biomonitoring of PFOA, PFOS and PFHxS in Germany (Göckener et al., 2020), that gave similar or lower values to what observed in Norway, Sweden, Netherland, Faroe Islands and China (Poothong et al., 2017; Worley et al., 2017; Hu et al., 2018; Duan et al., 2020; Gebbink and van Leeuwen, 2020; Glynn et al., 2020). We aimed to determine the impact of PFAS on the frequency and functionality of innate and adaptive immune cells upon various stimuli (anti-CD3/CD28 and *E. coli* K12) used for immune cell activation. We studied the immune response of immune cell subtypes by multi-parameter flow cytometry. In particular, peripheral blood mononuclear cells (PBMCs) from healthy donors were used, and the activation of T helper (CD4^{+}), cytotoxic T (CD8^{+}), Natural Killer T (NKT), and mucosal associated invariant T (MAIT) cells were assessed. In addition, basophils obtained from whole blood of healthy donors were exposed to PFAS and their activation level was assessed with flow cytometry. To get insights into the mechanism of action, the ability of PFAS to modify the expression of immune-related genes and genes coding for hormone receptors were also investigated.

Materials and Methods

<u>Tested chemicals</u>

PFAS, namely PFBA, PFHxA, PFHxS, PFOA, PFOS, and PFNA (Sigma-Aldrich, St. Louis, US) were dissolved in dimethyl sulfoxide (DMSO - Appli-Chem, Darmstadt, Germany) at 1 mg/mL stock solutions and stored at room temperature (RT) (Table 1).

Table 1
PFAS tested: name, acronym, formula, CAS number, and chain length.

Name	Acronym	Formula	CAS	Chain length
Perfluorobutanoic acid	PFBA	$C_4HF_7O_2$	375-22-4	Short
Perfluorohexanoic acid	PFHxA	$C_6HF_{11}O_2$	307-24-4	Short
Perfluorohexanesulfonic acid	PFHxS	$C_6HF_{13}O_3S$	355-46-4	Short
Perfluorooctanoic acid	PFOA	$C_8HF_{15}O_2$	335-67-1	Long
Perfluorooctanesulfonic acid	PFOS	$C_8HF_{17}O_3S$	1763-23-1	Long
Perfluorononanoic acid	PFNA	$C_9HF_{17}O_2$	375-95-1	Long

<u>PBMC treatment with PFAS</u>

Pseudonymous buffy coats from five male healthy adult volunteers were obtained from the blood bank at the University of Leipzig, after written informed consent. The study was approved by the Ethics Committees of the University of Leipzig (#079-15-09032015). Only male donors were selected to avoid intra cycle differences among women.

PBMCs were isolated by density-gradient centrifugation using Ficoll-Paque Plus (Cytiva Sweden AB, Uppsala, Sweden). Cells were then stored at -150 °C until use. For the treatment, PBMCs were thawed and cultured in IMDM (Gibco, Thermo Fisher Scientific, Waltham, US) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, US), 1X Penicillin-Streptomycin (Gibco, Thermo Fisher Scientific, Waltham, US), and 50 µM β-mercaptoethanol (Sigma-Aldrich, St. Louis, US). PBMCs were then plated at 10^6 cells/well in U-bottom 96-well microplates (Greiner Bio-One, Frickenhausen, Germany) for at least 2 h at 37 °C in a 5% CO2 incubator. PBMCs were afterwards exposed to PFAS mixtures at the concentrations of 0.02 ng/mL, 0.2 ng/mL, 2 ng/mL each (Table 2) for 20 h. For simplicity, the treatment conditions used and shown in Table 2 are mentioned as: DMSO, Short 2 ng/mL, Long 2 ng/mL, and Mix 2 ng/mL, throughout the manuscript.

After 20 h treatment with PFAS, PBMCs were stimulated either with anti-CD3 and anti-CD28 antibodies (anti-CD3/CD28) or with the bacterium *Escherichia coli* K12 strain for 6 h to induce activation. Concurrently, negative controls were performed, PBMCs stimulated with anti-CD28 alone or PBMCs without bacteria, respectively. The stimulation with anti-CD28 has been chosen to evaluate background activation levels of the different donors induced by an unspecific stimulus. In this regard, the five tested volunteers did not result in unspecific activation. Concurrently to each experiment, cell viability was evaluated following the exposure to PFAS and activation with proper stimulus (anti-CD3/CD28 or *E. Coli*) (Supplementary Fig. 1A).

Table 2
PFAS treatment: name, abbreviation, and composition (ng/ml per each single PFAS).

Treatment name	Abbreviation	Composition
Solvent control	DMSO	DMSO (0.0012%)
Short-chain 0.02 ng/ml	Short 0.02 ng/ml	PFBA, PFHxA, PFHxS (0.02 ng/ml)
Short-chain 0.2 ng/ml	Short 0.2 ng/ml	PFBA, PFHxA, PFHxS (0.2 ng/ml)
Short-chain 2 ng/ml	Short 2 ng/ml	PFBA, PFHxA, PFHxS (2 ng/ml)
Long-chain 0.02 ng/ml	Long 0.02 ng/ml	PFOA, PFOS, PFNA (0.02 ng/ml)
Long-chain 0.2 ng/ml	Long 0.2 ng/ml	PFOA, PFOS, PFNA (0.2 ng/ml)
Long-chain 2 ng/ml	Long 2 ng/ml	PFOA, PFOS, PFNA (2 ng/ml)
Mixture of short- and long-chain 0.02 ng/ml	Mix 0.02 ng/ml	PFBA, PFHxA, PFHxS, PFOA, PFOS, PFNA (0.02 ng/ml)
Mixture of short- and long-chain 0.2 ng/ml	Mix 0.2 ng/ml	PFBA, PFHxA, PFHxS, PFOA, PFOS, PFNA (0.2 ng/ml)
Mixture of short- and long-chain 2 ng/ml	Mix 2 ng/ml	PFBA, PFHxA, PFHxS, PFOA, PFOS, PFNA (2 ng/ml)

PBMC activation

Antibodies anti-CD3 (clone: OKT3) and anti-CD28 (clone: CD28.2) (BioLegend, San Diego, US) were added at a final concentration of 0.5 ng/mL and 0.5 μg/mL, respectively, and PBMCs were stimulated for 6 h. Prior experiments to determine the optimal concentrations of anti-CD3 and anti-CD28 were performed, indicating these as optimal to activate CD4$^+$, CD8$^+$, NKT and MAIT cells (Supplementary Figs. 2A, 2B, 2C, and 2D show the activation level

induced by the stimulation). To inhibit cytokine secretion, Brefeldin A (10 μg/mL) (Sigma-Aldrich, St. Louis, US) was added for the final 4 h of incubation.

In order to specifically activate MAIT cells within PBMCs, *Escherichia coli* K12 MG1655 samples (*E. coli* K12) were prepared as previously described (Krause et al., 2022) and stored at -80 °C until used. Prior experiments determined the optimal bacteria concentration, indicating 10 bacteria per cell (10 BpC) as sufficient to activate MAIT cells (Supplementary Fig. 3A). PBMCs were, therefore, stimulated with *E. coli* K12 (10 BpC) for 6 h. Brefeldin A (10 μg/mL) was added for the final 4 h of incubation.

Flow cytometric analysis of PBMCs

Following 6 h stimulation, PBMCs were transferred to V-bottom 96-well microplates (Thermo Fisher Scientific Waltham, US). To discriminate between alive and dead cells, the samples were stained with fixable viability dye-Zombie NIR™ (BioLegend) for 15 min at RT, thereafter stained for surface markers (CD3, CD4, CD8, CD19, CD56, CD161, and TCRVα7.2) for 20 min at RT (Supplementary Table S1). After that, PBMCs were fixed using FACS™ Lysing Solution (BD Biosciences, San Jose, US) for 10 min, and permeabilized with FACS™ Permeabilizing Solution 2 (BD Biosciences, San Jose, US) for further 10 min. Finally, PBMCs were stained for activation and intracellular markers (CD69, CD71, CD134, CD137, TNF-α, and IFN-γ, according to the protocol) for 20 min at RT (Supplementary Table S1).

Flow cytometrical acquisition was then performed using Cytek Aurora (Cytek Biosciences, California, US). A minimum of 100,000 viable T cells (according to Zombie NIR™ staining) were acquired per sample, then lymphocytes were identified among total PBMCs using FSC-A and SSC-A. The gating strategies for all lymphocytes and MAIT cells are presented in Supplementary Figs. 4 and 5. Following lymphocytes stimulation with anti-CD3/CD28, T helper cells were gated, after doublets and dead cells exclusion, as CD3$^+$CD56$^-$CD4$^+$, cytotoxic T cells were gated as CD3$^+$CD56$^-$CD8$^+$, NKT cells as CD3$^+$CD56$^+$, and MAIT cells as CD3$^+$CD56$^-$CD161$^+$TCRVα7.2$^+$ (Supplementary Fig. 4). Following stimulation with *E. coli* K12, MAIT cells were gated, after doublets and dead cells exclusion, as CD3$^+$CD8$^+$CD161$^+$TCRVα7.2$^+$ (Supplementary Fig. 5). After gating the lymphocyte population, they were analyzed for the expression of activation markers and intracellular pro-inflammatory cytokines. The expression levels of TNF-α, CD71, CD137, and the co-expression of TNF-α and CD69 were presented as % of positive cells for CD4$^+$, CD8$^+$, NKT and MAIT cells in anti-CD3/CD28 stimulated samples. The expression of TNF-α, IFN-γ, CD69, and the coexpression of CD69 with the pro-inflammatory cytokines was presented as % of positive cells

of MAIT cells, following stimulation with *E. coli* K12. Data analysis was then performed using FCS Express 7 (De Novo Software).

<u>t-SNE analysis of MAIT cells stimulated with *Escherichia coli* K12</u>

Following flow cytometry analysis of *E. coli* K12 stimulated PBMCs for the identification of activated MAIT cells, a further analysis using t-distributed stochastic neighbor embedding (t-SNE) transformation tool present in FCS Express 7 was performed. The FCS files of solvent control and of each treatment group at the highest tested concentration (2 ng/mL) were merged and, after manual gating of MAIT cells (Supplementary Fig. 5), the results were visualized in 2D t-SNE maps. Briefly, a sample size of 2,000,000 total events was selected to allow the fully representation of MAIT cells population. T-SNE was run with the downsampling algorithm as interval, with iteration number of 500, perplexity of 50 and approximation of 0.5. Furthermore, the optimized t-SNE was selected, and the plot was estimated also for unsampled events. After the obtainment of the 2D map, group gating (DMSO, Short 2 ng/mL, Long 2 ng/mL and Mix 2 ng/mL) and single gating were performed by sample ID. Clusters of MAIT cells, based on the expression level of TNF-α, IFN-γ, and CD69, were manually gated, and the % of gated cells for each single sample was obtained and analyzed to produce an heatmap.

<u>Whole blood treatment with PFAS and assessment of basophil activation</u>

Pseudonymous heparin-collected whole blood from five male healthy volunteers was obtained from the blood bank at the University of Leipzig, after written informed consent. The study was approved by the Ethics Committees of the University of Leipzig (#079-15-09032015). 100 μL of blood were transferred in FACS 5 mL polystyrene round-bottom tube (Falcon, Corning, New York, US). Blood was exposed to PFAS at the highest concentration (Short 2 ng/mL, Long 2 ng/mL, Mix 2 ng/mL) for 1 h at 37 °C in a 5% CO2 incubator. PFAS were diluted in a Mg Ca PBS buffer (BAT buffer) supplemented with IL-3 (2 ng/mL - Sigma-Aldrich, St. Louis, US). For this assay, a shorter timing was selected due to the shorter half-life of basophils compared to lymphocytes. As negative control, blood exposed to BAT buffer was used. After incubation, basophils were activated through the addition of anti-FcεR1α antibody (0.1 μg/mL, BioLegend) or with N-Formylmethionylleucyl-phenylalanine (fMLP, 0.05 μM, Sigma-Aldrich, St. Louis, US) and stained with anti-CCR3 and anti-CD63 antibodies (Supplementary Table S2).

Samples were incubated for 25 min. Previous experiments to set the optimal concentration of anti-FcεR1α and fMLP were conducted for appropriate basophil activation

(Supplementary Fig. 6). After 25 min, the reaction was stopped by adding EDTA 3.8% (Gibco, Thermo Fisher Scientific, Waltham, US). Erythrocytes were then lysed twice using erythrocytes lysis buffer (NH$_4$Cl – Sigma-Aldrich, NaCO$_3$ – KMF Laborchemie, Lohmar, Germany, EDTA – Thermo Fisher Scientific) and incubated 10 min at RT. To discriminate between alive and dead cells, fixable viability dye-eFluor™ 506 (eBioscience) was added and incubated for 20 min at 4 °C. Thereafter, cells were fixed in paraformaldehyde 1% (Sigma-Aldrich) and analyzed by flow cytometry using FACS Canto™ II (BD Biosciences, San Jose, US). Leukocytes were identified among whole blood cells using FSC-A and SSC-A, basophils were gated as CCR3$^+$ cells and within this population only viable cells were analyzed. Alive basophils were further analyzed for expression level of CD63 (% of positive cells) and divided in high expressing (CCR3$^+$CD63^{high}) and low expressing (CCR3$^+$CD63^{low}) cells (Supplementary Fig. 7). Data were analyzed using FCS Express 7.

Concurrently to each experiment, cell viability was evaluated following the exposure to PFAS and activation with proper stimulus (fMLP or anti-FcεR1α) (Supplementary Fig. 1B).

Gene expression analysis

Isolated PBMCs were exposed to PFAS mixtures at the concentration of 2 ng/mL each (Short 2 ng/mL, Long 2 ng/mL, Mix 2 ng/mL) for 20 h at 37 ∘C in a 5% CO2 incubator. Thereafter, PBMCs were stimulated with *E. coli* K12 (10 BpC) for 6 h After the treatment, PBMCs were transferred into 1.5 mL microfuge tubes and centrifuged. After that, 300 μL of Trizol (Invitrogen, Waltham, US) were added to each tube and mixed. Trizol samples were maintained at -80 °C until RNA extraction.

Total RNA was extracted according to manufacturer's instructions (Invitrogen). The cDNA synthesis was carried out with 200 ng of RNA by using 5U RevertAid™ H Minus Reverse Transcriptase (Fisher Scientific, Schwerte, Germany). Intron-spanning primers were designed and UPL probes were selected by the Universal Probe Library Assay Design Center (http://qpcr.probefinder.com/organism.jsp). 39 genes of interest and 3 reference genes (Supplementary Table S3) were pre-amplified for 12 cycles and quantitative PCR was performed on a 96 × 96 Dynamic array with BioMark™ HD System (Fluidigm, München, Germany). The cycling program consisted of 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. PCR was performed with FastStart Universal Probe Master Mix (Roche, Mannheim, Germany).

The genes analyzed (Table 3) were chosen accordingly to their involvement in immune responses, in particular focusing on MAIT cells. Furthermore, hormonal receptors were also measured.

Table 3
Genes analyzed and relative proteins.

Gene	Protein (I)	Gene	Protein (II)
AHR	Aryl hydrocarbon receptor	IL18R1	IL-18 receptor 1
CCL20	CCL20	IL21R	IL-21 receptor
CCR5	CCR5 (CD195)	IL22	IL-22
CCR6	CCR6 (CD196)	IL23R	IL-23 receptor
CD28	CD28	IL6	IL-6
CSF2	Colony stimulating factor 2	IRF4	IFN regulatory factor 4
CXCL8	IL-8	KLRB1	CD161
CXCL13	CXCL13 (BCA-1)	NFATC1	Nuclear factor of activated T-cells
CXCR4	CXCR4 (CD184)	NFKB2	NFκB p100
CXCR6	CXCR6 (CD186)	NFKBIA	NFκB inhibitor α
FASLG	Fas ligand	NFKBIB	NFκB inhibitor β
FOXP3	FoxP3	NR3C1	Glucocorticoid receptor
GZMB	Granzyme B	PPARA	PPARα
ICOS	Inducible T cell costimulator	PRF1	Perforin 1
IFNG	IFN-γ	RORC	RORγ
IKZF2	Helios	TBX21	T-bet
IL10	IL-10	TNF	TNF-α
IL12RB1	IL-12 receptor subunit β1	TNFSF15	TNF superfamily member 15
IL17A	IL-17A	ZBTB16	Plzf
IL17F	IL-17F		

Statistical analysis

Flow cytometrical data were presented as fold change (FC) relative to the concurrent stimulated solvent control (DMSO + anti-CD3/CD28 or DMSO + *E. coli* K12 or DMSO + anti-FcεR1α or DMSO + fMLP). Statistical analysis was performed using GraphPad Prism (version 9.4.0). Data were reported as mean ± standard deviation (SD) of 5 replicates (healthy

volunteers). Gene expression data were normalized on the average of reference genes and then to the minimum of each gene. The FC was then calculated relative to the concurrent stimulated solvent control as mentioned above. Normal distribution was assessed using the Shapiro-Wilk test, and therefore statistical test for normally distributed samples were performed. To calculate differences between the treatment, one-way ANOVA, followed by Dunnett's or Tukey's multiple comparison test, or t-test with Welch's correction (as stated in the figure legends), were performed. Differences were considered statistically significant with $p \leq 0.05$.

Results

PFAS mixtures reduced the activation of lymphocytes upon anti-CD3/CD28 stimulation

In this study, PBMCs were stimulated with anti-CD3/CD28 antibodies, leading to the activation of different lymphocyte populations, namely CD4^{+}, CD8^{+}, NKT and MAIT cells. PBMCs were pre-exposed to increasing concentrations of different PFAS mixtures, before their activation. PFAS were able to reduce the activation of the different T cells (Fig. 1). In particular, short-chain PFAS, at least at one tested concentration, were able to statistically significantly reduce the expression of CD71 in CD4^{+} cells (Fig. 1A), TNF-α and TNF-α co-expressed with CD69 in CD8^{+} cells (Fig. 1B), and TNF-α co-expressed with CD69 in NKT cells (Fig. 1C). Long-chain PFAS, instead exhibited no effects on CD4^{+} and NKT cells, conversely, reduced CD71 expression in CD8+ cells and in MAIT cells (Fig. 1B, D). Ultimately, the mixture of both short- and long-chain PFAS exhibited greater effects, inducing a down-regulation of all the analyzed T cell populations, decreasing all the activation markers expressed by CD4^{+} and MAIT cells (Fig. 1A, D) and the expression of TNF-α and TNF-α-CD69 in CD8^{+} and of CD71 in NKT cells (Fig. 1B and C). This indicates the ability of PFAS mixtures, above all when short- and long-chain PFAS are co-present, to reduce lymphocytes activation. Furthermore, in NKT cells the expression of CD137 was statistically significantly different between the treatment with long-chain PFAS and the mixture of short- and long-chain ones at 0.2 ng/mL.
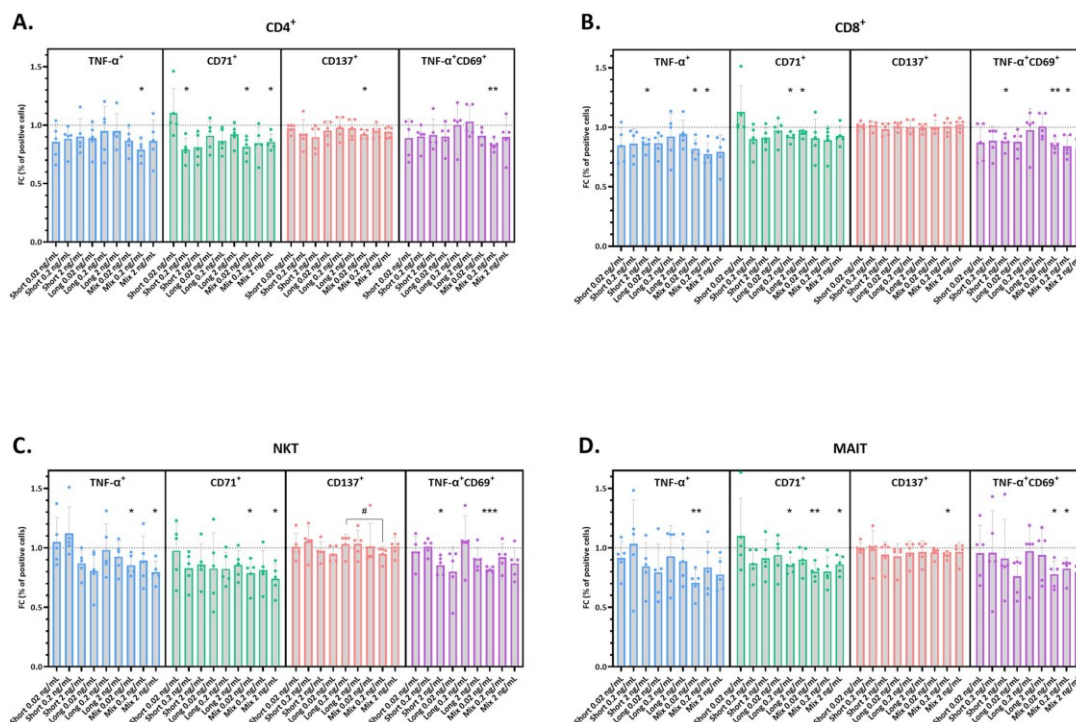
Figure 1. Graphical representation of the modulation of (A) CD4^{+}, (B) CD8^{+}, (C) NKT, and (D) MAIT cells expression of TNF-α (blue), CD71 (green), CD137 (orange), and co-expression of TNF-α and CD69 (violet) induced by PFAS mixtures. PBMCs were exposed to different PFAS mixtures at the concentration of 0.02, 0.2, and 2 ng/mL for 20 h and subsequently stimulated with anti-CD3 (0.5 ng/mL) + anti-CD28 (0.5 μg/mL) for 6 h. Brefeldin A was added for the last 4 h. The different lymphocyte populations were detected by flow cytometry, and cell activation was measured through the intracellular staining of the selected markers (% of positive cells). Results are expressed as FC calculated on anti-CD3/CD28-stimulated solvent-treated PBMCs (DMSO), which is set at 1.0 (dotted line). The bars represent the mean of 5 donors ± SD, and each dot represents the single value of the donor. Statistical analysis was performed following one-way ANOVA, followed by Dunnett's multiple comparison test, with $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ *vs* DMSO. Differences between treatments were assessed through one-way ANOVA, followed by Tukey's multiple comparison test, with $\#p \leq 0.05$ Mix 0.2 ng/mL *vs* Long 0.2 ng/mL. Representative contour density plots of CD4^{+} cell activation levels for DMSO, Short 2 ng/mL, Long 2 ng/mL, and Mix 2 ng/mL PFAS, following anti-CD3/CD28 stimulation are shown in Supplementary Fig. 8.

PFAS mixtures reduced the activation of MAIT cells upon *E. coli* K12 stimulation

Since MAIT cells were highly targeted by PFAS (as observed after CD3/CD28 stimulation) and being also the type of cells susceptible to other chemicals, such as bisphenols (Krause et al., 2022), we decided to be selective on these cells. For this purpose, we stimulated these cells within PBMCs in a specific way, using *E. coli* K12, since MAIT cells are activated by bacterial metabolites. The treatment of PBMCs to increasing concentrations of different PFAS mixtures before the activation with bacteria led to a decreased activation of MAIT cells (Fig. 2A). Interestingly, short-chain PFAS exposure induced a statistically significant decrease of IFN-γ production, long-chain PFAS reduced both IFN-γ and TNF-α-CD69 expression levels, and once again the impact of the mixture of short- and long-chain PFAS at the highest tested

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concentration was reflected in the downregulation of both pro-inflammatory cytokine levels and of their co-expression with CD69.
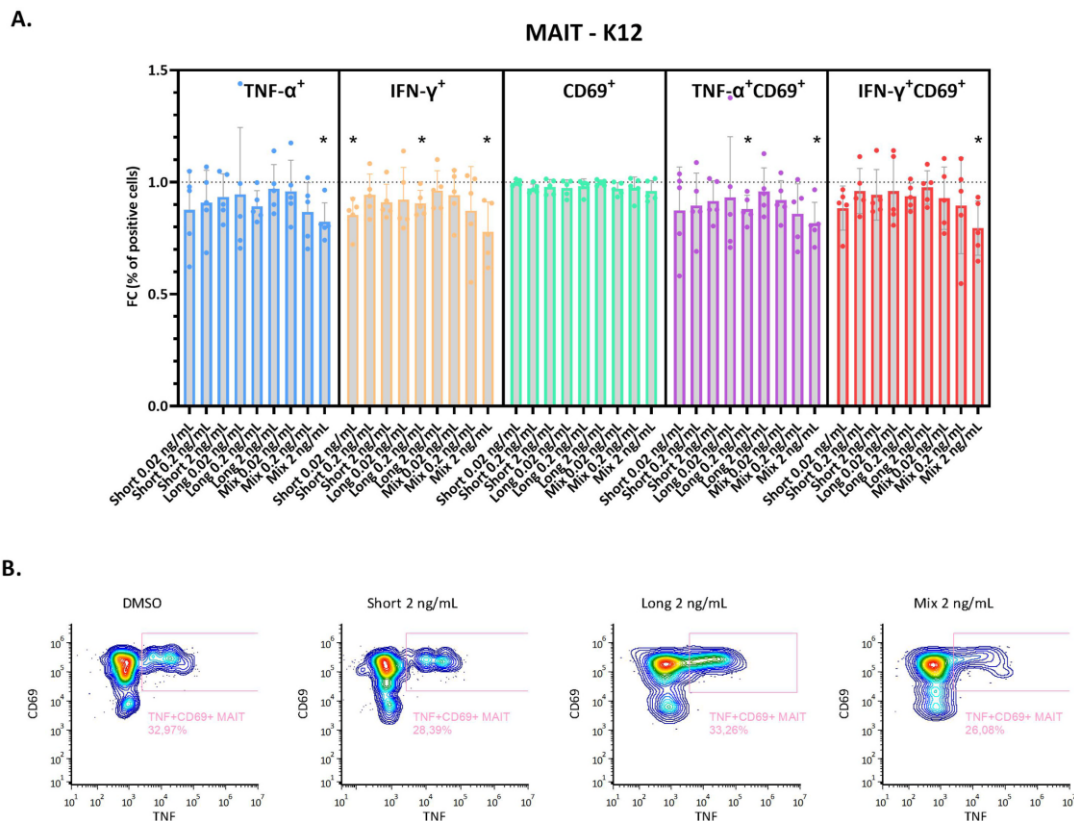
Figure 2. (A) Modulation of MAIT cell activation by expression of TNF-α (blue), IFN-γ (yellow), CD69 (light green), and co-expression of TNF-α and CD69 (violet), and of IFN-γ and CD69 (red), induced by PFAS. PBMCs were exposed to different PFAS and mixtures at the concentration of 0.02, 0.2, and 2 ng/mL for 20 h and subsequently stimulated with *E. coli* K12 (10 BpC) for 6 h. Brefeldin A was added for the last 4 h. MAIT cells population was detected by flow cytometry, and cell activation was measured through the intracellular staining of the selected markers (% of positive cells). Results are expressed as FC calculated on *E. coli* K12-
stimulated solvent-treated PBMCs (DMSO), which is set at 1.0 (dotted line). The bars represent the mean of 5 donors ± SD, and each dot represents the single value of the donor. Statistical analysis was performed following one-way ANOVA, followed by Dunnett's multiple comparison test, with $*p \leq 0.05$ *vs* DMSO. (B) Representative contour density plots of MAIT cell activation levels (TNF-α$^+$CD69$^+$) for DMSO, Short 2 ng/mL, Long 2 ng/mL, and Mix 2 ng/mL PFAS and subsequent *E. coli* K12 stimulation are reported.

The flow cytometric data obtained from the treatment at the highest PFAS concentration were further analyzed with the t-SNE transformation tool (Fig. 3). Clusters based on the expression of IFN-γ (Fig. 3A), TNF-α (Fig. 3B), and CD69 (Fig. 3C) were manually gated, and the % of gated cells were analyzed (Fig. 3D). T-SNE analysis revealed the ability of short-chain PFAS to statistically significantly reduce the % of cells present in cluster 1 TNF-α and in cluster 1 IFN-γ, whereas long-chain PFAS reduced only cluster 1 TNF-α (Fig. 3E). The mixture of

short- and long-chain PFAS was able to down-regulate all the activated clusters, and as consequence to increase the low activated and the inactivated cluster (Fig. 3E). In particular, there was a statistically significant difference in the inactivated cluster following treatment with long-chain PFAS and the mixture of both short and long.
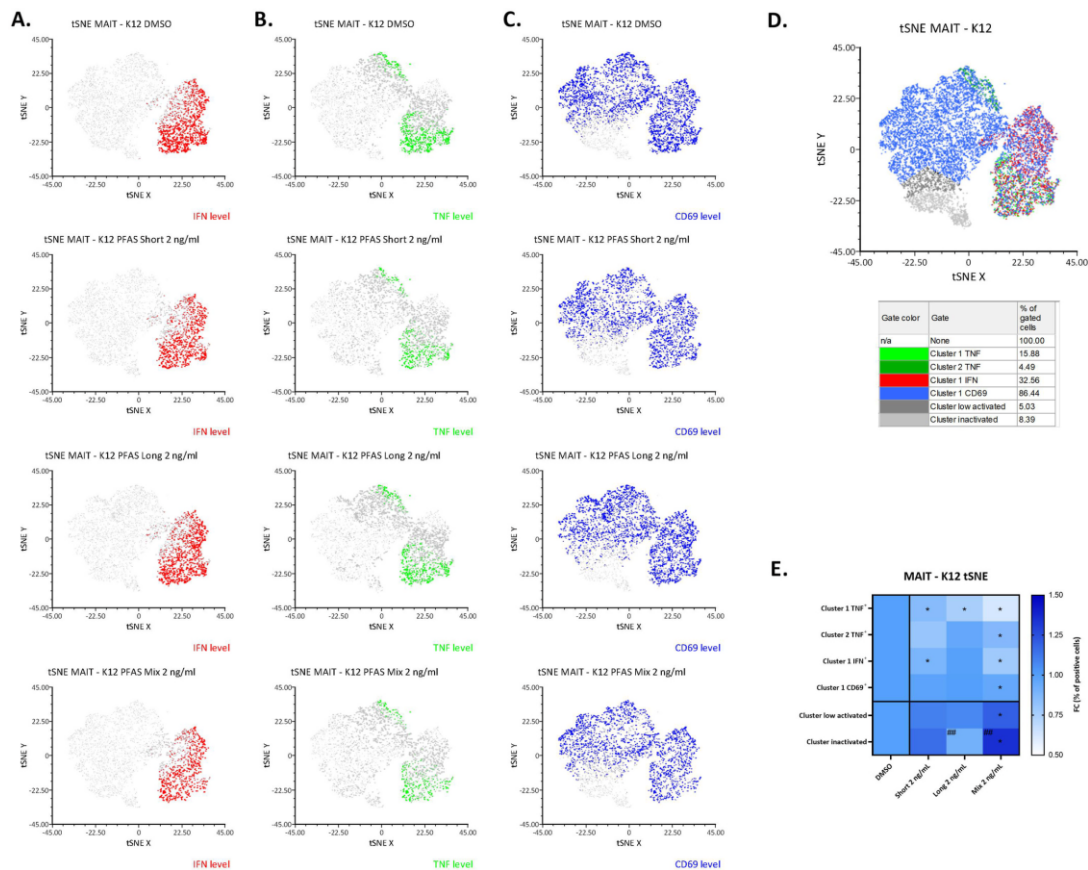
Figure 3. (2D t-SNE representation of MAIT cells subpopulations expressing (A) IFN-γ (red), (B) TNF-α (green), and (C) CD69 (blue). The different maps represent the 4 merged treatment conditions (DMSO, Short 2 ng/mL, Long 2 ng/mL, and Mix 2 ng/mL PFAS). Each dot represents a single event. PBMCs were exposed to different PFAS mixtures at the concentration of 2 ng/mL for 20 h and subsequently stimulated with *E. coli* K12 (10 BpC) for 6 h. Brefeldin A was added for the last 4 h. MAIT cells population was detected by flow cytometry using t-SNE transformation tool, and cell activation was measured setting a threshold level of 2000 for IFN-γ and TNF-α and of 20000 for CD69, to allow a good discrimination between positive and negative cells. Events with expression levels lower than the threshold value appear in grey, whereas events with expression levels higher than the threshold value appear colored (red, green or blue, with respect to the analyzed marker). (D) 2D t-SNE map of all merged samples (four conditions for the five donors) representing the 6 clusters of MAIT cells manually gated, based on the expression levels of the markers: Cluster 1 TNF (light green), Cluster 2 TNF (dark green), Cluster 1 IFN (red), Cluster 1 CD69 (blue), Cluster low activated (dark grey), and Cluster inactivated (light grey). The table shows the % of gated cells. The 2D t-SNE maps for the grouped treatment conditions are shown in Supplementary Fig. 9 (E) Heatmap with the data obtained from the % of gated cells of each donor and treatment condition. Results are expressed as FC calculated on *E. coli* K12-stimulated solvent-treated PBMCs (DMSO), which is set at 1.0 (light blue). Lower levels are represented in lighter blue to white (until 0.5), whereas higher levels are expressed in darker blue (until 1.5). The results represent the mean of 5 donors. Statistical analysis was performed following Welch's t-test, with

*$p \leq 0.05$ *vs* DMSO and following paired t-test to analyze the difference within the treatments with ##$p \leq 0.01$ Mix 2 ng/mL *vs* Long 2 ng/mL.

Gene expression analysis of PBMCs upon PFAS exposure and *E. coli* K12 stimulation

To unravel the possible mechanisms underneath MAIT cells' reduced activation induced by PFAS mixtures at the highest concentration (2 ng/mL), gene expression analysis following *E. coli* K12 stimulation was performed in PBMCs. Although no sorted MAIT cells have been used here, data gained by the flow cytometric analysis showed that in PBMCs stimulated via *E. coli* K12 the main populations activated were MAIT cells (Supplementary Figs. 3A, 3B, and 3C). Thus, it is reasonable that gene activation in PBMCs after *E. coli* treatment can be assigned mainly to MAIT cells. Out of the 39 analyzed genes, the expression of 18 genes was significantly impacted by the treatment with PFAS mixtures (Fig. 4), whereas the expression of 21 genes was not affected by PFAS treatment in a statistically significant way, although the general trend is of downregulation (Supplementary Table S4).

Most significant changes were observed when comparing treatment with mixtures of short-long PFAS to DMSO and to short PFAS-mixtures. The expression of all the analyzed genes was downregulated after PFAS treatment, above all following short-chain and short-long PFAS. The significantly highest changes of gene expression to DMSO were observed for NFKB2, pro-inflammatory cytokines, namely IFNG and TNFSF15, cytokines and chemokines receptors, namely CCR5, CCR6, CXCR4, IL12RB1, and IL23R, and several genes typically expressed by MAIT cells, such as GZMB, IRF4, and CSF2. The down-regulation profile of immune-related genes of MAIT cells induced by PFAS mixture supports the immunosuppressive behavior observed on T cells activation.
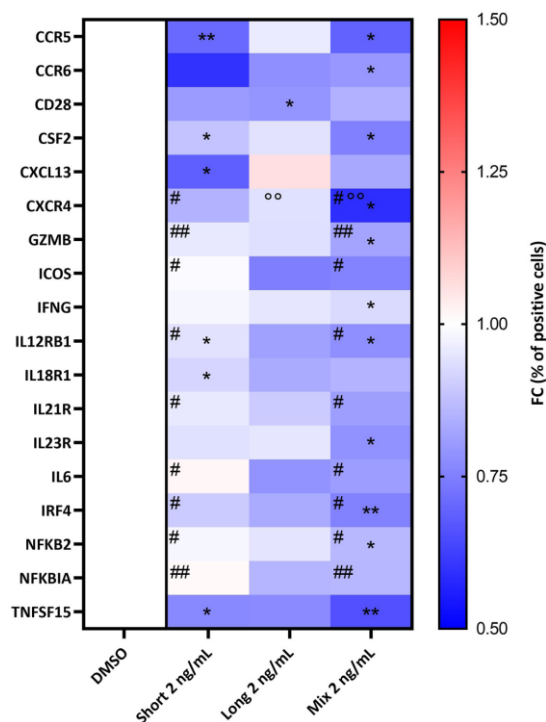
Figure 4. Heat map representing the statistically significant association between PFAS treatment and gene expression profile of PBMC. PBMCs were exposed to different PFAS mixtures at the concentration of 2 ng/mL for 20 h and subsequently stimulated with *E. coli* K12 (10 BpC) for 6 h to activate MAIT cells. Data are expressed as FC calculated on *E. coli* K12-stimulated solvent-treated PBMCs (DMSO), which is set at 1.0 (white). Color code represents up-regulation (toward red – 1.5) or down-regulation (toward blue – 0.5). The results represent the mean of 5 donors. Statistical analysis was performed following Welch's t test, with *$p \leq 0.05$, **$p \leq 0.01$ *vs* DMSO, and the differences between the treatment conditions were assessed following paired t-test with #$p \leq 0.05$, ##$p \leq 0.01$ for Mix 2 ng/mL *vs* Short 2 ng/mL and °$p \leq 0.01$ for Mix 2 ng/mL *vs* Long 2 ng/mL.

PFAS mixtures reduced the activation of basophils

In addition to T cells, another focus of this study were basophils as a proxy for innate immune responses. In this assay, whole blood samples were stimulated in two different ways to highlight the innate or the adaptive basophil response. When stimulated with fMLP, a chemotactic peptide involved in the innate immunity against pathogens, basophils innate response is activated, whereas anti-FcεR1α targets the IgE receptor, inducing the adaptive basophil response. Following the preexposure to different PFAS mixtures, based on their chain length, PFAS were able to reduce basophil activation, induced by anti-FcεR1α (Fig. 5A). In particular, long-chain PFAS were able to statistically significantly reduce the expression of the activation marker CD63 (Fig. 5A). When analyzing the different level of expression of the activation marker, in addition to long-chain PFAS, also the mixture of both short- and long-chain PFAS was able to reduce the % of cells highly expressing CD63 (Fig. 5A). In this case, the exposure to the mixture does not result into an additive effect, but the most effectiveness

was attributed to long-chain PFAS. Therefore, PFAS ability to reduce the adaptive response of basophils was demonstrated, instead regarding the innate response, no effect was observed (Fig. 5B).
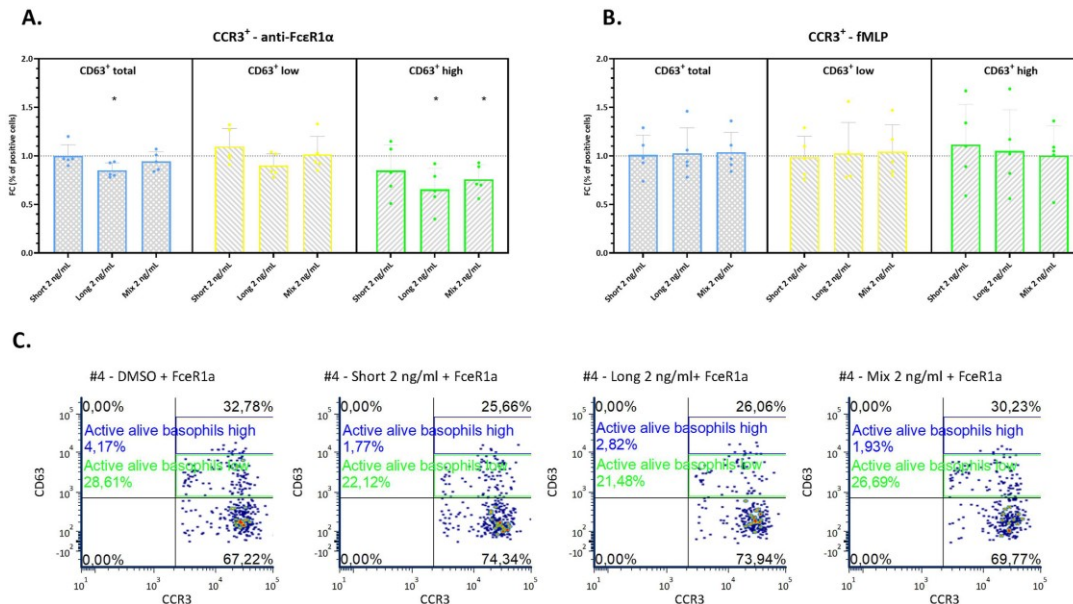
Figure 5. Modulation of basophil (CCR3$^+$ cells) activation by expression of CD63 analysis following stimulation with anti-FcεR1α (A) or with fMLP (B). The first panel represents the % of basophils positive to CD63 (CD63$^+$ total, blue), the second panel represents the % of basophils positive to CD63 with an expression level lower than 10^4 (CD63$^+$ low, yellow), whereas the last panel represents the % of basophils positive to CD63 with an expression level higher than 10^4 (CD63$^+$ high, green). Whole blood was exposed to different PFAS mixtures at the concentration of 2 ng/mL, or DMSO as control, for 1 h and subsequently stimulated with anti-FcεR1α (0.1 μg/mL) or fMLP (0.05 μM) for 25 min. Basophils population was detected by flow cytometry, and cell activation was measured through the surface staining of CD63 (% of positive cells). Results are expressed as FC calculated on anti-FcεR1α- or fMLP-stimulated solvent-treated whole blood (DMSO), which is set at 1.0 (dotted line). The bars represent the mean of 5 donors ± SD, and each dot represents the single value of the donor. Statistical analysis was performed following Welch's t-test, with *$p \leq 0.05$ *vs* DMSO. (C) Representative density plots of basophils activation level (CD63$^+$) for DMSO, Short 2 ng/mL, Long 2 ng/mL, and Mix 2 ng/mL PFAS and subsequent stimulation with anti-FcεR1α are reported.

Discussion

PFAS toxicity is of great concern considering the widespread environmental contamination and the daily exposure of humans through different ways. While some aspects of PFAS immunotoxicity are well established, others, like the effect on specific immune cell subtypes, have never been investigated. In our study, by using primary human immune cells and different stimulation strategies, i.e. anti-CD3/CD28 for general TCR activation and bacteria (*E. coli* K12), fMLP and anti- FcεR1α for specific immune activation, we were able to identify subtle effects on different immune cell subtypes. By using multi parameter flow cytometry

analysis, we observed a reduced activation of CD4^{+} T helper, CD8^{+} cytotoxic, NKT and MAIT cells after PFAS treatment. In addition, we detected a reduced expression of genes involved in T cell activation and regulation such as IFNG, TNFSF15, IRF4, IL23R, CCR5 CCR6, CXCR4 after PFAS treatment and bacterial activation of immune cells. To the best of our knowledge, this is the first study to show a direct effect of mixture of PFAS on human primary immune cells, demonstrating their ability, at concentrations mimicking real-life exposure, to reduce the activation of immune cells involved in both the innate and the adaptive immune response. A reduced activation of immune responses could, potentially, lead to adverse health outcomes due to the decreased capability to fight against pathogens (Medzhitov, 2007). Furthermore, the reduction of T lymphocytes activation can be also linked to the suppressive effects of PFAS on antibody levels following vaccination. Indeed, T cells activation is necessary for B cells activation and for the subsequent antibody production. The suppressive effects of PFAS (PFOS and PFOA, mainly) on antibody levels following vaccination, in children, have been widely assessed (Ehrlich et al., 2023; EFSA Panel on Contaminants in the Food Chain et al., 2018), although the clear underneath mechanism is not fully understood. Therefore, our findings on decreased T cells activation could be linked to this observation, and more investigations on this possible association should be performed.

As aforementioned, the main PFAS found in human samples are PFOA, PFOS, PFNA, and PFHxS (EFSA Panel on Contaminants in the Food Chain et al., 2020). The serum levels of Czech Republic population revealed their median levels between 0.2 and 2.4 ng/mL (Sochorová et al., 2017). Similarly, in UK women levels between 0.6 and 3.5 ng/mL have been found (Heffernan et al., 2018). Similar concentrations have been found also in German children and adolescents (Duffek et al., 2020). Regarding mixtures, a recent study analyzed the serum concentrations of 16 PFAS in 61 Italian children and the total level ranged between 1 and 13 ng/mL (Ledda et al., 2018). Based on these published serum concentrations, we chose the PFAS concentration for *in vitro* testing in a range from 0.02 to 2 ng/mL. The human exposure to long-chain PFAS and, above all, of PFOA and PFOS is decreasing worldwide (Okada et al., 2013; Gomis et al., 2017; East et al., 2021), due to the restrictions applied. Nevertheless, due to their difficult degradation they will be found for longer in the environment and human body compartments (Renner, 2001; Giesy and Kannan, 2002; Huset and Barry, 2018). Concomitantly, the exposure to short-chain PFAS will probably increase (Sunderland et al., 2019). Short-chain PFAS are characterized by a lower accumulation, but they are already widely present in the environment (Brendel et al., 2018). The few available studies regarding these substitutes suggest an equal toxicity level (Gomis et al., 2018). Regarding the mechanism,

the few information available suggest that both short- and long-chain PFAS are able to act on steroid hormone precursors and can bind to steroid hormone receptors, possibly altering the endocrine pathways (Mokra, 2021). As a results, several endocrine disrupting effects of PFAS have been observed in epidemiological studies (Lum et al., 2017; Blake et al., 2018; Byrne et al., 2018; Tian et al., 2019; Mancini et al., 2020). Regarding the putative targets, PFOA, PFOS, PFHxS, and PFNA are able to activate PPARα and related transcription factors, and to a less extent also PPARγ and estrogen receptor (Benninghoff et al., 2011; Rosen et al., 2017). In addition, PFOS can interact with thyroid receptor and glucocorticoid receptor (Du et al., 2013; Salgado-Freiría et al., 2018; Masi et al., 2022). Controversies regarding the effects on nuclear hormones resulted from the different cells analyzed. Due to the strict interconnection between the endocrine and the immune system, it is reasonable to hypothesize that immune effects could be a result of the effect of PFAS on hormone receptors. In our study, we showed that both, short- and long-chain PFAS, can lead to diminished activation of different immune cells. But the mixture of both short- and long-chain PFAS, as a proxy of real-life exposure, had a slightly higher effect. Regarding the effects on lymphocyte activation, where a concentration-response study was performed, it can be noted that generally higher concentrations induced a higher suppression of activation, therefore more in-depth studies were performed at the highest concentration of 2 ng/mL. In particular, PFAS induced a decreased MAIT cells activation, a T cell subtype present in human mucosa, acting as a first defense against bacteria (*Cowley, 2014; Legoux et al., 2020).* The t-SNE analysis of the effects of PFAS mixtures on *E. coli* K12-activated MAIT cells revealed a shift of population in favor of inactive cells. In addition, several genes related to MAIT cells functioning and activation were down-regulated following PFAS mixtures exposure. Activated MAIT cells express several cytokine and chemokine receptors, namely CCR5, CCR6, IL12R, IL18R, whose genes were all down-regulated by PFAS. Furthermore, IFNG and TNFSF15, which are typical Th1 cytokines, were down-regulated, together with IRF4, indicating an immunosuppressive action more on Th1-like MAIT cell subset. Due to their semi-invariant TCR Vα7.2, MAIT cells belong to the interface of innate and adaptive immune responses being protective to microbial agents (Ioannidis et al., 2020). Inhibitory effects on the activation of these cells might lead to increased numbers of infections (Rudak et al., 2018; Hinks and Zhang, 2020). Regarding CD8^{+} cytotoxic T cells, a reduced activation of these cells might be unfavorable for the elimination of infected host cells and have also consequences for tumor immune surveillance (Gulzar and Copeland, 2004; Ostroumov et al., 2018; Gupta et al., 2020). Also, the reduced activation of NKT cells can have important impact on immunity. This type of cells is involved in both innate and adaptive immunity, acting

as first line of defense against viruses and bacteria and being also involved in tumor growth surveillance and autoimmune disorders (Brutkiewicz and Sriram, 2002; Van Dommelen and Degli-Esposti, 2004; Vogt and Mattner, 2021).

One other important T cell subtype we analyzed is $CD4^+$ T helper cells that, within other activities, play an important role in sustaining B cells in their generation of antigen-specific antibodies (Zubler, 1984; Noelle and Snow, 1992). The few data available on this suggest that the reduction of antibody response induced by PFAS could be mediated by an effect on T helper cells, such as a defective T helper 2 response (Bell et al., 2021). In our study, we observed a decreased activation of $CD4^+$ T cells after PFAS treatment. Thus, a reduced $CD4^+$ T helper cell activation by PFAS might be the basis for less responsiveness to vaccines (Clem, 2011; Munier et al., 2016). In epidemiological studies, increased PFAS concentrations were associated with lower responses to vaccination against Tetanus, Diphtheria, measles, mumps, and rubella (Kielsen et al., 2016; Stein et al., 2016; Grandjean et al., 2017; Timmermann et al., 2020; Zhang et al., 2022). Overall, our study points out a reduced activation of all the analyzed T cell subsets: $CD4^+$, $CD8^+$, NKT and MAIT, making reasonable a link between PFAS exposure and noxious immune-related effects. Thus, our findings offer first possible mechanistic explanations for the reported health outcomes observed in epidemiological studies, suggesting T cell population activation as possible target of PFAS immunotoxic effects. It is noteworthy that the effect of PFAS was slightly different in MAIT cells activated by either polyclonal or bacterial stimulation. This suggests that the type of stimulation may influence the concentration threshold at which PFAS act and needs to be considered in the design of future immunological risk assessment studies.

The other cell type analyzed in this study is represented by basophils. Here, we stimulated them differently to specifically activate their innate or adaptive role. fMLP stimulation activates the innate role, whereas anti-FcεR1α induces the adaptive response. In our study, a reduced activation of basophils following anti-FcεR1α stimulation was observed. Therefore, no interference with the innate behavior of basophils, but a reduced adaptive response was highlighted. Contrary to T cells, for basophils, only few information on PFAS effect can be retrieved in literature. The only data associated children high PFAS levels with increased basophil number (Oulhote et al., 2017). It is widely known that basophils are involved in allergies, due to the high release of IL-4 and their ability to drive antibody responses (Sokol and Medzhitov, 2010; Schwartz and Voehringer, 2011; Yamanishi and Karasuyama, 2016; Kashiwakura et al., 2019; Miyake et al., 2021). FcεR1α-activated basophils are involved in T helper 2 differentiation and $CD8^+$ differentiation toward IL-10 secreting cells (Kim et al., 2009;

Sokol and Medzhitov, 2010). Indeed, in our study the reduced basophils activation is in line with the reduced T cells activation, and we can also speculate a link between the reduced IL-10 gene expression observed in PBMCs activated by *E. coli*. Therefore, a reduced basophil activation after PFAS-exposure might strengthen the effects observed for T cells in terms of proper adaptive immune responses. This study offers a first investigation of PFAS immunotoxicity on human primary cells and has some limitations. First of all, due to hormonal effects of PFAS, only samples from male donors were used to avoid the impact of female cycle variations. Thus, tests should be followed by proper evaluation of the effects on cells from female donors. Furthermore, the results should be validated in a larger donor cohort, ideally also including highly exposed individuals, vulnerable people and children. Adding also other incubation timing, to consider both acute or chronic exposure to PFAS, would allow to better understand the immunological background of PFAS-related epidemiological findings. In addition, our gene expression analysis was performed in PBMC. For a better understanding of PFAS effects at molecular level, analyses should be performed in purified immune cell subtypes. Also other PFAS, should be tested to increase the number and to have a wider view in terms of toxicological effect and immune system impairment. In particular, we limited our research on perfluoroalkyl carboxylic acids and sulfonic acids, which were mainly linked to immune outcomes (von Holst et al., 2021). Finally, other concentrations of PFAS, e.g. as found in complex real-life mixtures of human samples, should be used in a mixture risk assessment with human immune cells. Another important issue that is urgent to be solved is the estimation of the fraction of PFAS that reach the cells, and therefore the active concentration.

Conclusions

The exposure of human beings involves multiple chemicals (Braun et al., 2016; Escher et al., 2020), therefore it is essential to consider combined effects, when evaluating the harmful potential of substances, in particular in the case of PFAS which is a matter of mixtures. To deal with it, there is an urgent need of immunotoxicity assays for the assessment of the hazard of mixtures with the aim to regulate them. The assays proposed in this study could be used for this purpose. In the present study, we evaluated the immunotoxic potential of PFAS, in order to have an indication of their impact on the human immune system. Using *in vitro* assays employing human primary blood cells, we showed that PFAS mixtures have the capability to reduce immune cell activation, as observed for CD4$^+$, CD8$^+$ cells, as important players of the adaptive immune response. Further, we observed an impairment of NKT and MAIT cells, cells at the

interface between innate and adaptive immunity, that were negatively impacted by PFAS exposure. In conclusion, we observed a stronger effect of both short- and long-chain PFAS mixtures when exposed to T cells, suggesting a possible additive effect of mixtures.

Supplementary Material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.139204.

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Impact of chemical mixtures from wastewater treatment plant effluents on human immune cell activation: an effect-based analysis

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Abstract

Background: Humans are exposed to many different chemicals on a daily basis, mostly as chemical mixtures, usually from food, consumer products and the environment. Wastewater treatment plant effluent contains mixtures of chemicals that have been discarded or excreted by humans and not removed by water treatment. These effluents contribute directly to water pollution, they are used in agriculture and may affect human health. The possible effect of such chemical mixtures on the immune system has not been characterized.

Objective: The aim of this study was to investigate the effect of extracts obtained from four European wastewater treatment plant effluents on human primary immune cell activation.

Methods: Immune cells were exposed to the effluent extracts and modulation of cell activation was performed by multi-parameter flow cytometry. Messenger-RNA (mRNA)

expression of genes related to immune system and hormone receptors was measured by RT-PCR.

Results: The exposure of immune cells to these extracts, containing 339 detected chemicals, significantly reduced the activation of human lymphocytes, mainly affecting T helper and mucosal-associated invariant T cells. In addition, basophil activation was also altered upon mixture exposure. Concerning mRNA expression, we observed that 12 transcripts were down-regulated by at least one extract while 11 were up-regulated. Correlation analyses between the analyzed immune parameters and the concentration of chemicals in the WWTP extracts, highlighted the most immunomodulatory chemicals.

Discussion: Our results suggest that the mixture of chemicals present in the effluents of wastewater treatment plants could be considered as immunosuppressive, due to their ability to interfere with the activation of immune cells, a process of utmost importance for the functionality of the immune system. The combined approach of immune effect-based analysis and chemical content analysis used in our study provides a useful tool for investigating the effect of environmental mixtures on the human immune response.

Graphical abstract
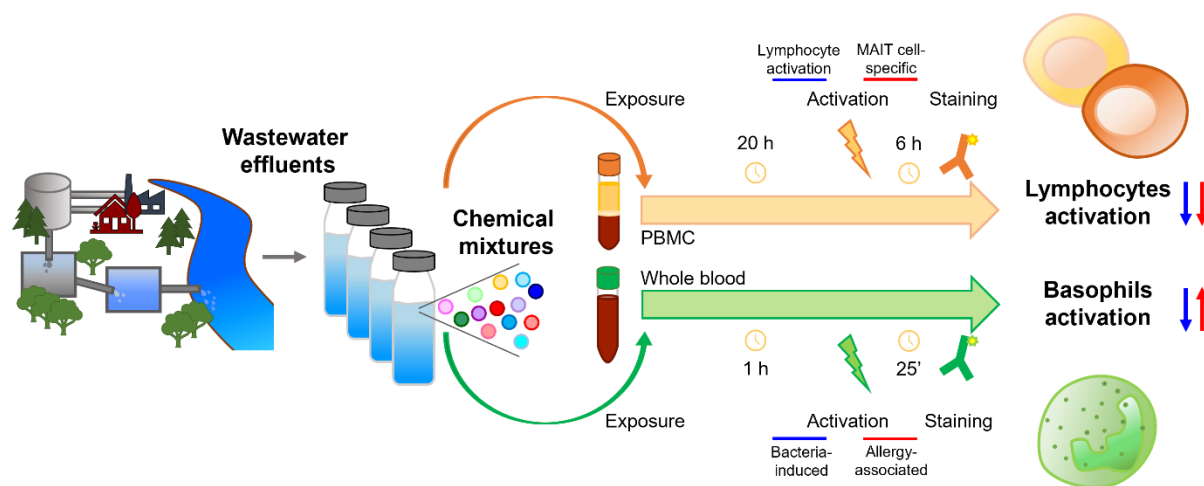
Keywords

Chemical mixtures, wastewater treatment plant, immunotoxicity, lymphocytes, MAIT cells, effect-based analysis

Introduction

The entire ecosystem and humans are constantly exposed to mixtures of potentially hazardous substances. More than 350'000 known chemicals have been registered in 2020 in the global market (Wang et al., 2020).

Although contaminating chemicals are usually present in low concentrations, their effects could be enhanced in mixtures. Many chemicals, including urban runoff chemicals, biocides and pesticides, personal care products, pharmaceuticals, and cleaning agents can pass the wastewater treatment plants (WWTP) and reach the water cycle as mixture of micropollutants with typical concentrations in the ng/L to µg/L range (Bourdat-Deschamps et al., 2014; Robles-Molina et al., 2014; Gorga et al., 2013; Ribeiro et al., 2015; Kümmerer, 2009). To date, these micropollutants are among the major contributors to aquatic pollution (Lee et al., 2022; Neale et al., 2020; Gago-Ferrero et al., 2020; Loos et al., 2013; Daughton, 2009). Even at such low concentrations, these compounds individually or as mixtures, may pose a toxic risk to aquatic life (Malaj et al., 2014), particularly due to the presence of pharmaceuticals (Yao et al., 2021; Wang et al., 2019; Giebułtowicz et al., 2018; Balakrishna et al., 2017; Ramos et al., 2016; Liu et al., 2015; Sim et al., 2010, Zhou et al., 2010; Kosonen and Kronberg, 2009; Daughton and Ternes, 1999). In addition to aquatic organisms, the chemical mixtures present in these effluents may also pose a risk to humans, due to treated wastewater reuse (Mukherjee & Chauhan, 2023). Currently, wastewater and treated wastewater irrigation is increasing to combat the depletion of freshwater resources and the water stress caused by climate change (Wu, 2020; Mishra et al., 2023; Dickin et al., 2016). This means that the general public could also be exposed, for example, by consuming food grown in contaminated areas or by bathing in contaminated recreational waters (Aftab et al., 2023; Khalid et al., 2018). Many publications show the chemical contamination of crops after irrigation with wastewater and treated wastewater, leading to contamination of the food chain (Othman et al., 2021; Xiaoqin Wu et al., 2014; Riemenschneider et al., 2016). For example, Xiaoqin Wu et al. measured the levels of 19 commonly occurring pharmaceutical and personal care products in 8 vegetables irrigated with treated wastewater under field conditions showing a high detection frequency for caffeine, meprobamate, primidone, DEET, carbamazepine, dilantin, naproxen, and triclosan (Xiaoqin Wu et al., 2014). Malchi and colleagues (2014) estimated the health risk associated with consumption of wastewater-irrigated root vegetables by using the threshold of toxicological concern (TTC) approach. The data showed that the TTC value of for example lamotrigine can be reached for a child at a daily consumption of half a carrot (~60 g) (Malchi et al., 2014). The use of untreated and treated wastewater to irrigate crops is common practice in arid and semi-

arid regions, and climate change will expand these regions and the reuse of wastewater. As a result, and also because of globalization, it is likely that larger parts of the population will become dependent on potentially contaminated food. Thus, there is an urgent need for analysis of the impact of the chemical mix present in wastewater and sewage treatment plants on human health, especially at the level of the immune system, where alterations are at the root of the development of many diseases.

To date, only few studies have investigated the effects of chemical mixtures on humans (reviewed in Bopp et al., 2016; Kumari and Kumar, 2020; Mustafa et al., 2023). The most commonly used approach is to analyze the interaction with nuclear receptors such as aryl hydrocarbon, estrogen, androgen or glucocorticoid receptors (Vinggaard et al., 2021), but complex cellular test systems are missing in humans. Endocrine disruption has been identified as a major effect in waste and surface water (Brion et al., 2019; Carvalho et al., 2019; Gómez et al., 2021; Kase et al., 2018; Leusch et al., 2018; Kunz et al., 2017; Wernersson et al., 2015).

It is known that the endocrine and the immune system are closely linked (Wensveen et al., 2019; Greives et al., 2017) and that hormones can lead to the activation or suppression of immune cells. This can then trigger a tissue/organ and organism response. Furthermore, both innate and adaptive immune cells express hormone receptors (Buskiewicz et al., 2016) and can be directly targeted by chemicals. Endocrine-mediated changes in the immune system and immunomodulation by single chemicals have already been demonstrated (Maddalon et al., 2022a; Maddalon et al., 2022b; Krause et al., 2022; Adegoke et al., 2021; Rogers et al., 2013), but the effects of complex chemical mixtures on the immune system have rarely been investigated (Bulka et al., 2021; Warner et al., 2021; Bonefeld et al., 2017; Thompson et al., 2015). The few data available suggest an immunotoxic effect of chemical mixtures (O'Dell et al., 2021; Maddalon et al., 2023), but the studies are limited to a small number of chemicals tested. It is important to study the effect of chemical mixtures on the immune system because increased activation of the immune system can lead to autoimmune diseases or allergies, while decreased activation (e.g. immunosuppression) can increase the risk of infection or neoplasia, both of which pose a risk to human health (Rijkers et al., 2021).

Our present investigation, building on studies by Finckh and colleagues (2022a; 2022b), integrates the characterization of four European WWTP effluent samples into an immune-effect-based analysis. We investigate the effect of chemical mixtures present in WWTP effluents on the activation of human immune cells, namely T helper (CD4$^+$), cytotoxic T (CD8$^+$), mucosal-associated invariant T (MAIT), natural killer (NK), NKT cells, B cells, and basophils. These cells cover both the innate and the adaptive immune response. With this study, we want

to point out the need to test the putative health effects, particularly at the level of immune cells, of treated wastewater prior to its reuse in order to identify chemicals of concern, and to improve the quality of wastewater treatment.

Material and Methods

Preparation of WWTP effluent extracts

From a set of 56 European WWTP effluent samples (Finckh et al., 2022a; 2022b), four of them were selected based on the overall chemical burden (e.g. including EDCs) and *in vitro* PPARγ and aryl hydrocarbon receptor responses previously obtained on SH-SY5Y cell line (Lee et al., 2022). WWTP effluents were sampled in four different European countries between summer 2018 and spring 2019 as specified in Table 1. Sampling, extraction and storage of the WWTP effluents has been previously described by Finckh et al. (2022b). In brief, WWTP effluents were extracted on site using a large-volume solid phase extraction (LVSPE) device with a polymer sorbent (Macherey-Nagle HR-X). For the preparation of blank samples, 1 L of liquid chromatography mass spectrometry grade water was extracted by LVSPE in the laboratory, transported during sampling and processed along with the sample cartridges. After elution, the extracts were dissolved in methanol at a relative enrichment factor (REF) of 1000 and stored at -20 °C until further analysis. The concentrations of the chemicals detected by LC-HRMS screening in the four WWTP extracts are shown in Supplementary File 1 (reprinted from Finckh et al., 2022b. For simplicity reasons, from now we name the WWTP effluent sample extracts "WWTP extracts".

Table 1
WWTP effluents tested: acronym, sample name, sample code by Finckh et al. (2022a; 2022b), sampling country and date. Note: For B (blank) the sampling country is not applicable (NA) since it is a machine blank.

Acronym	Sample name	Sample code	Sampling country	Sampling date
B	Blank	EU203	NA	NA
E1	Effluent 1	EU102	Czech Republic	16 July 2018
E2	Effluent 2	EU112	Germany	30 August 2018
E3	Effluent 3	EU120	Greece	9 January 2019
E4	Effluent 4	EU131	France	9 April 2019

Prior to cellular treatment, WWTP extracts were thawed, and methanol was evaporated under nitrogen. The extracts were resuspended in the same volume of culture medium (IMDM

or BAT buffer – explained below) to obtain the enrichment factor of 1000 compared to the original WWTP extracts. Several dilutions were tested (REF 25, 12.5, and 6.25).

Peripheral blood mononuclear cells (PBMCs) treatment with WWTP extracts

The study was approved by the Ethics Committees of the University of Leipzig (#079-15-09032015). Buffy coat blood from five pseudonymous healthy male donors was obtained from the blood bank at the University of Leipzig, after written informed consent. Only male donors were selected to avoid intra-cycle differences among women. PBMCs were isolated by density gradient centrifugation, using Ficoll-Paque Plus (Cytiva Sweden AB, Uppsala, Sweden) and PBMCs were stored at -150 °C in FCS/10%DMSO until use. For experiments, PBMCs were thawed and suspended in IMDM (Iscove's modified Dulbecco's medium - Gibco, Thermo Fisher Scientific, Waltham, US) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, US), 1X Penicillin-Streptomycin (Gibco, Thermo Fisher Scientific, Waltham, US), and 50 µM β-mercaptoethanol (Sigma-Aldrich, St. Louis, US). PBMCs were seeded at $1x10^6$ cells/well in 96-well U-bottom microplates (Greiner Bio-One, Frickenhausen, Germany) and rested for at least 2 hours at 37 °C in a 5% CO_2 incubator before treatment.

PBMCs were exposed to WWTP extracts at final REF of 6.25, 12.5, and 25. Initial experiments (Supplementary Figure 1A) proved that a final REF of 25 did not exhibit cytotoxicity on stimulated PBMCs, and concurrently to each experiment, cell viability was assessed for each exposure condition. As control for the WWTP extracts one column elution sample (B – EU203) was used as blank at a REF of 25. After 20 h exposure at 37 °C and 5% CO_2, PBMCs were stimulated with anti-CD3 and anti-CD28 antibodies (anti-CD3/CD28) or with *Escherichia coli* K12 (*E. coli* K12) for 6 hours, as better explained in the next paragraphs. Negative controls were included, namely PBMCs stimulated with anti-CD28 alone and with IMDM only, respectively. Stimulation with anti-CD28 allows to evaluate unspecific activation levels. None of the donors showed unspecific activation, therefore all donors were considered suitable for the analyses.

Lymphocytes activation

Lymphocytes activation was induced by activating CD3 and CD28 molecules expressed on lymphocytes. Soluble anti-CD3 (clone: OKT3; 0.5 ng/mL) and anti-CD28 (clone: CD28.2; 0.5 µg/mL) (BioLegend, San Diego, US) antibodies were added to PBMCs previously exposed to WWTP extracts and incubated for 6 hours at 37 °C and 5% CO_2. The optimal concentrations of anti-CD3 and anti-CD28 were previously determined, and the expression of the activation

markers induced by anti-CD3/CD28 stimulation are reported in Supplementary material (Suppl. Fig. 2 for CD4$^+$ and CD8$^+$, Suppl. Fig. 3 for MAIT and NKT cells, and Suppl. Fig. 4 for B, NK, and double negative – DN cells). To block cytokine secretion, Brefeldin A (10 µg/mL; Sigma-Aldrich, St. Louis, US) was added for the last 4 h of incubation.

MAIT cells specific activation

To specifically activate the MAIT cells, a T cell subtype reacting to bacterial metabolites, an activation with bacteria was used. The bacterial strain *Escherichia coli* K12 MG1655 was cultivated as previously described (Krause et al., 2022) and stored at -80 °C until use. Following 20 hours of exposure to WWTP extracts, PBMCs were stimulated with 10 BpC (bacteria per cell) of *E. coli* K12 for 6 hours at 37 °C in a 5% CO_2 incubator, and for the final 4 hours of incubation, Brefeldin A (10 µg/mL) was added. The degree of activation f MAIT cells induced by *E. coli* K12 (10 BpC) is reported in Suppl. Fig. 5.

Flow cytometrical analysis of PBMCs

Following stimulation, PBMCs were transferred to V-bottom 96-well microplates (Thermo Fisher Scientific Waltham, US). Dead cells were excluded by staining with fixable viability dye-Zombie NIR^{TM} (BioLegend) for 15 minutes at RT. Staining for surface markers (CD3, CD4, CD8, CD19, CD56, CD161, and TCRVα7.2,) was performed for 20 minutes at RT according to protocol (Suppl. Table S1). After staining, PBMCs were fixed using FACS^{TM} Lysing Solution (BD Biosciences, San Jose, US) for 10 minutes, and permeabilized with FACS^{TM} Permeabilizing Solution 2 (BD Biosciences, San Jose, US) for further 10 minutes. Finally, cells were stained for intracellular markers (CD69, CD71, CD134, CD137, TNF-α, and IFN-γ) for 20 minutes at RT (Suppl. Table S1). The cytometric analysis was performed using the flow cytometer Cytek Aurora (Cytek Biosciences, California, US). A minimum of 100,000 viable T cells (NIR$^-$CD3$^+$) were acquired per sample, then lymphocytes were identified among total PBMCs using FSC-A and SSC-A. The gating strategies for lymphocytes and MAIT cells are reported in Suppl. Fig. 6 and 7. Briefly, lymphocytes were detected, following stimulation with anti-CD3/CD28, as follows: regarding CD3$^+$ cells, T helper cells were gated as CD3$^+$CD56$^-$CD161$^-$TCRVα7.2$^-$CD4$^+$, cytotoxic T cells were gated as CD3$^+$CD56$^-$CD161$^-$TCRVα7.2$^-$CD8$^+$, double negative (DN) were gated as CD3$^+$CD56$^-$CD161$^-$TCRVα7.2$^-$CD4$^-$CD8$^-$, NKT cells as CD3$^+$CD56$^+$, and MAIT cells as CD3$^+$CD56$^-$CD161$^+$TCRVα7.2$^+$; for CD3$^-$ cells, instead, B cells were gated as CD3$^-$CD4$^-$CD19$^+$, and NK cells as CD3$^-$CD4$^-$CD56$^+$ (Suppl. Fig. 6). Regarding the specific activation of MAIT cells, with *E. coli* K12, MAIT cells were

gated as CD3$^+$CD8$^+$CD161$^+$TCRVα7.2$^+$ (Suppl. Fig. 7). The expression of activation markers and intracellular pro-inflammatory cytokines within these lymphocyte populations were analyzed individually for TNF-α, IFN-γ, CD69, CD71, CD134, CD137, and the double expression of TNF-α and CD69 were expressed as % of positive cells for CD4$^+$, CD8$^+$, NKT, MAIT, CD4$^-$CD8$^-$, NK, and B cells. Only the activation markers responding to the stimulus in the certain immune cell populations are shown. Data analysis was then performed using FCS Express 7 (De Novo Software).

t-SNE analysis of stimulated PBMCs

Following conventional flow cytometrical analysis, an in-depth analysis for high dimensional data using t-distributed stochastic neighbor embedding (t-SNE) transformation tool (FCS Express 7, De Novo Software) was performed on gated CD4$^+$ cells following anti-CD3/CD28 stimulation and on gated MAIT cells following *E. coli* K12 stimulation. These cells have been chosen due to the most prominent effect of WWTP extracts on them. As representative effect, the highest tested concentration of every WWTP extract at REF of 25, together with the blank were selected for each donor. For the t-SNE analysis, the samples were merged and gated as for conventional analysis – Suppl. Fig. 6 and 7. Briefly, 50,000 total events for CD4$^+$ cells and of 200,000 for MAIT cells were selected. The down-sampling was selected as interval, with iteration number: 500, perplexity: 50, and approximation: 0.5. Furthermore, the opt-SNE and the estimation for unsampled events were chosen to generate 2D t-SNE maps. The single treatment conditions (5 per donor) and the group conditions (B, E1, E2, E3, E4) were gated through the use of sample ID. Clusters of cells based on the expression level of the activation markers and of the cytokines were manually created, and the % of gated cells in each cluster was further analyzed.

Basophil activation

To activate basophils, following exposure to WWTP extracts, whole blood was stimulated in two different ways, to elicit both innate and adaptive response. Heparin-blood from eight male healthy pseudonymous volunteers was obtained from the blood bank at the University of Leipzig, after written informed consent (#079-15-09032015). For basophil activation, more blood samples were used than for lymphocyte activation experiments to provide more robust data due to the high variability in human basophil counts and reactivity. 100 µL of blood were transferred into flow cytometrical 5 mL polystyrene round-bottom tube (Falcon, Corning, New York, US) and exposed to the four WWTP extracts at a REF of 25 or to

the blank that were previously resuspended in BAT buffer (MgCa PBS buffer supplemented together with IL-3; 2 ng/mL – Sigma-Aldrich, St. Louis, US). In parallel, as a negative control, blood was exposed to BAT buffer. Again, cell viability was assessed concurrently with each experiment, (Suppl. Fig. 1B). After 1 h incubation at 37 °C in a 5% CO_2 incubator, the basophils contained in the whole blood were activated by the addition of anti-FcεR1α antibody (0.1 µg/mL, BioLegend) or N-formylmethionyl-leucyl-phenylalanine (fMLP, 0.05 µM, Sigma-Aldrich, St. Louis, US). Cells were stained with antibodies anti-CCR3 and anti-CD63 (Suppl. Table S2) for 25 minutes at 37 °C in a 5% CO_2 incubator. After 25 minutes, the reaction was stopped by adding EDTA 3.8% (Gibco, Thermo Fisher Scientific, Waltham, US), and erythrocytes were lysed by incubating the cells in erythrocytes lysis buffer (NH_4Cl – Sigma-Aldrich, $NaCO3$ – KMF Laborchemie, Lohmar, Germany, EDTA – Thermo Fisher Scientific) for 10 minutes at RT (twice). To discriminate between live and dead cells, cells were stained with fixable viability dye-eFluor^{TM} 506 (eBioscience) for 20 minutes at 4 °C. Cells were fixed in paraformaldehyde 1% (Sigma-Aldrich) and analyzed on a FACS Canto^{TM} II instrument (BD Biosciences, San Jose, US). Leukocytes were identified using scatter properties FSC-A and SSC-A, basophils were identified as CCR3$^+$ cells. The level of basophil activation was analyzed in CCR3$^+$ population as % CD63$^+$ cells and subdivided in CD63 high and low (the gating strategy is shown in Suppl. Fig. 7A). The basophil activation levels are reported in Suppl. Fig. 7B. Data were then analyzed using FCS Express 7 software.

Gene expression analysis (mRNA)

PBMCs were plated at a concentration of $1x10^6$ cells/well in IMDM and exposed to WWTP extracts at a REF of 25 or to the blank for 20 hours at 37 °C in a 5% CO_2 incubator. In order to specifically target the activation of MAIT cells, which were highly affected by WWTP extracts exposure, PBMCs were then stimulated with E. coli K12 (10 BpC) for 6 hours at 37°C and 5% CO_2. After the treatment, PBMCs were transferred to 1.5 mL microfuge tubes and centrifuged. PBMCs were suspended in 300 µL Trizol (Invitrogen, Waltham, US) and maintained at -80 °C prior to RNA extraction. Total RNA was extracted according to manufacturer's instructions (Invitrogen). cDNA synthesis was carried out with 200 ng of RNA by using 5U RevertAid™ H Minus Reverse Transcriptase (Fisher Scientific, Schwerte, Germany). Intron-spanning primers were designed and UPL probes were selected by the Universal Probe Library Assay Design Center. A number of 40 genes of interest and 3 reference genes (Suppl. Table S3 and S4) were pre-amplified in 12 cycles and quantitative PCR was performed on a 96x96 Dynamic array with BioMark™ HD System (Fluidigm, München,

Germany). The cycling program consisted of 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR was performed with FastStart Universal Probe Master Mix (Roche, Mannheim, Germany). The analyzed genes were chosen according to their involvement in the immune system, in particular on MAIT cells activation and functionality. Furthermore, some hormonal receptors were chosen.

Correlation between chemical composition and immune endpoints

The correlation between chemical concentrations of WWTP extracts and relative immune endpoints (lymphocytes activation, gene expression, and basophils activation) was evaluated using Kendall correlation method for non-normal distributed samples using the Corrr package in R and corrplot for P-value extraction. Correlation coefficients were visualized using the heatmap.3 function. Details are presented in Suppl. File 2.

Statistical analysis

Flow cytometric data were shown as fold change (FC) relative to the blank control. Statistical analysis was performed using GraphPad Prism (version 9.4.0). Data were reported as mean of 5 donors regarding lymphocytes and as mean ± standard deviation (SD) of 8 donors for basophils. Gene expression data were normalized to the average of the three reference genes and then to the minimum of the expression of each gene. FC was calculated on the blank. Normal distribution was assessed using the Shapiro-Wilk test. To calculate differences between treatments, one-way ANOVA, followed by Dunnett's multiple comparison test for several enrichment factors or ratio paired t-test for one enrichment factor were calculated. Unpaired t-test with Welch's correction was performed for gene expression analysis. Differences were considered statistically significant at $p \leq 0.05$.

Results

WWTP extracts reduce the activation of lymphocytes upon anti-CD3/CD28 stimulation

PBMCs were exposed to increasing REF of WWTP extracts and then stimulated with anti-CD3/CD28 to induce the activation of $CD4^{+}$, $CD8^{+}$, NKT, MAIT, and $CD4^{-}CD8^{-}$ (DN) cells. The stimulus can also induce $CD3^{-}$ lymphocytes activation indirectly, through the activation of T cells. In this case, we refer to B and NK cells. None of the WWTP extract concentrations used had an effect on cell viability (Suppl. Fig. 1). WWTP extracts reduced the activation of different T cell populations (Figure 1).
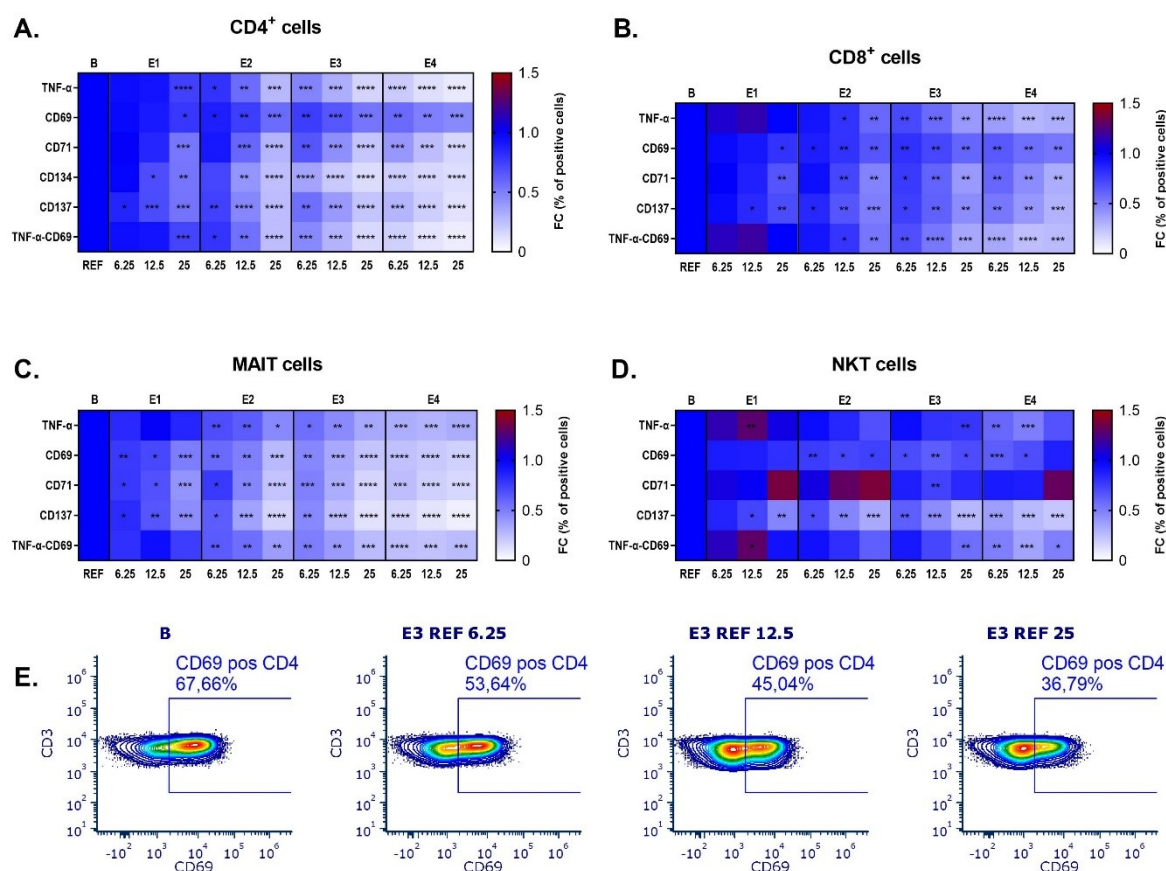
Figure 1. Modulation of CD4$^+$ (A), CD8$^+$ (B), MAIT (C), and NKT cell (D) cell activation. Expression of TNF-α, CD69, CD71, CD134, and CD137 was measured by flow cytometry after 20 h treatment of PBMCs with WWTP extracts at REF of 6.25, 12.5, 25, followed by stimulation with anti-CD3/CD28. Only activation markers responding to stimulation in the certain immune cell populations are shown. Results are presented as FC of anti-CD3/CD28 stimulated blank-treated PBMCs. The color change from white (0) to blue (1.0) to red (1.5) indicates a decrease (from 1.0 to 0), an increase (from 1.0 to 1.5), or no change (1.0) compared to blank. Data represents the mean of 5 donors. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparison test. Statistical significance: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 *vs* blank. (E) Representative density plots of CD69 expression in CD4$^+$ cells treated with blank or WWTP extract E3 at increasing concentrations. The same presentation for the other T cell populations is shown in Suppl. Fig. 14.

In particular, the activation of CD4$^+$ (Fig. 1A), MAIT cells (Fig. 1C), and CD8$^+$ cells activation was decreased (Fig. 1B), whereas no concentration-dependent effect could be observed for NKT cells (Fig. 1D). This indicates in general a reduction of cells activation by all WWTP extracts, except E1 (REF 12.5) which was able to increase the expression level of TNF-α and TNF-α-CD69 in CD8$^+$ and NKT cells. WWTP extracts E2, E3, and E4 reduced T cell activation already at the lowest concentration (REF 6.25), therefore potentially reducing T cell activation without enrichment, at the real concentration found in the effluent. In contrast, WWTP extract E1 at the lowest REF mostly affected MAIT cells and to a lower extent CD4$^+$

cells. Finally, all WWTP extracts reduced T cells activation, with a clear concentration-dependent response.

Regarding the other CD3$^+$ subpopulation, CD4$^-$CD8$^-$ cells, which resemble with high probability γδT cells (Ribot et al., 2021), WWTP effluents also decreased the activation of these cells, mainly acting on CD137 (Suppl. Fig. 8C). Instead, regarding CD3$^-$ lymphocytes, they also induced a decrease in NK and B cells activation (Suppl. Fig. 8A and B) already at the lowest concentration, indicating that the immunomodulating effect of the WWTP extracts was not exclusive on T cells.

As CD4$^+$ and MAIT cell activation was strongly affected by exposure to the chemical mixtures in the analyzed WWTP extracts, a detailed t-SNE analysis was performed for these cell populations (Fig. 2 and 4).
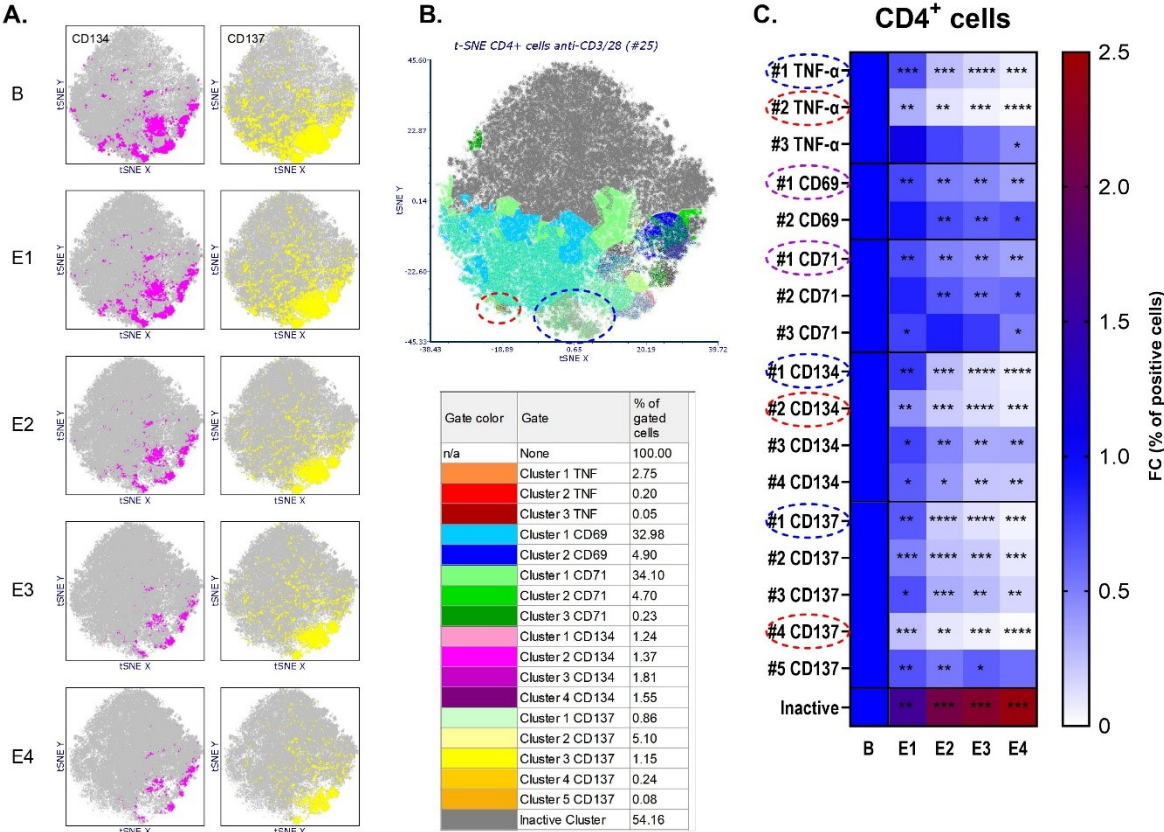
Figure 2. t-SNE analysis of CD4$^+$ cells. Expression of TNF-α, CD69, CD71, CD134, and CD137 was measured by flow cytometry after 20 h treatment of PBMCs with WWTP extracts at REF of 6.25, 12.5, 25, followed by stimulation with anti-CD3/CD28. Only data for CD4+ cells and REF 25 are shown. (A) Representative 2D t-SNE maps, representing the merged treatment and donor condition, for the expression of CD134 and CD137 showing the blank (B) and the WWTP extract (E1-E4)-treatments. CD4$^+$ population was detected using t-SNE transformation tool, and cell activation was visualized by setting a threshold level of 1000 for CD134 (pink) and of 2000 for CD137 (yellow). (B) 2D t-SNE map of all CD4$^+$ merged events showing, based on the expression levels of all activation markers and

manually gated, the 17 clusters of activated cells (active clusters) and 1 inactive cluster. The red and blue dotted oval represent the highly targeted populations. The table presents the gate color, name and % of gated cells. Due to low cell numbers in some clusters and overlap, not all colors are visible in the 2D t-SNE map. (C) Heatmap with the data obtained from the % of gated cells of each donor and treatment condition. Results are expressed as FC calculated on blank-treated PBMCs anti-CD3/CD28 stimulated (1.0, blue). The color changes from white (0) to blue (1.0) to red (2.5) indicates a reduction (from 1.0 to 0), an increase (from 1.0 to 2.5), or no change (1.0) compared to blank. Violet dotted clusters belong to both red and blue dotted ovals. Data represents the mean of 5 donors. Statistical analysis was performed by paired t-test. Statistical significance: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ *vs* blank.

Regarding CD4$^+$ cells, 17 clusters of activated cells were identified in the map containing the merged donor and treatment conditions (Fig. 2B). The colors and the % of cells in these clusters are shown in the table of Fig. 2B. Note, that due to low cell numbers in some clusters and overlap, not all colors are visible in the 2D t-SNE map. All of these activated CD4$^+$ subpopulations were decreased by the exposure to WWTP extracts (REF 25), with a stronger effect from E1 to E4, as shown in the corresponding heat map (Fig. 2C) and exemplary 2D t-SNE maps for the expression of CD134 and CD137 (Fig. 2A). The maps for TNF-α, CD69, and CD71 expression are presented in Suppl. Fig. 9. More in depth, two highly affected cell populations were identified, and they are highlighted in red and blue dotted ovals in Fig. 2B. The most targeted population (red circle) includes mainly cells represented in cluster 2 of TNF-α, cluster 2 of CD134 and cluster 4 of CD137. The blue dotted population consists mainly of cells shown as cluster 1 TNF-α, cluster 1 CD134 and cluster 1 CD137. Cluster 1 of CD69 and of CD71 present some cells in both highly targeted populations, marked with violet circles in Fig. 2C. From Fig. 2C it can be seen that the two highly affected cell populations consisted of cells that were positive for the majority of activation markers. Thus, the selected WWTP extracts have a preferential effect on highly activated cells, and not on cells expressing only few activation markers.

WWTP extracts reduce the activation of MAIT cells upon E. coli K12 stimulation

Since we observed that MAIT cells were one of the most targeted immune cell populations, PBMCs were stimulated with *E. coli* K12 to further investigate the effect of WWTP extracts specifically on the activation of these cells. MAIT cells are T cells involved in the first defense to bacteria (Legoux et al., 2020). They have been found to be susceptible to several chemicals, such as bisphenols and perfluorinated substances (Krause et al., 2022; Maddalon et al., 2023).

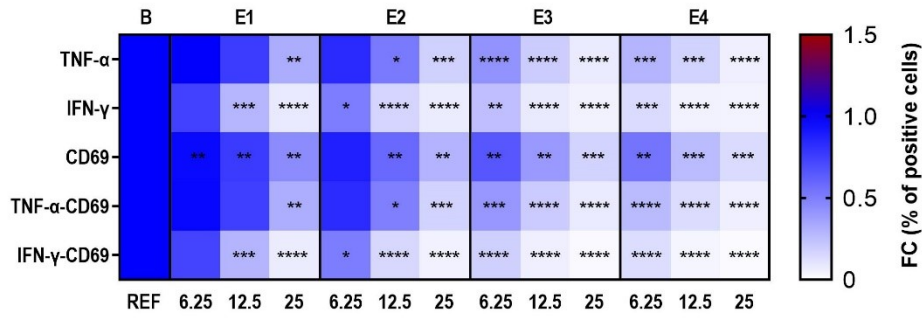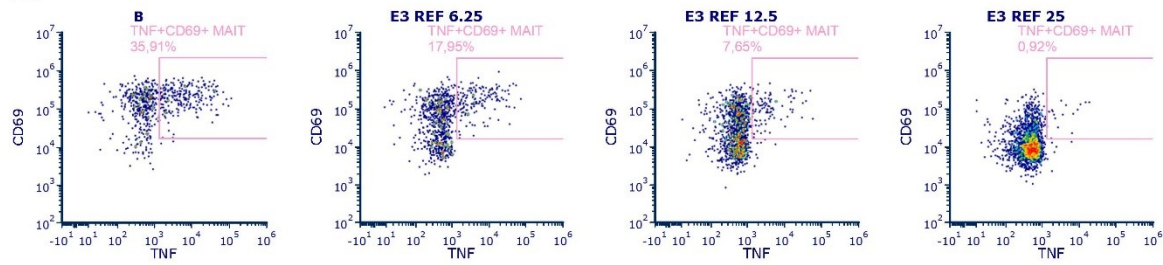**Figure 3. Modulation of MAIT cell activation (A)**. Expression of TNF-α, CD69, CD71, CD134, and CD137 was measured by flow cytometry after 20 h treatment of PBMCs with WWTP extracts at REF of 6.25, 12.5, 25, followed by stimulation with *E. coli* K12. Only activation markers responding to stimulation in this immune cell population are shown. Results are presented as FC of *E. Coli* K12 stimulated blank-treated PBMCs. The color change from white (0) to blue (1.0) to red (1.5) indicates a decrease (from 1.0 to 0), an increase (from 1.0 to 1.5), or no change (1.0) compared to blank. Data represents the mean of 5 donors. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparison test. Statistical significance: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 *vs* blank. (B) Representative density plots of CD69-TNF-α-co-expression in MAIT cells.

With the stimulation of MAIT cells by *E. coli*, the immunosuppressive effect of WWTP extracts was even more pronounced (Fig. 3B). For E3 and E4, the inhibitory effects were observed already at the lowest concentration, whereas WWTP extracts E1 and E2 exhibited inhibitory effects on MAIT cell activation at REF of 12.5 (Fig. 3A). Again, a clear dose-dependent immunosuppression was observed for all WWTP extracts.

MAIT cells were also further analyzed using t-SNE dimensionality reduction (Fig. 4A), as they were among the cells mainly affected by WWTP extracts exposure, as were CD4^{+} cells.
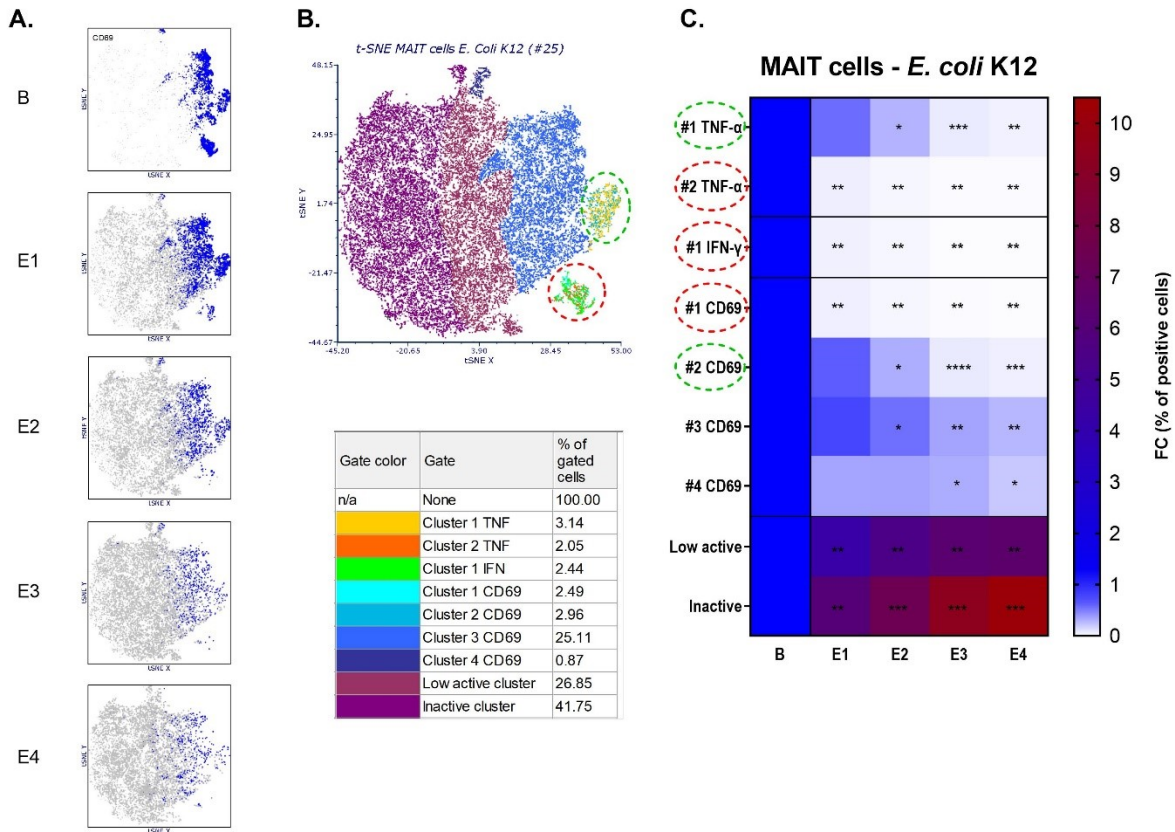
Figure 4. t-SNE analysis of MAIT cells. Expression of TNF-α, CD69, CD71, CD134, and CD137 was measured by flow cytometry after 20 h treatment of PBMCs with WWTP extracts at REF of 6.25, 12.5, 25, followed by stimulation with *E. coli* K12. Only data for MAIT cells and REF 25 are shown. (A) Representative 2D t-SNE maps, representing the merged treatment and donor condition, for the expression of CD69 showing the blank (B) and the WWTP extracts (E1-E4)-treatments. MAIT cell population was detected using t-SNE transformation tool, and cell activation was visualized by setting a threshold level of 5000 for CD69 (blue). (B) 2D t-SNE map of all MAIT cell merged events showing, based on the expression levels of all activation markers and manually gated, 7 clusters of activated cells (active clusters) 1 low active and 1 inactive cluster. The red and green dotted oval represent the highly targeted populations. The table presents the gate color, name and % of gated cells. Due to low cell numbers in some clusters and overlap, not all colors are visible in the 2D t-SNE map. (C) Heatmap with the data obtained from the % of gated cells of each donor and treatment condition. Results are expressed as FC calculated on blank-treated PBMCs *E. coli K12* stimulated (1.0, blue). The color changes from white (0) to blue (1.0) to red (2.5) indicates a reduction (from 1.0 to 0), an increase (from 1.0 to 2.5), or no change (1.0) compared to blank. Violet dotted clusters belong to both red and blue dotted ovals. Data represents the mean of 5 donors. Statistical analysis was performed by paired t-test. Statistical significance: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 *vs* blank.

Different clusters of activated MAIT cells were identified (Fig. 4B), all of which were decreased by WWTP extract (REF 25) exposure (Fig. 4C). In parallel, the percentage of low activated and inactive cells (grey, Fig. 4A and purple and violet in Fig. 4B) was highly increased after treatment with WWTP extracts (Fig. 4C). Notably, TNF-α cluster 2, IFN-γ cluster 1 and CD69 cluster 1 were composed of the same cells (Fig. 4B, red dotted circle) and represent highly activated MAIT cells expressing all three activation markers. Thus, these cells represent

the most affected population, showing the highest immunosuppressive effect upon exposure to WWTP extract. The second active population is represented by cluster 1 TNF-α⁺ and cluster 2 CD69⁺ (Fig. 4B, green dotted oval) and represents the second population of MAIT cells whose activation was highly reduced.

Transcript analysis of PBMCs activation upon E. coli K12 stimulation

Since MAIT cells were among the most severely suppressed immune cells, we investigated the effects of WWTP extracts at a REF of 25 on MAIT cell mRNA expression using the specific stimulation of PBMCs with *E. coli*. Among the 40 genes analyzed, the mRNA expression of 23 of them was statistically significantly modulated by at least one WWTP extract (Fig. 5). The expression of the remaining genes is presented as fold change to blank in Suppl. Table S5.

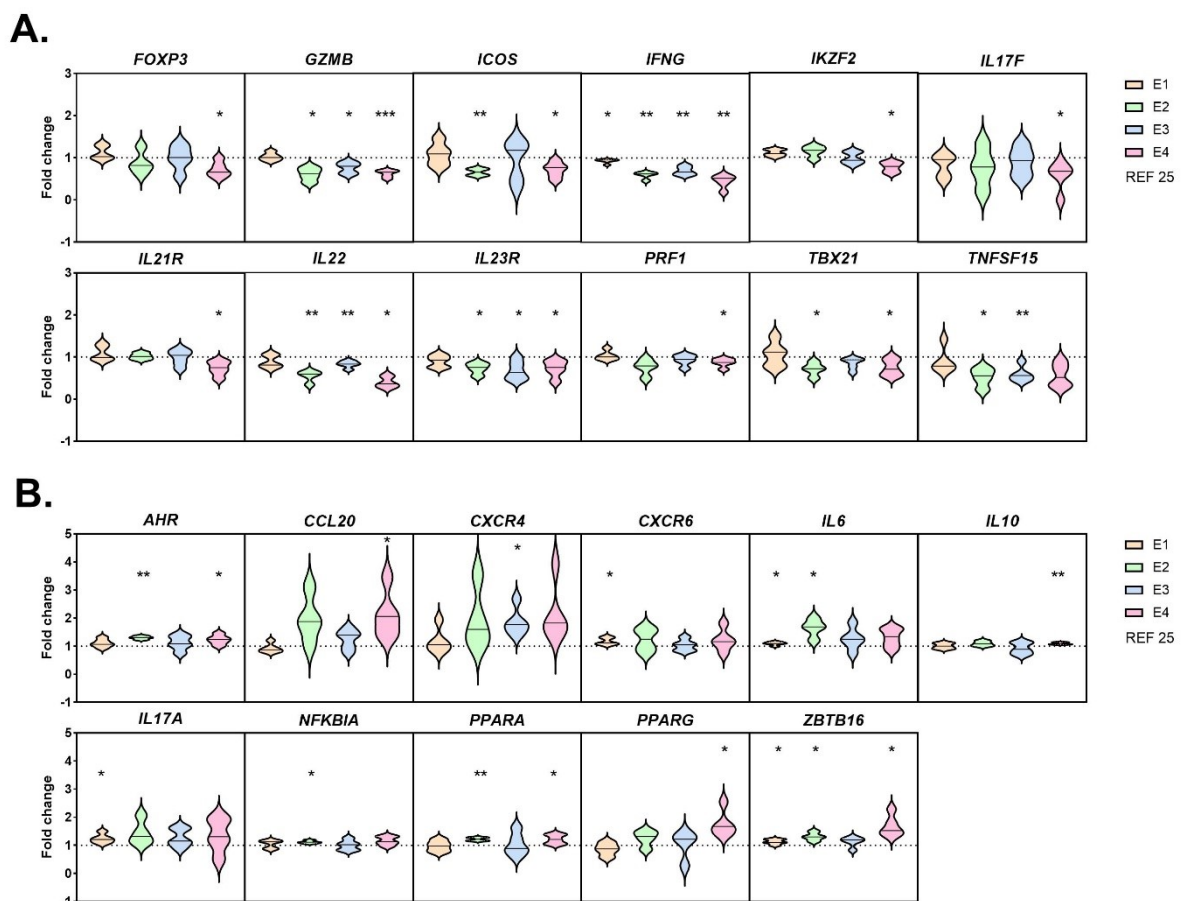**Figure 5. Modulation of mRNA expression in *E. coli*-stimulated PBMCs**. PBMCs were treated (20h) with WWTP extracts at REF 25, followed by stimulation with *E. coli* K12 to specifically stimulate the MAIT cells. Data are presented as FC calculated on *E. coli* K12-stimulated blank-treated PBMCs (1.0, dotted line). The presentation is divided in transcripts that were statistically significantly down-regulated

by at least one WWTP extract (A) and those statistically significantly up-regulated by at least one WWTP extract (B). Data represents the median of 5 donors (black line within each violin). Statistical analysis was performed following Welch's t-test. Statistical significance: with *p≤0.05, **p≤0.01, ***p≤0.001 *vs* blank.

Most significant changes were observed in the down-regulated genes (Fig. 5A) upon the treatment with E4, followed by E2. Notably, the expression of several cytokine transcripts, namely *IFNG, IL17F, IL22, TNFSF15*, cytokine receptors (*IL21R* and *IL23R*), and several typical transcription factors, such as *FOXP3* and *TBX21*, was reduced by WWTP extracts exposure. Furthermore, the expression of MAIT cell-specific transcripts, including *GZMB*, *ICOS*, *IKZF2*, and *PRF1,* was also down-regulated. These data suggest a preferred action against MAIT cell subsets especially releasing IFN-γ and IL-17. On the other hand, the up-regulation of genes induced by WWTP extracts was less pronounced (Fig. 5B). In particular, a slightly higher expression of certain cytokines (*IL6, IL10,* and *IL17A*), receptors (*CXCR4, CXCR6*), and other immune-related proteins (*CCL20, ZBTB16,* and *NFKBIA*) was observed. Furthermore, a higher expression of hormone receptors, namely *AHR*, *PPARA*, and *PPARG* was observed, mainly after treatment with E2 and E4, suggesting that these WWTP extracts could contain chemical mixtures and concentrations able to interfere with these receptors.

WWTP extracts modulated the activation of basophils

As all four WWTP extracts showed immunosuppressive effects on all lymphocyte subpopulations analyzed, we next assessed their immunotoxic potential to interfere with the activation of basophils, an immune cell type involved in allergic reactions (Suppl. Fig. 10). Whole blood was stimulated with anti-FcεR1α, which targets the IgE receptor, or with fMLP, a bacterial component capable of attracting and activating basophils. All WWTP extracts reduced basophil activation induced by fMLP (Suppl. Fig. 10A). We observed that fMLP-induced activation was significantly reduced in the CD63$^+$ high basophil population (Suppl. Fig. 10C). This was also reflected in the total CD63$^+$ population, which includes the CD63$^+$ high and low populations. Unlike what we observed with the lymphocytes, here the reduction was of the same magnitude for all four WWTP extracts. Conversely, with anti-FcεR1α stimulation, differences of magnitude were observed in the CD63$^+$ high population. In this case, the four WWTP extracts differentially increased the percentage of activated cells, although only E4 reached significance (Suppl. Fig. 10B). Notably, a reduction in anti-FcεR1α-induced activation was observed after treatment with E1 extract in the CD63$^+$ low and CD63$^+$ total basophil

populations. In conclusion, the different trends in modulation by WWTP extracts that we observed seem to be related to the mode of activation of these cells.

Correlation between identified chemicals and immune endpoints

Of 578 chemicals analyzed by LC-HRMS screening, 339 chemicals were identified in at least one of the four WWTP extracts E1, E2, E3 and E4 (Finckh et al., 2022a; 2022b). Concentrations of the detected chemicals are shown in the appendix (Suppl. File 1). The different categories of chemicals found in the analyzed WWTP extracts are reported in Suppl. Table S6.

In order to identify which chemicals were most implicated in the adverse immune effects caused by WWTP extracts, correlation analyses were performed with chemical concentrations and immune endpoints (Fig. 6, Suppl. Fig. 11 and 12).
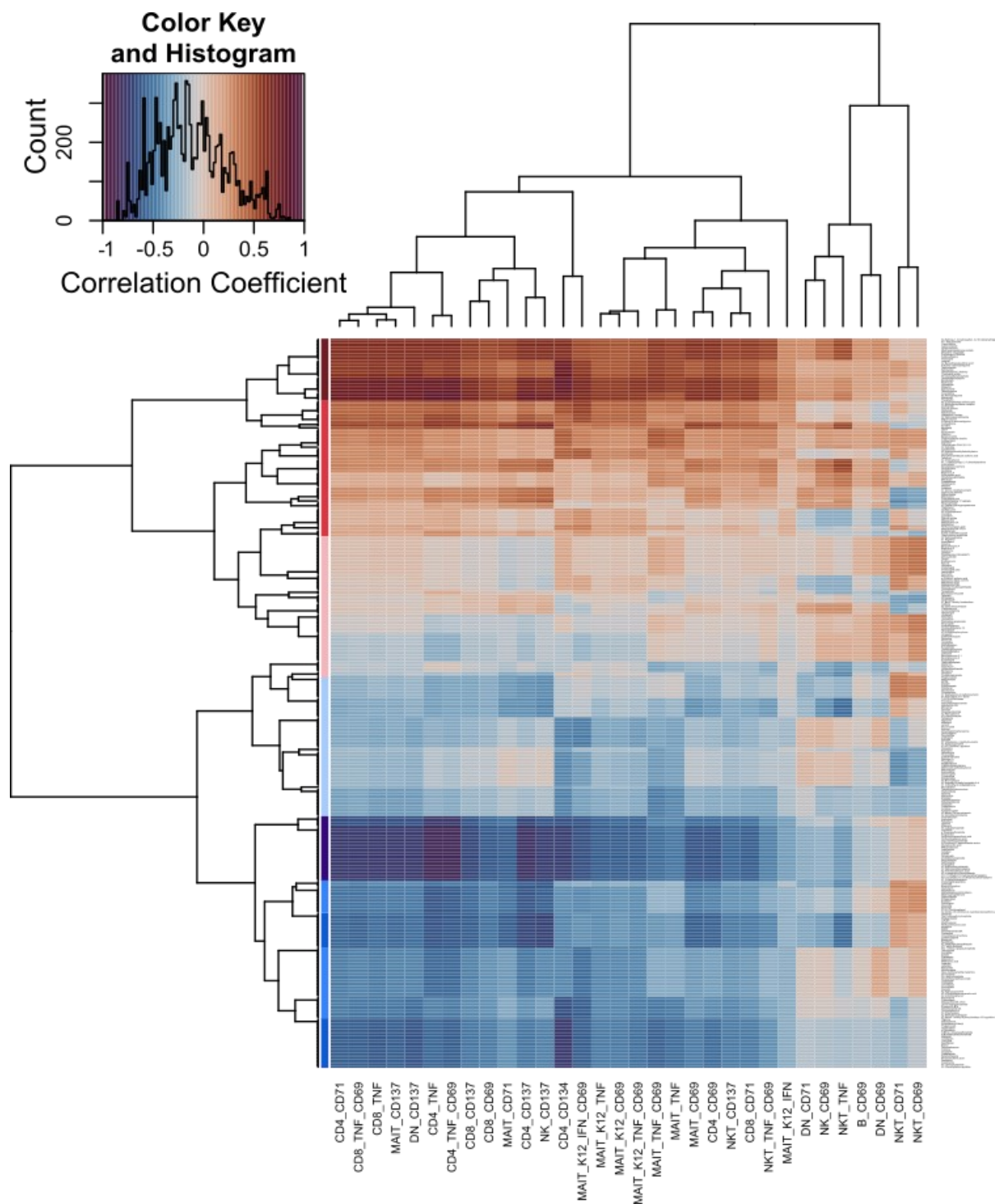
Figure 6. Correlation between chemicals and immune cell activation. The heat map shows the correlation coefficients between the concentration of WWTP extract chemicals and the % of activated immune cells, and the hierarchical clustering of similarly affected immune cells (horizontal) and similarly affected WWTP extract chemicals (vertical). Expression of TNF-α, CD69, CD71, CD134, and CD137 was measured by flow cytometry after 20 h treatment of PBMCs with WWTP extracts at REF of 6.25, 12.5, 25, followed by stimulation with anti-CD3/CD28 or *E. coli* K12. Data of the % of activated CD4^{+}, CD8^{+}, MAIT, NKT, NK, B and CD4^{-}CD8^{-} cells at REF 25 are shown. Color code: negative correlation (blue) to positive correlation (red). The color bars were used to group chemicals based on similar correlation coefficients with immune cell activation. The list of chemicals present in each color group is reported in Suppl. Fig. 16. The Kendall correlation coefficients and the p values are reported in Suppl. File 2.

The analyzed immune endpoints were: lymphocyte activation (activation markers TNF-α, CD69, CD71, CD134, and CD137 for all lymphocyte subpopulations and TNF-α, IFN-γ, and CD69 for MAIT cells), gene expression (40 genes) and basophil activation (high, low and total). The correlation coefficients between lymphocyte activation and chemical concentrations are shown (Fig. 6). The names of chemicals assigned to the color bares are better visualized in Suppl. Fig. 16.

Regarding lymphocyte activation, two third of correlations with chemical concentrations were negative (toward -1, blue), meaning that higher chemical concentrations associate with reduced lymphocyte activation. Chemicals clustering to the strongest negative correlation (dark blue bar, also see Suppl. Fig. 16A) include 13 pharmaceuticals, namely three carbamazepine metabolites (2-hydroxycarbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine and 10,11-dihydro-10-hydroxycarbamazepine), amitriptyline, azithromycin, efavirenz, loperamide, metoprolol acid, mycophenolic acid, propranolol, raloxifene, ranitidine, and ropinirole; 4 pesticides, namely 2,4-dichlorobenzoic acid, fenoxycarb, isoxaben, and thiamethoxam; 1 EDC: clobetasol propionate, and 12 chemicals belonging to other categories, like industrial chemicals mainly, namely 1,3-diphenylguanidine, 2(4-morpholinyl)benzothiazole, 4-hydroxybenzotriazole, benzothiazole, harmine, icaridin, N-cyclohexyl-2-benzothiazole-amine, octyl-methoxycinnamate, perfluoroheptanoic acid, perfluorooctanesulfonic acid, p-toluenesulfonamide, and tri-isobutylphosphate. The chemical concentrations positively correlated with lymphocyte activation (dark red bar, also see Suppl. Fig. 16B) were 9 pharmaceuticals (4-aminoantipyrine, amantadine, capecitabine, glimepiride, melperon, metoprolol, N-acetyl-4-aminoantipyrine, sulfamethazine, and telmisartan), 9 pesticides (bendiocarb, cyproconazole, desethylterbutylazine, flufenacet, prochloraz BTS40348, spiroxamine, tebuconazole, thiacloprid, and diacloprid amide), 4 EDCs (17-beta-estradiol, hydrocortisone, medroxyprogesterone acetate, and megestrol-17-acetate), and 4 other chemicals (2-benzothiazolesulfonic acid, 4-hydroxy-1-(2-hydroxyethyl)-2,2,6,6-tetramethylpiperidine, harman, and theophyllin). However, in vitro, the observed effects of STP extracts were in almost all cases a downregulation of lymphocyte activation. This suggests that although individual chemicals were positively correlated with lymphocyte activation, in the mixture the effect was a downregulatory one, overwhelmed by the negatively correlated chemicals.

The correlation between chemical concentrations and gene expression is presented in Suppl. Fig. 11. The gene expression was measured in PBMCs treated with WWTP extracts and

stimulated with *E. coli* K12 to specifically activate MAIT cells. The list of chemicals present in each color group is reported in Suppl. Fig. 17.

Cluster analyses revealed that individual chemicals strongly negatively correlating with half of the analyzed transcripts were positively correlated to the other half of the analyzed transcripts (dark blue bar, also see Suppl. Fig. 17A). In particular these chemicals comprised 30 pharmaceuticals (e.g. amitriptyline, azithromycin, bezafibrate, bisoprolol, bosentan, carbamazepine, carbamazepine metabolites, valsartan), 23 pesticides (e.g. 2,4-dichlorobenzoic acid, 2-amido-3,5,6-trichloro-4-cyanobenzenesulfonic acid, bromochlorophen, chlorothalonil-4-hydroxy, chlorotoluron, dichlorprop, dimetachlor ESA, simazine, thiabendazole), 10 EDCs (17-α-estradiol, 1H-benzotriazole, 2-phenylphenol, 3,4,5-trichlorophenol, 4-androstene-3,17-dione, clobetasol propionate, desonide, ethylparaben, medroxyprogesterone, and triclosan) and 19 chemicals belonging to other categories (e.g. 1,3-diphenylguanidine, 2(4-morpholinyl)benzothiazole, 2-(methylthio)benzothiazole, 4-hydroxybenzotriazole, 7-diethylamino-4-methylcoumarin, benzothiazole). The main transcripts involved in the negative correlations were *IFNG, GZMB, IL22, IKZF2, IL21R* (down-regulated mRNAs, Fig. 5A). Positively correlated genes were mainly *CCL20, ZBTB16*, and *PPARG* (up-regulated mRNAs, Fig. 5B), evidencing a positive association between higher chemical concentration and higher mRNA expression.

Chemicals that were positively correlated with gene expression (dark red bar, also see Suppl. Fig. 17B) included 16 pharmaceuticals (e.g.4-aminoantipyrine, amantadine, capecitabine, glimepiride, indometacin, melperon, metoprolol, sulfamethazine), 12 pesticides (bendiocarb, cyproconazole, desethylterbutylazine, fenpropimorph, spiroxamine, terbuthylazine), 9 EDCs (e.g. 17α-hydroxyprogesterone, 17-β-estradiol, 4-chlorophenol, androsterone, medroxyprogesterone acetate, megestrol-17-acetate), and 8 other chemicals (e.g. 5-chlorobenzotriazole, 7-amino-4-methylcoumarin, acridine, caffeine, cyclohexylamine, isophorone diamine, theophyllin). Contrary to the chemicals in the dark blue bar, these chemicals positively correlated with e *IFNG, GZMB, IL22, IKZF2, IL21R*, and negatively with *CCL20, ZBTB16*, and *PPARG*.

Regarding the correlation between chemicals and basophil activation, fewer significant correlations have been observed compared to lymphocytes (Suppl. Fig. 12). The list of chemicals present in each color group is reported in Suppl. Fig. 18.

It was visible that mostly the activation of basophils via anti-FcεR1α was associated to modulation by chemicals. The main chemicals that resulted in positive correlations (red bar, also see Suppl Fig. 18B), were similar to those that were negatively correlated with both

lymphocyte activation and mRNA expression. The main chemicals being negatively correlated to basophils activated by anti-FcɛR1α (blue bar, also see Suppl. Fig. 18A) were similar to the ones that positively correlated to lymphocyte activation and mRNA expression. Thus, it seems that the same chemicals differently modulate the activation of certain immune cell populations.

Prioritized chemicals for immunomodulation

A high similarity was observed between the chemicals that correlated highly with lymphocyte activation, basophil activation and modulation of mRNA expression (Figure 17A and B).

A total of 50 chemicals strongly correlated with all the three altered immune endpoints: 29 of them negatively correlated with lymphocyte activation and gene expression and positively with basophil activation; whereas 21 chemicals positively correlated with lymphocyte activation and gene expression and negatively with basophil activation. The chemicals associated with inhibitory effects on the immune endpoints (Fig. 7A), which were considered as prioritised chemicals due to the high consistency in the three correlation analyses, are listed in Suppl. Table S5 (together with use or mechanism of action).
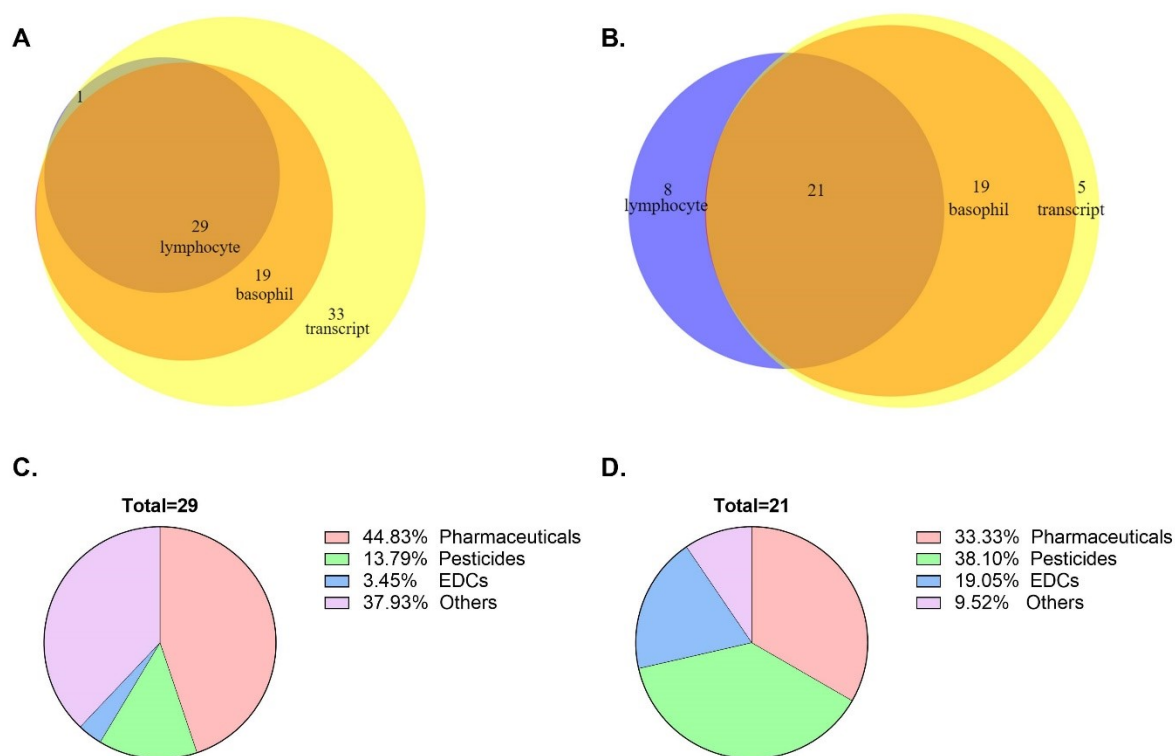

Figure 7. Venn diagrams showing the relationships between the three sets: lymphocyte activation (blue), basophil activation (red), and gene expression (yellow). The most highly correlated chemicals, highlighted from the heatmap with the highest correlation coefficients (dark blue and red bars for Fig.

6, suppl. Fig. 11 and 12), were evaluated. (A) The amount of chemicals negatively correlated with lymphocyte activation and gene expression (dark blue bars in Suppl. Fig. 16A and 17A) and positively correlated with basophil activation (red bars in Suppl. Fig. 18B) are shown. (B) The amount of chemicals positively correlated with lymphocyte activation and gene expression (dark red bars in Suppl. Fig. 16B and 17B) and negatively correlated with basophil activation (blue bars in Suppl. Fig. 19A) are shown. The diagrams were created using BioVenn (Hulsen et al., 2008 - https://www.biovenn.nl/index.php). Pie charts of the chemical categories of the chemicals resulting from the three correlation analyses. Categories of the common chemicals resulting from Fig. 7A (C) and from Fig. 17B (D), with their percentage.

The categories of these chemicals are reported in Fig. 7C. The 29 high concern chemicals highly correlated with the inhibitory effects on lymphocyte and basophil activation are mainly pharmaceuticals, and within them there are neuroactive (6), cardiovascular (2), antibiotics (2), antiviral (1), endocrine (1), and antihistamine (1). There is also one corticosteroid in the EDC class, 4 pesticides and 11 chemicals in other categories, mainly industrial chemicals such as perfluorinated compounds and rubber additives.

Discussion

A large number of chemicals have been quantified in treated effluent samples from wastewater treatment plants before discharge into rivers across European countries (Finckh et al., 2022a; 2022b). The four WWTP extracts used in the present study, differed in chemical composition and concentration (Suppl. File 1). The immunoassays were used to assess the putative adverse effects of these effluents on the human immune system, namely the modulation of the activation of different lymphocyte subpopulations, the modulation of basophil activation and finally the effect on the expression of different genes on lymphocytes, in particular MAIT cells.

Lymphocyte and basophil activation was differently affected by WWTP extracts. With regard to lymphocytes, our analysis showed differences between the four effluent extracts, and established a potency ranking for inhibition from E1 (the lowest in terms of potency) to E4 (the most potent). The WWTP extracts exerted their immune inhibitory effects already at the lowest REF tested, so it is highly likely that similar effects could be obtained at the real concentrations present in the effluent. The main *in vitro* effects, e.g. inhibition of TNF-α and IFN-γ production or expression of the activation markers CD134 and CD137 were observed on CD4$^+$ and MAIT cells. The latter were found to be more susceptible when highly activated following the stimulation with *E. coli*, a specific stimulus for these cells. WWTP extracts were also able to downregulate some transcripts indicative of MAIT cells functionality, such as *GZMB*, *ICOS*, *PRF1*, and *IKZF2*, highlighting an inhibitory effect on MAIT cells. MAIT cells represent a

bridge between the innate and adaptive immunity, acting mainly against microbial agents (Ioannidis et al., 2020). Therefore, a suppression of the activation of MAIT cells could represent a basis for an increased susceptibility to infections (Rudak et al., 2018; Hinks and Zhang, 2020). Other genes that we observed to be downregulated in our study are regularly involved in Th1 and Th17 responses, namely *IFNG, TNFSF15, TBX21, IL17F, IL22, IL21R* and *IL23R*. On the other hand, transcripts typical of the Th2 pathway, such as *IL6, IL10* and *CXCR4*, were upregulated. Lymphocytes can behave differently, based on the release of cytokines (Chiba et al., 2018). Thus, a modulation of immune responses for example towards Th2, may lead to the development of allergic diseases (Romagnani, 2004; Licona-Limón et al., 2013).

Overall, the lymphocyte activation test represents a promising effect-based analysis of mixtures of chemicals, since it allows the visualization of the effect on a wide range of immune cells and enables to distinguish the potency of the mixtures. Together with the assessment of lymphocyte functionality, also the evaluation of certain mRNAs is helpful to estimate the immunomodulatory capacity and also to observe differences between the different WWTP extracts. They have been previously associated to endocrine disruption, with sample E4 showing the highest activity on glucocorticoid and progesterone receptors (Finckh et al., 2022a). Interestingly, in our experiments on lymphocytes, E4 was able to up-regulate the expression of other hormone receptors, namely *AHR*, *PPARA*, and *PPARG,* more than the other WWTP extracts. Indeed, in E4 there are some EDCs higher concentrated than in the other WWTP extracts, like 4-androstene-3,17-dione, benzophenone-3, canrenone, ethylparaben, methylparaben, and medroxyprogesterone, which exhibited activity via one or more hormone receptors, like *AHR*, *PPAR*, and *PPARG* (Ford, 2013; Piccinni et al., 2019; Gouukon et al., 2020; Shin et al., 2020; Cha et al., 2021).

In terms of basophil activation, our study revealed less potent effects of WWTP extracts on cell activation, compared to lymphocytes. Basophils are cells of the innate immune system that are programmed for rapid response to foreign agents (Chirumbolo et al., 2018). Therefore, contrary to lymphocytes, only a short pre-incubation time with the WWTP extracts was indicated. We observed that in this case too, the modulatory effect was exerted on the more highly activated population of basophils. Stimulation of these cells with fMLP mimics bacterial invasion and the ability to respond to it appears to be reduced by pre-incubation with these chemical mixtures. In contrast, the activation of these cells was increased by WWTP extracts followed by stimulation with anti-FcεR1α, which mimics an allergic immune response. Thus, in both cases, the effect of WWTP extracts appears to be detrimental to the function of these cells.

The effect-based analysis proposed here, together with the chemical characterization, allowed the prioritization of chemicals of high concern in terms of immunomodulation. The chemical mixtures that were highly correlated with immune endpoints included both: chemicals directly involved in immune responses, such as antihistamines, and chemicals with other modes of action, such as antibiotics, neuroactive, endocrine or cardiovascular drugs, as well as pesticides and industrial chemicals. As these chemicals belong to widely used categories, their involvement in the immune system should be investigated in detail in future studies. Within pharmaceuticals, there are drugs acting on the immune system, like clobetasol propionate, mycophenolic acid and ranitidine, but there are also others acting on nervous system, endocrine system, and microbiota. Due to the strict interconnection between these organs and immunity, also these pharmaceutical agents could easily affect the immune system (Zefferino et al., 2021; Petra et al., 2015). Of the prioritized chemicals, amitriptyline, azithromycin, efavirenz, loperamide and ropinirole have already been associated to immunomodulation and mainly immunosuppression, supporting our results (Heaton et al., 2022; Isozaki et al., 2022; Juárez et al., 2018; Johnson et al., 2016; Lin et al., 2016). Furthermore, also cardiovascular pharmaceuticals, like beta-blockers, have been previously linked to immunomodulation (Shaw et al., 2009; Maisel et al., 1991). The cardiovascular pharmaceuticals on our prioritization list, metoprolol acid and propranolol, have not been evaluated by now for their immunological effects. From the category of pesticides, for example for fenoxycarb, which is also considered to be an EDC, it has been shown that it is able to alter the immunity of the gut (Attarianfar et al., 2023). For the other pesticides in the prioritization list, no or few information are present in literature. Regarding industrial chemicals, it is widely known that perfluorinated compounds can affect the immune system (EFSA CONTAM Panel, 2020). In our study perfluoroheptanoic acid and perfluorooctanesulfonic acid were highly associated with immunomodulation, supporting published data showing the interference with the two perfluorinated compounds with immune development and immune phenotyping (Torres et al., 2021; Maddalon et al., 2023; Weatherly et al., 2023). Furthermore, rubber additives such as benzothiazoles or widely used UV-filters (e.g. octyl-methoxycinnamate) we found, are also able to influence the immune response (Ferraris et al., 2020; Khan et al., 2016). Thus, there is a body of published data in support of the chemicals of immunological concern identified in our study. Certainly, several other chemicals contribute to the observed adverse immune effects, such as chemicals that were similarly concentrated in the different WWTP extracts, and were therefore not highlighted in the correlations, as well as other chemicals that were not detected. The characterization of the WWTP extracts revealed the concentrations of 339 chemicals, but even more chemicals are

likely to be present. Therefore, mixture effects are expected that exceed the effects of the individual mixture components (Escher et al., 2020). Compounds exhibiting similar modes of action typically act according to concentration addition (Kumari and Kumar, 2020; Altenburger et al., 2020; Kortenkamp, 2014), whereas, chemicals with different mode of actions usually act according to the independent action model (Kortenkamp, 2014). Contribution of single compounds to mixture toxicity can be observed at very low concentrations, even below detection limits (Kortenkamp and Faust, 2018). Therefore, the ultimate immunosuppressive effect observed may be due to the action of individual chemical entities, but also to the synergism and antagonism between them. The characterization of the chemical content and concentration of different agents is an important step in identifying the causal factors leading to adverse health effects in humans (Escher et al., 2017). To date, a risk assessment approach for chemical mixtures is lacking (Liew and Guo, 2022), especially in terms of immunomodulation. For this reason, we propose a combinatorial method that combines effect-based and chemical-based analysis to fill the gap in the risk assessment of mixtures. WWTP extracts are a mixture of chemicals that reflect human activities and also environmental impacts, representing so-called "real life mixtures". Their effects are relevant to the ecosystem and are becoming increasingly relevant to humans due to climate change and the need to conserve fresh water. Undoubtedly, the earth's limited water resources will lead to a global trend towards the use of reclaimed water, for example in agriculture, but the safe use of reclaimed water for human health is still in its infancy. Here, we developed specific *in vitro* assays to unravel the effect of chemical mixtures present in four European effluents of WWTP on the human immune response. We have demonstrated the ability of wastewater treatment plant effluents to adversely affect immune cell activation in vitro, which could potentially lead to adverse health outcomes, as dysregulated immune responses underlie many diseases, including infections, cancer, autoimmunity and other chronic inflammatory diseases.

Conclusions

In this study, we combined chemical characterization of WWTP extracts with effect-based methods, namely immunoassays together with gene expression analysis, in order to provide information on the immunomodulatory effects of the chemical mixtures in European WWTP effluents. We found several chemicals that were highly correlated with reduced immune activation, indicating that these substances may be chemicals of very high concern that should be further investigated and monitored. More broadly, we believe that our approach can

contribute to the hazard identification of WWTP effluents that are highly contaminated with chemicals of concern for immunotoxicity. Our data also highlight the need for action to improve wastewater treatment to reduce the risk to the environment and human health from reuse of this water.

Supplementary Material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.167495.

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Conclusion

The objective of this thesis was to assess whether the chosen chemicals, either known or suspected EAS/EDs, or combinations of chemicals, interfered with the activation and functioning of the immune system.

Starting from glyphosate, its ability, at concentrations relevant to human exposure, to directly interfere with immune cells was investigated. Specifically, it disrupted the equilibrium between T helper 1 and 2 cells, diminishing the former while augmenting the latter. We discovered that the mechanism underneath this action was through the estrogen receptor-α pathway. Another effect of glyphosate on the immune system was the up-regulation of miR-500a-5p, which was involved in the reduced IFN-γ production observed following exposure to the herbicide's active ingredient. The data indicate that glyphosate possesses the capacity to interfere with the endocrine system, particularly through the estrogen pathway, and consequently, it may perturb the immune system.

Regarding the investigation on ATR, CYP, DEP, EE, PFOS, and VIN, their effects on different immune parameters were assessed. We investigated their interaction with the endocrine system and their impact on immune activity. In detail, ATR, CYP, and VIN through different mechanisms involving androgen receptor were able to reduce RACK1 expression and related pro-inflammatory markers. On the contrary, DEP and PFOS reduced RACK1 expression and related markers, with a mechanism involving glucocorticoid receptor. On the other hand, EE was able to increase RACK1 expression and related markers, through an estrogenic pathway, involving GPER. The data were also confirmed in primary cells, and some sex-related effects were also observed. In addition, these EAS also interfered with NK lytic activity and with T helper and cytotoxic cells differentiation.

To gain a more comprehensive understanding of the exposome, we examined the impact of a blend of six PFAS on the activation of immune cells. The results showed a decrease in both lymphocytes and basophils activation, with a more pronounced effect observed on MAIT cells and genes associated with them. Additionally, various extracts derived from WWTP effluents were analyzed for their chemical composition, revealing a mixture of 339 detected substances. These extracts were then exposed to various human immune cells, leading to a broad inhibition of lymphocyte activation and an altered response in basophils. Correlation analysis highlighted several chemicals strongly associated with immunotoxicity, primarily pharmaceuticals, EDs, pesticides, and other industrial chemicals, such as PFAS.

The case studies analyzed covered a wide range of endocrine effects, with different targets and potency. In addition, the majority of substances have pleiotropic effects, thus acting on different pathways, involving both endocrine and non-endocrine-mediated effects. Therefore, the immunomodulatory effects cannot be solely attributed to their endocrine disturbances. Furthermore, due to the broad range of roles covered by the immune system, it is necessary to perform a battery of tests to assess the interference with the different immune components.

In conclusion, the chemicals we examined, known or suspected to have endocrine-active properties, demonstrated the capacity to modulate the human immune system *in vitro*. This further underscores the interconnectedness between the endocrine and immune systems. Consequently, there is a growing need to prioritize the hazard assessment of chemicals, particularly EAS and EDs, while also considering their behavior in combination rather than as isolated entities. Finally, the proposed *in vitro* testing battery has shown promise as a valuable tool for assessing the potential hazards posed by chemicals on the human immune system.

List of abbreviations

The objective of this

AR	Androgen receptor
ATR	Atrazine
BAT	Basophil activation test
BpC	Bacteria per cell
CYP	Cypermethrin
DEP	Diethylphthalate
DMSO	Dimethyl sulfoxide
E2	17β-estradiol
EAS	Endocrine active substances
ECHA	European Chemical Agency
EDs	Endocrine disruptors
EDCs	Endocrine disrupting chemicals
EE	17α-ethynyl estradiol
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
ER	Estrogen receptor
FC	Fold change
fMLP	N-Formylmethionylleucyl-phenylalanine
G	Glyphosate
GPER	G-protein estrogen receptor
GR	Glucocorticoid receptor
LBD	Ligand binding domain
LPS	Lipopolysaccharide
MAIT	Mucosal-associated invariant T
MFI	Mean fluorescence intensity
miRNA	Micro RNA
NK	Natural killer
NKT	Natural killer T
PAEs	Phthalate esters
PBMCs	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
PD	Parkinson's disease
PFAS	Perfluoroalkyl substances
PFBA	Perfluorobutanoic acid
PFBS	Perfluorobutane sulfonic acid
PFHxA	Perfluorohexanoic acid
PFHxS	Perfluorohexanesulfonic acid
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctanesulfonic acid
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate
POP	Population
RA	Rheumatoid arthritis
RACK1	Receptor for activated C kinase 1
REF	Relative enrichment factor
RT	Room temperature
SD	Standard deviation
SEM	Standard error of mean
SI	Stimulation index
Tc	T cytotoxic
Th	T helper
Treg	T regulatory
t-SNE	t-distributed stochastic neighbor embedding
VIN	Vinclozolin
WHO	World Health Organization
WWTP	Wastewater treatment plants

Research activity during the three years of PhD

Publications

1. **Maddalon**, A., Galbiati, V., Colosio, C., Mandić-Rajčević, S., & Corsini, E. (2021). Glyphosate-based herbicides: Evidence of immune-endocrine alteration. Toxicology, 459, 152851. https://doi.org/10.1016/j.tox.2021.152851

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9. Pfannes, R., Pierzchalski, A., **Maddalon**, A., Simion, A., Zouboulis, C. C., Behre, G., Zenclussen, A. C., Westphal, S., Fest, S., & Herberth, G. (2023). Characterization of post-vaccination SARS-CoV-2 T cell subtypes in patients with different hematologic malignancies and treatments. Frontiers in immunology, 14, 1087996. https://doi.org/10.3389/fimmu.2023.1087996

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Book chapter

1. **Maddalon**, A., Galbiati, V., & Corsini, E. Glyphosate-based herbicides: evidence of immune-endocrine-microbiome alteration. In Toxicological Risk Assessment and Multi-System Health Impacts from Exposure (pp. 569-578). 2021 Academic Press.

Conferences participation with poster or oral presentation

1. Oral presentation: Glyphosate and T cells: an immunotoxicity in vitro evaluation. A **Maddalon**. (ITCASS, Virtual Congress, 3-5 February 2021).

2. Oral presentation: Glyphosate and T cells: an immunotoxicity in vitro evaluation. A **Maddalon**. (EUROTOX, 56th Congress of the European Societies of Toxicology Virtual Congress, 26-29 September 2021).

3. Poster: Valutazione in vitro degli effetti diretti del glifosato sui linfociti T helper. A **Maddalon**, M Iulini, V Galbiati, E Corsini. (SITOX, 20th Congresso Nazionale Bologna, 25-27 October 2021).

4. Poster: Investigation of the effects of endocrine disruptors on T cells differentiation – A **Maddalon**, M Naghavi, L Cari, M Iulini, V Galbiati, M Marinovich, G Nocentini, E Corsini. (ICT, XVI international congress of toxicology, Maastricht, 18-21 September 2022).

5. Oral presentation: In vitro immunotoxicity: study of the effects of xenobiotics on T helper cells differentiation – A **Maddalon**. (ICT, XVI international congress of toxicology, Maastricht 19 September 2022).

6. Poster: Effetto degli interferenti endocrini sul differenziamento dei linfociti T – A **Maddalon**, M Naghavi, L Cari, M Iulini, V Galbiati, M Marinovich, G Nocentini, E Corsini. (SITOX, Bologna, 20-22 February 2023).

7. Poster: Impact of Endocrine Disruptors on In Vitro T Cell Differentiation – A **Maddalon**, M Naghavi, L Cari, M Iulini, V Galbiati, M Marinovich, G Nocentini, E Corsini. (SOT 62nd Annual Meeting & ToxExpo, Nashville, 19-23 March 2023).
8. Poster: Impact of endocrine disruptors on peripheral blood mononuclear cells in vitro: sex-related effects – A **Maddalon**, M Naghavi, L Cari, M Iulini, V Galbiati, M Marinovich, G Nocentini, E Corsini (EUROTOX, Ljubljana, 10-13 September 2023).

Honours and awards:

- Elsevier Best Poster Award 23rd March 2023 from In vitro and Alternative Methods Specialty Section of SOT, Nashville
- Study Bursary for the Best Poster at the 20th National Congress SITOX, Bologna (27th October 2021) from SITOX and Nutrition Foundation of Italy

Other activities

- Membership to SITOX society from 2019.
- Membership to SOT society from 2023.
- Reviewer for the journals: Toxicology and Applied Pharmacology, Archiv der Pharmazie, Archives of Toxicology, and Environmental Toxicology and Pharmacology.
- Intensive Course in Dermato Cosmetic Sciences, Vrije Universiteit Brussel 35 edition 1 September - 10 October 2021, with exam certification (5 ECTS).
- Organization and participation to TWINALT 2021 S&T Course 2 Immunotoxicity: theoretic al and practical course on the use of the whole blood assay. Presentation: In vivo and in vitro effects of glyphosate on T helper cells, November 2021.
- Tutor of several Bachelor and Master degrees students during the laboratory activity.
- Co-supervisor of 12 Master Thesis students from Pharmacy, Pharmaceutical Chemistry and Technology, and Safety Assessment of Xenobiotics and Biotechnological Products.
- Laboratory assistant for the course of Environmental Microbiology and Biotechnological Remediation (Safety Assessment of Xenobiotics and Biotechnological Products, for the years 2021 and 2023).
- Seminar activities for the courses of Toxicology (Pharmacy and Pharmaceutical Chemistry and Technology), Environmental Toxicology (Environmental Science and Policy), Nanotechnology Based Medicinal Products (Pharmaceutical Biotechnology) from 2020 to 2023.
- Examination assistant for the courses of Toxicology (Pharmacy and Pharmaceutical Chemistry and Technology) and Genotoxicology, Cancerogenicity, Immunotoxicology, Reproductive and Developmental Toxicity (Safety Assessment of Xenobiotics and Biotechnological Products) from 2020 to 2023.