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Differential influences of the BPA, BPS and BPF on *in vitro* IL-17 secretion by mouse and human T cells

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Abstract

The endocrine disruptor and food contaminant bisphenol A (BPA) is frequently present in consumer plastics and can produce several adverse health effects participating in the development of inflammatory and autoimmune diseases. Regulatory restrictions have been established to prevent risks for human health, leading to the substitution of BPA by structural analogues, such as bisphenol S (BPS) and F (BPF). In this study, we aimed at comparing the *in vitro* impact of these bisphenols from 0.05 to 50000 nM on Th17 differentiation, frequency and function in mouse systemic and intestinal immune T cells and in human blood T cells. This study reports the ability of these bisphenols, at low and environmentally relevant concentration, *i.e*, 0.05 nM, to increase significantly IL-17 production in mouse T cells but not in human T lymphocytes. The use of an aryl hydrocarbon receptor (AhR) specific inhibitor demonstrated its involvement in this bisphenol-induced IL-17 production. We also observed an increased IL-17 secretion by BPS and BPF, and not by BPA, in mouse naive T cells undergoing *in vitro* Th17 differentiation. In total, this study emphasizes the link between bisphenol exposures and the susceptibility to develop immune diseases, questioning thus the rational of their use to replace BPA.

Keywords: Bisphenols, Mice, Human, IL-17 secretion, Th17 differentiation, *In vitro*

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1. Introduction

Endocrine disruptors (EDs) are compounds known to impair the functioning of the endocrine system, and their bioaccumulation in humans may cause adverse health effects (Kabir et al., 2015). Among EDs, bisphenol A (BPA) is widely used as a component of epoxy resins and polycarbonate plastics by industry. BPA is present in plastic food containers, metal cans as epoxy coatings, kitchenware toys, medical devices, and dental composites and sealants (Becher et al., 2018). In humans, BPA has been shown to have developmental, reproductive, cardiovascular, immune and metabolic adverse outcomes (Rochester, 2013). In 2017, BPA was identified as a substance of very high concern in the list of the European Chemical Agency (ECHA). Regarding the recent regulations to restrict the use of BPA in food contact materials (Andújar et al., 2019), food packaging companies are exploring BPA substitutes (García-Córcoles et al., 2018). Among these analogues, bisphenol S (BPS), more heat- and photo-resistant, has been chosen by the industry as a replacement for BPA in the production of polycarbonates and epoxy resins for the manufacturing of industrial and consumer products (Chen et al., 2012). Bisphenol F (BPF) is also a BPA analogue with a wide spectrum of industrial uses. BPF is used in epoxy resins for several consumer products especially for systems needing increased thickness and durability (*i.e.*, high-solid/ high-build systems) (Rochester and Bolden, 2015). All these bisphenols have been shown to be released from such products into food and water, contributing then to human exposure through diet or drinking water (Cao et al., 2019). The analysis conducted by the *National Health and Nutrition Examination Survey* showed BPA concentrations in the range of 1.8–660 nM in urine samples of the majority of US residents (Calafat et al., 2005). In human plasma, the predominant bisphenols were BPA, BPS, with mean concentrations of 0.40 and 0.15ng/mL respectively (Jin et al., 2018).

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Emerging evidences suggest that exposure to bisphenols has been associated with altered immune function, and a variety of studies have explored this, in particular for BPA (Robinson and Miller, 2015). Among these studies, human cross-sectional and prospective studies demonstrate that prenatal and postnatal exposure to BPA has been linked to the development of asthma and inflammatory and allergic diseases (Donohue et al., 2013; Gascon et al., 2015; Spanier et al., 2012; Vaidya and Kulkarni, 2012). Investigations using experimental animals showed that prenatal exposure to BPA contributes to different effects on airway inflammation and allergic sensitization (Bauer et al., 2012; Nakajima et al., 2012; O'Brien et al., 2014). Previously, we also showed that perinatal exposure to BPA increased the risk of food intolerance at adulthood, as well as the susceptibility to intestinal infection and/or exacerbation of mucosal inflammation through deregulation of Th1/Th2 cytokine profiles in rats (Menard et al., 2014). Such effects of BPA on Th1/Th2 balance has also been reported in *in vitro* studies using murine T cells (Lee et al., 2003).

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor present in almost every tissue of animals and humans, and is involved in several metabolic processes including xenobiotic metabolization. AhR can bind aromatic hydrocarbon compounds derived from plastics including BPA (Krüger et al., 2008). Moreover, a crosstalk exists between AhR, the estrogen receptor (ER) and androgen receptor (AR), classically targeted by bisphenols (Grimaldi et al., 2019; Pocar et al., 2005). However, studies that have examined the potential role of AhR in mediating the toxic effects of BPA have been equivocal. Bonefeld-Jorgensen *et al.* pointed out weak antagonistic effects of BPA with AhR (Bonefeld-Jørgensen et al., 2007) whereas, in more recent studies, Ziv-Gal *et al.* showed also that BPA may inhibit follicle growth partially *via* the AhR pathway (Ziv-Gal et al., 2013). More recently, Deluca *et al.* have shown that BPA is able to alter tryptophan and microbiota metabolites affecting gut physiology to exacerbate inflammation (DeLuca et al., 2018). Although direct evidence

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indicates a role of tryptophan metabolites on AhR activation (Rannug and Rannug, 2018), the potential involvement of AhR in these BPA-induced effects has not been investigated. Interestingly, AhR has emerged as a critical physiological regulator of immunity and development of Th17 cells (Veldhoen et al., 2009), which are key effector T cells in several inflammatory diseases such as inflammatory bowel diseases and colorectal cancers (Cho et al., 2018; Hurtado et al., 2018). Indeed, Th17 cells are particularly enriched in the gastrointestinal tract and AhR pathway activation is important for the production of IL-17 and IL-22 cytokines by this T cell subset. Interestingly, we have recently reported that perinatal exposure to BPA induced intestinal and systemic immune imbalances in female offspring mice at adulthood, through an increase of Th17 cell frequencies in the small intestine *lamina propria* (si*LP*) (Malaisé et al., 2018). Such an increase in the development of Th17 cells in the offspring has also been described by Luo *et al.* after gestational and lactational BPA exposure (Luo et al., 2016). However, the involvement of AhR on this effect has not been investigated.

Restrictions have been imposed on BPA, but substitutes having similar chemical structures like BPS and BPF, with very low regulations, are now used leading to the question whether those substitutes are safe. Indeed, the considerable use of BPA analogues requires studies to better characterize their potential effects on human health. While perturbations of immune responses, especially linked to Th17 immunity, have been documented *in vivo* in animal models (Malaisé et al., 2017), the effects of *in vitro* exposure to BPA substitutes, in animal and human cell models, are poorly described. To this aim, we analysed the dose-response relationship of BPS and BPF, in comparison to BPA, on Th17 differentiation, frequency and function in mouse T cells isolated from systemic and intestinal compartments and in human T cells from blood donors with a special interest for the involvement of AhR on these Th17 immune responses.

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2. Materials and methods

2.1. Chemical

BPA, BPS and BPF (purity > 99%, CAS number: 80-05-7, 80-09-1, 620-92-8, respectively) Sigma-Aldrich, Saint Quentin Fallavier, France) were dissolved in ethanol, which therefore was included as vehicle control in all exposures. Each bisphenol (stock solution 1M) was added into cell culture at 0.05 to 50000 nM. AhR agonist FICZ (6-Formylindolo[3,2-b]carbazole, CAS number: 172922-91-7) and TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxine, CAS number: 1746-01-6) (Sigma-Aldrich) were added at 10 nM, and the AhR inhibitor CH-223191 (Sigma-Aldrich), at 3 nM to cultures.

2.2. Bisphenol exposure in mouse cellular models

2.2.1. Animals

All experiments were approved by the Local Animal Care and Use Committee (TOXCOM 0035/EH-2013), in compliance with the European directive 2010/63/UE. Eight weeks old C3H/HeN (Janvier, Roubaix, France) mice were used. All animals were kept at a constant temperature (22+/-1°C) and maintained on a 12:12 h light/dark cycle (light on at 7:30 am). Si*LP* and spleens were sampled for primary cell cultures.

2.2.2. Cell isolation from mouse spleens and si*LP* and chemical exposure

Spleens were collected and cells were isolated through 70µm cell strainer to make a single-cell suspension in PBS-1% KnockOutTM SR (KO SR) (Gibco, lifetechnologies, Paisley, UK). Small intestines were washed in cold PBS, cut into 0.5 cm pieces, incubated four times in 30 ml of PBS with 3mM EDTA (Sigma-Aldrich) and digested in 20 ml of Dulbecco

modified Eagle medium (DMEM) added with 20% Fetal Calf Serum (FCS) and 100 U/mL of collagenase (Sigma-Aldrich) for 40 min at 37°C. Si*LP* cells were purified on a 40%–80% Percoll gradient centrifuged for 15 min at 1800g at room temperature. Cells were cultured in Cerrotini culture medium as DMEM supplemented with 8% Knockout serum replacement, (Gibco), 36 mg/l asparagine, 116 mg/l arginine, 10 mg/l folic acid, 1 g/l 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, 0.05 mmol/l β-mercapto-ethanol, 100 U/ml penicillin, 100 mg/ml streptomycin and 1µg/ml fungizone. Cells were seeded in 96-well plates at $1x10^6$ cells/ml, pre-coated with 5µg/ml hamster anti-mouse CD3 and hamster anti-mouse CD28 antibodies (BD Biosciences, San Jose, CA, USA). AhR agonists FICZ and TCDD or the AhR inhibitor CH-223191 were added into cells 1h prior bisphenol incubations. BPA, BPS and BPF exposure were started 1h after plating cells by adding 125 µl of Cerrotini medium containing 2X concentrations of each bisphenol. After 4 days of stimulation, culture supernatants were collected and frozen at -80° C prior to cytokine measurements.

2.2.3. Naïve T cell purification from splenocytes, Th17 cell differentiation and bisphenol exposures

Naïve $CD4^+$ T cells were isolated from mouse spleens using Naïve $CD4^+$ T cells isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of naïve T cells was checked using anti-CD4 antibody by flow cytometry. The purity was > 97 % of naïve $CD4^+$ T cells. All cells have been checked for viability by trypan blue staining before cell cultures. These cells were polarized *in vitro* into Th17 cells adding a combination of several cytokines and neutralizing Abs (cytobox Th17 kit, Miltenyi biotech) in Cerrotini culture medium as described above. Cells were dispensed on 96-well plates at $1x10^6$ cells/ml in 125 µl of Cerrotini medium for differentiation process, pre-coated with 5µg/ml hamster anti-mouse

CD3 and hamster anti-mouse CD28 antibodies as described above. BPA, BPS and BPF exposure were started 1 day after plating cells by adding 125 µl of Cerrotini medium containing 2X concentrations of each bisphenol. After 4 days of stimulation, culture supernatants were collected and frozen at -80°C prior to cytokine measurements.

2.2.4. Fluorescence-Activated Cell Sorter Analysis

All cultures have been checked for viability using Viobility™ fixable dyes staining (Miltenyi biotec). For Th17 intracellular staining, isolated cells from spleens and si*LP* were stimulated with a cocktail of phorbol 12-myristate 13-acetate (PMA) (15 nM, Sigma-Aldrich) and ionomycin (1 µg/ml, Sigma-Aldrich) for 5h at 37 °C. Brefeldin A (10 µg/ml; Sigma-Aldrich) was added for 2h after PMA-ionomycin stimulation. Cells were then stained with antibodies to the following markers: anti-CD4 (1 µg/ml, GK1.5, BD), anti-CD3 (2 µg/ml, 145-2C11, BD), anti-IL-17 (2 µg/ml TC11-18H10, BD), anti-RORγt (2 µg/ml Q31-378, BD) (BD Biosciences). Specificity of each labelling was checked using specific isotype controls. The intracellular staining was realized by using a Cytofix/Cytoperm Kit Plus (BD Biosciences) according to the manufacturer's instructions. Flow cytometry data were collected on MACSQuant Analyzer 10 and the data were analyzed using FlowLogic software (Miltenyi Biotec).

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2.2.5. Cytokine measurements

IL-22 and IL-17 present in supernatants of cell cultures were assayed using commercial enzyme linked immunosorbent assays (ELISA kits ref DY421, DY582, respectively; Duoset R&D Systems, Lille, France).

2.3. Bisphenol exposures in human cellular models

2.3.1. Cell isolation and exposure

Peripheral human blood mononuclear cells (PBMC) were isolated from blood donor buffy coats (written consent for the use of blood samples for the research protocol obtained according to the regulation for blood transfusion of the French blood organization Etablissement Français du Sang, Rennes (France) by Ficoll (Thermofischer Scientific) gradient centrifugation. After separation of monocytes by a 1-h adhesion step, $CD4^+$ T lymphocytes were purified from non-adherent cells by negative selection using Dynabeads® human CD4 T cell kit (Thermofischer Scientific). T lymphocytes were cultured at $1x10^6$ cells/ml in RPMI medium without phenol red (Eurobio, Les Ulis, France) supplemented with 20 IU/mL penicillin, 20 µg/mL streptomycin, and 10% decomplemented fetal calf serum (Thermofischer Scientific), and stimulated with Dynabeads® T-Expander beads coated with anti-CD3 and anti-CD28 antibodies (Thermofischer Scientific) as previously reported (Liamin et al., 2018, 2017) during 16h before a 72h treatment with bisphenols. Cultured lymphocytes were then harvested for intracellular cytokine staining and culture supernatants were collected and frozen at -80°C prior cytokine measurements.

2.3.2. Cytokine measurements

The concentrations of IL-17, and IL-22 cytokines in supernatants of T lymphocyte cultures were assayed by ELISA using specific Duoset system kits ((ELISA kits ref DY317, DY782, respectively, R&D Systems) as previously reported (Prigent et al., 2014).

2.3.3. Intracellular cytokine staining and analysis by flow cytometry

All cultures have been checked for viability using fixable viability dye eFluor 660 (Thermofischer Scientific). For intracellular staining, T lymphocyte cultures were stimulated with a cocktail of PMA (50 ng/ml) and ionomycin (1 µM) for 5h at 37 °C. Brefeldin A (10 µg/ml) was added for 2h after PMA-ionomycin stimulation. T lymphocytes were then stained with antibodies to the following markers: anti-IL-17 (2.4 µg/ml, 12-7179-42) and anti-IL-22 (1.24 µg/ml, 11-7229-41) (Thermofischer Scientific). Specificity of each labelling was checked using specific isotype controls. The intracellular staining was realized by using a Cytofix/Cytoperm Kit Plus (BD Biosciences) according to the manufacturer's instructions. Data were acquired using a BD LSR Fortessa flow cytometer and the data were analysed using FlowLogic software (Miltenyi Biotec).

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2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.00 (GraphPad Software, San Diego, California, USA). Results were expressed as means +/- SEM. Multiple group was compared to control group used one-way ANOVA, Kruskal wallis Dunn's multiple comparison tests. P-values < 0.05 were considered significant (indicated by asterisks): *p <

0.05; **p < 0.01; ***p < 0.001; ****p < 0.000: differences between bisphenol treatments *vs* vehicle control. $ <0.05: differences between bisphenol concentrations.

3. Results

3.1. *In vitro* effects of bisphenols on Th17 polarization of mouse activated naïve CD4+ T cells

To investigate the impact of BPS and BPF in comparison with BPA on Th17 differentiation, naïve CD4+ T cells were purified from mouse spleens, activated and cultured under Th17 differentiation conditions for 5 days and then exposed to a dose-response of BPS, BPF, or BPA for the last 4 days. Since Th17 cells are defined by the expression of the RORγt transcription factor and the secretion of IL-17, the secretion of this cytokine was first determined in CD4+ T cells undergoing Th17 cells differentiation (Fig 1).

We observed that BPS and BPF exposures significantly increased IL-17 production at low concentrations of 0.05 and 5 nM, whereas the highest concentration (50000 nM) did not alter this secretion. By contrast, only a high concentration of BPA, *i.e.* 50000 nM, significantly decreased IL-17 secretion (Fig 1A).

Figure 1.

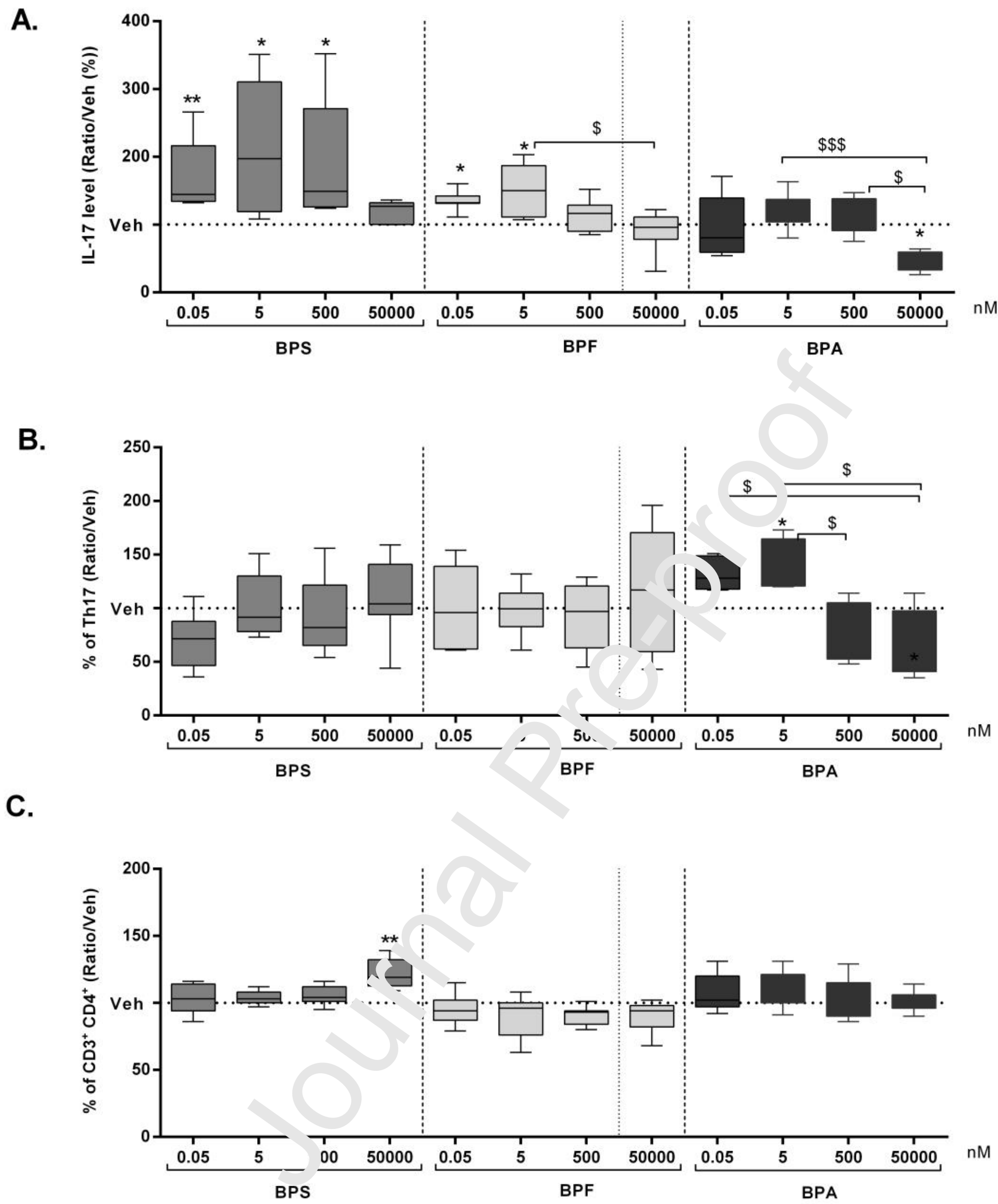

Figure 1. *In vitro* effects of bisphenols on Th17 polarization of mouse activated naïve CD4$^+$ T cells isolated from mouse spleens. Naïve CD4$^+$ T cells, isolated from mouse spleens, were stimulated with anti-CD3 and anti-CD28 antibodies, in the presence of Th17-polarizing cytokines for 5 days and then treated with vehicle (ethanol) or with various increasing bisphenol concentrations (0.05 to 50000 nM) for the last 4 days of culture. (A) IL-

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17 secretion measured in cell supernatants by ELISA. (B) Frequency of Th17 cells ($CD3^+$ $CD4^+$ RORγt$^+$ $IL17^+$/total lived cells) and (C) frequency of T cells (% of $CD3^+$ $CD4^+$/total lived cells) measured by flow cytometry. Data are expressed relative to IL-17 concentration (A), to $CD3^+CD4^+$RORγt$^+$$IL17^+$ cells (B) and to $CD3^+CD4^+$ T cells (C) found in vehicle-treated T cells arbitrarily set at 100% and designed as "Veh". Box and whisters (min to max), of 5 independent experiments, each being in 2 replicates. *$p < 0.05$: differences between bisphenol treatments *vs* vehicle control; $^\$p < 0.05$: differences between bisphenol concentrations.

Flow cytometry was next performed to measure the percentage of CD3, CD4, RORγt and IL-17 expressing cells. Surprisingly, neither BPS nor BPF altered the frequency of Th17-polarized $CD4^+$ T cells, measured by the IL-17-positive cells, whereas BPA exposure induced an increased Th17 differentiation from $CD4^+$ naïve T cells at low concentrations with a significant effect at 5 nM (Fig 1B). Only BPS exposure at high concentration (50000 nM) provoked an increase in percentages of $CD3^+$ $CD4^+$ T cells (Fig 1C). Measurement of viability reveals that exposure to bisphenols for all used concentrations did not alter cellular viability (data not shown).

3.2. *In vitro* effects of bisphenols on frequency and function of Th17 isolated from mouse splenocytes

To further examine the impact of bisphenols in T cells, we isolated splenocytes from mouse, activated and exposed them to various increasing bisphenol concentrations as previously described for 4 days and analyzed the effects on Th17 responses. As shown in Fig 2, BPF, as BPA, is able to increase significantly IL-17 production (Fig 2A) at 0.05 nM in this spleen cell

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suspension. IL-22 secretion is also induced by both BPF and BPA at this low concentration but without reaching a significant level (Fig 2B). In comparison with Fig 1, BPS did not produce any effect on IL-17 secretion by T cells isolated from mouse splenocytes (Fig 2A). Interestingly, BPS, BPF and BPA had no effect on Th17 frequency measured by the percentage of CD3, CD4, RORγt and IL-17 expressing cells (Fig 2C). In parallel and as already described in mouse T cells (Schiering et al., 2018), we noticed a high cytokine production of both IL17 and IL-22 after incubation with FICZ. The level of IL-17 and IL-22 is higher or at an equivalent level after exposure to bisphenols, especially BPF and BPA, than after stimulation by FICZ, which is demonstrated as a strong activator of these cytokines (Ehrlich et al., 2018). Concordantly with our previous results, no alteration in cell viability was reported whatever bisphenol concentrations used (data not shown).

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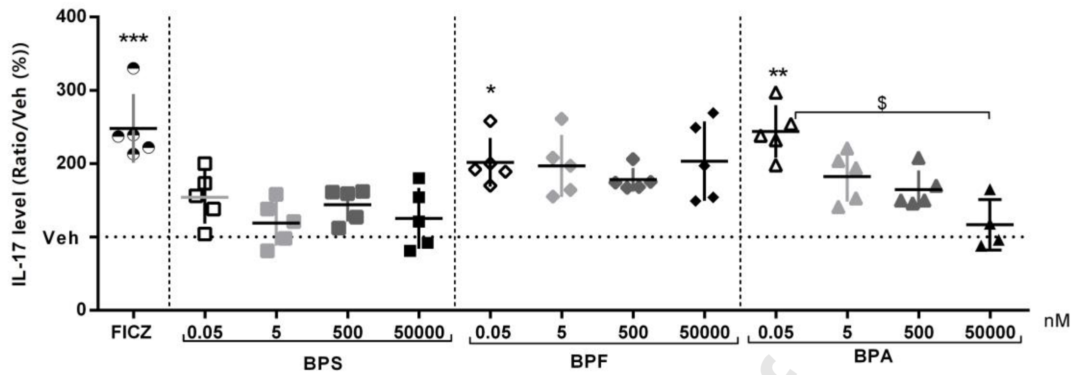
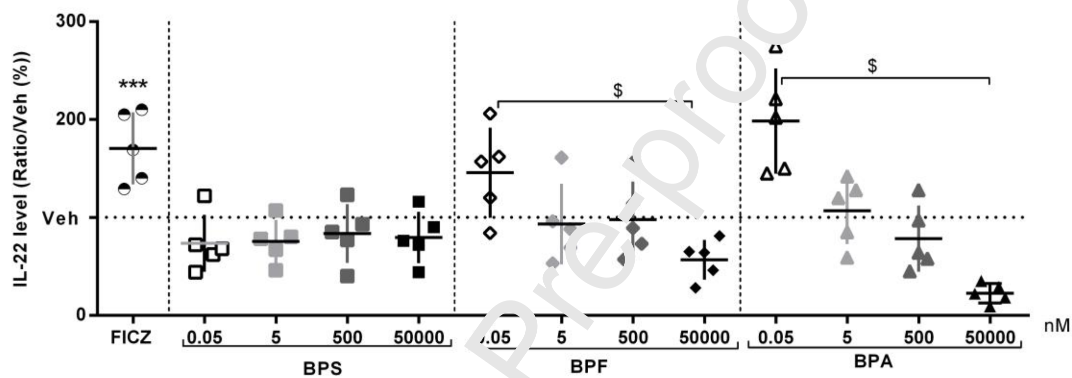
Figure 2.

A.



B.



C.

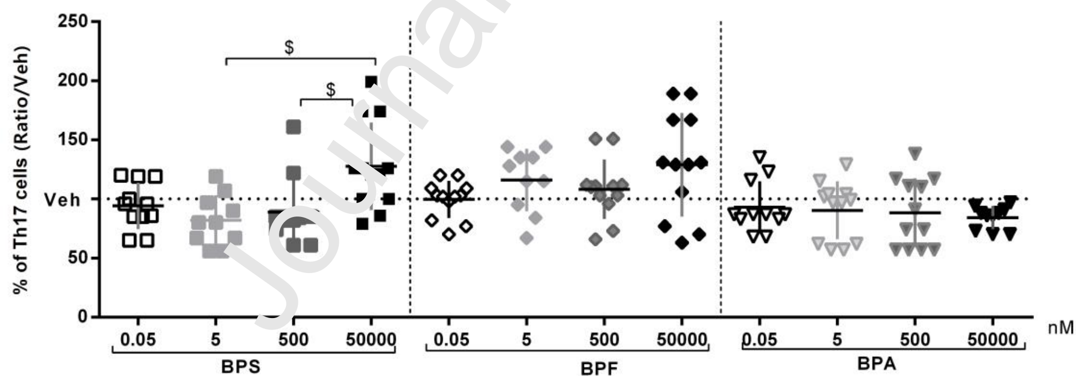


Figure 2. ***In vitro*** **effect of bisphenols on frequency and function of Th17 isolated from mouse splenocytes.** Total cell suspensions, isolated from mouse spleens, were stimulated with anti-CD3 and anti-CD28 antibodies for 4 days and then treated with vehicle (ethanol), with the agonist AhR agonist FICZ, or with various increasing bisphenol concentrations (0.05 to 50000 nM) for the last 3 days. IL-17 (A) and IL-22 (B) secretions were measured in cell

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supernatants by ELISA. Data are expressed relative to concentrations found in vehicle-treated T cells arbitrarily set at 100% designed as "Veh". (C) Frequency of Th17 cells ($CD3^+CD4^+ROR\gamma t^+IL17^+$/total lived cells) was measured by flow cytometry. Data are expressed relative to $CD3^+CD4^+ROR\gamma t^+$ $IL17^+$ cells found in vehicle-treated T cells arbitrarily set at 100% designed as "Veh". Results were expressed as means +/- SEM of 5 independent experiments made in duplicate. *$p < 0.05$: differences between bisphenol treatments *vs* vehicle control; $^{\$}$ $p < 0.05$: differences between bisphenol concentrations.

3.3. In vitro effects of bisphenols on function of Th17 isolated from mouse si*LP*

Since Th17 cells preferentially were accumulated in the intestine (Omenetti et al., 2019) and that bisphenols were preferentially administered by oral route, we isolated, activated and exposed T cells from si*LP* to the same concentrations of bisphenols. We then analyzed their effects on Th17 responses in this small intestine cell suspension by measuring IL-17 and IL-22 cytokine productions. In agreement with Figure 2, BPF and BPA, at 0.05 nM, significantly increased IL-17 concentrations (Fig 3A and 3B). IL-22 secretion is also significantly induced by both BPF and BPA at this low concentration. Interestingly and unlike Fig 2, BPS exposure at such low concentration provoked a significant increase of both IL-17 and IL-22 secretion (Fig 3A and 3B). As in T cells isolated from mouse splenocytes, FICZ is able to increase both IL-17 and IL-22 secretions in T cells isolated from si*LP* (Fig 3A and 3B). None concentration of bisphenol used affects cellular viability (data not shown).

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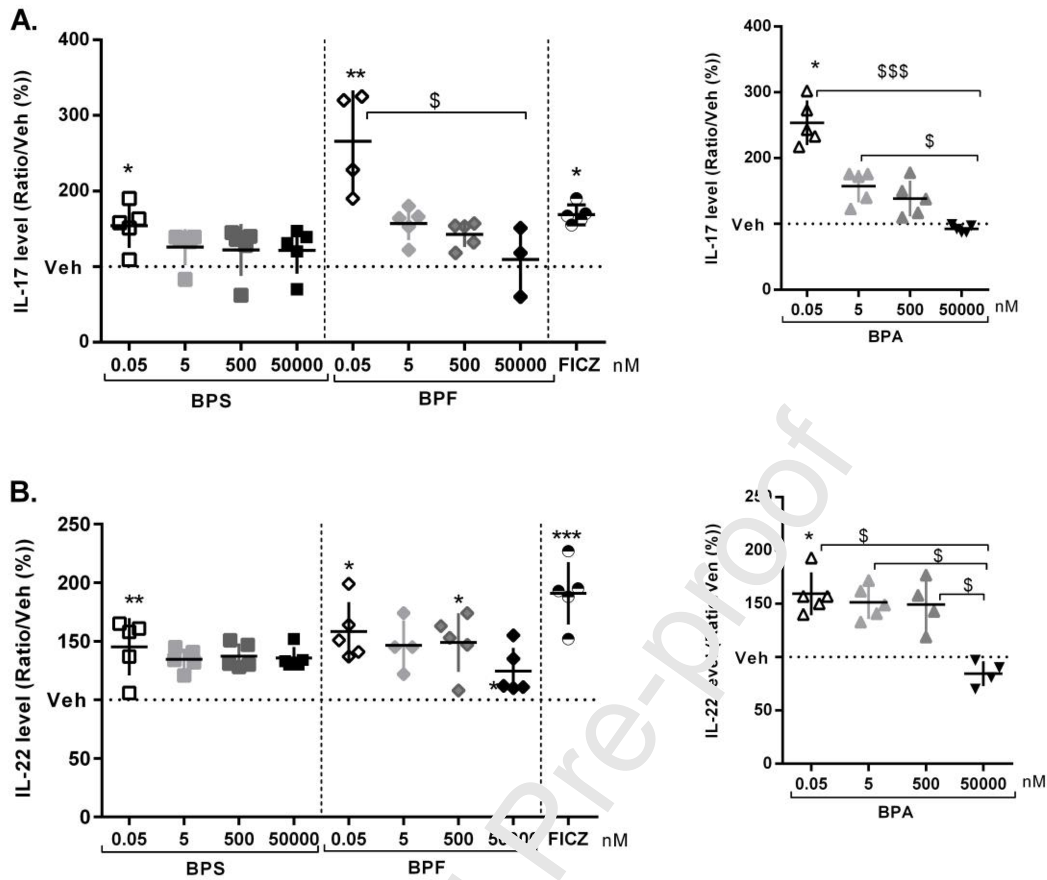

Figure 3. ***In vitro*** **effects of bisphenols on function of Th17 isolated from mouse si*LP*.**

Total cell suspensions, isolated from mouse si*LP*, was stimulated with anti-CD3 and anti-CD28 antibodies for 4 days and then treated with vehicle (ethanol), with the AhR agonist FICZ or with various increasing bisphenol concentrations (0.05 to 50000 nM) for the last 3 days. IL-17 (A) and IL-22 (B) secretions were measured in cell supernatants by ELISA. Data are expressed relative to concentrations found in vehicle-treated T cells arbitrarily set at 100% designed as "Veh". Results were expressed as means +/- SEM of 5 independent experiments made in duplicate. *p < 0.05: differences between bisphenol treatments *vs* vehicle control; $^{\$}$p < 0.05: differences between bisphenol concentrations.

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3.4. Involvement of AhR on BPA and BPF-induced IL-17 and IL-22 cytokine secretions by murine T cells

Given the role of AhR in modulating T cell responses and its expression in Th17 cell subset (Baricza et al., 2016), we tested whether BPA and BPF-enhanced IL-17 and IL-22 secretions were AhR-dependent. In Fig 2 and 3, we have shown that the potent AhR agonist, FICZ (Wu et al., 2020), is able to increase both IL-17 and IL-22 productions by T cells isolated from spleens and from si*LP*. Then, these T cells were pretreated or not with the AhR antagonist CH-223191 for 1 h and then exposed to 0.05 and 5 nM BPA and BPF, or vehicle. CH-223191 is a specific antagonist of AhR without detectable AhR agonist-like activity or estrogenic potency, even at high doses (Kim et al., 2006).

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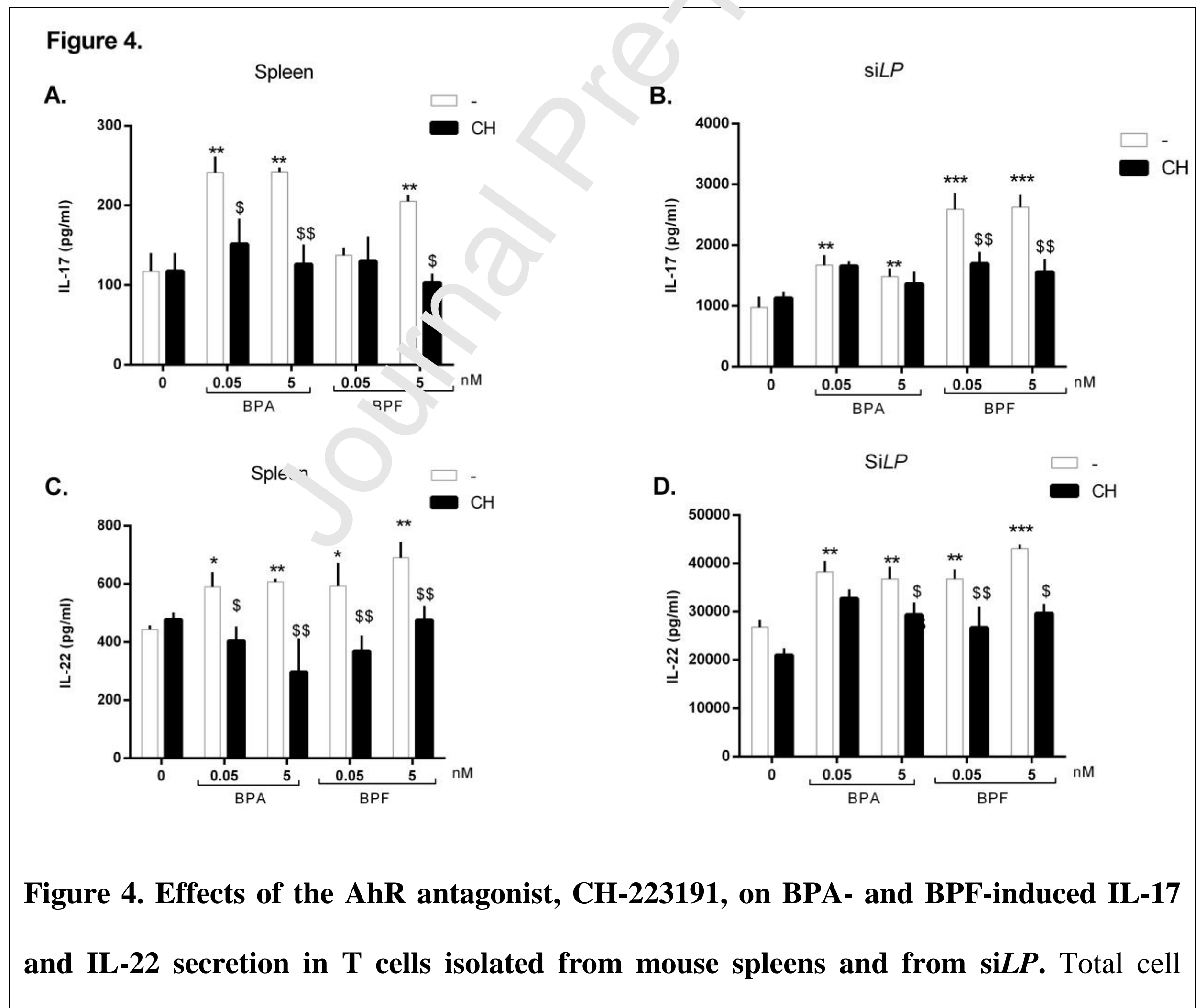

Figure 4. Effects of the AhR antagonist, CH-223191, on BPA- and BPF-induced IL-17 and IL-22 secretion in T cells isolated from mouse spleens and from si*LP*. Total cell

suspensions, isolated from mouse spleens (A, C) or from mouse si*LP* (B, D) were stimulated with anti-CD3 and anti-CD28 antibodies for 4 days, and then pre-treated or not with CH-223191 for 1 hour before vehicle (ethanol) or bisphenol treatment at 0.05 and 5 nM for the last 3 days. IL-17 (A, B) and IL-22 (C, D) secretions were measured in cell supernatants by ELISA. Data are expressed in pg/ml. Results were expressed as means +/- SEM of 4 independent experiments. *$p < 0.05$: differences between bisphenol treatments *vs* vehicle control; $^{\$}p < 0.05$: differences between bisphenol concentrations.

As shown in Fig 4A and 4C, CH-223191 is able to inhibit significantly the BPA and BPF-dependent production of IL-17 and IL-22 in T cells from spleen when treated with 0.05 or 5 nM of bisphenol. Although it is less visible in the production of IL-17 by si*LP* T cells treated with BPA, CH-223191 also inhibits IL-17 and IL-22 production induced by BPA and BPF at low concentrations in T cells isolated from *si*LP (Fig 4B and 4D). Interestingly, CH-223191 did not significantly counteract IL-17 and IL-22-induced secretions by the potent AhR ligand, FICZ whereas it significantly counteracted IL-17 production induced by TCDD, another AhR agonist (Cervantes-Barragan and Colonna, 2018), in both murine splenocytes and T cells from the intestine (Fig S1). Altogether, these findings suggest that BPA and BPF may contribute to increased IL-17 and IL-22 production in both spleen and small intestine T cells through mechanism involving an AhR-dependent pathway.

3.5. *In vitro* effects of bisphenols on Th17 frequency and on IL-17 and IL-22 secretions in CD4^{+} T cells isolated from human lymphocytes

Lastly, human CD4^{+} T cells were purified from blood donors, activated as previously reported (Liamin et al., 2018, 2017), and then exposed to the same dose-response of BPS, BPF, or BPA for the last 3 days of culture as described for mouse T cells. First, we tested the ability of

bisphenols to affect IL-17 and IL-22 secretions by these human T cells. Interestingly and unlike the data obtained with mouse T cells, low concentrations of BPS, BPF, as well as BPA, did not significantly modify both IL-17 and IL-22 productions in human T cells (Fig 5A and 5B).

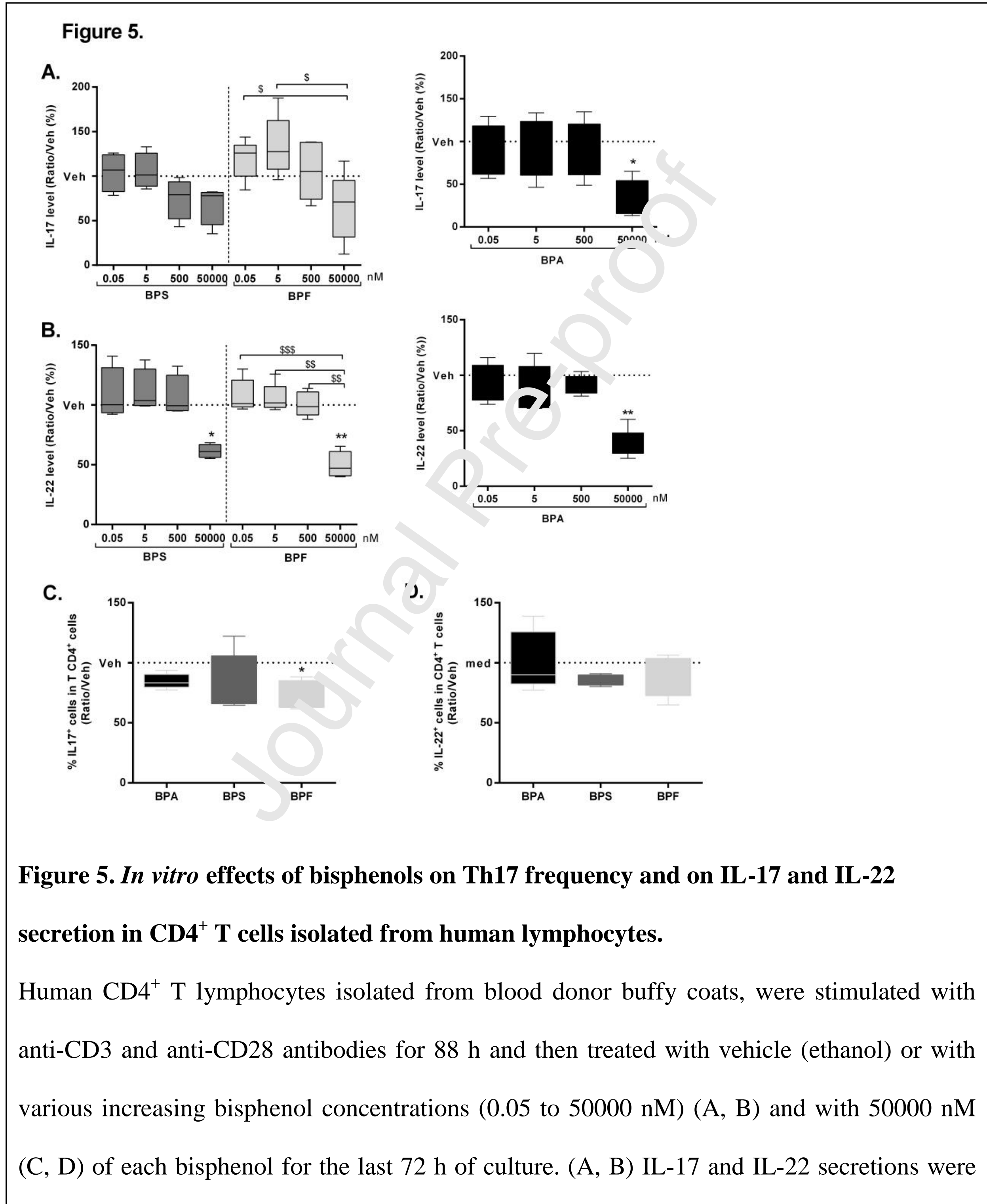

Figure 5. ***In vitro*** **effects of bisphenols on Th17 frequency and on IL-17 and IL-22 secretion in CD4^{+} T cells isolated from human lymphocytes.**

Human CD4^{+} T lymphocytes isolated from blood donor buffy coats, were stimulated with anti-CD3 and anti-CD28 antibodies for 88 h and then treated with vehicle (ethanol) or with various increasing bisphenol concentrations (0.05 to 50000 nM) (A, B) and with 50000 nM (C, D) of each bisphenol for the last 72 h of culture. (A, B) IL-17 and IL-22 secretions were

measured in cell supernatants by ELISA. (C, D) Intracellular staining of IL-17 and IL-22 was determined by flow cytometry. Data are expressed relative to concentrations and to positive $CD4^+$ cells found in vehicle-treated T lymphocytes arbitrarily set at 100% designed as "Veh". Representation in box and whiskers, min to max, of 5 independent experiments made in duplicate. *$p < 0.05$: differences between bisphenol treatments *vs* vehicle control; $^{\$}p < 0.05$: differences between bisphenol concentrations.

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Only the highest concentration tested, *i.e.* 50000 nM, decreased both IL-17 and IL-22 productions, with a significant effect for IL-17 after BPA treatment and for IL-22 in BPS-, BPF- and BPA-exposed T lymphocytes (Fig 5A and 5B). Such a high concentration of bisphenol did not alter T lymphocyte viabilities in our conditions (data not shown) but slightly decreased the percentages of IL-17 and IL-22 positive cells measured by intracellular staining (Fig 5C and 5D). Altogether, these data suggest that *in vitro* exposure to the high concentration, *i.e.* 50000 nM of BPA, and its analogues BPS and BPF, could decrease Th17 differentiation from $CD4^+$ human T lymphocytes.

4. Discussion

Because of government restrictions on the use of BPA in some countries, selected commercial applications now employ various BPA structural analogues such as BPS and BPF. Even if it is believed that BPA substitutes have similar effects to BPA (Rosenmai et al., 2014), there is limited information about their adverse effects, especially on immune system. Our recent studies revealed that BPA is able to alter immune responses *in vivo*, with a particular focus on Th17 immunity (Braniste et al., 2010; Malaisé et al., 2018; Menard et al., 2014). However, such effects on the host immune status depend on various parameters,

including the period of exposure (perinatal *versus* adult), sex, animal model as well as the dose and route used for bisphenol treatments. Well-designed *in vitro* studies, representing a complementary tool to human and animal studies, will enable reproducible additional data of the effects of bisphenol exposures. Here, we extended our previous *in vivo* studies to analyze the *in vitro* impacts of BPA and its structural analogues BPS and BPF on Th17 differentiation, frequency and function, using mice immune T cells from systemic and intestinal compartments as well as human isolated T lymphocytes.

We showed that an *in vitro* exposure at an environmentally concentration, *i.e.*, 0.05 nM of BPF and BPA, is able to increase significantly IL-17 production in T cells isolated from mouse spleens. At such a concentration, BPS also increases IL-17 secretion by these T cells, but without reaching a significant difference. Since the primary pathway to bisphenols exposure in the human body is considered to be dietary exposure (Liao et al., 2012), we also examined the regulation of IL-17 secretion by bisphenols in T cells isolated from si*LP*. Interestingly, an *in vitro* exposure at 0.05 nM of BPF, BPA and BPS significantly increased IL-17, as well as IL-22, secretions by these T cells. Even if BPA and BPS have been already shown to activate inflammatory signals in cultured immune cells (Huang et al., 2019), to the best of our knowledge, this is the first experimental evidence for a specific effect on IL-17 and IL-22 secretions by *in vitro* exposure of T lymphocytes to BPA and its substitutes, especially BPF. In link with these *in vitro* data, our recent *in vivo* work revealed the ability of the three bisphenols to provoke an increase of IL-17 levels in splenocytes as well as in intestinal immune cells isolated from female offspring mice exposed at low doses of BPA, BPS and BPF (manuscript in preparation). We also observed an increased IL-17 secretion after BPS or BPF, but not after BPA treatment, at low concentrations in murine naïve T cells under *in vitro* Th17 differentiation. Such differences observed between total splenocytes and Th17-polarized $CD4^+$ T cells in IL-17 production after BPA exposure *in vitro* could be due to

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the presence of antigen-presenting cells (APC), such as dendritic cells or macrophages, in the cell suspensions. Indeed, we and other have already reported that BPA is able to modulate dendritic cells, which can induce IL-23 production and therefore ultimately IL-17 enhancing Th17 cell responses (Camarca et al., 2016; Malaisé et al., 2018). Then, whether the other bisphenols, such as BPF or BPS, can modulate the action of APC to induce an enhanced Th17 response will deserve further studies.

Chen *et al.* (2015) reported that estradiol inhibits Th17 cell differentiation (Chen et al., 2015). So, the increase of IL-17 production that we observed by bisphenols suggests another way of receptor activation than the classical estradiol. AhR is well known to modulate T cell responses and to be largely expressed in Th17 cell subset associated with IL-17 and IL-22 secretions (Baricza et al., 2016; Prigent et al., 2014). In addition, AhR can bind compounds derived from plastics including BPA resulting in its activation (Kruger et al., 2008). In more recent studies, Ziv-Gal *et al.* showed also that BPA may inhibit follicle growth partially *via* the AhR pathway (Ziv-Gal et al., 2015). BPS was not able to activate AhR in reporter gene assay (Zenata et al., 2017). However, more recently, Park *et al.* have shown that only high concentrations of BPA, BPF and BPS had significant AhR-mediated activity (Park et al., 2020). Since *in vitro* models are designed to assess mechanistic data, we were next interested in analysing the involvement of AhR in bisphenol-mediated IL-17 and IL-22 secretions in murine T cells. Expression of AhR was not detectable in non-polarized CD4$^+$ T cells, but high in *in vitro* differentiated Th17 cells (Prigent et al., 2010) and in *ex vivo* isolated Th17 cells (Liu et al., 2017). In the present study, we used T cells isolated from systemic and intestinal compartments expressing a functional AhR. First, we exposed these T cells to AhR potent ligands, such as FICZ and TCDD, leading to strong IL-17 and IL-22 secretions in agreement with previous findings (Schiering et al., 2018). We have then analysed AhR involvement in IL-17 secretion by these T cells isolated from both spleens and si*LP* exposed to bisphenols by

using CH-223191, a potent AhR antagonist inhibiting TCDD-mediated nuclear translocation and DNA binding of AhR (Kim et al., 2006). We observed a decrease of IL-17 and IL-22 productions after treatment by CH-223191 and low concentrations of BPA and BPF, indicating that AhR is partly involved in these IL-17 and IL-22 secretions. Actually, AhR involvement in IL-17 and IL-22 secretions by immune cells is clearly recognized but the exact mechanism remains unelucidated (Baricza et al., 2016). Since forced expression of AhR did not impact the expression of RORγτ transcription factor driving Th17 differentiation (Veldhoen et al., 2009), other mechanisms might be involved in this IL-17 secretion. Analysis of the *Il17* loci revealed a putative binding site for AhR on an E-box element at the *Il17* promoter, suggesting that AhR might directly regulate IL-17 expression (Cui et al., 2011). By contrast, van Voorhis et al. (2013) have suggested that AhR serves as a repressor to IL-17 transcription in a model where "unliganded" AhR is associated with an unknown transcription factor controlling IL-17 expression (Van Voorhis et al., 2013). They propose that after ligand binding, the AhR dissociates from this transcription factor, which is now free to activate the promoter for IL-17 expression. Altogether, these data indicate that the extent and duration of AhR activation by bisphenols, which can lead to increased secretion of IL-17, remain to be determined.

In contrast with BPA exposure, the present study did not find any significant changes in Th17 cell frequencies from mice immune cells isolated from both splenic and intestinal compartments after *in vitro* exposure to BPS and BPF. Since Th17 differentiation does not require IL-17 but cytokines such as IL-6 or IL-23, it would be interesting to determine the effects of BPA on these cytokines promoting differentiation of Th17 cell lineage *via* an autocrine action. Additionally, and in contrary to results obtained in mouse T cells, low concentrations of BPS, BPF, as well as BPA, did not significantly modify neither IL-17 nor IL-22 productions by human T cells isolated from blood donors. However, we found that the

highest concentration of BP, *i.e.*, 50 000 nM, is able to decrease both IL-17 and IL-22 secretions and percentages of IL-17- and IL-22-positive cells in these activated human CD4$^+$ T cells. We also observed such a decrease, at this high concentration, in BPA-exposed activated naïve mouse CD4$^+$ T cells undergoing *in vitro* Th17 differentiation. Importantly, such a concentration was not associated with a decreased viability in both human and mouse CD4$^+$ T cells (data not shown). To the best of our knowledge, the effects of bisphenols on human T lymphocytes exposed *in vitro* to these contaminants have never been described. In literature, BPA was reported to decrease significantly human PBMC proliferation, after cell-activation by anti-CD3 and anti-CD28 antibodies only at low concentration and for a long-term exposure (Herz et al., 2017). In an *in vitro* study, BPA, at concentrations comparable to those found in human serum, might also affect the human immune system homeostasis and reactiveness to external stimuli, by altering both PBMC and monocyte-derived dendritic cells functions (Camarca et al., 2016). Such apparent differences between mouse and human cells linked to Th17 cell phenotype have already been reported (Tuomela et al., 2016). For example, TCDD, a strong AhR ligand, has been reported to have opposite effects on mouse and human immune responses of B cells (Kovalova et al., 2017). In addition, the affinity of AhR for its ligands is generally higher in mice than in humans (Flaveny, 2009). Altogether, these studies indicate that the differences observed could be attributed to discrepancies between the mouse and human AhR pathways and may complicate the interpretation of results as previously reported (Zhou, 2016).

Finally, we observed that the increase in IL-17 production by murine T cells exposed to bisphenols occurs at low doses with a trend to follow non-monotonic dose-response curves (NMDRCs) commonly described for endocrine disrupting chemicals (Lagarde et al., 2015; Menard et al., 2014). As illustrated by Rhomberg and Goodman, 2012, NMDRC issue has been questioned and merits further sufficient evidences. In our study, we observed non-

monotonic effects using 4 doses (Figures 2 and 3) while using 2 doses (Figure 4), we did not find this non-monotonic effect. In addition, our results presented in these figures 2, 3 and 4 illustrate the challenge to work with low doses to see significant biological effects. Altogether, these data demonstrate the importance of the dose choices as well as that of the number of doses to use when working on low doses in order to show non-monotonic effects.

Numerous evidences have shown that bisphenols can have significant metabolic and immune effects at environmental low doses, which may not be apparent at higher doses used in traditional toxicological studies (Vandenberg et al., 2013). For example, micromolar concentrations of BPA, could regulate expression of a distinct set of genes involved in growth and development, inducing direct toxic effects in immune cells such as PBMC at these sublethal concentrations (Gostner et al., 2015). In comparison, previous works showed that, at environmental low doses, such as 1 nM (0.23 ng/ml), in the range of human serum concentrations (Savastano et al., 2015), BPA may modified several cellular responses to increase susceptibility to inflammatory and autoimmune disease, and reproductive and metabolic defects (Alonso-Magdalena et al., 2010; Angle et al., 2013). Then, our results were observed at relevant concentrations for humans (Beausoleil et al., 2018; Calafat et al., 2005) and as recently reported for treatment of cultured pre-adipocyte cell lines (De Filippis et al., 2018).

The present study reported that *in vitro* exposure to bisphenols increases IL-17 secretion in mouse immune T cells and that this effect is mediated in part by AhR. Our results revealed for the first time the strong effect of BPA substitutes, such as BPF and BPS, at low and environmentally relevant concentrations, on Th17 differentiation and function, questioning their safety and the rational of their use to replace BPA. Thus, our *in vitro* findings reinforce the relationship between immune cell alterations by environmental exposure to bisphenols and the potential increased susceptibility to develop inflammatory and autoimmune diseases.

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Declaration of competing interest

The authors declare they have no conflict of interest.

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Authorship contribution statement

Yann Malaisé: Formal analysis, Investigation, Writing - original draft, Writing – review & editing**, Hélène Le Mentec**: Formal analysis, Investigation, Writing – review & editing**, Lydie Sparfel:** Conceptualization, Funding acquisition, Formal analysis, Investigation, Writing - original draft, Writing – review & editing, **Laurence Guzylack-Piriou:** Conceptualization, Funding acquisition, Formal analysis, Investigation, Writing - original draft, Writing – review & editing**.**

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Supplementary data legend

Fig S1 Effects of the AhR antagonist, CH-223191, on FICZ- and TCDD-induced IL-17 and IL-22 secretion in T cells isolated from mouse spleens and from si*LP*

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Total cell suspensions, isolated from mouse spleens (A, B) or from mouse si*LP* (C, D) were stimulated with anti-CD3 and anti-CD28 antibodies, and then pre-treated or not with CH-223191 before treatments with FICZ at 10 nM or TCDD at 10 nM for 4 days. IL-17 (A, C) and IL-22 (B, D) secretions in cell supernatant after 4 days of culture with bisphenol treatment. Results were expressed as means +/- SEM of 4 replicates. *$p < 0.05$: differences between bisphenol treatments *vs* vehicle control; $^{\$}p < 0.05$: differences between bisphenol concentrations.

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Highlights

- *In vitro* exposure to bisphenols A, S, F differentially alter IL-17 secretion by mouse T cells
- Such effect occurs at low and environmentally relevant concentrations
- Such effect is partly mediated by aryl hydrocarbon receptor activation
- Effects of bisphenols A, S, F on IL-17 secretion are different in human and mouse T cells

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