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Abstract

Bisphenol (BP) A, a known food contaminant, is a possible risk factor in the epidemic of non-communicable diseases (NCD) including food intolerance and inflammatory bowel diseases (IBD). Regulatory restrictions regarding BPA usage led to BPA removal and replacement by poorly described substitutes, like BPS or BPF (few data on occurrence in food and human samples and biological effect). Oral tolerance protocol to ovalbumin (OVA) in WT mice and *Il10*$^{-/-}$ mice prone to IBD were used respectively to address immune responses towards food and microbial luminal antigens following BP oral exposure. Both mice models were orally exposed for five weeks to BPA, BPS or BPF at 0.5, 5 and 50 µg/kg of body weight (bw)/day (d). Oral exposure to BPs at low doses (0.5 and 5 µg/kg bw/d) impaired oral tolerance as indicated by higher humoral and pro-inflammatory cellular responses in OVA-tolerized mice. However, only BPF exacerbate colitis in *Il10*$^{-/-}$ prone mice associated with a defect of fecal IgA and increased secretion of TNF-α in colon. These findings provide a unique comparative study on effects of adult oral exposure to BPs on immune responses and its consequences on NCD related to intestinal luminal antigen development.

Keywords: Bisphenols, Immune response, colitis, food intolerance

1. Introduction

Human health in modern societies is being threatened by a plethora of chronic non-communicable diseases (NCDs) that have in common an underlying inflammation. Among NCDs, two pathologies are associated with a rupture of immune tolerance toward intestinal luminal antigens, food in the case of adverse food reactions or microbial antigens in the case of inflammatory bowel diseases (IBD). Adverse food reactions are increasing worldwide with > 20% of the population suffering from food allergy or food intolerance in industrialized countries (Skypala, 2011; Turnbull et al., 2015). The incidence of IBD, a complex collection of gastrointestinal disorders, is not only on the rise but also a concerning trend. Indeed, colitis-associated inflammation is a risk factor for developing colon cancer. Increased prevalence of both diseases has been observed in North American and European nations for decades. A growing body of data suggests that environmental xenobiotic exposures significantly influence IBD development (Legaki and Gazouli, 2016; Molodecky et al., 2012; Sairenji et al., 2017). The endocrine disrupting compounds (EDCs) are of particular interest within this matter. EDCs alter the normal functioning of the endocrine system, and their bioaccumulation in humans may cause adverse health effects (Kabir et al., 2015). Indeed, human exposure to EDCs increases the risk of immune vulnerability, including loss of tolerance to food antigens (Dietert and Zelikoff, 2008) and IBD (de Silva et al., 2017).

Bisphenol A (BPA) is a well-known endocrine disruptor that is industrially produced, and largely used as a component of epoxy resins and polycarbonate plastics (Vandenberg et al., 2007). BPA-based plastics and resins are used for manufacturing food contact materials such as packaging and crockery. Actually, BPA has been found in plastic food containers, epoxy coatings of metal cans, kitchenware toys, medical devices, and dental composites and sealants (Becher et al., 2018). In humans, BPA was proven to have developmental, reproductive, cardiovascular, immune, and metabolic effects (Rochester, 2013). Based on

several studies, the United States Environmental Protection Agency (EPA) set a reference dose of 50 μg/kg bw/d for BPA. As a response to a refined risk assessment of BPA and its unwanted health effects, the European Food Safety Authority (EFSA) decreased the tolerable daily intake (TDI) from 50 μg/kg bw/d to 4 μg/kg bw/d in 2015 (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), 2015). However, the EFSA also claimed that the average levels of BPA exposure are far below the TDI and BPA will not increase the health risks for any population including unborn children, infants and youngsters. In 2017, BPA was listed as a substance of very high concern by the European Chemical Agency (ECHA). In view of recent regulations that further restrict the use of BPA in food contact materials (Andújar et al., 2019), food packaging companies are exploring substitutes to gradually eliminate BPA from their products (García-Córcoles et al., 2018; Liao and Kannan, 2013). Then, commercialization of BPA-free labeled products is expanded while BPA analogues are being increasingly used in the manufacturing of consumer products. However, some BPA analogues, such as bisphenol S (BPS) and bisphenol F (BPF), share the basic bisphenol structure of two benzene rings separated by a short carbon or other chemical chain, which question their safety as BPA substitutes (García-Córcoles et al., 2018).

BPS, which is more heat- and photo-resistant than BPA, was chosen by the industry as a replacement for BPA in the production of polycarbonates and epoxy resins (Kitamura et al., 2005), for the manufacturing of industrial and consumer products (Chen et al., 2012). BPF, other BPA analogue, is used to make epoxy resins and coatings, due to its increased thickness and durability (i.e., high-solid/ high-build systems) (Fiege et al., 2000).

The Human exposure to bisphenols occurs mainly through diet (food and food contact materials) (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), 2015). However, there is little information regarding the occurrence of bisphenol A analogues in foodstuffs. Liao and Kannan (2013) analyzed the presence of BPA, BPF, and

BPS in nine categories of foodstuffs in the U.S., and showed that 75% of the samples contained BPs. In the same study, the presence of eight BPs (n = 289) was detected in 13 categories of foodstuffs in China, BPA and BPF being the most frequently BPs found.

Data on BPA analogues occurrence in human samples are scarce. Liao et al. (2012) determined the total concentration of BPS in 315 urine samples collected from the U.S. and different Asian countries, and detected BPS in 81% of the samples analyzed. Measurement of BPs in urine samples collected at eight time points between 2000 and 2014 (n=616) in U.S. adult volunteers showed that detection frequency of BPS increased in time whereas BPA one decreased (Ye et al., 2015). This study reflects the reality of substituting BPA with BPS. Free and conjugated BPF were detected in 55% of tested urine samples of anonymous adults in the U.S. (n=100) (Zhou et al., 2017) investigating the association between the presence of BPA, BPF, and BPS in urine samples from adults (n = 1808) and children (n = 868). In this study, BPA, BPS, and BPF levels were measured in 95.7%, 89.4%, and 66.5% of total population, respectively.

There is a limited number of studies on the hormonal effects of BPA analogues (Liao et al., 2012; Pelch et al., 2019). *In vitro* studies demonstrated that BPS has a lower affinity to human estrogen receptor (ER)α and ERβ (Molina-Molina et al. 2013 ; Kojima et al. 2019) but can bind to membrane ERs (Viñas and Watson, 2013). BPF showed comparable estrogen and anti-androgen activities to those of BPA (Fic et al., 2013).

Exposure to EDCs has been associated with altered immune functions, typically depending on the context by either suppressing immunity, thereby increasing susceptibility to infections, or by enhancing the immune response and participating to the growing incidence of NCD like inflammation, allergies, or autoimmune diseases (Bansal et al., 2017). In animals, previous studies from our laboratory highlighted a deleterious effect of BPA oral exposure on intestinal physiology. Indeed, we previously showed that daily gavage with BPA

of rodent mothers from gestation day 15 to pups weaning, increased the risk of food intolerance (Menard et al., 2014b) and colitis (Braniste et al., 2010) at adulthood. Similarly, BPA treatment impaired systemic immune response in young rats, hence predisposed them to a parasitic infection in the gut (Menard et al., 2014a). More recently, we reported that perinatal exposure to BPA (50 µg/kg bw/d) induced intestinal and systemic immune imbalances in male offspring mice at adulthood, through a decrease of Th1/Th17 cell frequencies in the *lamina propria*, concomitant to an increase of splenic Th1/Th17 immune responses (Malaisé et al., 2017). In comparison, the same BPA perinatal exposure led to a defect in dendritic cell maturation in the *lamina propria* and spleen associated with activated- and regulatory- T cells decrease in the *lamina propria* of female offspring mice (Malaisé et al., 2018). Moreover, in this study, a sharp increase in interferon-γ and interleukin-17 production by intestinal immune cells and a T helper 17 profile in the spleen were observed. However, there is limited information on BPS or BPF impacts on the development of food intolerance and IBD symptoms.

In the present study, we analysed the consequences of direct oral exposure of adult mice to BPA, BPS and BPF administered at 0.5, 5 and 50 µg/kg bw/d on the development of food intolerance and IBD symptoms. We hypothesized that BPs exposure might be associated with a loss of immune tolerance toward luminal contents (food and/or bacteria) and contribute to the increasing of prevalence of NCDs.

2. Methods

2.1. Animal models and BP treatment

We used C3H/HeN (Janvier, Roubaix, France) female mice or C57BL/6 *Il10*$^{-/-}$ male and female mice (kindly provided by Dr Manuela Büettner). In all experiments, mice 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). Food (Harlan, Gannat, France) and water were available *ad libitum*. At 8 weeks of age, mice were daily treated orally with 0.5, 5 or 50 µg/kg bw/d of BPA, BPS or BPF or vehicle alone (0.1 % ethanol in corn oil) as control group for six weeks (as described in table 1). The groups were named as follows: BP0.5, 5 or 50 µg/kg bw/d.

2.2. Ethics approval

All experiments were approved by the Institutional Animal Care and Use Committee (TOXCOM 0035/EH-2013), in compliance with the European directive 2010/63/UE.

2.3. Oral tolerance to OVA

At 9 weeks of age, C3H/HeN females exposed to BPA, BPS, BPF, or vehicle (0.1% ethanol in corn oil, controls), were tested for oral tolerance induction (OVA-tolerized) and immunization (OVA-immunized) as previously described (Menard et al., 2014b) (Table 1). To address oral tolerance to luminal food antigen *vs* immunization, we chose to work with the OVA model (for reference: Pabst and Mowat, 2012). Briefly, OVA-tolerized mice were orally exposed to 20 mg of OVA (in 200 µL of 0.2 M bicarbonate buffer) (pH 8.0) for oral tolerance protocol, while OVA-immunized mice were orally given bicarbonate buffer alone. After 1 week, all mice were primed subcutaneously with 100 µg OVA in complete Freund adjuvant (CFA) and boosted 2 weeks later by subcutaneous injection of 100 µg OVA. After 1 more week, mice were euthanized by cervical dislocation, blood was collected by intra-cardiac puncture and spleens were sampled.

2.3.1. Measurement of OVA-specific IgG (humoral response)

Plasma anti-OVA IgG titers were determined using an ELISA as follows: 96-well flat-bottomed plates (Nunc, Roskilde, Denmark) were coated overnight with OVA (Sigma-

Aldrich, St.Louis, MO, USA) in phosphate-buffered saline (PBS). After washes with PBS-0.05% Tween 20 (PBS-Tween), non-specific binding sites were blocked with PBS-5% fetal calf serum (FCS). Plates were then incubated with serial dilution of plasma. Horseradish peroxidase-conjugated polyclonal goat anti-mouse IgG (Southernbiotech) was added. Finally, TMB substrate (ThermoScientific, Suwanee, GA, USA) was added to each well. The reaction was stopped with 2N H_2SO_4, and plates were read at 450 nm using an automatic Infinite M200microplate reader (Tecan, Maennedorf, Switzerland). Titers were expressed as the highest plasma dilution, giving an optical density at least twice the blank value.

2.3.2. In vitro cell restimulation

After washing, splenic cells were seeded in 24-well plates at 2×10^6 cells/well for cytokine assays in Cerottini culture medium (Dulbecco's modified Eagle medium without red phenol, supplemented with 8% heat inactivated fetal calf serum (FCS), 36 µg/mL asparagine, 116 µg/mL arginine 10 µg/mL folic acid, 1 mg/mL 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid, 0.05 mM-mercaptoethanol, 100 U/mL penicillin, 100 kg/mL streptomycin, and 1 µg/mL fungizone) in the presence or absence of : (a) the specific antigen OVA (2 mg/mL), (b) 5 µg/mL hamster anti-mouse CD3 and hamster anti-mouse CD28 (BD) coated wells. Supernatants were collected after 72h.

2.3.3. Cytokine measurement

Cytokine secretion was measured in supernatants of primary cell cultures of spleen. IFN-γ and IL-17 in primary cell culture supernatants were assayed using commercial ELISA kits (Duoset R&D Systems, Lille, France) according to the manufacturer's instructions. Data are expressed as picograms per milliliter of supernatant of cell culture medium.

2.4. *Il10*$^{-/-}$ mouse model of colitis

At 9 weeks of age, C57BL/6 *Il10*$^{-/-}$ female and male mice were exposed to BPA, BPS, BPF, or vehicle (0.1% ethanol in corn oil, controls) for 6 weeks. Data from male and female were compiled because previous studies have shown no incidence of sex in the susceptibility to develop colitis in this mice model (Riba et al., 2017). Previous studies in C57BL/6 wild-type mice showed no sign of spontaneous colitis after BPA direct exposure (DeLuca et al., 2018). Mice were exposed to bisphenols for 6 weeks to reproduce comparable exposure BPs treatment as oral tolerance. Mice were euthanized by cervical dislocation; blood was collected by intra-cardiac puncture and spleens and colons were sampled. Colon length was measured.

2.4.1. Histology

Colon samples were collected, fixed with 10% formalin for 24 h, dehydrated and embedded in paraffin according to standard histological procedures. Sections (5 µm) were mounted on SuperFrost® Plus Slides then dewaxed in a xylene bath for 10 min and rehydrated in graded alcohol baths. Slides were stained with hematoxylin and eosin (H&E) and analyzed for intestinal morphometry. Erben et al. histomorphological scoring was used (Erben et al., 2014).

2.4.2. Commensal E. coli lysate preparations

Pellets from an overnight culture of one of the *E. coli* isolates previously characterized (Riba et al., 2017) were washed in 0.9% NaCl, suspended in 1 mL of distilled water, sonicated for 1 h, frozen in liquid nitrogen, melt and centrifuged. Supernatants were filtered in 0.22 µm and conserved at -20 °C as the *E. coli* lysate used for cellular response. Lysate protein concentration was measured using BCA protein Assay kit, Uptima (Interchim, Montluçon, France) according to the manufacturer's instructions.

2.4.3. In vitro cell restimulation

After washing, splenic cells were seeded in 24-well plates at 2×10^6 cells/well for cytokine assays in Cerottini culture medium in the presence or absence of : (a) 5 µg/mL hamster anti-mouse CD3 and hamster anti-mouse CD28 (BD) coated wells, (b) 1µg/mL of protein from commensal *E. coli* lysate or (c) 100 ng/mL Lipopolysaccharide (LPS; Sigma). Supernatants were collected after (a/b) 72h or (c) 24h.

2.4.4. Cytokine measurements

Cytokines were measured in supernatants of primary cell cultures of spleen, in feces and in colonic tissue segments. Frozen colonic fragments or feces were suspended in RIPA buffer (0.5% deoxycholate, 0.1% SDS, and 1% Igepal in TBS) containing complete anti-protease cocktail (Roche, Indianapolis, IN, USA), and protein concentrations were determined using BCA protein Assay kit, Uptima (Interchim, Montluçon, France). IFN-γ, IL-17 and TGF-β in primary cell culture supernatants, and lipocalin and TNF-α in fecal and colonic tissues, were assayed using commercial ELISA kits (Duoset R&D Systems, Lille, France) according to the manufacturer's instructions. Data are expressed as picograms of cytokine per milligram of protein in colons or feces, and in picograms per milliliter of supernatant of cell cultures.

2.4.5. Immunoglobulin specificity against commensal E. coli lysate

Maxisorp 96-wells plates were coated with 5µg/mL of protein from mouse commensal *E. coli* lysate, incubated with plasma (10 µg/mL IgG), and revealed as above-mentioned. Results were expressed as arbitrary units (AU) per 10 µg/mL of IgG, in comparison with a standardized immune serum.

2.4.6. Quantification of fecal IgA concentrations

Fecal IgA concentrations were measured by ELISA in MaxiSorp 96-wells flat-bottomed plates (NUNC, Thermo Scientific, Dutcher, Brumath, France). Plates were coated with 50 µL of 5 µg/mL sheep anti-mouse IgA (Sigma) in PBS and kept overnight at +4 °C. Plates were blocked with PBS-5% FCS (GIBCO, Invitrogen, Life Technology, St Aubin, France) before incubation with diluted samples. Horseradish-peroxidase (HRP)-conjugated goat anti-mouse IgA (Sigma) were added, HRP was revealed using TMB (Becton Dickinson; BD, Le Pont de Claix, France) and the reaction was stopped with 25 µL of 2N H_2SO_4 before reading at 450 nm using an automatic Infinite M200 microplate reader (Tecan). Results were expressed as µg/mg of fecal proteins for IgA.

2.5. 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 groups were compared to control group by using two-way ANOVA. Differences were considered significant for $p<0.05$.

3. Results

3.1. Consequences of oral BPs exposure on oral tolerance

3.1.1. Oral exposure to BPs exacerbates humoral response

Oral tolerance is manifested by the absence of humoral response (anti-OVA IgG) to the orally administered antigen (OVA) corresponding to the OVA-tolerized mice. In control mice (i.e., not exposed to BP, Vehicle (Veh) group, OVA tolerization (i.e., OVA oral ingestion prior to

OVA systemic immunization) significantly decreased serum anti-OVA IgG titers in comparison to immunized mice, validating our protocol for induction of oral tolerance to OVA (Figure 1 A, B and C). Anti-OVA IgG titers in OVA-tolerized mice remained significantly higher in BPA5 (Figure 1A), BPS0.5 and BPS5 (Figure 1B), and BPF0.5 and BPF5 (Figure 1C) compared to OVA-tolerized veh group, demonstrating a non-monotonic dose-response relationship.

The loss of the significant difference between OVA-tolerized group and corresponding OVA-immunized group suggested a defect of oral tolerance to food antigens.

3.1.2. Oral exposure to BPs impairs cellular tolerance to OVA

Oral tolerance is manifested not only by the absence of humoral response (anti-OVA IgG) but also by absence of cellular response to the orally administered antigen (OVA) in OVA-tolerized group compared to OVA-immunized. Cellular response to OVA stimulation was here assessed by analyzing cytokine production (Figure 2) in splenocytes isolated from OVA-immunized and OVA-tolerized mice, with and without exposure to bisphenols. IFN-γ and IL-17 were studied for their respective role in induction (Kweon et al., 1998; Lee et al., 2000) or repression of oral tolerance (Kawakami et al., 2012).

Firstly, low to not detectable levels of IFN-γ or IL-17 were observed without OVA *in vitro* restimulation (Figure 2, medium *in vitro* restimulation group). As expected, in vehicle (Veh) mice after OVA-immunization, *in vitro* OVA restimulation induced an increase of IFN-γ concentration in spleen cell culture supernatants compared to OVA-tolerized group (Figure 2A). In BPA-treated mice at low doses of 0.5 in tolerized group and 5 µg/kg bw/d in tolerized and immunized group, *in vitro* OVA restimulation induced a sharp increase of IFN-γ release by splenocytes compared to the vehicle group (Figure 2A). In a same way, after BPS and BPF exposure to low dose (0.5 µg/kg bw/d), an increase of IFN-γ level in splenocyte supernatant

from tolerized mice was measured (Figure 2B and C). Furthermore, in OVA-immunized mice, splenocytes isolated from BPA5, BPS50 and BPF0.5-exposed animals displayed higher IFN-γ production following *in vitro* OVA restimulation compared to non-exposed controls (Figure 2A, B and C).

Investigating IL-17 secretion in our model, we noticed that immunization was not associated with increased IL-17 secretion after OVA *in vitro* restimulation, suggesting that immune response toward antigen in immunized mice is not dependent on IL-17. Interestingly, whereas BPA induced no difference in the production of IL-17 in tolerized or immunized animals, low doses of BPS and BPF (0.5 µg/kg bw/d) led to a significant increase of IL-17 production after *in vitro* OVA restimulation in OVA-tolerized mice (Figure 2E and Figure 2F).

In addition, the functionality of T lymphocytes was investigated by *in vitro* anti-CD3/CD28 restimulation. Because there was no consequence of tolerization and immunization protocol on the TcR-dependent cytokine response (supplementary data figure 1), the two groups of tolerized and immunized mice were pooled. Without stimulation, BPs did not modify either IFN-γ or IL-17 secretion by splenocytes (data not shown). After anti-CD3/CD28 antibody *in vitro* restimulation, higher level of IFN-γ was induced in BPA0.5 and BPS0.5 groups in comparison with vehicle (Figure 3A and B). In BPF-exposed mice, a significant increase of IFN-γ level was also observed, regardless of the dose of bisphenol (Figure 3C). Concerning IL-17 production, the level of this cytokine was also higher in splenocyte supernatants from BPA0.5, BPA5, BPF0.5 and BPF5 groups compared to vehicle group (Figure 3 D and F). In summary, bisphenols (A, F and S) not only altered splenic T cell specific immune response but also non-specific response at low dose.

3.2. Consequences of oral BPs exposure on colitis in *Il10*$^{-/-}$ mouse model

Oral exposure to BPA, BPS and BPF were done at 5 µg/kg bw/d.

3.2.1. *Oral exposure to BPF exacerbates colitis*

Direct oral treatment with bisphenols did not affect the body weight gain of *Il10*$^{-/-}$ mice (data not shown).

Then, we wondered if oral exposure to bisphenols could worsen colitis in genetically predisposed mice, housed on Specific and Opportunistic Pathogen Free (SOPF) sanitary status. First, we did not observe any differences between males and females in colonic TNF-α level from *Il10*$^{-/-}$ mice after BPs treatment (Supplementary data Figure 2). Since the effect of BPs on colitis in *Il10*$^{-/-}$ mice was not sex-dependent, we decided to present results as pooled data from males and females. We observed an increase of histology damage score only in BPF-treated mice compared to vehicle mice (Figure 4A and B) without any change in colon length (Figure 4C). Histologic scores indicating colitis in BPF group were associated with a decrease of IgA fecal levels (Figure 4F) and a significant increase of inflammatory cytokine TNF-α in colonic sample (Figure 4D) without modification of IFN-γ (Figure 4E). In addition, we observed a significant rise of lipocalin level in the feces (Fig 4G) of BPA-treated mice compared to vehicle mice associated with an increase of anti-*E. coli* IgG in plasma (Figure 4H).

3.2.2. *Oral exposure to bisphenols modulates systemic immune response*

We investigated innate response by measuring TNF-α production by splenocytes from *Il10*$^{-/-}$ mice treated or not with bisphenols, in response to lipopolysaccharide (LPS) stimulation. We noticed a significant increase of TNF-α level in splenocyte supernatants of BPF-treated mice after 24h *in vitro* restimulation with LPS (Fig 5A).

Then, we analyzed functionality of T lymphocytes by anti-CD3/CD28 *in vitro* restimulation of splenocytes. Without stimulation, BPs did not modify either IFN-γ or IL-17 secretion by

splenocytes (data not shown). None of the BP tested modified IFN-γ secretion in response to anti-CD3/CD28 (Figure 5B). Anti-CD3/CD28 stimulation induced a significant decrease of TGF-β after BPS treatment of *Il10*$^{-/-}$ mice in comparison with vehicle group (Fig 5F), and also a higher IL-17 production in BPF (Fig 5D). Moreover, *in vitro* restimulation by *E. coli* lysate induced also a decrease of TGF-β in splenocytes of BPS-treated mice (Fig 5G) and higher IL-17 production by splenocytes of BPA-treated mice (Fig 5E) but none of the BPs tested altered IFN-γ secretion in response to *E. coli* lysate stimulation (Figure 5C).

4. Discussion

~~As a consequence of regulatory restrictions on the use of BPA in some countries, selected commercial applications now employ various BPA structural analogues such as BPS and BPF. The effects of BP on the host immune status depend on various parameters, including the period of exposure (adult vs perinatal), sex and animal model as well as the dose and route used for BP treatments.~~

In this present study, we investigated the impacts of BPA and its structural analogues BPS and BPF, administered orally, on the development of NCDs such as food intolerance and IBD in different mouse models. The chronic exposure to endocrine disruptors contributes to the increasing incidence of adverse food reactions, such as food allergy and food intolerance, in industrialized countries (Dunbar et al., 2012). A defect in dietary antigen recognition in the digestive tract may cause hypersensitivity reactions, both at local (i.e., gut-associated lymphoid tissue, GALT) and systemic levels, known as food intolerance. We analyzed the consequences of oral BPA, BPS or BPF exposure on the immune system, by performing either immunization or oral tolerance protocols to OVA (used as a food antigen model). In our study, oral tolerance and immunization protocols were performed in parallel in untreated wild-type mice.

Our work demonstrated that direct oral exposure in adult mice to low doses of BPA, BPS and BPF (at the same level or below the human safety limit defined by U.S. and E.U. food safety authorities) induced the impairment of oral tolerance to dietary antigens, inducing food intolerance. Indeed, we reported that direct oral BPs exposure at low doses increased humoral response in OVA-tolerized mice. Higher anti-OVA IgG titers were observed in OVA tolerized mice exposed to 0.5 or 5 µg/kg bw/d of BPS or BPF and for BPA5 compared to control mice, demonstrating a non-monotonic dose-response relationship that is characteristic of EDC response (Lagarde et al., 2015; Menard et al., 2014b). Similarly, in transgenic mice carrying a specific OVA TcR (*i.e.*, favoring OVA recognition), direct exposure to BPA induced an increase of plasmatic anti-OVA IgG titers (Ohshima et al., 2007) and an enhanced production of anti-OVA IgG by splenocytes restimulated *in vitro* with OVA (Goto et al., 2007), respectively. Yoshino et al. (2004) reported an increase of plasmatic specific IgG titers in adult mice treated for 20 days with BPA, immediately after immunization (Yoshino et al., 2003). Moreover, in a previous study, we also showed that perinatal exposure to BPA resulted in an increase of anti-OVA IgG titers at all BPA dosages (0.5, 5 or 50 µg/kg bw/d) in OVA-tolerized rats and at 5 µg/kg bw/d in OVA immunized rats compared to control rats treated with vehicle (Menard et al., 2014b), supporting present data. In the current study, we found that direct BPA, BPS or BPF oral exposure at low dose (0.5 µg/kg of bw/d) induced a marked increase of OVA-induced IFN-γ secretion in splenocytes subjected to oral tolerance protocol. This finding is in line with our previous data obtained in OVA-tolerized wild type rats treated with BPA (Menard et al., 2014b). The increase of humoral and cellular response in BPA-exposed mice following an oral tolerance protocol was expected, as we demonstrated an overactivation of splenic T cells. Indeed, the different BPs induced a Th1 immune response characterized by increased production of IFN-γ in response to OVA stimulation and anti-OVA IgG. These results are consistent with the role of IFN-γ in triggering Th1 humoral Ig response

in vivo (Finkelman et al., 1988). Other studies revealed an imbalance in immune responses after exposure of pregnant female rodents to varying relevant human-exposure levels of BPA (Menard et al., 2014b; O'Brien et al., 2014; Yoshino et al., 2004). Interestingly, these four studies reported an increase in a pro-inflammatory Th1 response in the offspring.

A previous study showed that administration of IL-17 as well as the transfer of Th17 cells have the potential to abolish the therapeutic effects of oral tolerance to antigen, thereby suppressing the expansion of Treg cells (Kawakami et al., 2012). Our study reveals a significant increase of IL-17 production OVA-induced in OVA-tolerized mice after direct exposure to low dose of BPS and BPF but not BPA, suggesting a break of oral tolerance in these animals, and a specific mechanism of oral tolerance breakdown for BPA. Moreover, at systemic level, we also report an increase of IFN-γ and IL-17 cytokines production after anti-CD3/CD28 restimulation of splenocytes from BPA and BPS at low dose (0.5 µg/kg bw/d) and BPF (all dose tested)-exposed mice. This effect was associated to an increase of IL-17 with bisphenol dose of 0.5 and 5 µg/kg bw/d. Luo et al. (2016) recently reported similar observation after gestational and lactational exposure to BPA. Our study reveals an inflammatory Th1/Th17 cytokine profile in adult mice after only 5 weeks of oral exposure to a low dose of bisphenols.

We showed that BP oral exposure led to intolerance to luminal food antigens, and then wondered if BP exposure would exacerbate colitis, a pathological condition associated with a rupture of tolerance toward luminal microbiota antigens. Here, we used *Il10*$^{-/-}$ mice, a well-described model of IBD, which develop spontaneous Crohn's disease-like intestinal inflammation in the presence of conventional intestinal microbiota (Schultz et al., 2002; Sellon et al., 1998), or a mild patchy colitis with incomplete disease penetrance under SOPF conditions (Berg et al., 1996). Therefore, *Il10*$^{-/-}$ mice in SOPF condition represent an ideal model to study the role of oral bisphenol exposure on the initiation and/or exacerbation of

colitis. Here, BPF oral exposure at low dose (5 µg/kg bw/d) during only 5 weeks aggravated colitis as observed by the increasing histology damage score. It has been shown that intestinal IgA are involved in the development and maintenance of the homeostasis between microbiota and the host immune system (Reikvam et al., 2012). In this model, only direct oral exposure to BPF at 5 µg/kg bw/d induced a fall of fecal IgA in adult mice, but no effect was observed after BPA or BPS exposure. This observation is in accordance with the increase of pro-inflammatory cytokine TNF-α in colons of these animals. Furthermore, related to this data, a significant increase of TNF-α level in splenocytes after LPS *in vitro* restimulation was observed in BPF-exposed mice, revealing an inflammatory innate systemic response against microbial compounds. In addition, systemic exposure to LPS with inflammatory cytokine production has been linked also to clinical disease of IBD in adults (Hollander et al., 1986; Pasternak et al., 2010; Pastor Rojo et al., 2007).

In mice, we previously showed that BPA exposure decreased the frequency of IgA^+ plasma cells in small intestine *lamina propria* and colon but we did not investigate the class switch to IgA (Malaisé et al., 2018). We also demonstrated that BPA exposure impaired dendritic cells frequency in small intestine *lamina propria* and spleen and decreased markers involved in antigens presentation (MHCII and CD80) (Malaisé et al., 2018). The same mechanism may apply in this study, but this needs to be further investigated. Indeed, in this study, depending on the BP, we observed different responses with OVA and TcR stimulations suggesting that antigen presenting cells (APC) are affected by BPs exposure and this effect is dependent on the type of BP used.

A significant increase of lipocalin level, another inflammatory marker (Abella et al., 2015) in feces and colon was detected after BPA direct exposure at the low dose of 5 µg/kg bw/d; it was associated with an increase of anti-*E. coli* IgG in these animals, suggesting a BPA specific side-effect on gut barrier, involving a disruption of immune response toward

commensal antigens, even though no aggravation of colitis was observed. Moreover, previous studies linked changes in fecal microbiota with colonic inflammation and IBD development (Le Gall et al., 2011). In connection with these data, dietary exposure to BPA resulted in a decrease of gut microbial diversity, which is associated with intestinal inflammation (DeLuca et al., 2018; Lai et al., 2016). These results suggest that BPs depending on their chemical nature induced different immunological signature and gut barrier functions.

Among the mechanisms involved in colitis pathophysiology, the production of IL-17 by immune cells was initially described in animal models of intestinal inflammation and later on in patients suffering from IBD (Fujino et al., 2003; Rovedatti et al., 2009). These increased bacterial responses were identified within the peripheral $CD4^+$ T cell compartment of Crohn disease (CD) patients compared to controls, and were characterized by the production of larger amounts of IL-17A and overexpression of a number of Th17 signature transcripts while showing similar expression of classical Th1 genes. Evidence of Th17 axis involvement is also strongly supported by the fact that IL-23 (a cytokine involved in stabilization and further maturation of the IL-17 response) has also been critically involved in experimental models of intestinal inflammation (Ahern et al., 2010; Yen et al., 2006). Thus, at the systemic level, we reported an increase of IL-17 cytokines production after anti-CD3/CD28 restimulation of splenocytes from BPA and BPF-exposed mice, and after *in vitro E. coli* lysate restimulation only for BPA exposure. No effect was observed on Th1 immune response after exposure to bisphenols in this mouse model. Then, a decrease of TGF-β cytokine involved in tolerance homeostasis was observed specifically in BPS-exposed mice after anti-CD3/CD28 and *E. coli* lysate *in vitro* restimulation without consequence on inflammatory cytokine production in these mice, suggesting that BPS uses a specific pathway. Mechanistic studies must be performed to explain the specific effects of each bisphenol on the development of oral tolerance and the consequence on colitis exacerbation. Finally, we compared exposure to

three different BPs at three different doses on immune response toward food and microbial luminal intestinal antigens, and our results revealed for the first time the strong effect of BPA's analogues at low doses and after a short exposure time on food intolerance and IBD, demonstrating a potential role of BPs exposure on NCDs epidemic.

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, Corinne Lencina: Formal analysis, Investigation, Fanny Placide: Formal analysis, Investigation, Valérie Bacquié: Formal analysis, Investigation, Maïwenn Olier: Formal analysis, Investigation, Manuela Buettner: Resources, Markus Wallbrecht: Resources, Sandrine Ménard: Conceptualization, 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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Legends

Figure 1. OVA-specific IgG titers in OVA-tolerized or OVA-immunized mice exposed to increasing doses of bisphenols.

Tolerization was achieved in vehicle (Veh) group, as attested by a significant decrease of anti-OVA IgG titers in OVA-tolerized mice compared to OVA-immunized mice. Daily oral exposure to BPA (A), BPS (B) or BPF (C) for five weeks increased anti-OVA IgG titers through a nonlinear dose-response relationship. N= 10 (two batches of separate experiments). *P < 0.05, *** P<0.005 vs vehicle OVA-tolerized groups. $p<0.05 vs vehicle OVA-immunized groups. # P<0.05 vs corresponding OVA-tolerized group. BP0.5: BP 0.5 µg/kg bw/d; BP5: 5 µg/kg bw/d; BP50: 50 µg/kg bw/d.

Figure 2. OVA-specific cellular immune response in spleen of mice exposed to increasing doses of BPs.

Cellular tolerization was achieved in vehicle (Veh) group, as attested by an absence of IFN-γ secretion in response to OVA stimulation in OVA-tolerized mice compared to OVA-immunized mice. Daily oral exposure to BPs for five weeks increased IFN-γ secretion through a nonlinear dose-response relationship.

Adult mice were orally exposed to various doses of BPA (A, D), BPS (B, E) or BPF (C, F). Concentrations of IFN-γ (A, B and C) and IL-17 (D, E, F) in cell cultures of spleens isolated from OVA-tolerized or -immunized conditions and stimulated or not with OVA. N= 10 (two batches of separate experiments). *P < 0.05, ** P<0.01 vs vehicle OVA-tolerized groups. $^{\$}$p<0.05 vs vehicle OVA-immunized groups. BP0.5: BP 0.5 µg/kg bw/d; BP5: 5 µg/kg bw/d; BP50: 50µg/kg bw/d.

Figure 3. Cytokine profile in splenocytes of mice exposed to increasing doses of BPs.

Adult mice were orally exposed daily for 5 weeks to various doses of BPA (A, D), BPS (B, E) or BPF (C, F). Concentrations of IFN-γ (A, B and C) and IL-17 (D, E, F) in cell cultures of spleen after anti-CD3/CD28 *in vitro* restimulation. Spleens from OVA-tolerized and OVA-immunized mice were pooled. Daily oral exposure to BPs increased IFN-γ and IL-17 secretion through a nonlinear dose-response relationship.

N= 10 (two batches of separate experiments). *P < 0.05, ** P<0.01 *** P<0.005 vs vehicle (Veh). BP0.5: BP 0.5 µg/kg bw/d; BP5: 5 µg/kg bw/d; BP50: 50 µg/kg bw/d.

Figure 4. BPF aggravates susceptibility to colitis in adult *Il10*$^{-/-}$ C57BL/6 genetically predisposed mice.

Adult *Il10* $^{-/-}$ mice were orally exposed daily during 5 weeks with BPA, BPS or BPF at 5 µg/kg bw/d.

(A) Histological scores in colons, (B) Representative macroscopic images of colon in Vehicle (Veh), BPA, BPS or BPF-treated *Il10*$^{-/-}$ mice colon, (C) Ratio of colon length (cm)/total mouse length (cm), (D) TNF-α levels in colons of *Il10*$^{-/-}$ mice, (E) IFN-γ levels in colon of IL-10$^{-/-}$ mice, (F) Total IgA concentration measured by ELISA in fecal samples (G) Lipocalin level determined in fecal supernatants and (H) Anti- *E. coli* IgG in plasma measured in arbitrary units (AU)/10 µg/mL in comparison with a standard immune serum. N= 8-9/group (mixed female and male mice). *$P < 0.05$ *vs* vehicle (Veh).

Figure 5. Oral exposure to bisphenols modulates systemic immune response in adult *Il10*$^{-/-}$ C57BL/6 genetically predisposed mice.

Adult *Il10* $^{-/-}$ mice were orally exposed daily during 5 weeks to various bisphenol at 5 µg/kg bw/d. Immune response was assessed in spleen.

(A) TNF-α level in splenocytes supernatants after 24h of culture with LPS. IFN-γ (B, C), IL-17 (D, E) or TGF-β (F, G) secretion after 72H of anti-CD3/CD28 *in vitro* restimulation (B, D, F) or *E. coli* lysate *in vitro* restimulation (C, E, G) of isolated lymphocytes from splenic IL-10$^{-/-}$ mice. N= 8-9/group (mixed female and male mice). *$P < 0.05$ *vs* vehicle (Veh).

Supplementary data Figure 1. Cytokine profile in splenocytes of mixed OVA-tolerized and OVA-immunized mice stimulated for 72H with anti CD3/28.

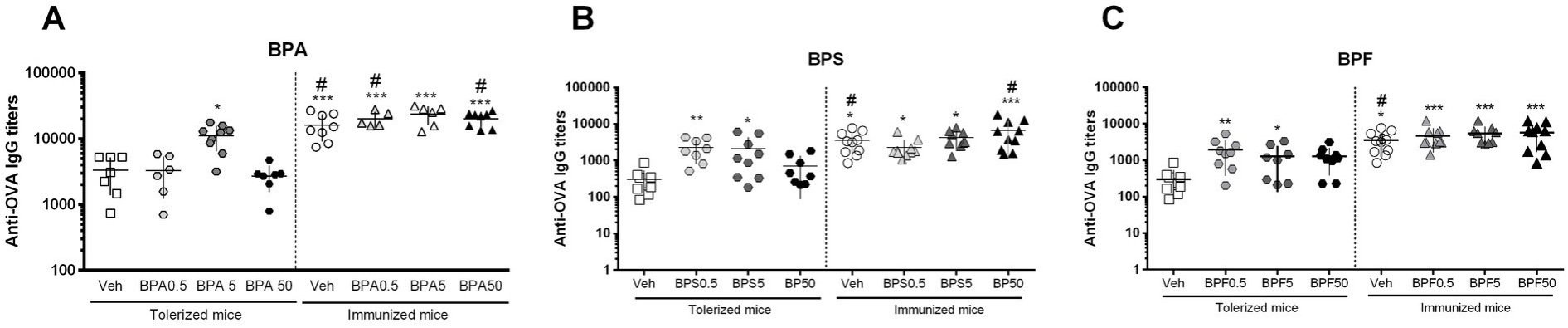
Adult mice were orally exposed to various doses of BPA. Concentration of IFN-γ in cell cultures of spleen after anti-CD3/CD28 *in vitro* restimulation from tolerized (triangle) and

immunized (round) mice. N= 10 (two batches of separate experiments). *P < 0.05 vs vehicle (Veh).

Supplementary data Figure 2. Sex-independent response of TNF-α production in colons of adult *Il10*$^{-/-}$ mice after oral exposure to bisphenols.

Adult mice were orally exposed to 5 µg/kg bw/d of BPA, BPS or BPF. Concentration of TNF-α in colons of male (black circle) or female (white circle) mice. N= 8-9/group. *$P < 0.05$ *vs* vehicle (Veh).

Figure 1


A
BPA
Anti-OVA IgG titers
100000
10000
1000
100
Veh
BPA0.5
BPA 5
BPA 50
Veh
BPA0.5
BPA5
BPA50
Tolerized mice
Immunized mice
B
BPS
Anti-OVA IgG titers
100000
10000
1000
100
10
1
Veh
BPS0.5
BPS5
BP50
Veh
BPS0.5
BPS5
BP50
Tolerized mice
Immunized mice
C
BPF
Anti-OVA IgG titers
100000
10000
1000
100
10
1
Veh
BPF0.5
BPF5
BPF50
Veh
BPF0.5
BPF5
BPF50
Tolerized mice
Immunized mice

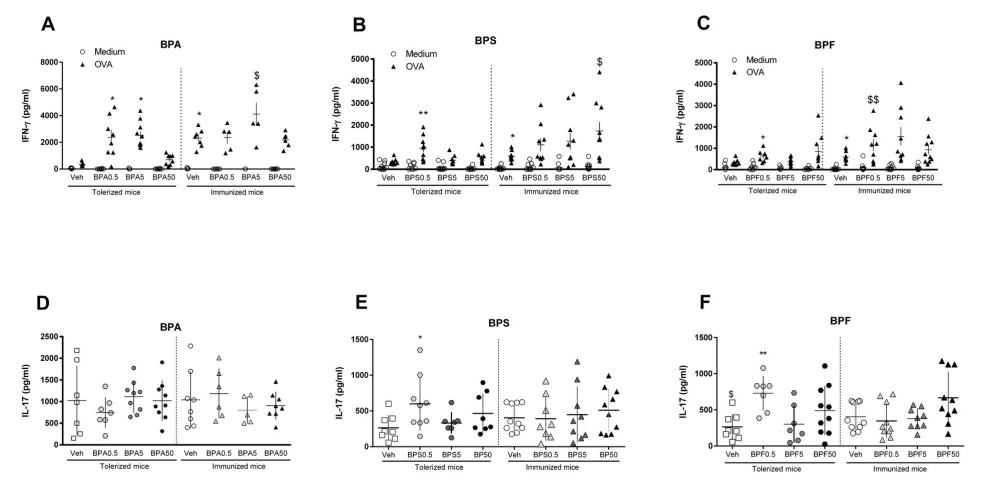
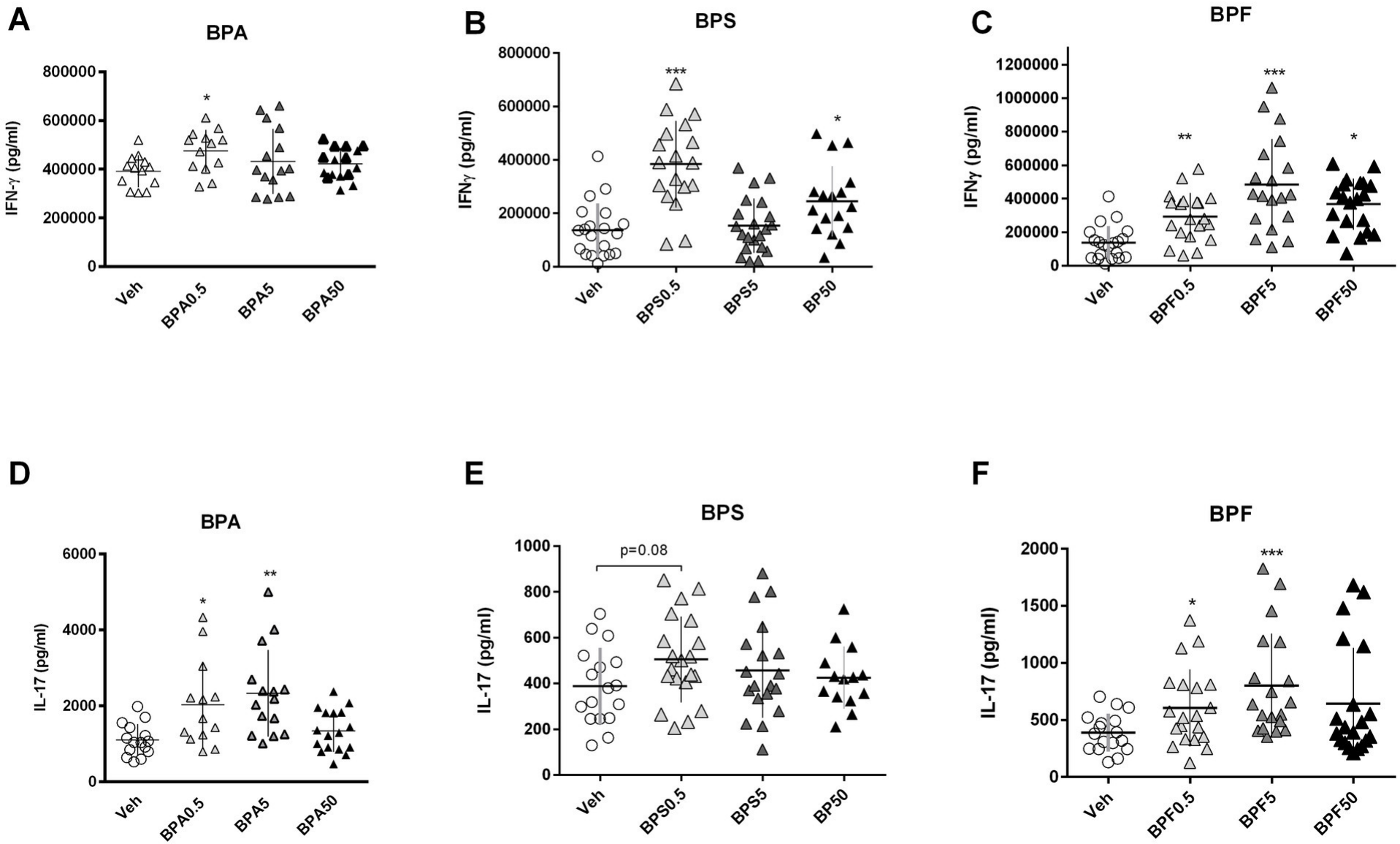

A
BPA
Medium
OVA
IFN-γ (pg/ml)
8000
6000
4000
2000
0
*
*
*
$
Veh
BPA0.5
BPA5
BPA50
Veh
BPA0.5
BPA5
BPA50
Tolerized mice
Immunized mice
B
BPS
Medium
OVA
IFN-γ (pg/ml)
5000
4000
3000
2000
1000
0
**
*
$
Veh
BPS0.5
BPS5
BPS50
Veh
BPS0.5
BPS5
BPS50
Tolerized mice
Immunized mice
C
BPF
Medium
OVA
IFN-γ (pg/ml)
5000
4000
3000
2000
1000
0
*
*
$$
Veh
BPF0.5
BPF5
BPF50
Veh
BPF0.5
BPF5
BPF50
Tolerized mice
Immunized mice
D
BPA
IL-17 (pg/ml)
2500
2000
1500
1000
500
0
Veh
BPA0.5
BPA5
BPA50
Veh
BPA0.5
BPA5
BPA50
Tolerized mice
Immunized mice
E
BPS
IL-17 (pg/ml)
1500
1000
500
0
*
Veh
BPS0.5
BPS5
BPS50
Veh
BPS0.5
BPS5
BPS50
Tolerized mice
Immunized mice
F
BPF
IL-17 (pg/ml)
1500
1000
500
0
$
**
Veh
BPF0.5
BPF5
BPF50
Veh
BPF0.5
BPF5
BPF50
Tolerized mice
Immunized mice

Figure 3


A
BPA
IFN-γ (pg/ml)
800000
600000
400000
200000
0
*
Veh
BPA0.5
BPA5
BPA50
B
BPS
IFNγ (pg/ml)
800000
600000
400000
200000
0
***
*
Veh
BPS0.5
BPS5
BP50
C
BPF
IFNγ (pg/ml)
1200000
1000000
800000
600000
400000
200000
0
**
***
*
Veh
BPF0.5
BPF5
BPF50
D
BPA
IL-17 (pg/ml)
6000
4000
2000
0
*
**
Veh
BPA0.5
BPA5
BPA50
E
BPS
IL-17 (pg/ml)
1000
800
600
400
200
0
p=0.08
Veh
BPS0.5
BPS5
BP50
F
BPF
IL-17 (pg/ml)
2000
1500
1000
500
0
*
***
Veh
BPF0.5
BPF5
BPF50

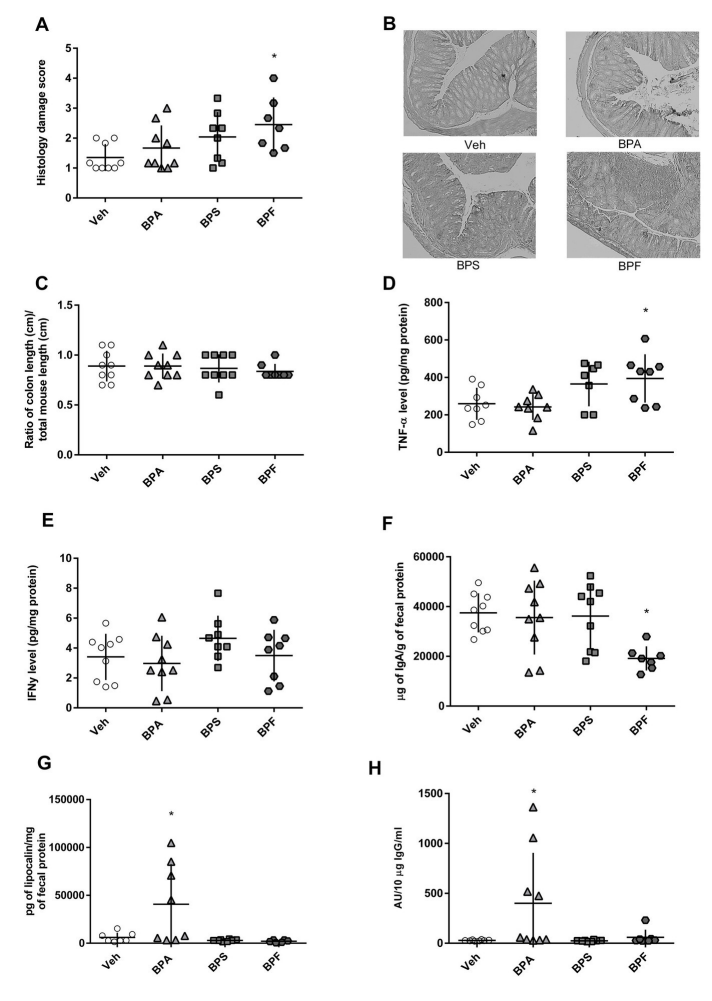
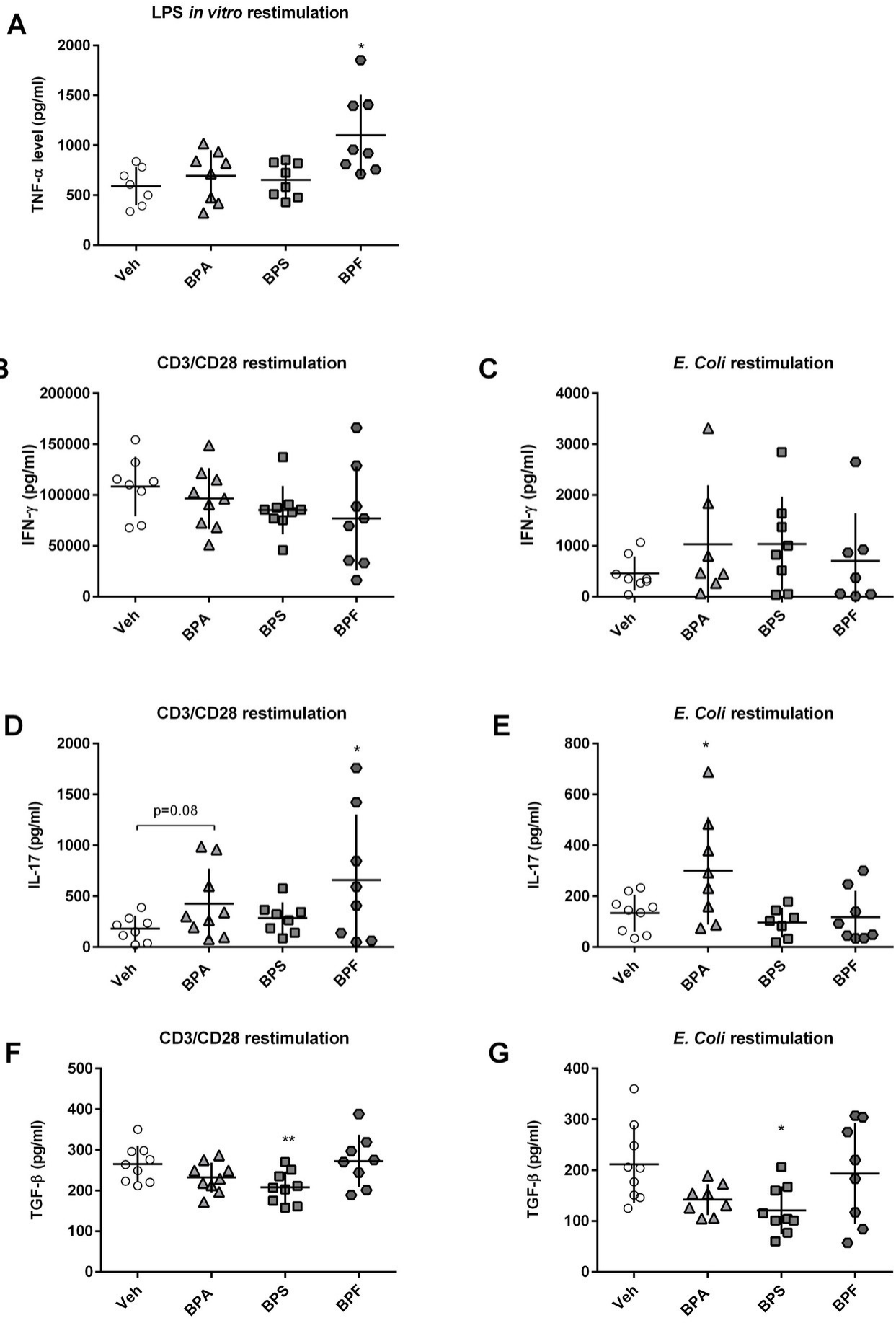

A
Histology damage score
5
4
3
2
1
0
*
Veh
BPA
BPS
BPF
B
Veh
BPA
BPS
BPF
C
Ratio of colon length (cm)/ total mouse length (cm)
1.5
1.0
0.5
0.0
Veh
BPA
BPS
BPF
D
TNF-α level (pg/mg protein)
800
600
400
200
0
*
Veh
BPA
BPS
BPF
E
IFNγ level (pg/mg protein)
10
8
6
4
2
0
Veh
BPA
BPS
BPF
F
μg of IgA/g of fecal protein
60000
40000
20000
0
*
Veh
BPA
BPS
BPF
G
pg of lipocalin/mg of fecal protein
150000
100000
50000
0
*
Veh
BPA
BPS
BPF
H
AU/10 μg IgG/ml
1500
1000
500
0
*
Veh
BPA
BPS
BPF

Figure 5


A
LPS *in vitro* restimulation
TNF-α level (pg/ml)
2000
1500
1000
500
0
*
Veh
BPA
BPS
BPF
B
CD3/CD28 restimulation
IFN-γ (pg/ml)
200000
150000
100000
50000
0
Veh
BPA
BPS
BPF
C
E. Coli restimulation
IFN-γ (pg/ml)
4000
3000
2000
1000
0
Veh
BPA
BPS
BPF
D
CD3/CD28 restimulation
IL-17 (pg/ml)
2000
1500
1000
500
0
p=0.08
*
Veh
BPA
BPS
BPF
E
E. Coli restimulation
IL-17 (pg/ml)
800
600
400
200
0
*
Veh
BPA
BPS
BPF
F
CD3/CD28 restimulation
TGF-β (pg/ml)
500
400
300
200
100
0
**
Veh
BPA
BPS
BPF
G
E. Coli restimulation
TGF-β (pg/ml)
400
300
200
100
0
*
Veh
BPA
BPS
BPF

Age of mice	8 Weeks	9 Weeks	11 Weeks	12 Weeks	14 Weeks	15 Weeks
BP gavage (0.5, 5 or 50 µg/ kg bw/d)	- (acclimatization)	+	+	+	+	+
OVA-tolerized			Gavage OVA 20mg in Bicarbonate buffer	OVA in CFA 100 µg sc	OVA boost 100 µg sc	Sacrifice Humoral and cellular response
OVA-immunized			Gavage Bicarbonate buffer			

CFA: Complete Freund Adjuvant
OVA: ovalbumin
sc: subcutaneous
µg/ kg bw/d: µg/ kg of body weight/ day
+ : daily oral gavage with BP