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Flame Retardants, Hexabromocyclododecane (HCBD) and Tetrabromobisphenol A (TBBPA), Alter Secretion of Tumor Necrosis Factor Alpha (TNFα) from Human Immune Cells

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Abstract

Hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA) are flame retardants, used in a variety of applications, which contaminate the environment and are found in human blood. HBCD and TBBPA have been shown to alter the tumor killing function of natural killer (NK) lymphocytes and the secretion of the inflammatory cytokines interferon gamma (IFNγ) and interleukin 1 beta (IL-1β). The current study examined the effects of HBCD and TBBPA on secretion of the critical pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) from human immune cells. Preparations of human immune cells that ranged in complexity were studied to determine if the effects of the compounds were consistent as the composition of the cell preparation became more heterogeneous. Cell preparations studied were: NK cells, monocyte-depleted (MD) peripheral blood mononuclear cells (PBMCs), and PBMCs. Exposure of NK cells to higher concentrations of HBCD (5 and 2.5 μM) caused decreased secretion of TNFα. However, when the cell preparation contained T lymphocytes (MD-PBMCs and PBMCs) these same concentrations of HBCD increased TNFα secretion as did nearly all other concentrations. This suggests that HBCD's ability to increase TNFα secretion from immune cells was dependent on the presence of T lymphocytes. In contrast, exposures to TBBPA decreased the secretion of TNFα from all immune cell preparations regardless of the composition of the cell preparation. Further, HBCD-induced increases in TNFα secretion utilized the p38 MARK pathway. Thus, both HBCD and TBBPA may have the capacity to disrupt the inflammatory response with HBCD having the potential to cause chronic inflammation.

Keywords

NK cells; PBMCs; Granulocytes; hexabromocyclododecane; tetrabromobisphenol A; TNFα

INTRODUCTION

Pro-inflammatory cytokines such as Tumor necrosis factor alpha (TNFα) are crucial in the immune response to invading pathogens. If levels of this key regulator of the inflammatory response are too low, an inadequate immune response occurs. Conversely, if levels of TNFα are elevated in the absence of infection then chronic inflammation can develop (Guicciardi and Gores 2009; Khalil et al., 2006; Silke 2011; Goetz et al., 2004). TNFα is produced predominantly by lymphocytes and monocytes/macrophages (Khalil et al., 2006). TNFα exists in soluble and membrane-bound forms. The soluble form is produced by the metalloprotease TNFα-converting-enzyme (TACE) cleavage of membrane TNF (Khalil et al., 2006). The signaling pathways that regulate TNFα production include nuclear factor kappa B (NFκB), and mitogen activated protein kinase (MAPK) (ERK 1/2, p38, and JNK) pathways (Gaestel et al., 2009). TNFα has the capacity to increase the proliferation and invasiveness of tumor cells (Vajdic, 2009). Due to its capacity to cause chronic inflammation, TNFα has been linked to the development of a number of diseases including Crohn's diseases and certain cancers (Chowers and Allez, 2010; Macarthur et al., 2004; Balkwill and Mantovani, 2001).

Flame retardants are used in the manufacture of plastics, textiles, upholstery and electronic products (Birnbaum and Staskal, 2004). Halogenated hydrocarbons are the most commonly used flame retardants in industry (Birnbaum and Staskal, 2004). Hexabromocyclododecane (HBCD) is a non-aromatic, brominated cyclic alkane which is primarily used as an additive flame retardant (Schecter et al., 2012). It is used in foam and expanded polystyrene insulation (Birnbaum and Staskal, 2004). Additionally, HBCD is applied to upholstery textiles used in furniture, drapery and wall coverings (Kajiwara et al., 2009). Since HBCD is not chemically bound to plastics or textiles, it can separate and leach from the surface of these products into the environment (de Wit, 2002). Due to its hydrophobic nature, it binds to soil, sediments and sewage sludge and is found in dust particles in the air (Hale et al., 2006; Abdallah et al., 2008; Remberger et al., 2004). Human exposure to HBCD occurs through dust ingestion and dietary exposure (Abdallah et al., 2008; Knutsen et al., 2008; van Leeuwen et al., 2008). Significant concentrations of (0.01–1.36 ng/g w/w) HBCD have been found in food products including beef, fish, peanut butter, pork, and turkey collected from grocery stores (Schecter et al., 2012). Studies have reported moderate concentrations of HBCD in human blood, adipose tissue, and breast milk (Knutsen et al., 2008; Pulkrabora et al., 2009; Kakimoto et al., 2008). Levels in human blood serum of 100 pg/g serum (approximately 0.16 nM, using a conversion factor of 400 mg lipid/100 mL serum) have been found (Covaci et al., 2006; Knutsen et al., 2008). Van der Ven et al. (2006) reported an increase in liver and pituitary weight and levels of cholesterol in rats exposed to HBCD. Tetraboromobisphenol A (TBBPA) is mainly used as a reactive flame retardant in epoxy resin circuit boards (IPCS/WHO, 1995). TBBPA enters the environment from treated products (Sellström and Jansson, 1995). It has been found in commercial drinking water stored in polycarbonate containers (Peterman et al. 2000). Studies in Japan and Norway have shown TBBPA in human serum samples (approximately 1.8–5.3 pM), averaging 4.5 ng/g lipid (approximately 33.8 pM) respectively (Nagayama et al., 2001; Thomsen et al., 2002). In mice, it has been shown to cause decreases in serum proteins and red blood cells as well as increases in spleen weight (Ronisz et al., 2004).

Previous studies showed that exposures to both HBCD and TBBPA decreased the ability of human NK cells to destroy tumor target cells (Hinkson and Whalen 2009; Kibakaya et al., 2009) while decreasing expression of several key cell-surface proteins needed for NK cells to bind to target cells (Hinkson and Whalen, 2010; Hurd and Whalen, 2011). Other studies showed that exposing immune cells to HBCD and TBBPA altered the secretion of the inflammatory cytokines interferon gamma (IFNγ) and interleukin 1β (IL-1β) (Almughamsi and Whalen, 2015; Anisuzzaman and Whalen, 2016). As mentioned earlier, chronic inflammation associated with elevated levels of TNFα has been associated with diseases such as Crohn's disease and certain cancers (Chowers and Allez, 2010; Macarthur et al., 2004; Balkwill and Mantovani, 2001); therefore it is important to study if exposure to HBCD or TBBPA, would affect the secretion of TNFα.

The current study examines the effects of exposures to a range of HBCD and TBBPA concentrations on secretion of TNFα from preparations of human immune cells of varying complexity: NK cells, monocyte-depleted (MD)-peripheral blood mononuclear cells (PBMCs) (T and NK lymphocytes), and PBMCs (monocytes and lymphocytes). This allowed us to determine if the effects of the compounds were altered as the composition of the cell preparation became more heterogeneous. Additionally, the signaling pathways involved in compound-induced increases in TNFα secretion were examined using selective inhibitors of pathways known to be involved in the secretion of TNFα (Gaestel et al., 2009).

MATERIALS AND METHODS

Preparation of PBMCs, and monocyte-depleted PBMCS

PBMCs were isolated from Leukocyte filters (PALL-RCPL or RC2D) obtained from the Red Cross Blood Bank Facility (Nashville, TN) as described in Meyer et al., 2005. Leukocytes were retrieved from the filters by back-flushing them with an elution medium (sterile PBS containing 5 mM disodium EDTA and 2.5% [w/v] sucrose) and collecting the eluent. The eluent was layered onto Ficoll-Hypaque (1.077g/mL) and centrifuged at 1200g for 30 min. Granulocytes and red cells pelleted at the bottom of the tube while the PBMCs floated on the

Ficoll-Hypaque. Mononuclear cells were collected and washed with PBS (500g, 10min). Following washing, the cells were layered on bovine calf serum for platelet removal. The cells were then suspended in RPMI-1640 complete medium which consisted of RPMI-1640 supplemented with 10% heat-inactivated BCS, 2 m*ML*-glutamine and 50 U penicillin G with 50 μg streptomycin/mL. This preparation constituted PBMCs. Monocyte-depleted PBMCs (10–20% $CD16^+$, 10–20 % $CD56^+$, 70–80% $CD3^+$, 3–5% $CD19^+$, 2–20% $CD14^+$) were prepared by incubating the cells in glass Petri dishes (150 in diameter) at 37 °C and air/CO_2, 19:1 for 1 h. This cell preparation is referred to as MD-PBMCs cells.

Preparation of NK cells

Leukocytes were retrieved from the filters by back-flushing them with an elution medium (sterile PBS containing 5 mM disodium EDTA and 2.5% [w/v] sucrose) and collecting the eluent as described above. RosetteSep human NK cell enrichment antibody cocktail (0.6–0.8 mL) (StemCell Technologies, Vancouver, British Columbia, Canada) was added to 45 mL of filter eluent. The mixture was incubated for 25 min at room temperature (~ 25º C). Approximately 8 mL of the mixture was layered onto 4 mL of Ficoll-Hypaque (1.077 g/mL) (MP Biomedicals, Irvine, CA) and centrifuged at 1200 g for 30 min. NK cells were collected and washed twice with phosphate buffered saline (PBS) pH 7.2 and stored in complete media (RPMI-1640 supplemented with 10% heat-inactivated bovine calf serum (BCS), 2 m*M L*-glutamine and 50 U penicillin G with 50 µg streptomycin/ml) at 1 million cells/mL at 37 °C and air/CO_2, 19:1.

Chemical Preparation

HBCD and TBBPA were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions were prepared as 100 mM solutions in Dimethylsulfoxide (DMSO). Desired concentrations of either HBCD or TBBPA were prepared by dilution of the stock into media.

Inhibitor Preparation

Enzyme inhibitors were purchased from Fischer Scientific (Pittsburgh, PA). Each inhibitor was prepared as a 50 mM stock solution in DMSO. JNK inhibitor (JNK X BI78D3) at a final concentration of 50 µM, MEK 1/2 pathway inhibitor (PD98059) at a final concentration of 50 µM, NFκB inhibitor (BAY11-7085) at a final concentration of 1.25–0.3 µM, p38 inhibitor (SB202190) at a final concentration of 25 µM, and TACE inhibitor (Batimastat) at a final concentration of 25 µM were prepared by appropriate dilution of the stock solution into cell culture media.

Cell Treatments

NK cells, MD-PBMCS, and PBMCs were treated with HBCD or TBBPA at concentrations of 0.05–5 µM for 24 h, 48 h, or 6 days. Following the incubations, the cells were pelleted and supernatants were collected and stored at −70° C until assaying for TNFα.

For pathway inhibitor experiments, MD-PBMCs (at a concentration of 1.5 million cells/mL) were treated with enzyme inhibitors 1h before adding HBCD at concentrations of 2.5, 1, 0.5 µM for 24 h. Following the incubations, the cells were pelleted and supernatants were collected and stored at −70° C until assaying for TNFα.

Cell Viability

Cell viability was assessed at the end of each exposure period. Viability was determined using the trypan blue exclusion method. Briefly, cells were mixed with trypan blue and counted using a hemocytometer. The total number of cells and the total number of live cells were determined for both control and treated cells to determine the percent viable cells. Exposure to the compounds caused no changes in viability of any of the cell preparations compared to that of the control (data not shown).

TNFα Secretion Assay

TNFα levels were measured using the BD OptEIA^{TM} Human TNFα enzyme-linked immunosorbent assay (ELISA) kit (BD-Pharmingen, San Diego, CA). Briefly, a 96-well micro well plate, designed for ELISA (Fisher, Pittsburgh, PA), was coated with a capture antibody for TNFα diluted in coating buffer. The plate was incubated with the capture antibody overnight at 4° C. After incubation, the capture antibody was removed by washing the plate three times with wash buffer (PBS and 0.05% Tween-20). Assay diluent (PBS and bovine calf serum) was added to each well (blocking non-specific binding) and incubated at room temperature for 1h. The assay diluent was removed by washing the plate three times, and the cell supernatants and TNFα standards were added to the coated plated and incubated for 2h at room temperature. Following this incubation, the plate was thoroughly washed five times and then incubated for 1h with a detection antibody linked to horseradish peroxidase (HRP). The detection antibody-HRP complex was removed by washing the plate seven times and then the plate was incubated for 30 min with substrates for horseradish peroxidase. The incubation with the substrates was ended by addition of acid and the absorbance was measured at 450 nm on a Thermo Labsystems Multiskan MCC/340 plate reader (Fisher Scientific).

Statistical Analysis

Statistical analysis of the data was performed by using ANOVA and Student's t test. Data were initially compared within a given experimental setup by ANOVA. A significant ANOVA was followed by pair wise analysis of control versus exposed data using Student's t test, a p-value of less than 0.05 was considered significant.

RESULTS

Effects of HBCD Exposure on Secretion of TNFα by NK cells

Table 1 shows the effects of exposing NK cells from 4 separate donors to 0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5 μM HBCD for 24 h, 48 h and 6 days on TNFα secretion (KB = Key Biologic buffy coat; F = filter obtained from the Red Cross). Cells from all 4 donors showed a significant decrease in TNFα secretion when exposed to 5 μM HBCD after 24 h, and at 2.5 and 5 μM after 48 h and 6 days. Additionally, all donors demonstrated increased TNFα secretion in response to HBCD. However, these increases were usually quite small and the concentrations and lengths of exposure where they occurred varied from one donor to the next. For instance, cells from donor F429 showed the greatest HBCD-induced increase (3.6 fold) after 6 days of exposure at the 1 μM exposure, while cells from donor F430 showed a maximum HBCD-stimulated increase of only 1.14 fold after 48 h. In contrast, the decreases in TNFα secretion seen with 2.5 and 5 μM HBCD were very substantial. Figure 1A shows the effects of HBCD exposures at each of the lengths of exposure for an individual donor (F429).

Effects of HBCD Exposure on Secretion of TNFα by MD-PBMCs

The effects of exposing MD-PBMCs from 4 individual donors to 0 to5 μM HBCD for 24 h, 48 h and 6 days on the secretion of TNFα are shown in Table 2. This preparation is mostly NK cells and T cells. In stark contrast to the effects of HBCD on NK cells, there were significant increases in TNFα secretion from MD-PBMC from all donors examined after all lengths of exposure to HBCD. Significant increases in TNFα secretion were induced by HBCD for all donors at the 0.1, 0.25, 0.5, 1, 2.5, and 5 μM concentrations after 24 h and/or 48 h of exposure. There was considerable variation in the fold increase in secreted TNFα among different donors. For example, cells from donor F283 treated with 2.5 μM HBCD showed an increase of 1.7 fold after 24 h while cells from donor F291 exhibited and increase 7.9 fold after this same treatment. The results after a 6 day exposure to HBCD were similar to those seen after 24 and 48 h exposures. Figure 1B shows the effects of HBCD exposures at each of the lengths of exposure for an individual donor (F284).

Effects of HBCD Exposure on Secretion of TNFα by PBMCs

The effects of exposures to HBCD on TNFα secretion from PBMCs (a preparation including NK cells, T cells, and monocytes) are shown in Table 3 and are very similar to those seen with MD-PBMCs. Cells from all donors showed increased TNFα secretion in response to 2.5 and/or 5 μM HBCD after the 24 h and 48 h exposures. As with MD-PBMCs, the magnitude of the increases seen at a given HBCD concentration varied from one donor to the next. For instance, the fold increases in TNFα secretion seen with a 24 h exposure to 5 μM HBCD were 2.5 (F189), 1.4 (F192), 2.0 (F195), and 18 (F356). Figure 1C shows the effects of HBCD exposures at each of the lengths of exposure for an individual donor (F195).

Effects of TBBPA Exposure on Secretion of TNFα by NK cells

Table 4 summarizes the effects of exposing NK cells to 0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, and 5 μM TBBPA for 24 h, 48 h and 6 days on the secretion of TNFα. Cells prepared from 4 separate donors were tested. Overall results show that TNFα secretion decreased as the concentration of TBBPA increased. This pattern of decreases was seen in all donors over all lengths of incubation. Figure 2A shows the effects of TBBPA exposures at each of the lengths of exposure for an individual donor (KB386).

Effects of TBBPA Exposure on Secretion of TNFα by MD-PBMCs

As was seen with NK cells, MD-PBMCs exposed to 0–5 μM TBBPA also showed decreased secretion of TNFα in response to exposure. MD-PBMCs (predominantly T and NK lymphocytes) from all donors showed very significant decreases in TNFα secretion at 2 or more TBBPA exposure concentrations after 24 h and 48 h (Table 5). A similar trend was seen at 6 days. Figure 2B shows the effects of TBBPA exposures at each of the lengths of exposure for MD-PBMCs from individual donor (F284).

Effects of TBBPA Exposure on Secretion of TNFα by PBMCs

The effects of exposing PBMCs (lymphocytes +monocytes) to TBBPA were similar to those seen with NK cells and MD-PBMCs (Table 6). Cells from all donors showed TBBPA-induced decreases in TNFα secretion at the 1, 2.5 and/or 5 μM concentrations after 24 h and 48 h. This pattern persisted out of 6 days of exposure. Figure 2C shows the effects of TBBPA exposures at each of the lengths of exposure for an individual donor (F381).

Effects of HBCD Exposure on Secretion of TNFα by MD-PBMCs with Selective Enzyme Inhibitors

JNK Inhibitor (JNK Inhibitor X BI78D3)—The effects of exposure to 0.5, 1, and 2.5 μM HBCD on secretion of TNFα from MD-PBMCs where JNK had been inhibited with B178D3 are shown in Table 7. These results indicate that JNK is not needed for HBCD-induced increases in TNFα secretion. For example, in Figure 3A, there are 1.9, 2.1, and 7.8 fold increases when MD-PBMCs (donor F412) are exposed to 0.5, 1 and 2.5 μM HBCD in the absence of JNK inhibitor. When the inhibitor is present those same HBCD exposures are able to cause 2.3, 2.7, 7.9 fold increases in TNFα secretion.

MEK Inhibitor (PD98059)—The results of experiments examining the ability of 0.5, 1, and 2.5 μM HBCD to stimulate TNFα secretion from MD-PBMCs where the ERK 1/2 pathway has been inhibited with the MEK inhibitor PD98059 are shown in Table 7. PD98059 did not tend to diminish the ability of HBCD to increase TNFα release from MD-PBMCs. For example, MD-PBMCs from donor F421exposed to 0.5, 1 and 2,5 μM HBCD showed increases in TNFα secretion of 2.3, 4.6, and 6.1 fold, respectively in the absence of PD98059. In the presence of the inhibitor, these same HBCD concentration caused increased secretion of TNFα of 2.6, 6.4, and 11 fold (Figure 3B). Thus, the ERK1/2 pathway does not appear to be essential for HBCD-induced increases in TNFα secretion.

NFκB Inhibitor (BAY 11-7085)—Cells from 4 of the 5 donors tested showed diminished HBCD-induced secretion of TNFα when the NFκB pathway was inhibited (Table 7) at a minimum of one exposure concentration. Figure 3C shows the data from donor F412 where the increases in TNFα secretion in response to 005, 1, and 2.5 μM HBCD were 1.6, 2.2, and 8.9 when BAY 11-7085 was absent, those same HBCD concentrations caused increases of 1.9, 2.1, and 6.4 when the inhibitor was present. These data suggest that the NFκB pathway may contribute to the HBCD induction of TNFα secretion, but that it is not the principle pathway being utilized by HBCD to increase TNFα secretion.

p38 Inhibitor (SB202190)—HBCD-induced increases in TNFα were diminished or blocked at one or more concentration in cells from all donors (Table 7). Figure 3D shows that cells from donor F412 had increases of 1.3, 1.6, and 4.1 fold in the secretion of TNFα after exposure to 0.5, 1, and 2.5 μM HBCD. These increases in TNFα secretion were completely blocked in the presence of SB202190. These results indicated that the p38 pathway may play a role in HBCD-induced stimulation of TNFα from immune cells.

TACE and MMP Inhibitor (Batimastat)—The effects of exposure to 0.5, 1, and 2.5 μM HBCD on secretion of TNFα from MD-PBMCs where TACE had been inhibited with Batimastat are shown in Table 7. MD-PBMCs from donor F417 showed 1.9, 1.5, and 5.5 fold increases in TNFα secretion when exposed to 0.5, 1 and 2.5 μM HBCD in the absence of TACE inhibitor (Figure 4E). When the inhibitor was present those same HBCD exposures were able to cause 3.4, 2.6, and 3.5 fold increases in TNFα secretion. Although inhibition of TACE decreases baseline secretion of TNFα dramatically, HBCD was still able to cause similar fold increases in TNFα secretion except at the 2.5 μM exposure. There was at least one concentration of HBCD where inhibition of TACE did result in decreased HBCD-induced TNFα secretion from cells from each donor (Table 7). These results indicate that HBCD may be utilizing the pathway associated with the TACE and MMP to cause the secretion of TNFα in a concentration dependent manner.

DISCUSSION

TNFα is considered a master regulator of inflammatory cytokine production (Parameswaran and Patial, 2010). Dysregulation of TNFα has been associated with rheumatoid arthritis, Crohn’s disease, and cancers such as gastrointestinal cancer. (Macarthur et al., 2004 and Shurety et al., 2000). Flame retardants, HBCD and TBBPA, are used in manufacturing goods such as plastics, textiles, and electronic products (Birnbaum and Staskal, 2004). Both are found in human blood (Germer et al., 2006; Covaci et al., 2006; Knutsen et al., 2008; Van der Ven et al., 2006; Nagayama et al., 2001; Thomsen et al., 2002). Previous studies showed that exposing immune cells to HBCD and TBBPA altered the secretion of the inflammatory cytokines interferon gamma (IFNγ) and interleukin 1β (IL-1β) (Almughamsi and Whalen, 2015; Anisuzzaman and Whalen, 2016). The current study shows the effects of these contaminants on the ability of human immune cells to secrete TNFα.

Exposure of MD-PBMCs and PBMCs to HBCD caused increased TNFα secretion. Unlike MD-PBMCs and PBMCs, NK cells exposed to HBCD showed decreased secretion of TNFα at the 2 highest concentrations, 2.5 and 5 μM. NK cells from all donors also showed HBCD-induced increases in TNFα release but only at a few concentrations of HBCD and the time that it took to see these increases varied among cells from different donors. For example, NK cells from two donors showed increases at only 2 concentrations of HBCD after 24 h of exposure. In contrast, MD-PBMCs from all donors showed increased TNFα secretion at nearly all HBCD concentrations after a 24 h exposure. Thus, there appears to be a difference in effect of HBCD on TNFα secretion depending on the composition of the cell preparation. Those preparations containing T and NK lymphocytes (MD-PBMCs) and lymphocytes and monocytes (PBMCs) showed a markedly different patterns of response to the same HBCD exposures than did a preparation that was predominantly NK lymphocytes. The more complex preparations (MD-PBMCs and PBMCs) may be more reflective of the situation in vivo and thus, HBCD-induced stimulation of TNFα secretion might be more likely than the decreases. Interestingly, this distinction between the least complex preparation (NK cells) and the more complex, MD-PBMCs and PBMCs, was not seen when measuring the effects of HBCD on IFNγ or IL-1β secretion (Almughamsi and Whalen, 2015; Anisuzzaman and Whalen, 2016) but was seen when examining the effects of organotin contaminants, tributyltin and dibutyltin, on TNFα secretion (Hurt et al., 2013). This suggests that the regulation of secretion and/or production of TNFα from NK cells may be different than that of T cells and/or monocytes (Stanley and Lacy, 2010; Gaestel et al., 2009).

When immune cells were exposed to TBBPA, secretion of TNFα was significantly decreased at the highest concentrations in cells from all donors at all lengths of exposure regardless of the complexity of the cell preparation. Unlike HBCD, each of the different cell preparations showed similar responses to TBBPA in terms of TNFα secretion. Previous studies examining the effects of TBBPA on secretion of IFNγ and IL-1β from human immune cells also showed decreased TNFα secretion in response to TBBPA exposures (Almughamsi and Whalen, 2015; Anisuzzaman and Whalen, 2016).

After establishing that HBCD-induced increases in TNFα release were occurring, inhibitors of pathways utilized in the production/secretion of TNFα were examined for their ability to alter this effect. Mitogen-activated protein kinase (MAPK) pathways including JNK, p38 and ERK1/2 as well as the nuclear factor kappa B (NFκB) pathway and the metalloprotease TNFα converting enzyme (TACE) TACE are known to regulate the secretion/production of TNFα (Gaestel et al., 2009; Khalil et al., 2006; Wajant, 2003). MD-PBMCs were chosen for the pathway studies due to their consistent HBCD-induced increases in the secretion of TNFα after a 24 h exposure. The JNK and ERK1/2 MAPK pathways were not essential to the HBCD-induced increases in TNFα secretion. TACE activity appeared to be needed for

HBCD to stimulate increased secretion of TNFα, but only at certain concentrations of HBCD (which varied from donor to donor). The NFκB pathway appears to contribute to HBCD stimulation of the secretion of TNFα, but blocking its function only somewhat inhibits the process. In contrast to the other pathways, the p38 MAPK pathway appears to be consistently utilized by HBCD in its stimulation of TNFα secretion. Previous studies examining the role of MAPK and NFκB pathways on HBCD-induced increases in IFNγ and IL-1β indicated that the ERK1/2 pathway was needed for HBCD-induced secretion of IFNγ (Almughamsi and Whalen, 2015) and that both the p38 and ERK1/2 pathways were utilized when HBCD increased the secretion of IL-1β (Anizussaman and Whalen, 2015).

TNFα has the capacity to cause chronic inflammation, which may lead to diseases such as rheumatoid arthritis, Crohn’s disease, and cancers (Macarthur et al., 2004; Shurety et al., 2000). Studies suggest that TNFα inhibitors may provide a first-line biologic therapy in managing rheumatoid arthritis and Crohn’s disease; these studies showed successful treatment of rheumatoid arthritis and Crohn’s disease when treated with anti-TNFα antibody (Barnabe et al., 2011; Khalil et al., 2006; Michaud et al., 2014).

The current study indicates that flame retardants, HBCD and TBBPA, alter secretion of TNFα from human immune cells. Both flame retardants have the ability to disrupt TNFα release from increasingly complex human immune cells preparations. HBCD increased TNFα secretion from MD-PBMCs (T cells + NK cells) and PBMCs (T cells + NK cells + monocytes). As mentioned above, HBCD-induced stimulation of TNFα secretion might be more likely than the decreases since the more complex preparations (MD-PBMCs and PBMCs) may be more reflective of the situation in vivo. In contrast, TBBPA decreased secretion of TNFα in all cell preparations. Further studies with selective enzyme inhibitors suggested that HBCD is utilizing the p38 MAPK pathway to produce a pro-inflammatory signal. As mentioned in the introduction, p38 is a pathway that leads to transcription of the TNFα gene (Gaestel et al., 2009). Thus, TBBPA may have the capacity to cause inadequate immune responsiveness by decreasing the ability of immune cells to secrete TNFα while HBCD may contribute to chronic inflammation by increasing TNFα secretion, which may lead to several diseases and increased tumor invasiveness.

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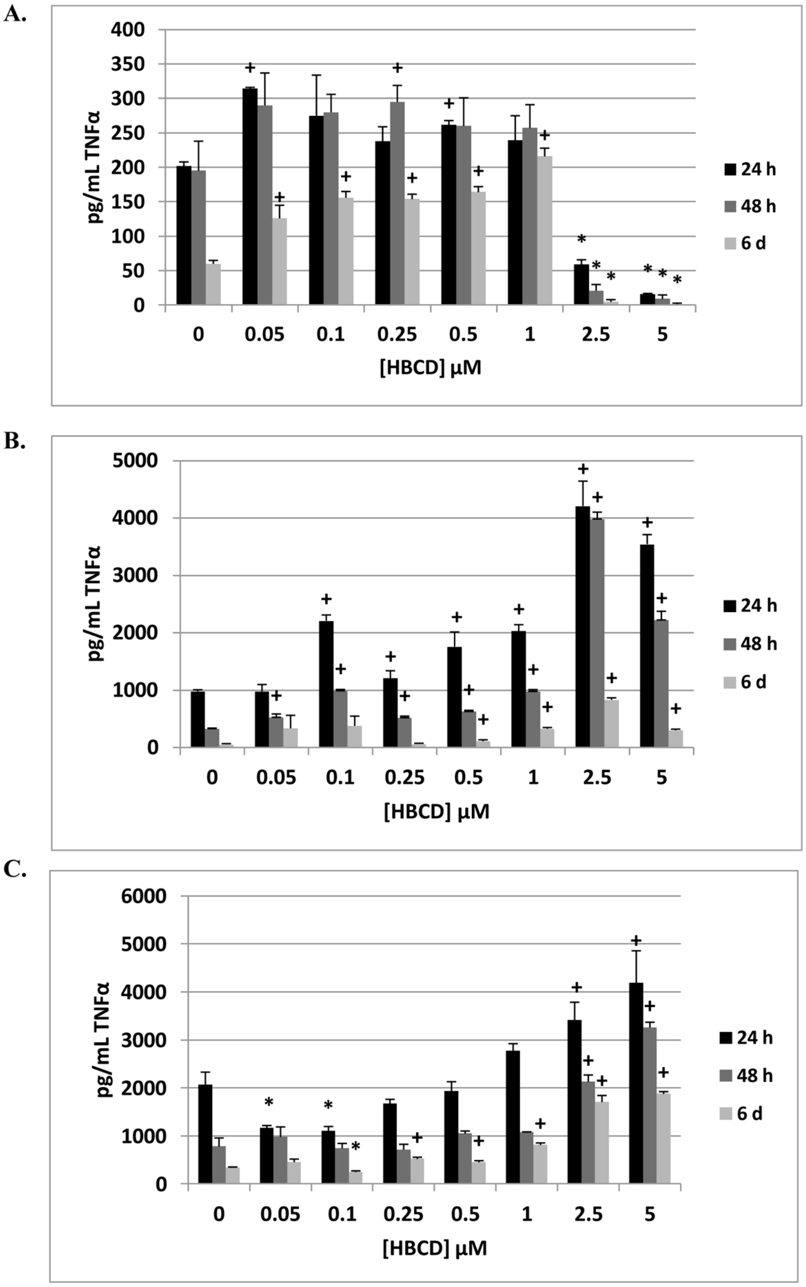


Figure 1.

Effects of 24 h, 48 h and 6 days exposures to HBCD on TNFα secretion from highly purified human NK cells, monocyte-depleted PBMCs, and PBMCs. A) NK cells exposed to 0.05–5μM HBCD (donor F429). B) Monocyte-depleted PBMCs exposed to 0.05–5μM HBCD (donor F284). C) PBMCs exposed to 0.05–5μM HBCD (donor F195). (+) indicates a significant increase compared to control and (*) indicates a significant decrease compared to control.

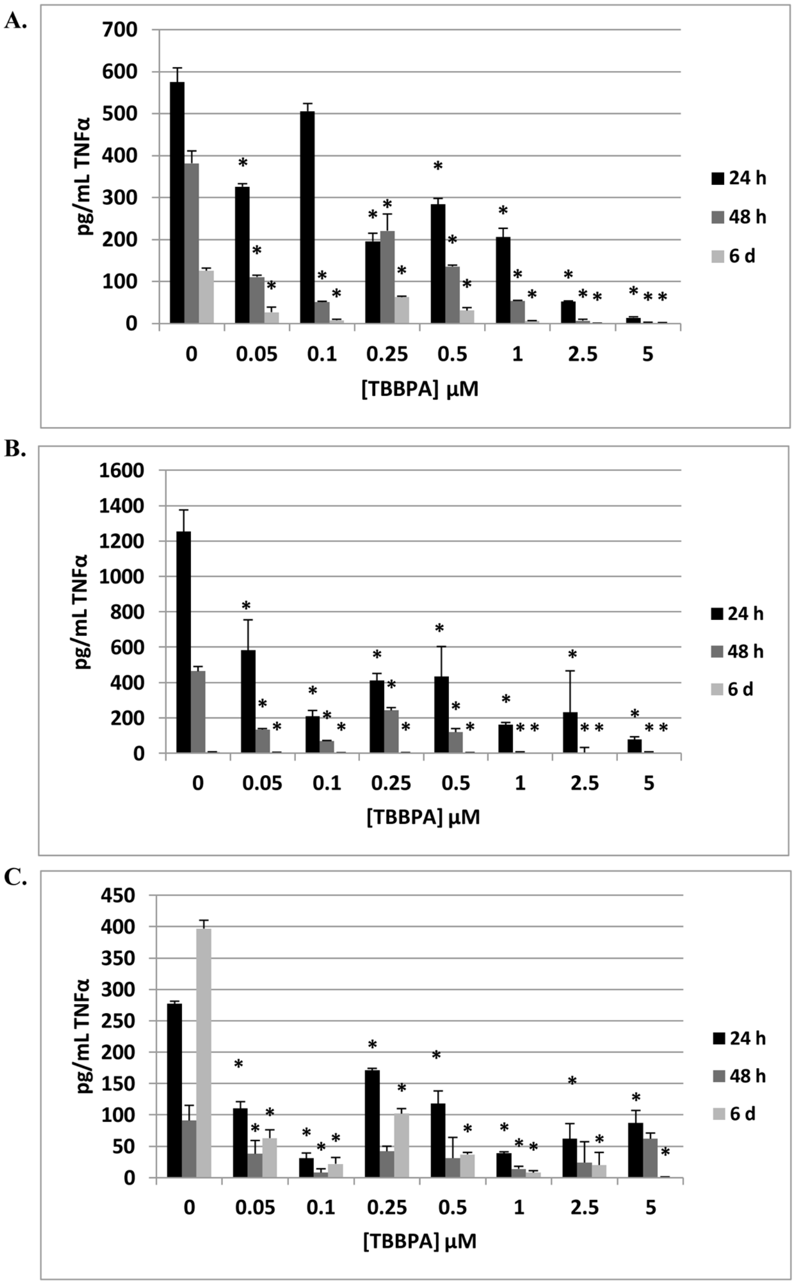
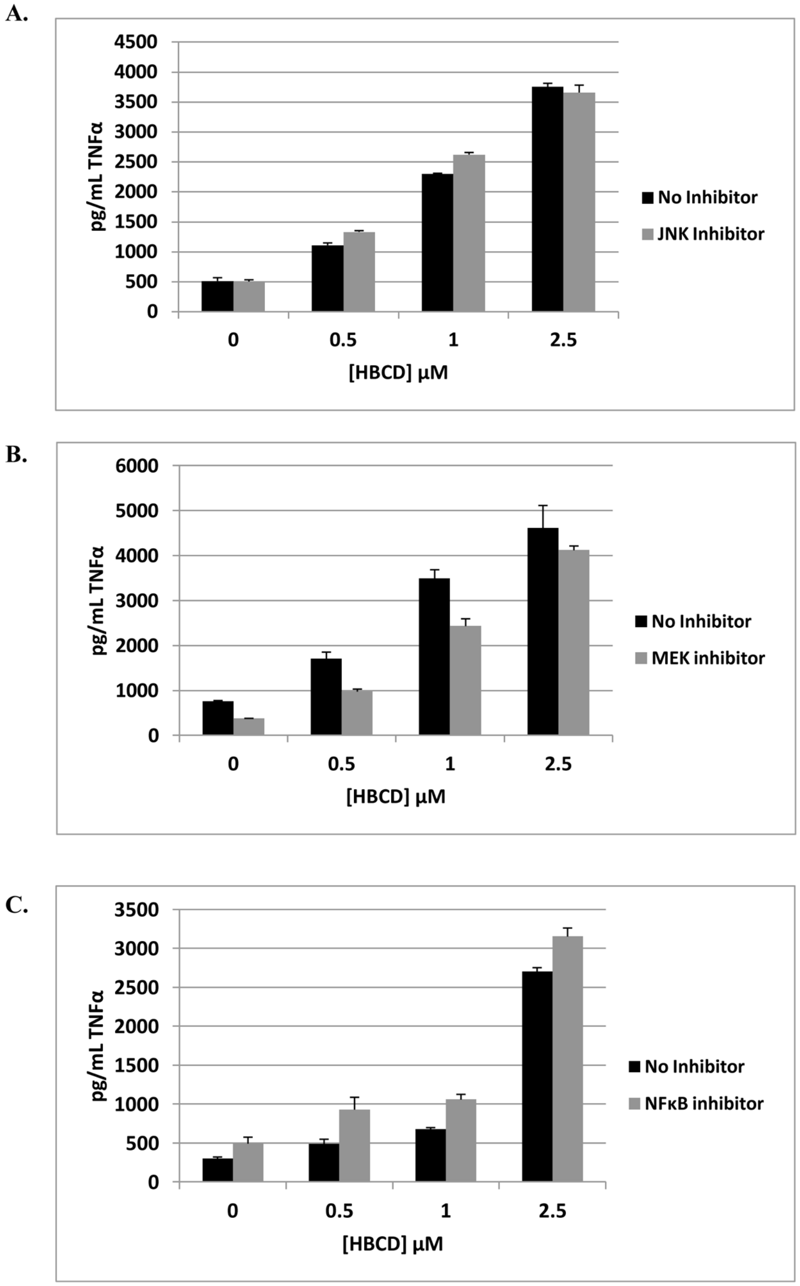


Figure 2.

Effects of 24 h, 48 h and 6 days exposures to TBBPA on TNFα secretion from highly purified human NK cells, monocyte-depleted PBMCs, and PBMCs. A) NK cells exposed to 0.05–5μM TBBPA (donor KB386). B) Monocyte-depleted PBMCs exposed to 0.05–5μM TBBPA (donor F284). C) PBMCs exposed to 0.05–5μM TBBPA (donor 381). (*) indicates a significant decrease compared to control.


A.
4500
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pg/mL TNFα
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0.5
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2.5
[HBCD] µM
■ No Inhibitor
■ JNK Inhibitor
B.
6000
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3000
2000
1000
0
pg/mL TNFα
0
0.5
1
2.5
[HBCD] µM
■ No Inhibitor
■ MEK inhibitor
C.
3500
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pg/mL TNFα
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2.5
[HBCD] µM
■ No Inhibitor
■ NFκB inhibitor

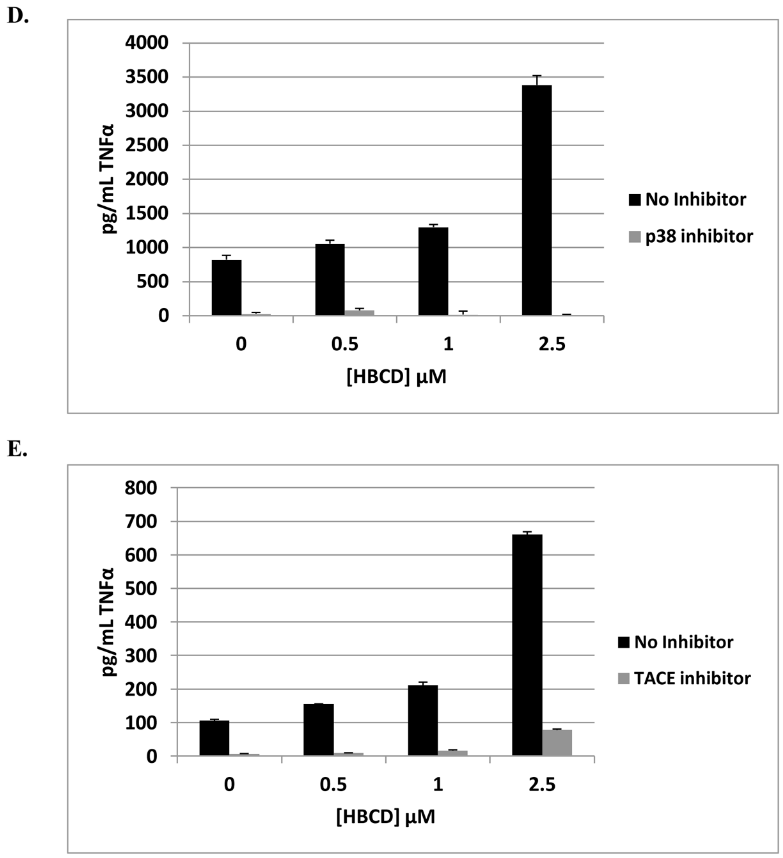

Figure 3.

Effects of 24 h exposure to 0.5, 1, and 2.5 μM HBCD on TNFα secretion from monocyte-depleted PBMCs with selective enzyme inhibitors in an individual donor. A) JNK inhibitor (donor F421). B) MEK 1/2 Inhibitor (PD98059) (donor F421). C) NFκB Inhibitor (BAY 11-7085) (donor F412). D) p38 Inhibitor (SB202190) (donor F412). E) TACE and MMP Inhibitor (Batimastat) (donor F412).

Table 1

Effects of 24 h, 48 h, and 6 days exposures to HBCD on TNFα secretion from highly purified human NKs.

24 h	**TNFα secretion in pg/mL (mean±S.D.)**			
[HBCD] μM	**KB-182**	**F410**	**F429**	**F430**
0	1207±93	2669±54	202±6	81±11
0.05	1289±93	2494±55*	314±2$^{\#}$	99±3
0.1	1218±166	2586±130	275±59	80±5
0.25	1324±68	2775±93	238±21	94±5
0.5	1315±116	2989±125$^{\#}$	262±6$^{\#}$	90±8
1	1249±130	3047±97$^{\#}$	239±36	51±6*
2.5	1115±56	400±38*	59±7*	18±1*
5	736±18*	139±17*	16±1*	18±6*

48 h	**TNFα secretion in pg/mL (mean±S.D.)**			
[HBCD] μM	**KB-182**	**F410**	**F429**	**F430**
0	1017±12	826±232	195±43	244±3
0.05	1112±94	1102±57	290±47	275±4$^{\#}$
0.1	1019±27	1095±33	280±26	191±3*
0.25	1005±12	1187±42	295±24$^{\#}$	268±5$^{\#}$
0.5	1086±32$^{\#}$	1052±40	260±41	287±16$^{\#}$
1	1113±136	1146±40	257±34	182±2*
2.5	873±24*	111±32*	21±9*	19±1*
5	482±38*	55±30*	9±6*	7±2*

6 d	**TNFα secretion in pg/mL (mean±S.D.)**			
[HBCD] μM	**KB-182**	**F410**	**F429**	**F430**
0	827±64	2001±58	60±5	63±9
0.05	1354±287	1726±53*	126±19$^{\#}$	72±25
0.1	1230±191	1874±17	156±9$^{\#}$	40±3*
0.25	1264±271	2088±72	154±7$^{\#}$	77±7
0.5	1482±345	2202±97*	164±8$^{\#}$	56±1
1	1260±85$^{\#}$	2042±35	216±12$^{\#}$	39±2*
2.5	792±79	206±9*	5±3*	11±10*
5	702±33	145±58*	0±3*	8±5*

Values are mean±S.D. of triplicate determinations.

[#] Indicates a significant increase and

[*] indicates a significant decreases in secretion compared to control cells, $p<0.05$

Table 2

Effects of 24 h, 48 h, and 6 days exposures to HBCD on TNFα secretion from monocyte-depleted PBMCs (T and NK Lymphocytes).

24 h	**TNFα secretion in pg/mL (mean±S.D.)**			
[HBCD]μM	**F283**	**F284**	**F286**	**F291**
0	1718±162	974±29	734±25	924±41
0.05	2746±77[#]	975±119	950±79[#]	1577±51[#]
0.1	3599±223[#]	2207±99[#]	1294±15[#]	2712±15[#]
0.25	2128±152[#]	1205±130	865±16[#]	1410±36[#]
0.5	2608±94[#]	1756±254[#]	1023±21[#]	1690±56[#]
1	3592±130[#]	2028±109[#]	1518±41[#]	2861±64[#]
2.5	2907±50[#]	4206±435[#]	3882±58[#]	7292±282[#]
5	1796±121	3541±167[#]	4472±111[#]	4958±262[#]

48 h	**TNFα secretion in pg/mL (mean±S.D.)**			
[HBCD]μM	**F283**	**F284**	**F286**	**F291**
0	684±42	327±8	201±16	98±6
0.05	1334±69[#]	524±57[#]	847±876	352±46[#]
0.1	2107±96[#]	988±21[#]	386±10[#]	1220±27[#]
0.25	876±67[#]	517±29[#]	399±280	236±38[#]
0.5	1496±121[#]	625±19[#]	366±65[#]	362±19[#]
1	2443±63[#]	977±30[#]	444±60[#]	1044±87[#]
2.5	2037±274[#]	3981±119[#]	1750±198[#]	6752±639[#]
5	1110±167[#]	2221±150[#]	1763±136[#]	3898±126[#]

6 d	**TNFα secretion in pg/mL (mean±S.D.)**			
[HBCD]μM	**F283**	**F284**	**F286**	**F291**
0	81±3	51±14	0±1	81±6
0.05	496±17[#]	332±227	293±115[#]	267±110
0.1	1355±71[#]	374±170	71±35	841±28[#]
0.25	281±20[#]	61±10	30±30	219±45[#]
0.5	505±11[#]	107±24[#]	103±158	233±39[#]
1	1187±54[#]	326±19[#]	0±1	818±63[#]
2.5	1038±19[#]	830±34[#]	28±33	4009±246[#]
5	323±1[#]	297±21[#]	52±10[#]	2468±132[#]

Values are mean±S.D. of triplicate determinations.

[#] Inidcates a significant increases and

[*] indicates a significant decrease in secretion compared to control cells, $p<0.05$

Table 3

Effects of 24 h, 48 h, and 6 days exposures to HBCD on TNFα secretion from PBMCs (lymphocytes and monocytes).

24 h	**TNFα secretion in pg/mL (mean±S.D.)**			
[HBCD]μM	**F189**	**F192**	**F195**	**F356**
0	801±16	1695±175	2074±258	358±10
0.05	1788±106#	1486±155	1172±49*	710±84#
0.1	1385±71#	1553±227	1110±91*	851±38#
0.25	2045±60#	1556±36	1679±88	423±18#
0.5	1826±106#	1870±179	1936±198	540±86
1	2491±108#	1779±107	2274±151	810±48#
2.5	3184±191#	2406±18#	3415±375#	2378±190#
5	1977±137#	2383±266#	4190±671#	6548±103#

48 h	**TNFα secretion in pg/mL (mean±S.D.)**			
[HBCD]μM	**F189**	**F192**	**F195**	**F356**
0	153±14	493±87	786±176	41.2±1
0.05	264±25#	494±62	983±209	883±128#
0.1	304±20#	632±411	745±104	78±1#
0.25	528±62#	240±95*	718±111	58±4#
0.5	533±14#	567±124	1052±54	55±4#
1	581±658	802±469	1071±18	122±1#
2.5	2388±217#	898±57#	2129±144#	431±297#
5	1258±89.5#	676±8	32641±110#	1174±43#

6 d	**TNFα secretion in pg/mL (mean±S.D.)**			
[HBCD]μM	**F189**	**F192**	**F195**	**F356**
0	72±21	45±68	342±16	0±2
0.05	141±115	69±20	460±62	208±260
0.1	123±41	58±8	253±23*	34±101#
0.25	385±46#	58±13	524±38#	63±11#
0.5	306±37#	75±11	456±32#	22±21
1	1596±89#	107±15	816±42#	11±11
2.5	1833±168#	142±22	1709±138#	64±19#
5	1021±17#	146±4	1880±47#	330±15#

Values are mean±S.D. of triplicate determinations.

# Indicates a significant increase and

* indicates a significant decrease in secretion compared to control cells, p<0.05

Table 4

Effects of 24 h, 48 h, and 6 days exposures to TBBPA on TNFα secretion from highly purified human NKs.

24h	**TNFα secretion in pg/mL (mean±S.D.)**			
[TBBPA] μM	**KB-130**	**KB-183**	**F378**	**F386**
0	486±7	40±5.7	365±13	575±34
0.05	463±20	48±13	214±4*	326±7*
0.1	405±5*	41±15	97±6*	506±18
0.25	261±5*	22±11	266±10*	196±19*
0.5	140±52*	48±8	169±3*	284±14*
1	68±7*	4±2*	70±3*	206±21*
2.5	15±2*	0±1*	19±1*	53±1*
5	0±2*	8±9*	3±2*	14±2*
48h	**TNFα secretion in pg/mL (mean±S.D.)**			
[TBBPA] μM	**KB-130**	**KB-183**	**F378**	**F386**
0	342±10	233±62	267±10	382±29
0.05	350±25	206±91	126±8*	111±4*
0.1	283±14*	167±12	92±59*	51±2*
0.25	217±3*	117±6	188±4*	221±40*
0.5	95±7*	584±518	98±4*	136±3*
1	40±10*	51±46*	43±1*	54±1*
2.5	0±2*	30±32*	4±1*	7±3*
5	0±2*	18±17*	0±1*	0±3*
6d	**TNFα secretion in pg/mL (mean±S.D.)**			
[TBBPA] μM	**KB-130**	**KB-183**	**F378**	**F386**
0	329±116	87±96	39±10	126±6
0.05	310±197	73±70	53±20	27±12*
0.1	250±88	48±47	34±12	8±2*
0.25	267±165	11±4	41±7	63±2*
0.5	160±186	55±38	41±5	31±7*
1	112±150	5±8	29±0	6±1*
2.5	191±311	32±25	58±7	0±1*
5	0±2*	48±50	32±9	0±2*

Values are mean±S.D. of triplicate determinations.

* Indicates a significant decrease in secretion compared to control cells, p<0.05

Table 5

Effects of 24 h, 48 h, and 6 days exposures to TBBPA on TNFα secretion from monocyte-depleted PBMCs (T and NK Lymphocytes).

24 h	**TNFα secretion in pg/mL (mean±S.D.)**			
[TBBPA]μM	F175	F284	F291	F292
0	1127±68	1255±121	484±56	1326±33
0.05	1046±59	582±173*	224±109*	293±10*
0.1	807±17*	209±34*	48±34*	164±16*
0.25	597±39*	413±39*	245±10*	615±19*
0.5	399±42*	433±171*	237±98*	297±18*
1	178±4*	161±14*	41±35*	158±10*
2.5	123±29*	232±235*	111±187	116±6*
5	157±71*	78±16*	95±55*	107±9*

48 h	**TNFα secretion in pg/mL (mean±S.D.)**			
[TBBPA]μM	**F175**	**F284**	**F291**	**F292**
0	460±13	464±27	237±21	753±86
0.05	629±254	135±5*	55±11*	356±127*
0.1	302±4*	69±4*	26±9*	158±70*
0.25	369±77	243±16*	76±22*	220±9*
0.5	229±207	120±20*	70±24*	106±18*
1	147±20*	0±9*	18±5*	50±37*
2.5	193±138	0±34*	43±15*	107±19*
5	40±7*	0±9*	106±5*	138±81*

6 d	**TNFα secretion in pg/mL (mean±S.D.)**			
[TBBPA]μM	**F175**	**F284**	**F291**	**F292**
0	327±15	8±1	117±92	221±9
0.05	534±308	5±1*	28±4	1435±287
0.1	234±31*	3±1*	0±10	692±185*
0.25	147±20*	4±1*	90±6	482±195*
0.5	22±44*	4±1*	12.8±3	133±50*
1	0±23*	0±1*	0±1	48±15*
2.5	0±14*	0±1*	0±4	50±13*
5	707±1361	0±1*	110±172	18±12*

Values are mean±S.D. of triplicate determinations.

*Indicates a significant decrease in secretion compared to control cells, p<0.05

Table 6

Effects of 24 h, 48 h, and 6 days exposures to TBBPA on TNFα secretion from PBMCs (lymphocytes and monocytes).

24 h	**TNFα secretion in pg/mL (mean±S.D.)**			
[TBBPA]μM	**F188**	**F356**	**F381**	**F385**
0	522±21	106±2	277±4	1384±130
0.05	614±391	199±122	110±11*	434±76*
0.1	576±346	104±37	31±8*	284±54*
0.25	292±4*	234±68	171±3*	801±289
0.5	205±3*	64±21	118±20*	934±688
1	189±7*	38±8*	39±2*	245±48*
2.5	182±3*	49±4*	62±24*	193±10*
5	197±7*	79±21	87±20*	205±36*

48 h	**TNFα secretion in pg/mL (mean±S.D.)**			
[TBBPA]μM	**F188**	**F356**	**F381**	**F385**
0	87±19	14±1	91±24	216±44
0.05	161±11	8±1*	38±21*	110±30*
0.1	817±1378	6±1*	8±6*	66±26*
0.25	152±172*	9±1*	42±8	203±31
0.5	1436±2451*	5±1*	31±33	105±97
1	0±20*	5±1*	14±4*	35±18*
2.5	0±16*	7±1*	24±33	51±27*
5	0±3*	10±1*	62±9	99±59

6 d	**TNFα secretion in pg/mL (mean±S.D.)**			
[TBBPA]μM	**F188**	**F356**	**F381**	**F385**
0	204±16	105±26	397±13	479±207
0.05	341±197	122±130	63±13*	114±77
0.1	132±3	134±180	22±10*	67±21
0.25	98±18*	315±460	102±8*	345±100
0.5	34±0*	48±12*	37±3*	59±31
1	8±1*	27±5*	8±3*	54±17
2.5	0±2*	50±5	20±20*	51±29
5	0±3*	6±3*	0±1*	14±2

Values are mean±S.D. of triplicate determinations.

*Indicates a significant decrease in secretion compared to control cells, p<0.05

Table 7

Effects of 24 h exposure to HBCD +/− Pathway inhibitors.

JNK Inhibitor X BI78D3

24 h	TNFα secreted in pg/mL (mean±S.D.)				
[HBCD] μM	**F412**	**F414**	**F417**	**F421**	**F423**
0	439±25	741±260	290±62	512±54	3578±278
0 + JNK	445±17	1179±80	357±56	503±29	3278±70
0.5	862±118*	1592±170*	515±57*	1109±38*	4325±157*
0.5 + JNK	1026±234*	1850±155*	598±81*	1332±20*	5358±74*
1	910±98*	3628±872*	769±38*	2301±9*	6015±577*
1 + JNK	1195±125*	4056±213*	802±12*	2620±36*	6325±174*
2.5	3445±270*	6936±323*	1898±226*	3754±56*	8408±490*
2.5 + JNK	3545±107*	7339±93*	2094±107*	3657±123*	8348±387*

MEK1/2 Pathway Inhibitor (PD98059)

24 h	TNFα secreted in pg/mL (mean±S.D.)				
[HBCD] μM	**F412**	**F414**	**F417**	**F421**	**F423**
0	654±88	529±48	113±18	756±27	3065±194
0 + PD	226±97	442±221	8±2	380±7	1791±113
0.5	907±21*	1461±347*	135±16	1708±152*	4100±186*
0.5 + PD	476±50*	955±99*	174±41*	982±57*	2775±221*
1	1332±129*	1916±50*	195±12*	3497±196*	5766±453*
1 + PD	490±51*	721±57	106±40	2438±162*	4130±269*
2.5	3988±92*	3704±543*	484±13*	4615±506*	7925±60*
2.5 + PD	2146±146*	3217±84*	284±44*	4127±95*	7128±192*

NFκB Inhibitor (BAY 11-7085)

24 h	TNFα secreted in pg/mL (mean±S.D.)				
[HBCD] μM	**F412**	**F414**	**F417**	**F421**	**F423**
0	302±20	793±124	550±43	591±28	1032±48
0 + BAY	495±83	1369±336	512±111	927±53	795±50
0.5	492±59*	2640±522*	1163±87*	1513±91*	1468±66*
0.5 + BAY	931±158**	2831±550*	946±137*	1680±68*	1482±271*
1	676±24*	3574±116*	496±7.2	3125±160*	2085±44*
1 + BAY	1059±69**	5326±457*	412±38	3655±113*	2787±124*
2.5	2701±53*	7960±761*	3062±98*	4162±185*	3112±277*
2.5 + BAY	3154±109**	7245±156*	3608±569*	4119±93*	2433±130*

p38 Inhibitor (SB202190)

24 h	TNFα secreted in pg/mL (mean±S.D.)			
[HBCD] μM	**F412**	**F421**	**F423**	**F437**
0	820±63	66±2	1316±45	1216±40
0 + SB	29±17	0±1	22±4	334±206
0.5	1054±52*	167±4*	1954±60*	1988±116*
0.5 + SB	78±27	3±1	44±14	307±173
1	1298±36*	281±5*	2163±60*	2392±219*
1 + SB	15±52	8±3*	82±10*	200±71
2.5	3381±136*	295±32*	3717±1856	4448±185*
2.5 + SB	0±18	0±2	218±20*	132±6

TACE and MMP Inhibitor (Batimastat)

24 h	TNFα secreted in pg/mL (mean±S.D.)			
[HBCD] μM	**F412**	**F417**	**F421**	**F423**
0	106±4	171±5	758±27	1872±104
0 + TACE	7±1	39±6	42±18	139±24

TACE and MMP Inhibitor (Batimastat)

24 h	**TNFα secreted in pg/mL (mean±S.D.)**			
[HBCD] μM	**F412**	**F417**	**F421**	**F423**
0.5	155±1[*]	316±5[*]	1462±44[*]	2950±181[*]
0.5 + TACE	9±1	133±30[*]	69±37	253±59
1	212±9[*]	266±17[*]	2257±94[*]	4219±180[*]
1 + TACE	17±2[*]	103±18[*]	116±9[*]	269±42[*]
2.5	661±8[*]	935±46[*]	2776±58[*]	6325±161[*]
2.5 + TACE	78±3[*]	136±7[*]	1258±37[*]	1244±149[*]

Values are mean±S.D. of triplicate determinations.

[*] Indicates a significant increase compared to the appropriate control p<0.05. For concentrations of HBCD without inhibitor “0” is the appropriate control and for concentrations of HBCD with inhibitor “0+TACE” is the appropriate control