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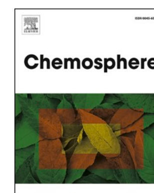

Mixtures of per- and poly-fluoroalkyl substances (PFAS) reduce the *in vitro* activation of human T cells and basophils

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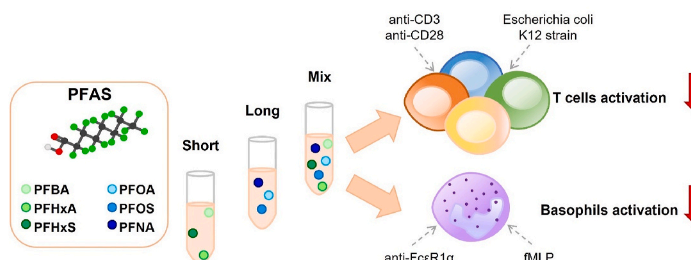
HIGHLIGHTS

- per- and poly-fluoroalkyl substances (PFAS), are ubiquitous and may modulate immune responses.
- PFAS and PFAS mixtures have the ability to reduce the activation of innate and adaptive immune cells.
- Stronger effect of both short- and long-chain PFAS mixtures on T cells, suggesting a possible additive effect of mixtures.

GRAPHICAL ABSTRACT



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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.

Abbreviations: PFAS, per- and poly-fluoroalkyl substances; MAIT cells, mucosal-associated invariant T cells.

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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.

1. 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.

2. Material and methods

2.1. Tested chemicals

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).

2.2. PBMC treatment with PFAS

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.

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

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% CO_2 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).

2.2.1. 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.

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/mlL)

2.3. 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 $CD3^+CD56^-CD161^+TCRV\alpha7.2^+$ (Supplementary Fig. 4). Following stimulation with *E. coli* K12, MAIT cells were gated, after doublets and dead cells exclusion, as $CD3^+CD8^+CD161^+TCRV\alpha7.2^+$ (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 co-expression 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).

2.3.1. t-SNE analysis of MAIT cells stimulated with Escherichia coli K12

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 down-sampling 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.

2.4. Whole blood treatment with PFAS and assessment of basophil activation

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% CO_2 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*-Formylmethionyl-leucyl-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_4Cl – Sigma-Aldrich, NaCO3 – 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).

2.5. 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% CO_2 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.

2.6. 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 $p \leq 0.05$.

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-17 A	ZBTB16	Plzf
IL17F	IL-17 F		

3. Results

3.1. 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.

3.2. 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

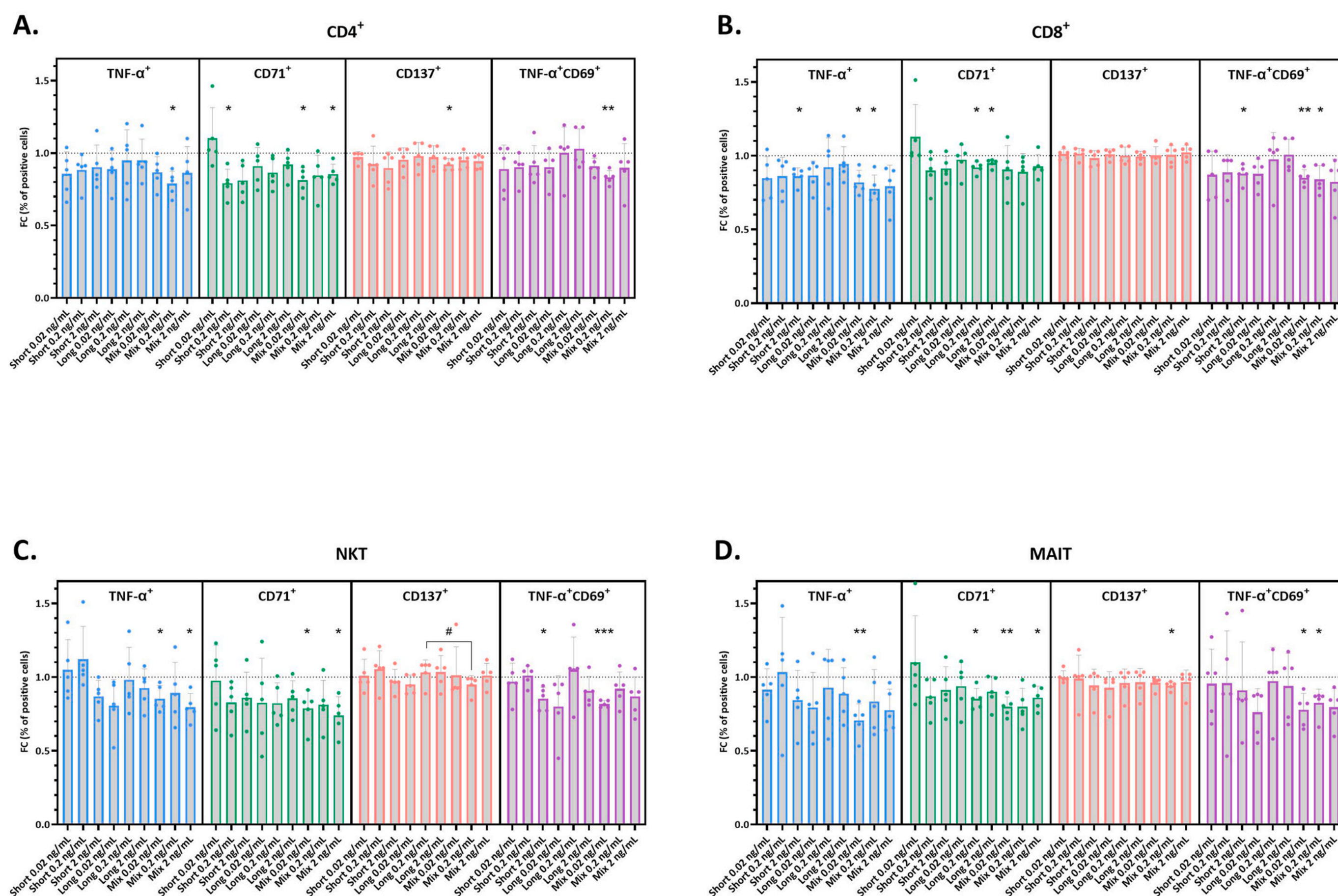


Fig. 1. Graphical representation of the modulation of (A) CD4$^+$, (B) CD8$^+$, (C) NKT, and (D) MAIT cells expression of TNF-α (blue), CD71 (green), CD137 (orange), and co-expression of TNF-α and CD69 (violet) induced by PFAS mixtures. PBMCs were exposed to different PFAS mixtures at the concentration 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.

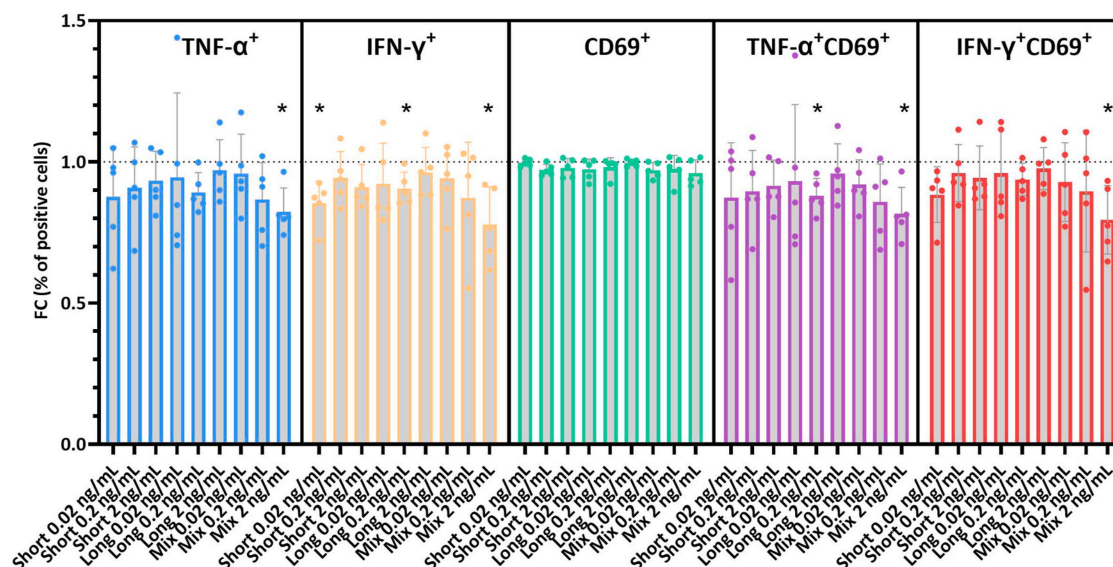
other chemicals, such as bisphenols (Krause et al., 2022), we decided to be selective on these cells. For this purpose, we stimulated these cells within PBMCs in a specific way, using *E. coli* K12, since MAIT cells are activated by bacterial metabolites. The treatment of PBMCs to increasing concentrations 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 concentration was reflected in the downregulation of both pro-inflammatory cytokine levels and of their co-expression with CD69.

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.

3.3. 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



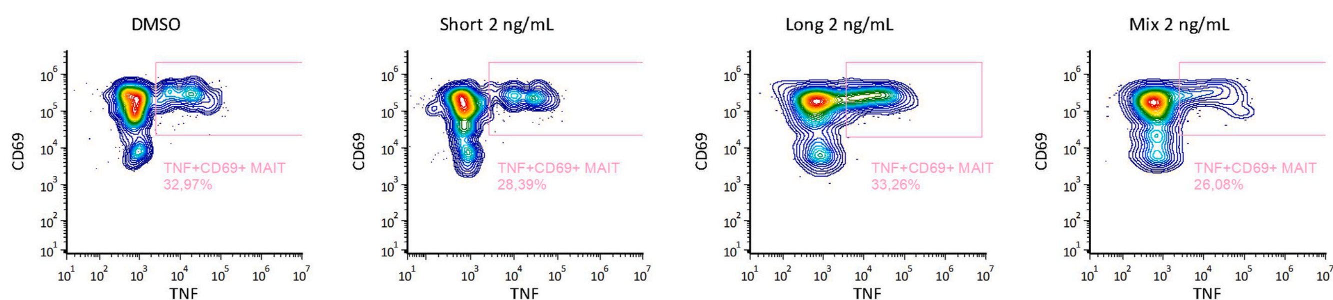


Fig. 2. (A) Modulation of MAIT cell activation by expression of TNF-α (blue), IFN-γ (yellow), CD69 (light green), and co-expression of TNF-α and CD69 (violet), and of IFN-γ and CD69 (red), induced by PFAS. PBMCs were exposed to different PFAS and mixtures at the concentration of 0.02, 0.2, and 2 ng/mL 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 ≤ 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.

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.

3.4. 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 pre-exposure 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).

4. 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

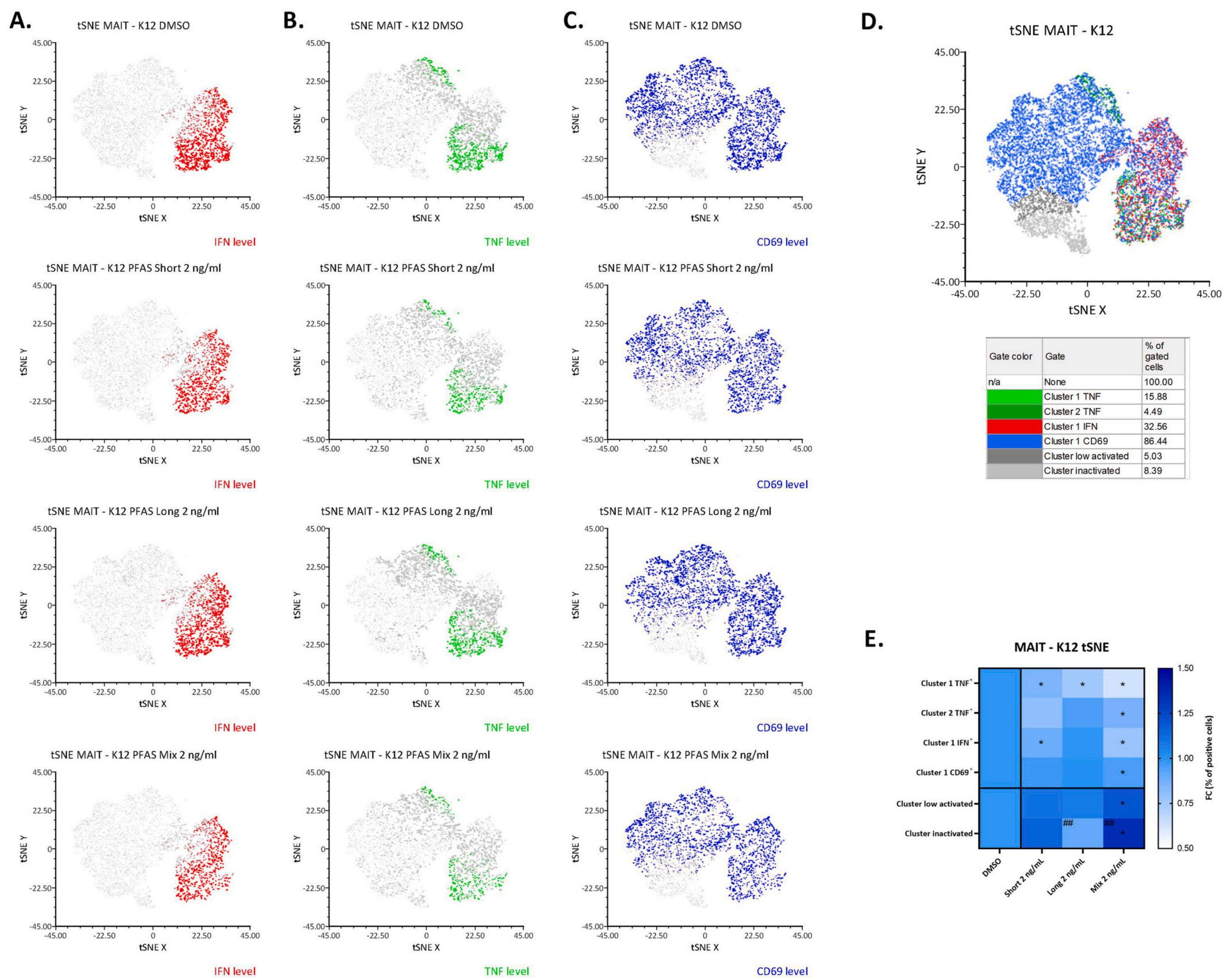


Fig. 3. 2D t-SNE representation of MAIT cells subpopulations expressing (A) IFN-γ (red), (B) TNF-α (green), and (C) CD69 (blue). The different maps represent the 4 merged treatment conditions (DMSO, Short 2 ng/mL, Long 2 ng/mL, and Mix 2 ng/mL PFAS). Each dot represents a single event. PBMCs were exposed 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.

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

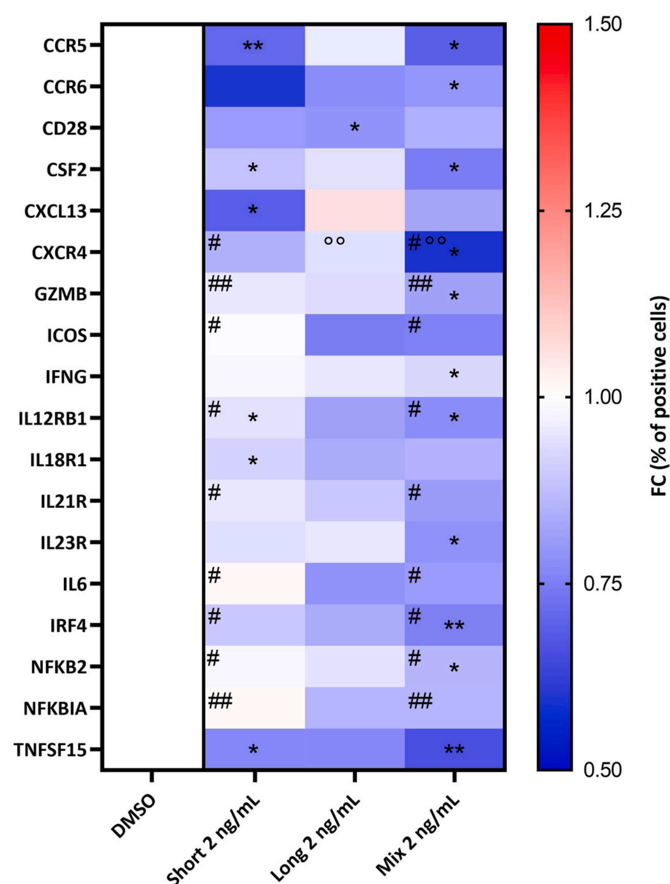


Fig. 4. Heat map representing the statistically significant association between PFAS treatment and gene expression profile of PBMC. PBMCs were exposed to different PFAS mixtures at the concentration of 2 ng/mL for 20 h and subsequently stimulated with *E. coli* K12 (10 BpC) for 6 h to activate MAIT cells. 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.

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

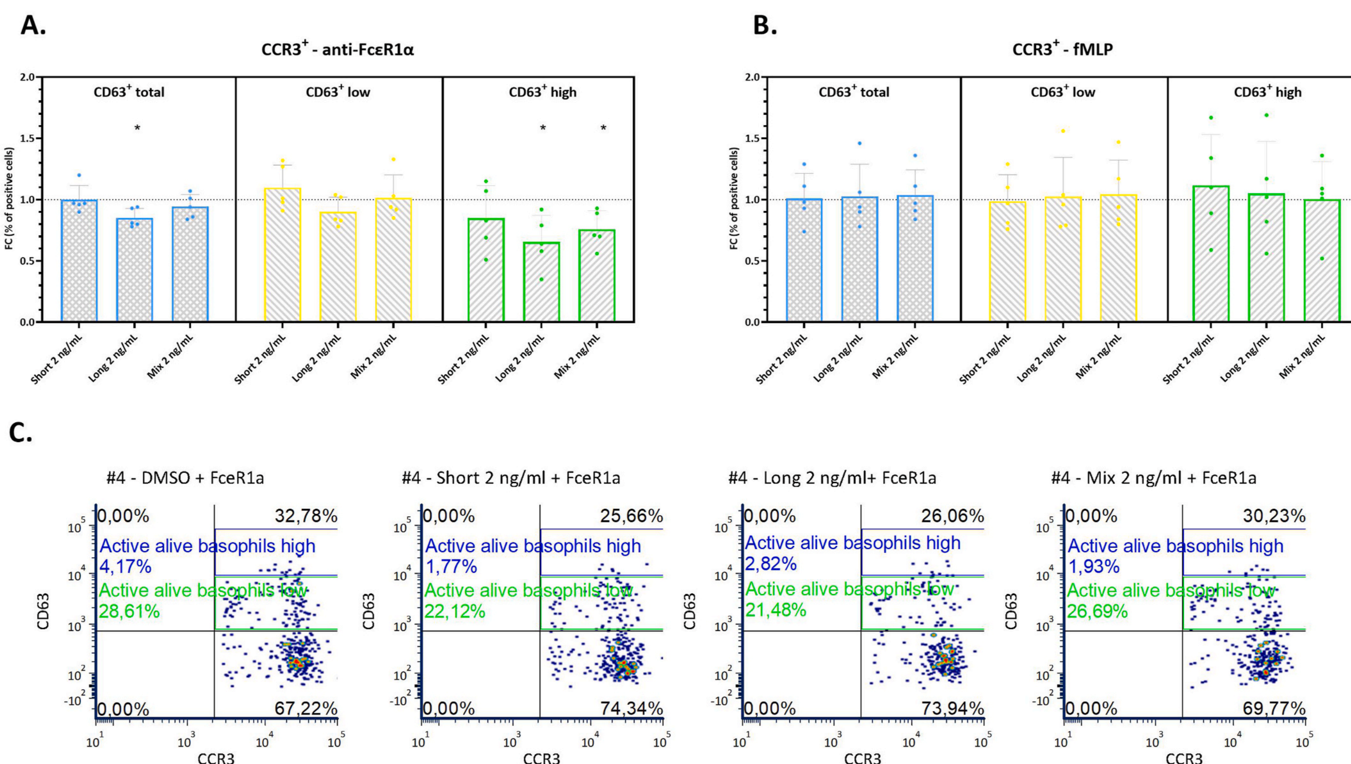


Fig. 5. Modulation of basophil (CCR3$^+$ cells) activation by expression of CD63 analysis following stimulation with anti-FcεR1α (A) or with fMLP (B). The first panel represents the % of basophils positive to CD63 (CD63$^+$ total, blue), the second panel represents the % of basophils positive to CD63 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.

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 indiviaduals, 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 backgound of PFAS-related epidemilological findings. In addition, our gene expression anlysis 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.

5. 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.

Author contributions statement

Ambra Maddalon: Investigation, Data curation, Formal analysis, Visualisation, Writing – original draft; Arkadiusz Pierzchalski: Methodology, Supervision, Writing – review & editing; Tobias Kretschmer: Supervision, Review & editing; Mario Bauer: Formal analysis, Data curation; Ana C Zenclussen: Funding acquisition, Resources, Writing – review & editing; Marina Marinovich: Review & Editing; Emanuela Corsini: Supervision, Writing – review & editing; Gunda Herberth: Conceptualization, Supervision, Writing – review & editing.

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Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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

References

Abraham, K., Mielke, H., Fromme, H., Volkel, W., Menzel, J., Peiser, M., Zepp, F., Willich, S.N., Weikert, C., 2020. Internal exposure to perfluoroalkyl substances (PFASs) and biological markers in 101 healthy 1-year-old children: associations between levels of perfluorooctanoic acid (PFOA) and vaccine response. Arch. Toxicol. 94, 2131–2147. https://doi.org/10.1007/s00204-020-02715-4.

Ateia, M., Maroli, A., Tharayil, N., Karanfil, T., 2019. The overlooked short- and ultrashort-chain poly- and perfluorinated substances: a review. Chemosphere 220, 866–882. https://doi.org/10.1016/j.chemosphere.2018.12.186.

Barbarossa, A., Masetti, R., Gazzotti, T., Zama, D., Astolfi, A., Veyrand, B., Pession, A., Pagliuca, G., 2013. Perfluoroalkyl substances in human milk: a first survey in Italy. Environ. Int. 51, 27–30. https://doi.org/10.1016/j.envint.2012.10.001.

Bell, E.M., De Guise, S., McCutcheon, J.R., Lei, Y., Levin, M., Li, B., Rusling, J.F., Lawrence, D.A., Cavallari, J.M., O'Connell, C., Javidi, B., Wang, X., Ryu, H., 2021. Exposure, health effects, sensing, and remediation of the emerging PFAS contaminants - Scientific challenges and potential research directions. Sci. Total Environ. 780, 146399 https://doi.org/10.1016/j.scitotenv.2021.146399.

Benninghoff, A.D., Bisson, W.H., Koch, D.C., Ehresman, D.J., Kolluri, S.K., Williams, D.E., 2011. Estrogen-like activity of perfluoroalkyl acids in vivo and interaction with human and rainbow trout estrogen receptors in vitro. Toxicol. Sci. 120 (1), 42–58. https://doi.org/10.1093/toxsci/kfq379.

Blake, B.E., Pinney, S.M., Hines, E.P., Fenton, S.E., Ferguson, K.K., 2018. Associations between longitudinal serum perfluoroalkyl substance (PFAS) levels and measures of thyroid hormone, kidney function, and body mass index in the Fernald Community Cohort. Environ. Pollut. (Barking, Essex: 1987) 242 (Pt A), 894–904. https://doi.org/10.1016/j.envpol.2018.07.042.

Boiteux, V., Dauchy, X., Rosin, C., Munoz, J.F., 2012. National screening study on 10 perfluorinated compounds in raw and treated tap water in France. Arch. Environ. Contam. Toxicol. 63 (1), 1–12. https://doi.org/10.1007/s00244-012-9754-7.

Braun, J.M., Gennings, C., Hauser, R., Webster, T.F., 2016. What can epidemiological studies tell us about the impact of chemical mixtures on human health? Environ. Health Perspect. 124, A6–A9. https://doi.org/10.1289/ehp.1510569.

Brendel, S., Fetter, E., Staude, C., Vierke, L., Biegel-Engler, A., 2018. Short-chain perfluoroalkyl acids: environmental concerns and a regulatory strategy under REACH. Environ. Sci. Eur. 30, 9. https://doi.org/10.1186/s12302-018-0134-4.

Brutkiewicz, R.R., Sriram, V., 2002. Natural killer T (NKT) cells and their role in antitumor immunity. Crit. Rev. Oncol.-Hematol. 41 (3), 287–298. https://doi.org/10.1016/s1040-8428(01)00198-6.

Byrne, S.C., Miller, P., Seguinot-Medina, S., Waghiyi, V., Buck, C.L., von Hippel, F.A., Carpenter, D.O., 2018. Exposure to perfluoroalkyl substances and associations with serum thyroid hormones in a remote population of Alaska Natives. Environ. Res. 166, 537–543. https://doi.org/10.1016/j.envres.2018.06.014.

Centers for Disease Control and Prevention, 2017. Per- and polyfluorinated substances (PFAS) factsheet. https://www.cdc.gov/biomonitoring/PFAS_FactSheet.html. (Accessed 19 October 2022).

Clem, A.S., 2011. Fundamentals of vaccine immunology. J. Global Infect. Dis. 3 (1), 73–78. https://doi.org/10.4103/0974-777X.77299.

Cowley, S.C., 2014. MAIT cells and pathogen defense. Cell. Mol. Life Sci. 71 (24), 4831–4840. https://doi.org/10.1007/s00018-014-1708-y.

Du, G., Hu, J., Huang, H., Qin, Y., Han, X., Wu, D., Song, L., Xia, Y., Wang, X., 2013. Perfluorooctane sulfonate (PFOS) affects hormone receptor activity, steroidogenesis, and expression of endocrine-related genes in vitro and in vivo. Environ. Toxicol. Chem. 32 (2), 353–360. https://doi.org/10.1002/etc.2034.

Duan, Y., Sun, H., Yao, Y., Meng, Y., Li, Y., 2020. Distribution of novel and legacy per-/polyfluoroalkyl substances in serum and its associations with two glycemic biomarkers among Chinese adult men and women with normal blood glucose levels. Environ. Int. 134, 105295 https://doi.org/10.1016/j.envint.2019.105295.

Duffek, A., Conrad, A., Kolossa-Gehring, M., Lange, R., Rucic, E., Schulte, C., Wellmitz, J., 2020. Per- and polyfluoroalkyl substances in blood plasma - results of the German Environmental Survey for children and adolescents 2014-2017 (GerES V). Int. J. Hyg Environ. Health 228, 113549. https://doi.org/10.1016/j.ijheh.2020.113549.

East, A., Egeghy, P.P., Hubal, E.A.C., Slover, R., Vallero, D.A., 2021. Computational estimates of daily aggregate exposure to PFOA/PFOS from 2011 to 2017 using a basic intake model. J. Expo. Sci. Environ. Epidemiol. https://doi.org/10.1038/s41370-021-00374-w.

ECHA, 2021. Committee for Risk Assessment (RAC) Committee for Socio-Economic Analysis (SEAC) Opinion on an Annex XV Dossier Proposing Restrictions on Undecafluorohexanoic Acid (PFHxA), its Salts and Related Substances ECHA/RAC/RES-O-0000006976-57-01/F ECHA/SEAC/RES-O-0000007039-72-01/F, 8 December. https://echa.europa.eu/documents/10162/97eb5263-90be-ede5-0dd9-7d8c50865c7e.

Ehrlich, V., Bil, W., Vandebriel, R., Granum, B., Luijten, M., Lindeman, B., Grandjean, P., Kaiser, A.M., Hauzenberger, I., Hartmann, C., Gundacker, C., Uhl, M., 2023. Consideration of pathways for immunotoxicity of per- and polyfluoroalkyl substances (PFAS). Environ. Health : Global Access Sci. Source 22 (1), 19. https://doi.org/10.1186/s12940-022-00958-5.

Escher, B.I., Stapleton, H.M., Schymanski, E.L., 2020. Tracking complex mixtures of chemicals in our changing environment. Science 367, 388–392. https://doi.org/10.1126/science.aay6636.

EU, 2019. Regulation (EU) 2019/1021 of the European parliament and of the Council of 20 June 2019 on persistent organic pollutants. Off. J. Eur. Union L 169, 45–77.

EU, 2020. Commission delegated regulation (EU) of 8 April 2020 amending annex I to regulation (EU) 2019/1021 of the European parliament and of the Council as regards the listing of perfluorooctanoic acid (PFOA), its salts and PFOA-related compounds. Off. J. Eur. Union L 188, 1–5.

Fenton, S.E., Ducatman, A., Boobis, A., DeWitt, J.C., Lau, C., Ng, C., Smith, J.S., Roberts, S.M., 2021. Per- and polyfluoroalkyl substance toxicity and human health review: current state of knowledge and strategies for informing future research. Environ. Toxicol. Chem. 40, 606–630. https://doi.org/10.1002/etc.4890.

Gebbink, W.A., van Leeuwen, S.P.J., 2020. Environmental contamination and human exposure to PFASs near a fluorochemical production plant: review of historic and

current PFOA and GenX contamination in The Netherlands. Environ. Int. 137, 105583 https://doi.org/10.1016/j.envint.2020.105583.

Giesy, J.P., Kannan, K., 2002. Perfluorochemical surfactants in the environment. Environ. Sci. Technol. 36, 146A–152A. https://doi.org/10.1021/es022253t.

Glüge, J., Scheringer, M., Cousins, I.T., DeWitt, J.C., Goldenman, G., Herzke, D., Lohmann, R., Ng, C.A., Trier, X., Wang, Z., 2020. An overview of the uses of per- and polyfluoroalkyl substances (PFAS). Environmental science. Process. Impacts 22 (12), 2345–2373. https://doi.org/10.1039/d0em00291g.

Glynn, A., Kotova, N., Dahlgren, E., Lindh, C., Jakobsson, K., Gyllenhammar, I., Lignell, S., Nalsen, C., 2020. Determinants of serum concentrations of perfluoroalkyl acids (PFAAs) in school children and the contribution of low-level PFAA-contaminated drinking water. Environ. Sci. Process Impacts 22, 930–944. https://doi.org/10.1039/c9em00497a.

Göckener, B., Weber, T., Rüdel, H., Bücking, M., Kolossa-Gehring, M., 2020. Human biomonitoring of per- and polyfluoroalkyl substances in German blood plasma samples from 1982 to 2019. Environ. Int. 145, 106123 https://doi.org/10.1016/j.envint.2020.106123.

Gomis, M.I., Vestergren, R., MacLeod, M., Mueller, J.F., Cousins, I.T., 2017. Historical human exposure to perfluoroalkyl acids in the United States and Australia reconstructed from biomonitoring data using population-based pharmacokinetic modelling. Environ. Int. 108, 92–102. https://doi.org/10.1016/j.envint.2017.08.002.

Gomis, M.I., Vestergren, R., Borg, D., Cousins, I.T., 2018. Comparing the toxic potency in vivo of long-chain perfluoroalkyl acids and fluorinated alternatives. Environ. Int. 113, 1–9. https://doi.org/10.1016/j.envint.2018.01.011.

Goudarzi, H., Miyashita, C., Okada, E., Kashino, I., Chen, C.J., Ito, S., Araki, A., Kobayashi, S., Matsuura, H., Kishi, R., 2017. Prenatal exposure to perfluoroalkyl acids and prevalence of infectious diseases up to 4years of age. Environ. Int. 104, 132–138. https://doi.org/10.1016/j.envint.2017.01.024.

Grandjean, P., Andersen, E.W., Budtz-Jorgensen, E., Nielsen, F., Molbak, K., Weihe, P., Heilmann, C., 2012. Serum vaccine antibody concentrations in children exposed to perfluorinated compounds. JAMA 307, 391–397. https://doi.org/10.1001/jama.2011.2034.

Grandjean, P., Heilmann, C., Weihe, P., Nielsen, F., Mogensen, U.B., Budtz-Jørgensen, E., 2017. Serum vaccine antibody concentrations in adolescents exposed to perfluorinated compounds. Environ. Health Perspect. 125 (7), 077018 https://doi.org/10.1289/EHP275.

Granum, B., Haug, L.S., Namork, E., Stolevik, S.B., Thomsen, C., Aaberge, I.S., van Loveren, H., Lovik, M., Nygaard, U.C., 2013. Pre-natal exposure to perfluoroalkyl substances may be associated with altered vaccine antibody levels and immune-related health outcomes in early childhood. J. Immunot. 10, 373–379. https://doi.org/10.3109/1547691X.2012.755580.

Gulzar, N., Copeland, K.F., 2004. CD8+ T-cells: function and response to HIV infection. Curr. HIV Res. 2 (1), 23–37. https://doi.org/10.2174/1570162043485077.

Gupta, S.S., Wang, J., Chen, M., 2020. Metabolic reprogramming in CD8+ T cells during acute viral infections. Front. Immunol. 11, 1013. https://doi.org/10.3389/fimmu.2020.01013.

Heffernan, A.L., Cunningham, T.K., Drage, D.S., Aylward, L.L., Thompson, K., Vijayasarathy, S., Mueller, J.F., Atkin, S.L., Sathyapalan, T., 2018. Perfluorinated alkyl acids in the serum and follicular fluid of UK women with and without polycystic ovarian syndrome undergoing fertility treatment and associations with hormonal and metabolic parameters. Int. J. Hyg Environ. Health 221, 1068–1075. https://doi.org/10.1016/j.ijheh.2018.07.009.

Hinks, T.S.C., Zhang, X.W., 2020. MAIT cell activation and functions. Front. Immunol. 11, 1014. https://doi.org/10.3389/fimmu.2020.01014.

Hu, X.C., Andrews, D.Q., Lindstrom, A.B., Bruton, T.A., Schaider, L.A., Grandjean, P., Lohmann, R., Carignan, C.C., Blum, A., Balan, S.A., Higgins, C.P., Sunderland, E.M., 2016. Detection of poly- and perfluoroalkyl substances (PFASs) in U.S. Drinking water linked to industrial sites, military fire training areas, and wastewater treatment plants. Environ. Sci. Technol. Lett. 3, 344–350. https://doi.org/10.1021/acs.estlett.6b00260.

Hu, X.C., Dassuncao, C., Zhang, X., Grandjean, P., Weihe, P., Webster, G.M., Nielsen, F., Sunderland, E.M., 2018. Can profiles of poly- and Perfluoroalkyl substances (PFASs) in human serum provide information on major exposure sources? Environ. Health 17, 11. https://doi.org/10.1186/s12940-018-0355-4.

Huset, C.A., Barry, K.M., 2018. Quantitative determination of perfluoroalkyl substances (PFAS) in soil, water, and home garden produce. MethodsX 5, 697–704. https://doi.org/10.1016/j.mex.2018.06.017.

Ioannidis, M., Cerundolo, V., Salio, M., 2020. The immune modulating properties of mucosal-associated invariant T cells. Front. Immunol. 11, 1556. https://doi.org/10.3389/fimmu.2020.01556.

Kashiwakura, J.I., Ando, T., Karasuyama, H., Kubo, M., Matsumoto, K., Matsuda, T., Kawakami, T., 2019. The basophil-IL-4-mast cell axis is required for food allergy. Allergy 74 (10), 1992–1996. https://doi.org/10.1111/all.13834.

Kempisty, D.M., Racz, L., 2021. Forever Chemicals: Environmental, Economic, and Social Equity Concerns with PFAS in the Environment, first ed. ed. CRC Press.

Kielsen, K., Shamim, Z., Ryder, L.P., Nielsen, F., Grandjean, P., Budtz-Jørgensen, E., Heilmann, C., 2016. Antibody response to booster vaccination with tetanus and diphtheria in adults exposed to perfluorinated alkylates. J. Immunot. 13 (2), 270–273. https://doi.org/10.3109/1547691X.2015.1067259.

Kim, S., Shen, T., Min, B., 2009. Basophils can directly present or cross-present antigen to CD8 lymphocytes and alter CD8 T cell differentiation into IL-10-producing phenotypes. J. Immunol. (Baltimore, Md 183 (5), 3033–3039. https://doi.org/10.4049/jimmunol.0900332, 1950.

EFSA Panel on Contaminants in the Food Chain (CONTAM), Knutsen, H.K., Alexander, J., Barregård, L., Bignami, M., Brüschweiler, B., Ceccatelli, S., Cottrill, B., Dinovi, M., Edler, L., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L.R., Nebbia, C.S., Oswald, I.P., Petersen, A., Rose, M., Roudot, A.C., Vleminckx, C., Vollmer, G., et al., 2018. Risk to human health related to the presence of perfluorooctane sulfonic acid and perfluorooctanoic acid in food. EFSA J. Eur. Food Safety Authority 16 (12), e05194. https://doi.org/10.2903/j.efsa.2018.5194.

Krause, J.L., Engelmann, B., Nunes da Rocha, U., Pierzchalski, A., Chang, H.D., Zenclussen, A.C., von Bergen, M., Rolle-Kampczyk, U., Herberth, G., 2022. MAIT cell activation is reduced by direct and microbiota-mediated exposure to bisphenols. Environ. Int. 158, 106985 https://doi.org/10.1016/j.envint.2021.106985.

Kvalem, H.E., Nygaard, U.C., Lodrup Carlsen, K.C., Carlsen, K.H., Haug, L.S., Granum, B., 2020. Perfluoroalkyl substances, airways infections, allergy and asthma related health outcomes - implications of gender, exposure period and study design. Environ. Int. 134, 105259 https://doi.org/10.1016/j.envint.2019.105259.

Ledda, C., La Torre, G., Cina, D., Paravizzini, G., Vitale, E., Pavone, P., Iavicoli, I., Rapisarda, V., 2018. Serum concentrations of perfluorinated compounds among children living in Sicily (Italy). Toxicol. Lett. 298, 186–193. https://doi.org/10.1016/j.toxlet.2018.09.001.

Legoux, F., Salou, M., Lantz, O., 2020. MAIT cell development and functions: the microbial connection. Immunity 53 (4), 710–723. https://doi.org/10.1016/j.immuni.2020.09.009.

Llorca, M., Farré, M., Picó, Y., Müller, J., Knepper, T.P., Barceló, D., 2012. Analysis of perfluoroalkyl substances in waters from Germany and Spain. Sci. Total Environ. 431, 139–150. https://doi.org/10.1016/j.scitotenv.2012.05.011.

Looker, C., Luster, M.I., Calafat, A.M., Johnson, V.J., Burleson, G.R., Burleson, F.G., Fletcher, T., 2014. Influenza vaccine response in adults exposed to perfluorooctanoate and perfluorooctanesulfonate. Toxicol. Sci. 138, 76–88. https://doi.org/10.1093/toxsci/kft269.

Lum, K.J., Sundaram, R., Barr, D.B., Louis, T.A., Buck Louis, G.M., 2017. Perfluoroalkyl chemicals, menstrual cycle length, and fecundity: findings from a prospective pregnancy study. Epidemiology (Cambridge, Mass.) 28 (1), 90–98. https://doi.org/10.1097/EDE.0000000000000552.

Mancini, F.R., Cano-Sancho, G., Gambaretti, J., Marchand, P., Boutron-Ruault, M.C., Severi, G., Arveux, P., Antignac, J.P., Kvaskoff, M., 2020. Perfluorinated alkylated substances serum concentration and breast cancer risk: evidence from a nested case-control study in the French E3N cohort. Int. J. Cancer 146 (4), 917–928. https://doi.org/10.1002/ijc.32357.

Masi, M., Maddalon, A., Iulini, M., Linciano, P., Galbiati, V., Marinovich, M., Racchi, M., Corsini, E., Buoso, E., 2022. Effects of endocrine disrupting chemicals on the expression of RACK1 and LPS-induced THP-1 cell activation. Toxicology 480, 153321. https://doi.org/10.1016/j.tox.2022.153321.

Medzhitov, R., 2007. Recognition of microorganisms and activation of the immune response. Nature 449 (7164), 819–826. https://doi.org/10.1038/nature06246.

Miyake, K., Shibata, S., Yoshikawa, S., Karasuyama, H., 2021. Basophils and their effector molecules in allergic disorders. Allergy 76 (6), 1693–1706. https://doi.org/10.1111/all.14662.

Mokra, K., 2021. Endocrine disruptor potential of short- and long-chain perfluoroalkyl substances (PFASs)-A synthesis of current knowledge with proposal of molecular mechanism. Int. J. Mol. Sci. 22 (4), 2148. https://doi.org/10.3390/ijms22042148.

Mudumbi, J.B.N., Ntwampe, S.K.O., Matsha, T., Mekuto, L., Itoba-Tombo, E.F., 2017. Recent developments in polyfluoroalkyl compounds research: a focus on human/environmental health impact, suggested substitutes and removal strategies. Environ. Monit. Assess. 189, 402. https://doi.org/10.1007/s10661-017-6084-2.

Munier, C.M.L., van Bockel, D., Bailey, M., Ip, S., Xu, Y., Alcantara, S., Liu, S.M., Denyer, G., Kaplan, W., , PHIIDO Study group, Suzuki, K., Croft, N., Purcell, A., Tscharke, D., Cooper, D.A., Kent, S.J., Zaunders, J.J., Kelleher, A.D., 2016. The primary immune response to Vaccinia virus vaccination includes cells with a distinct cytotoxic effector CD4 T-cell phenotype. Vaccine 34 (44), 5251–5261. https://doi.org/10.1016/j.vaccine.2016.09.009.

Noelle, R., Snow, E.C., 1992. T helper cells. Curr. Opin. Immunol. 4 (3), 333–337. https://doi.org/10.1016/0952-7915(92)90085-s.

OECD, 2018. Toward a New Comprehensive Global Database of Per- and Polyfluoroalkyl Substances (PFAS): Summary Report on Updating the OECD 2007 List of Per and Polyfluoroalkyl Substances (PFAS).

Okada, E., Kashino, I., Matsuura, H., Sasaki, S., Miyashita, C., Yamamoto, J., Ikeno, T., Ito, Y.M., Matsumura, T., Tamakoshi, A., Kishi, R., 2013. Temporal trends of perfluoroalkyl acids in plasma samples of pregnant women in Hokkaido, Japan, 2003-2011. Environ. Int. 60, 89–96. https://doi.org/10.1016/j.envint.2013.07.013.

Ostroumov, D., Fekete-Drimusz, N., Saborowski, M., Kühnel, F., Woller, N., 2018. CD4 and CD8 T lymphocyte interplay in controlling tumor growth. Cell. Mol. Life Sci. 75 (4), 689–713. https://doi.org/10.1007/s00018-017-2686-7.

Oulhote, Y., Shamim, Z., Kielsen, K., Weihe, P., Grandjean, P., Ryder, L.P., Heilmann, C., 2017. Children's white blood cell counts in relation to developmental exposures to methylmercury and persistent organic pollutants. Reprod. Toxicol. (Elmsford, N.Y.) 68, 207–214. https://doi.org/10.1016/j.reprotox.2016.08.001.

Perez, F., Llorca, M., Farré, M., Barceló, D., 2012. Automated analysis of perfluorinated compounds in human hair and urine samples by turbulent flow chromatography coupled to tandem mass spectrometry. Anal. Bioanal. Chem. 402 (7), 2369–2378. https://doi.org/10.1007/s00216-011-5660-5.

Poothong, S., Thomsen, C., Padilla-Sanchez, J.A., Papadopoulou, E., Haug, L.S., 2017. Distribution of novel and well-known poly- and perfluoroalkyl substances (PFASs) in human serum, plasma, and whole blood. Environ. Sci. Technol. 51, 13388–13396. https://doi.org/10.1021/acs.est.7b03299.

REACH, 2017. Commission regulation (EU) 2017/1000 of 13 june 2017 amending annex XVII to regulation (EC) No 1907/2006 of the European parliament and of the council concerning the registration, evaluation, authorisation and restriction of chemicals (REACH) as regards. Perfluorooctanoic Acid (PFOA), Its Salts and PFOA related

Substances. Available at: http://data.europa.eu/eli/reg/2017/1000/oj. (Accessed 19 October 2022).

Renner, R., 2001. Growing concern over perfluorinated chemicals. Environ. Sci. Technol. 35, 154A–160A. https://doi.org/10.1021/es012317k.

Rosen, M.B., Das, K.P., Rooney, J., Abbott, B., Lau, C., Corton, J.C., 2017. PPARα-independent transcriptional targets of perfluoroalkyl acids revealed by transcript profiling. Toxicology 387, 95–107. https://doi.org/10.1016/j.tox.2017.05.013.

Rudak, P.T., Choi, J., Haeryfar, S.M.M., 2018. MAIT cell-mediated cytotoxicity: roles in host defense and therapeutic potentials in infectious diseases and cancer. J. Leukoc. Biol. 104 (3), 473–486. https://doi.org/10.1002/JLB.4RI0118-023R.

Salgado-Freiría, R., López-Doval, S., Lafuente, A., 2018. Perfluorooctane sulfonate (PFOS) can alter the hypothalamic-pituitary-adrenal (HPA) axis activity by modifying CRF1 and glucocorticoid receptors. Toxicol. Lett. 295, 1–9. https://doi.org/10.1016/j.toxlet.2018.05.025.

EFSA Panel on Contaminants in the Food Chain (EFSA CONTAM Panel), Schrenk, D., Bignami, M., Bodin, L., Chipman, J.K., Del Mazo, J., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L.R., Leblanc, J.C., Nebbia, C.S., Nielsen, E., Ntzani, E., Petersen, A., Sand, S., Vleminckx, C., Wallace, H., Barregård, L., Ceccatelli, S., Cravedi, J.P., Schwerdtle, T., 2020. Risk to human health related to the presence of perfluoroalkyl substances in food. EFSA J. Eur. Food Safety Authority 18 (9), e06223. https://doi.org/10.2903/j.efsa.2020.6223.

Schwartz, C., Voehringer, D., 2011. Basophils: important emerging players in allergic and anti-parasite responses. Bioessays : news and reviews in molecular, cellular and developmental biology 33 (6), 423–426. https://doi.org/10.1002/bies.201100028.

Sochorová, L., Hanzlíková, L., Černá, M., Drgáčová, A., Fialová, A., Švarcová, A., Gramblička, T., Pulkrabová, J., 2017. Perfluorinated alkylated substances and brominated flame retardants in serum of the Czech adult population. Int. J. Hyg Environ. Health 220 (2 Pt A), 235–243. https://doi.org/10.1016/j.ijheh.2016.09.003.

Sokol, C.L., Medzhitov, R., 2010. Emerging functions of basophils in protective and allergic immune responses. Mucosal Immunol. 3 (2), 129–137. https://doi.org/10.1038/mi.2009.137.

Stein, C.R., McGovern, K.J., Pajak, A.M., Maglione, P.J., Wolff, M.S., 2016. Perfluoroalkyl and polyfluoroalkyl substances and indicators of immune function in children aged 12-19 y: National Health and Nutrition Examination Survey. Pediatr. Res. 79 (2), 348–357. https://doi.org/10.1038/pr.2015.213.

Sunderland, E.M., Hu, X.C., Dassuncao, C., Tokranov, A.K., Wagner, C.C., Allen, J.G., 2019. A review of the pathways of human exposure to poly- and perfluoroalkyl substances (PFASs) and present understanding of health effects. J. Expo. Sci. Environ. Epidemiol. 29, 131–147. https://doi.org/10.1038/s41370-018-0094-1.

Szilagyi, J.T., Avula, V., Fry, R.C., 2020. Perfluoroalkyl substances (PFAS) and their effects on the placenta, pregnancy, and child development: a potential mechanistic role for placental peroxisome proliferator-activated receptors (PPARs). Current Environ. Health Rep. 7 (3), 222–230. https://doi.org/10.1007/s40572-020-00279-0.

Tian, Y.P., Zeng, X.W., Bloom, M.S., Lin, S., Wang, S.Q., Yim, S.H.L., Yang, M., Chu, C., Gurram, N., Hu, L.W., Liu, K.K., Yang, B.Y., Feng, D., Liu, R.Q., Nian, M., Dong, G.H., 2019. Isomers of perfluoroalkyl substances and overweight status among Chinese by sex status: isomers of C8 Health Project in China. Environ. Int. 124, 130–138. https://doi.org/10.1016/j.envint.2019.01.006.

Timmermann, C.A.G., Jensen, K.J., Nielsen, F., Budtz-Jørgensen, E., van der Klis, F., Benn, C.S., Grandjean, P., Fisker, A.B., 2020. Serum perfluoroalkyl substances, vaccine responses, and morbidity in a cohort of Guinea-bissau children. Environ. Health Perspect. 128 (8), 87002 https://doi.org/10.1289/EHP6517.

Van Dommelen, S.L., Degli-Esposti, M.A., 2004. NKT cells and viral immunity. Immunol. Cell Biol. 82 (3), 332–341. https://doi.org/10.1111/j.0818-9641.2004.01261.x.

Vogt, S., Mattner, J., 2021. NKT cells contribute to the control of microbial infections. Front. Cell. Infect. Microbiol. 11, 718350 https://doi.org/10.3389/fcimb.2021.718350.

von Holst, H., Nayak, P., Dembek, Z., Buehler, S., Echeverria, D., Fallacara, D., John, L., 2021. Perfluoroalkyl substances exposure and immunity, allergic response, infection, and asthma in children: review of epidemiologic studies. Heliyon 7, e08160. https://doi.org/10.1016/j.heliyon.2021.e08160.

Wilhelm, M., Bergmann, S., Dieter, H.H., 2010. Occurrence of perfluorinated compounds (PFCs) in drinking water of North Rhine-Westphalia, Germany and new approach to assess drinking water contamination by shorter-chained C4-C7 PFCs. Int. J. Hyg Environ. Health 213 (3), 224–232. https://doi.org/10.1016/j.ijheh.2010.05.004.

Worley, R.R., Moore, S.M., Tierney, B.C., Ye, X., Calafat, A.M., Campbell, S., Woudneh, M.B., Fisher, J., 2017. Per- and polyfluoroalkyl substances in human serum and urine samples from a residentially exposed community. Environ. Int. 106, 135–143. https://doi.org/10.1016/j.envint.2017.06.007.

Yamanishi, Y., Karasuyama, H., 2016. Basophil-derived IL-4 plays versatile roles in immunity. Semin. Immunopathol. 38 (5), 615–622. https://doi.org/10.1007/s00281-016-0568-y.

Zhang, X., Xue, L., Deji, Z., Wang, X., Liu, P., Lu, J., Zhou, R., Huang, Z., 2022. Effects of exposure to per- and polyfluoroalkyl substances on vaccine antibodies: a systematic review and meta-analysis based on epidemiological studies. Environ. Pollut. (Barking, Essex 306, 119442. https://doi.org/10.1016/j.envpol.2022.119442, 1987.

Zheng, G., Schreder, E., Dempsey, J.C., Uding, N., Chu, V., Andres, G., Sathyanarayana, S., Salamova, A., 2021. Per- and polyfluoroalkyl substances (PFAS) in breast milk: concerning trends for current-use PFAS. Environ. Sci. Technol. 55, 7510–7520. https://doi.org/10.1021/acs.est.0c06978.

Zubler, R., 1984. Roles of the specific T-helper signal and the antigen signal in B-cell activation. Ann. Immunol. (Paris) 135D (1), 79–82. https://doi.org/10.1016/s0769-2625(84)80158-0.