Published in final edited form as:

J Appl Toxicol. 2018 February ; 38(2): 201–218. doi:10.1002/jat.3514.

BUTYLTIN COMPOUNDS ALTER SECRETION OF INTERLEUKIN 6 FROM HUMAN IMMUNE CELLS

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Abstract

Butyltins (BTs), tributyltin (TBT) and dibutyltin (DBT) are organotin compounds that have been used in a variety of industrial applications; as a result, these compounds have been found in human blood. IL-6 is a pro-inflammatory mediator that is produced by T lymphocytes and monocytes. It is responsible for immune response regulation as well as tissue repair and cellular growth. Both BTs decrease the ability of human natural killer (NK) cells to destroy tumor cells and alter the secretion of pro-inflammatory cytokines tumor necrosis factor alpha (TNF α), interferon gamma (IFN γ), and IL-1 beta (β) from human lymphocytes *ex vivo*. Here, we show that BTs alter the secretion of IL-6 from increasingly reconstituted preparations of human immune cells. IL-6 secretion was examined after 24h, 48h, or 6 day exposures to TBT and DBT in highly enriched human NK cells, monocyte-depleted (MD) peripheral blood mononuclear cells (MD-PBMCs), PBMCs, granulocytes, and a preparation combining both PBMCs and granulocytes (PBMCs +granulocytes). The results indicated that both BTs altered IL-6 secretion from all cell preparations. Significant decreases of IL-6 secretion were seen at the highest concentration of TBT (200 nM) and DBT (5–2.5 μ M) while the lower concentrations of DBT (0.05 and 0.1 μ M) caused elevation of IL-6 secretion. The data indicates that BT-induced alterations of IL-6 secretion from immune cells may be a significant consequence of BT exposures that may potentially affect immune competence.

Keywords

NK cells; PBMCs; Granulocytes; Dibutyltin; Tributyltin; Interleukin 6

Introduction

Interleukin 6 (IL-6) is a glycosylated protein (22–27 kDa) (Kishimoto, 1989), and is mainly produced by monocytes/macrophages, fibroblasts, and vascular endothelial cells (Akira *et al.*, 1993). It is also produced by T and NK lymphocytes (Li and He, 2006; Hall *et al.*, 2010) and granulocytes (Riedemann *et al.*, 2004; Zimmermann *et al.*, 2014; 2016). MAP kinase and NF κ B signaling pathways are utilized in the regulation of its production (Gaestel *et al.*, 2009). IL-6 belongs to a family of cytokines that consists of IL-11, leukemia inhibitory

factor, oncostatin M, ciliary neurotropic factor, cardiotrophin-1, cardiotrophin-like related cytokine, neuropoietin and IL-27. The similarity with IL-6 and this cytokine family is the utilization of the transmembrane glycoprotein gp130 in its signal transduction pathway (Gearing *et al.*, 1992; Heinrich *et al.*, 2003; Hirano *et al.*, 1994; Ip *et al.*, 1992; Liu *et al.*, 1992; Pennica *et al.*, 1995; Yin *et al.*, 1993). Activation of JAK/STAT and MAPK pathways can occur through the activation of a membrane-bound IL-6 receptor (IL-6R) and gp130 expressed on the cell surface (termed *classic signaling*) (Heinrich *et al.*, 2003; Scheller *et al.*, 2006). Cells that express IL-6R on the cell surface include macrophages, neutrophils, some T-cells, and hepatocytes (Scheller *et al.*, 2011). Those same pathways can also be activated by a soluble IL-6R in conjunction with gp130 in cells that have no endogenous IL-6R expressed on their surface (termed *trans-signaling*) (Scheller *et al.*, 2006; Scheller *et al.*, 2011; Rose-John, 2012). Trans-signaling has been demonstrated in chronic inflammatory diseases such as colon cancer, colitis, and peritonitis (Atreya *et al.*, 2000; Hurst *et al.*, 2001). IL-6 regulates endometrial tissue growth, bone formation, and hormone synthesis (Naitoh *et al.*, 1988; Spangelo *et al.*, 1989). Elevation of IL-6 can be seen in inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, psoriasis, and Crohn's disease (Gabay, 2006). High IL-6 levels have been shown in patients with carcinomas such as breast, lung, and lymphoma (Hong *et al.*, 2007). Elevation of IL-6 levels correlates with tumor progression (Culig *et al.*, 2005; Nilsson *et al.*, 2005).

Butyltins (BTs) are man-made organotins that include tributyltin (TBT) and its metabolites dibutyltin (DBT) and monobutyltin (MBT). TBT and DBT have been used in a range of industrial and consumer applications, thus leading to environmental contamination (Kimbrough, 1976; Tanabe *et al.*, 1998; Roper, 1992; Gipperth, 2009; Takahashi *et al.*, 1999; Kannan *et al.*, 1995; Forsyth *et al.*, 1992; Forsyth and Jay, 1997; Sadiki *et al.*, 1996). TBT has been used for wood preservation, slime control in paper mills, and antifungal agent in industrial cooling water systems and breweries (Antizar-Ladislao, 2008; Gipperth, 2009; Kannan *et al.*, 1995; Kannan *et al.*, 1999). It has contaminated the marine and fresh water environment due to antifouling paint usage on boats and ships. Although TBT use as a marine antifouling agent was restricted in the United States in the 1980s (Loganathan *et al.*, 2001; Loganathan, 2016) and banned by the International Convention on the Control of Harmful Antifouling Systems on Ships in 2008 (Gipperth, 2009), it is still contaminating the environment due to its chemical stability (Gipperth, 2009) and use by those who do not acknowledge the ban. As a result, TBT has been found in human blood samples (ranging as high as 261 nM) and other tissues through the consumption of fish and other foods (Antizar-Ladislao, 2008; Kannan *et al.*, 1995; Kannan *et al.*, 1999; Whalen *et al.*, 1999). It is also found in some household goods such as siliconized-paper baking parchments and shower curtains (Yamada *et al.*, 1993). Mammals with TBT exposure show increased incidences of tumors (Wester *et al.*, 1990).

DBT is used as a stabilizer in polyvinyl chloride (PVC) plastics such as pipes that distribute drinking water (Forsyth *et al.*, 1992; Forsyth and Jay, 1997; Sadiki *et al.*, 1996) and some plastic food containers (Nakashima *et al.*, 1990; Yamada *et al.*, 1993). DBT can leach from PVC plastics (Fent, 1996); as a result, it has been found in water samples (Sadiki and Williams, 1999). DBT is also used as de-worming agent in certain poultry and is found in some poultry products (Epstein *et al.*, 1991). Due to these routes of exposure, DBT has been

found in human blood samples ranging as high as 300 nM (Kannan *et al.*, 1999; Whalen *et al.*, 1999).

Both BTs decrease the ability of human NK cells to destroy tumor cells with accompanying decreases in cytotoxic and cell surface protein expression (Dudimah *et al.*, 2007a; Dudimah *et al.*, 2007b; Thomas *et al.*, 2004; Whalen *et al.*, 2002). In addition, both TBT and DBT inhibit and stimulate the secretion of tumor necrosis factor alpha (TNF α), interferon gamma (IFN γ), and IL-1 β from human lymphocytes (depending on the exposure concentration) (Hurt *et al.*, 2013; Lawrence *et al.*, 2015; Brown and Whalen, 2015; Brown *et al.*, 2017). TBT is more potent than DBT in causing these immunomodulatory alterations. The importance of introducing TBT and DBT as stress inducers to the cell environment is to understand whether exposure to BTs disrupt the secretion interleukin 6 (IL-6). Based on the effect of both BTs on TNF α , IFN γ , and IL-1 β (Hurt *et al.*, 2013; Lawrence *et al.*, 2015; Brown and Whalen, 2015; Brown *et al.*, 2017), we hypothesize that exposure to TBT and DBT will alter the ability of human lymphocytes to secrete IL-6.

In this study, increasingly complex preparations of immune cells were examined for the effects of BT exposures on the secretion of IL-6. The preparations studied included: human NK cells, human monocyte-depleted (MD) peripheral blood mononuclear cells (PBMCs) (MD-PBMCs), PBMCs, PBMCs combined with granulocytes (PBMCs+granulocytes), and granulocytes. The use of increasingly reconstituted immune cell preparations allowed us to investigate the influence that various immune cell types may have on the ability of BTs to induce alteration in secretion of IL-6 which gives a better account for physiological circumstances. This work is significant because any disruption of the cytokines may indicate that TBT and DBT exposures may potentially affect immune competence as well as diseases that are influenced by increased levels of this pro-inflammatory cytokine as mentioned above.

Materials and Methods

Preparation of PBMCs, MD- PBMCs, and Granulocytes

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 30–50 min. Granulocytes and red blood 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 mM L-glutamine and 50 U penicillin G with 50 μ g streptomycin/mL. This preparation constituted PBMCs. PBMCs are composed of 70–90% lymphocytes and 10–30% monocytes. 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 $\times$ 15 mm) at 37 $^{\circ}$ C and air/CO₂, 19:1 for 1.5 h. This cell preparation is referred to as MD-PBMCS cells.

Granulocytes were isolated with the removal of mononuclear and red blood cells as described in Kuijpers *et al.*, 2013. Granulocytes were collected and washed with PBS (2500 rpm, 15min). Red blood cells were lysed with NH_4Cl isotonic solution (155mM NH_4Cl , 10mM KHCO_3 , 0.1 mM EDTA) for 10 min. Cells were then washed with PBS and centrifuged at 800g for 10 min. This preparation was greater than 90% granulocytes. The cells were then suspended in RPMI-1640 complete medium supplemented with 10% heat-inactivated BCS, 2 mM *L*-glutamine and 50 U penicillin G with 50 μg streptomycin/mL.

Preparation of NK cells

NK cells were prepared from buffy coats (from healthy adult donors) purchased from Key Biologics, LLC (Memphis, TN). Highly purified NK cells were prepared using a rosetting procedure. RosetteSep human NK cell enrichment antibody cocktail (0.6–0.8 mL) (StemCell Technologies, Vancouver, British Columbia, Canada) was added to 45 mL of buffy coat. The mixture was incubated for 20 min at room temperature ($\sim 25^\circ\text{C}$). 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 30–50 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 mM *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. The preparation was greater than 85% NK cells.

Chemical Preparation

The organotin compounds tributyltin chloride (TBT) and dibutyltin dichloride (DBT) were purchased from Sigma-Aldrich (96%) (St. Louis, MO). A DBT stock solution was prepared by dissolving DBT in dimethylsulfoxide (DMSO). Desired concentrations of DBT were prepared by dilution of the stock into cell culture media. The final concentration of DMSO for DBT exposures did not exceed 0.01%. Appropriate DMSO controls were run. Desired concentrations of TBT were prepared by dilution of the stock into cell culture media. TBT was a neat standard, dissolved initially in deionized water to give a 1 mM solution.

Cell Treatments

NK cells, MD-PBMCs, PBMCs, granulocytes (at a concentration of 1.5 million cells/mL), or PBMCs+granulocytes (at a concentration of 1.5 million PBMCs/mL + 1.5 million granulocytes/mL) were treated with TBT at concentrations of 2.5–200 nM TBT 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 IL-6.

NK cells, MD-PBMCs, PBMCs, granulocytes (at a concentration of 1.5 million cells/mL), or PBMCs+granulocytes (at a concentration of 1.5 million PBMCs/mL + 1.5 million granulocytes/mL) were treated with DBT 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 IL-6.

$\text{TNF}\alpha$ and IL- 1β were tested for their ability to stimulate secretion of IL-6. MD-PBMCs were treated with $\text{TNF}\alpha$ or IL- 1β (10,000 pg/mL, 1000 pg/mL and 100 pg/mL) for 24 h.

Following the incubations supernatants were collected and stored as described above until being tested for IL-6 secretion.

Antibodies to TNF, IL-1 β , and an isotype control (BD-Pharmingen, San Diego, CA) were added to PBMCs 1 h prior to exposing the cells to DBT (0.25–0.05 μ M) for 24 h. Following the incubations, supernatants were collected and stored as described above until assaying for IL-6.

Cell Viability

Cell viability was assessed at the beginning and 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.

IL-6 Secretion Assay

IL-6 levels were measured using the BD OptEIA™ Human IL-6 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 IL-6 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 IL-6 standards were added to the coated plate and incubated for 2 h at room temperature. Following this incubation, the plate was thoroughly washed five times and then incubated for 1h with a detection antibody to IL-6 which was conjugated with horseradish peroxidase. The excess detection antibody was removed by washing seven times and a substrate solution was added for 30 min at room temperature to produce a colored product. The incubation with the substrate 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

Viability of NK cells, MD-PBMCs, PBMCs, PBMCs+ Granulocytes, and Granulocytes exposed to TBT

Table 1 shows the effects of TBT exposures (2.5–200 nM) on the viability of highly purified NK cells, MD-PBMCs, PBMCs, PBMCs+granulocytes, and granulocytes. Exposure of NK

cells to 2.5–100 nM TBT had no effect on their viability as compared to the control after 24 h length of exposure. A slight decrease in NK cell viability was noticed at the highest concentration of TBT (200 nM). Exposure to these same concentrations for 48 h caused some decrease (about 20%) only at the highest concentration of TBT while the remaining concentrations showed no decrease in viability compared to control cells. NK cells exposed to 2.5–50 nM TBT for 6 days showed no change in viability as compared to the control. A significant reduction in viability of 50% was seen with 200 nM TBT exposures. MD-PBMCs showed a very slight decrease in viability with exposure to 200 nM TBT while PBMCs showed significant decreases at 100 nM (about 18%) and 200 nM (about 38%) for 24 h. Both MD-PBMCs and PBMCs showed significant decreases in viability at the highest concentration after 48 h exposure. Significant decreases were also seen after 6 days for MD-PBMCs and PBMCs for TBT concentrations of 100, and 200 nM. Exposure of PBMCs +granulocytes to TBT did not significantly decrease viability compared to control cells after any length of incubation. Exposure to 200 nM TBT for 48 h and 6 days significantly diminished the viability of granulocytes.

Viability of NK cells, MD-PBMCs, PBMCs, PBMCs+ Granulocytes, and Granulocytes exposed to DBT

Table 2 shows the effects of DBT exposures (0.05–5 μ M) on the viability of the various immune cell preparations. NK cells exposed to DBT for 48 h showed significant reductions in viability (around 35%) at 2.5 and 5 μ M. No statistically significant change in viability as compared to control cells was seen after 6 days of exposure. MD-PBMCs and PBMCs both showed a significant decrease in viability with exposure to 5 μ M DBT for 24 h. The viability of MD-PBMCs at 48 h was significantly reduced by exposure to 1 (15%), 2.5 (40%), and 5 μ M (41%) DBT. After 6 days of exposure 0.5 μ M DBT also caused significant reductions in viability of MD-PBMCs. The viability of PBMCs+granulocytes and granulocytes alone was not significantly affected by exposure to DBT.

Effects of TBT Exposure on Secretion of IL-6 by NK cells

Table 3 shows the effects of TBT (0, 2.5, 5, 10, 25, 50, 100, and 200 nM) exposure on IL-6 secretion from NK cells. Cells from 4 different donors were exposed to TBT for 24 h, 48 h, and 6 days (KB=Key Biologic buffy coat). All donors showed significant decreases in IL-6 secretion when exposed to 100 and 200 nM TBT at 24 h, 48 h, and 6 days. Figure 1A shows the decreases from a representative experiment (KB169).

Effects of TBT Exposure on Secretion of IL-6 by MD-PBMCs

Table 4 summarizes the effects of exposing MD-PBMCs from 4 individual donors to TBT on IL-6 secretion (F=filter obtained from the Red Cross). Significant decreases in IL-6 secretion were seen in cells from all donors at 200 nM TBT. Additionally, cells from 3 of 4 donors showed an increase in secretion after exposure for 24 h, 48 h, or 6 days at one or more concentration of TBT. The maximum increase of IL-6 in cells from one donor was 1.34 fold (F137 at 2.5 nM after 48 h). Figure 1B shows the effects of TBT exposures for an individual donor (F156).

Effects of TBT Exposure on Secretion of IL-6 by PBMCs

The effects of exposures to TBT on secretion of IL-6 from PBMCs from 4 donors are shown in Table 5a. All donors showed significant decreases in IL-6 secretion after 24 h and 6 days of exposure to 200 nM TBT. Cells from each of the donors showed increased secretion of IL-6 at one or more TBT exposure after 24 and 48 h. The range of maximum increases after 24 h was 1.1 fold (F252, 2.5 nM), 1.4 fold (F257, 50 nM), 1.4 fold (F261, 2.5 nM), and 2.4 fold (F264, 2.5 nM). The effects of TBT exposures for a representative experiment are shown in Figure 1C (F261).

Effects of TBT Exposure on Secretion of IL-6 by PBMCs + Granulocytes

Effects of exposing PBMCs+granulocytes (prepared from the same 4 donors as PBMCs) to TBT on IL-6 secretion are shown in Table 5b. Cells from 3 of the 4 donors showed a significant decrease in IL-6 secretion at 200 nM TBT after either 24 h or 48 h of exposure, with 3 of the 4 donors showing a significant decrease at this concentration after 48 h. As with PBMCs alone, the combination of PBMCs+granulocytes also showed TBT-induced increases in IL-6 secretion in cells from all donors at each length of exposure. However, TBT-induced increases were of the greatest magnitude after 6 days (all donors) (11.8 fold for F252, 6.8 fold for F257, 7.5 fold for F261, 18.4 fold for F264). The increases are shown from an individual donor in Figure 1D (F261).

Effects of TBT Exposure on Secretion of IL-6 by Granulocytes

The effects of exposures to TBT on secretion of IL-6 were examined in granulocytes prepared from the same 4 donors as were PBMCs (results shown in Table 5c). All donors showed significant decreases of IL-6 secretion at 200 nM TBT after 24 h length of exposure. Significant increases in IL-6 secretion were seen at TBT levels ranging from 2.5–100 nM after 24 h and TBT levels ranging from 5–50 nM after 48 h for all donors. The fold increases seen after 48 h exposures to 5 nM TBT were 273 (F252), 8 (F257), 3.5 (F261), and 1.1 (F264). Minimal IL-6 secretion was noted after 6 days of exposure. However, 2.5 nM TBT induced substantial increases in the granulocytes from each of the donors after 6 days of exposure (2.8 fold for F252, 9.6 fold for F257, 4.8 fold for F261, and 3.5 fold for F264). Figure 1E shows the effects of TBT exposures from an individual donor (F261).

Effects of DBT Exposure on Secretion of IL-6 by NK cells

The effects of DBT (0, 0.05, 0.1, 0.25, 0.05, 1, 2.5 and 5 μ M) exposures on IL-6 secretion from NK cells are shown in Table 6. Cells from 3 different donors were exposed to DBT for the same lengths of exposure as were used in the TBT studies. All donors showed significant decreases in IL-6 secretion when exposed to 0.5, 1, 2.5, and 5 μ M DBT at all lengths of exposure. Additionally, all donors showed increased IL-6 secretion with exposures to one or more concentration of DBT in the range of 0.05 to 0.25 μ M DBT after 24 h and/or 48 h exposures. The increases were rather modest ranging from 1.2–1.5 fold. Figure 2A shows the effects of DBT exposures at each length of exposure for a representative experiment (KB169).

Effects of DBT Exposure on Secretion of IL-6 by MD-PBMCs

Table 7 shows the effects of exposing MD-PBMCs to DBT on IL-6 secretion. Cells from 5 different donors were exposed to DBT. All donors showed significant decreases at 2.5 and 5 μM DBT at all lengths of exposure. Additionally, all donors showed DBT-induced increases in secretion at one or more length of exposure with concentrations of DBT in the range of 0.5–0.25 μM . The effects of DBT exposures for a representative experiment are shown in Figure 2B (F152).

Effects of DBT Exposure on Secretion of IL-6 by PBMCs

Table 8a summarizes the effects of exposing PBMCs from 4 individual donors to DBT on IL-6 secretion. Significant decreases were seen at the 5 μM concentration in cells from each of the donors at all lengths of exposure. Cells from the vast majority of donors also showed very significant decreases with exposures to 1 and 2.5 μM DBT. Significant increases were seen at lower concentrations of DBT (0.05 to 0.5 μM) in cells from all donors after 48 h and 6 day exposures. Cells from 3 of the 4 donors also showed increases after 24 h of exposure. Figure 2C shows the effects of DBT exposures from an individual donor (F285).

Effects of DBT Exposure on Secretion of IL-6 by PBMCs + Granulocytes

Effects of exposing PBMCs+granulocytes (prepared from the same 4 donors as PBMCs) to DBT on IL-6 secretion are shown in Table 8b. After 24 and 48 h of exposure, all donors showed significant decreases in IL-6 secretion at 2.5 and 5 μM DBT. All lengths of exposure displayed significant increases at 0.25 μM DBT for all donors. Figure 2D shows the effects of TBT exposures for an individual donor (F285).

Effects of DBT Exposure on Secretion of IL-6 by Granulocytes

The effects of exposures to DBT on secretion of IL-6 were examined in granulocytes prepared from the same 4 donors as were PBMCs (results shown in Table 8c). Cells from all donors showed significant decreases in IL-6 secretion at 2.5 and 5 μM DBT after 24 h and 48 h exposures. However, significant increases were seen at DBT concentrations of 0.05, 0.1, and 0.5 μM for 3 of the 4 donors after 24 h of exposure. Cells from the fourth donor (F294) showed a small but significant increase in IL-6 secretion after 48 h. Cells from 3 of the 4 donors continued to show DBT-stimulated increases in IL-6 secretion after 6 days while the secretion from control cells was nonexistent or very low. Cells from the fourth donor (F294) showed no IL-6 secretion under any condition after 6 days. Figure 2E shows the effects of TBT exposures for an individual donor (F285).

Ability of TNF α and IL-1 β to stimulate IL-6 secretion

The effects of TNF α to elicit secretion of IL-6 from MD-PBMCs were examined. Figure 3A shows that incubation of MD-PBMCs for 24 h with 10,000 pg/mL of TNF α increased the secretion of IL-6 by 2.5 fold. When the amount of TNF α was 1000 pg/mL the fold increase in IL-6 secretion was 1.3 fold. When MD-PBMCs were treated with 1000 pg/mL of IL-1 β for 24 h there was a 2 fold increase in IL-6 secretion. A 24 h exposure of the cells to 100 pg/mL IL-1 β caused a 1.4 fold increase (Figure 3B). Antibodies to TNF α (TA β), IL-1 β (IA β) and an isotype control (CA β) were added to PBMCs prior to exposure to DBT in order to

see if DBT-induced secretion of either of these cytokines was responsible for a portion of the increase seen with BT exposure. As seen in Figure 3C, there was no decrease in DBT-induced elevation of IL-6 when either TNF α or isotype control antibodies were present. There was a very slight decrease in DBT-induced IL-6 secretion when IL-1 β antibody was present. However, when both TNF and IL-1 β antibodies were present there was no decrease.

Discussion

BTs, TBT and DBT, have been used in a variety of industrial applications such as antifouling paints (TBT) (Gipperth, 2009) and stabilizer of plastics (DBT) (Forsyth *et al.*, 1992; Forsyth and Jay, 1997; Sadiki *et al.*, 1996). Levels of BTs have been found as high as 261 nM TBT (Kannan *et al.*, 1995; Whalen *et al.*, 1999) and 0.3 μ M DBT in human blood (Kannan *et al.*, 1999; Whalen *et al.*, 1999). Both BTs decrease the ability of human NK cells to destroy tumor cells (Dudimah *et al.*, 2007a; Dudimah *et al.*, 2007b; Thomas *et al.*, 2004; Whalen *et al.*, 2002) and alter the secretion of TNF α , IFN γ , and IL-1 β from immune cells (Brown and Whalen, 2015; Brown *et al.*, 2017; Hurt *et al.*, 2013; Lawrence *et al.*, 2015). IL-6 is necessary for normal immune functions (Naitoh *et al.*, 1988; Spangelo *et al.*, 1989); however, elevation of this cytokine can lead to inflammatory diseases and carcinomas (Gabay, 2006; Hong *et al.*, 2007). We investigated whether BTs alter the secretion of IL-6 from immune cells in an *ex vivo* human model. Increasingly complex cell preparations were used (NK cells, MD-PBMCs, PBMCs, PBMC+granulocytes, and granulocytes) to investigate whether certain cell types or combinations of cell types were more susceptible to alterations of IL-6 secretion by either TBT or DBT.

TBT induced both increases and decreases in IL-6 secretion from human immune cells. The highest concentration of TBT (200 nM) decreased IL-6 secretion from all cell preparations at one or more length of incubation. These decreases were seen in NK cells and monocyte-depleted (MD)-PBMCs (predominantly T and NK lymphocytes) from all donors after a 24 h exposure to TBT. However, in PBMCs, PBMCs+granulocytes, and granulocytes alone the length of exposure at which a decrease was seen with exposure to 200 nM TBT varied among cells from different donors. This indicated that the presence of monocytes and granulocytes changed the effectiveness of 200 nM TBT in inhibiting IL-6 secretion. Increases in IL-6 secretion seen with exposures to TBT were consistently seen in MD-PBMCs and PBMCs. However, while TBT induced increases in 3 of 4 donors in NK cells, the length of exposure and concentration of TBT at which the increases occurred varied from donor to donor.

When monocytes and granulocytes were present the TBT-induced decreases in IL-6 became less consistent but TBT-induced increases were seen in all donors at all lengths of exposure. While PBMCs and granulocytes prepared from every donor showed TBT-induced increases in IL-6 secretion, it was the granulocytes that showed the greatest sensitivity to TBT. The baseline secretion of IL-6 is much lower in granulocytes than in PBMCs. For example, PBMCs from donor F257 had a baseline secretion of 123,182 pg/mL at 24 h and a maximal TBT-induced increase in IL-6 secretion of 1.4 fold (50 nM). Granulocytes from this same donor had a baseline secretion of 137 pg/mL with an increase of 3.7 fold in IL-6 secretion induced by 2.5 nM TBT. This same pattern was seen across donors. Thus, results of this

study indicate that higher concentrations of TBT are most effective at decreasing IL-6 secretion in cell preparations that do not contain monocytes or granulocytes. Conversely, concentrations of TBT of 100 nM and below are most effective at elevating IL-6 secretion when monocytes and granulocytes are present. Granulocytes are the most sensitive cell type to TBT-induced elevation of IL-6 secretion. As neutrophils are the most abundant white blood cell, the ability of TBT to induce very large increases in their normally low secretion of the important pro-inflammatory cytokine, IL-6, could lead to very significant disruption of the immune response leading to unwarranted inflammation.

DBT also altered IL-6 secretion from each of the immune cell preparations that were examined. The two highest concentrations of DBT (5 and 2.5 μ M) induced a nearly complete blockage of IL-6 secretion from NK cells, MD-PBMCs, PBMCs, PBMCs +granulocytes, and granulocytes. These DBT concentrations caused some decrease in viability, but the decrease in IL-6 secretion was far greater than any decreases seen in viability at any of the time points or concentrations. Lower concentrations of DBT (ranging from 0.05 to 0.5 μ M) induced elevation of IL-6 secretion in NK cells, MD-PBMCs, PBMCs, PBMCs+granulocytes and granulocytes at one or more length of incubation. As was seen with TBT, the length of exposure and the concentration of DBT at which the increases in IL-6 occurred in NK cells varied from donor to donor. Past studies have shown that DBT (at lower concentrations) is able to increase both IL-1 β and TNF α secretion from human immune cell preparations that contain T-lymphocytes and monocytes (Hurt *et al.*, 2013; Brown *et al.*, 2017). Again, similar to TBT, the increases induced by DBT exposures occurred most consistently when monocytes were present. As mentioned above, granulocyte secretion of IL-6 was more sensitive to TBT-induced elevation than were other cell types. This was not the case for DBT. For instance, PBMCs from donor F294 showed an increase in IL-6 secretion of 2 fold when exposed to 0.25 μ M DBT for 24 h. PBMCs+granulocytes from this same donor showed only a 1.2 fold increase under the same conditions and granulocytes alone showed no increase. Thus, unlike the results seen with TBT, DBT appears to be most effective at increasing IL-6 elevation from monocytes rather than granulocytes.

It is known that other cytokines such as IL-1 β and TNF α are able to stimulate IL-6 production in a variety of cells including skeletal muscle cells (Luo *et al.*, 2003; McGee *et al.*, 1995; Ostrowski *et al.*, 1998b; Ostrowski *et al.*, 1998a; Pedersen *et al.*, 2001; Tilg *et al.*, 1997; Tosato and Jones, 1990). Monocyte production of IL-6 is stimulated by IL-1 β (Tosato and Jones, 1990). Thus, we investigated whether BT-stimulated T lymphocyte (or monocyte) production of one of these cytokines may be responsible for the increase in IL-6. Previously, we have shown that TBT and DBT are able to induce elevations in MD-PBMC production of both TNF α and IL-1 β (Hurt *et al.*, 2013; Lawrence *et al.*, 2015; Brown and Whalen 2015). Thus, either TNF α and/or IL-1 β secreted by monocytes or T lymphocytes in response to either TBT or DBT could explain, at least in part, the BT-induced increases in IL-6 secretion from immune cells seen in this study. Here we showed that when cells were treated with either TNF α or IL-1 β ("addition experiments") there was a significant increase in IL-6 production by these cells, IL-1 β was more effective than TNF α in its ability to stimulate this increase. However, there was no major loss of BT-induced increases in IL-6 when antibodies to IL-1 β or TNF α were added prior to exposure to DBT. Thus, while the "addition

experiments” suggest that IL-1 β and/ or TNF α might be in part responsible for the increases in IL-6 secretion stimulated by BTs, the results of experiments using antibodies to diminish levels of DBT-induced IL-1 β or TNF α did not corroborate those results. However, there are complications in the antibody experiments that could obscure the interpretation of the results. For instance, the incubation conditions allow for the cells to become closely packed (due to settling in the tube) and this might limit access of the antibody to areas where high concentrations of secreted IL-1 β and TNF α would occur. Thus, the competition between these cytokines’ receptors on the cell surface and the antibody for DBT-generated IL-1 β and TNF α might greatly favor the receptors. Additionally, it has been shown that the receptors for TNF generally have significantly higher affinities for the cytokine than do many antibodies (Lang *et al.*, 2016; Song *et al.*, 2008).

In addition to their effect on cytokine secretion shown here and in previous studies (Hurt *et al.*, 2013; Brown and Whalen, 2015; Lawrence *et al.*, 2015; Brown *et al.*, 2017), both TBT and DBT have been shown to induce apoptosis of thymocytes *in vitro* (Gennari *et al.*, 1997) and degeneration of the thymus (maturation site of T-cells) in rats (Snoeijs *et al.*, 1988). Comparison of TBT and DBT effects on IL-6 secretion in NK cells from the same donor at the same concentrations (50 and 100 nM) showed that TBT inhibited IL-6 secretion from NK cells by 97.7% at 100 nM whereas DBT inhibited secretion by 9%. When the relative effectiveness of TBT and DBT were compared in MD-PBMCs from the same donor, we found that TBT inhibited IL-6 secretion at 50 or 100 nM while DBT either had no effect or stimulated IL-6 secretion at these same concentrations. The greater effectiveness of TBT over DBT has been seen previously when comparing the effects of TBT and DBT on IL-1 β secretion (Brown *et al.*, 2017). Taken together, these comparisons indicate that DBT may be generally less effective at inducing an increase in both IL-6 and IL-1 β secretion than is TBT. This may be due to the fact that DBT is less hydrophobic than TBT due to the loss of the third butyl group (Laughlin *et al.*, 1986; Harper, 2005) and is less able to enter the cell to bind to intracellular targets (such as MAPKs (Brown *et al.*, 2017) that appear to be involved in the compound-induced increases in IL-1 β secretion which appear to be at least in part responsible for inducing increases in IL-6 as discussed above. This study also suggests that DBT has a lower affinity for those intracellular components that are responsible for causing decreased IL-6 secretion and thus is somewhat less effective at inducing that effect.

A previous study demonstrated that DBT alters IL-1 β secretion from increasingly reconstituted preparations of human immune cells (Brown *et al.*, 2017). As was seen with IL-6 secretion in the current study, higher concentrations of DBT (5 and 2.5 μ M) decreased the secretion of IL-1 β , while lower concentrations (0.1 and 0.05 μ M) increased the secretion of IL-1 β . However, in contrast to the effects of DBT on IL-6 secretion, the secretion of IL-1 β was increased when NK cells were exposed to lower concentrations of DBT for at each length of incubation. As mentioned above, there was no consistent increase in secretion of IL-6 by NK cells in response to DBT exposures. Additionally, the greater sensitivity of granulocytes to TBT-induced increases in IL-6 secretion that was seen in the current study did not occur when looking at granulocyte secretion of IL-1 β (Brown and Whalen, 2015).

These results suggest that TBT and DBT-induced alterations of IL-6 secretion as well as other pro-inflammatory cytokines from immune cells may potentially affect immune

function and cancer invasiveness. Monobutyltin (MBT), which can be a breakdown product of both TBT and DBT, was not examined in this study as it showed very limited effects on immune cell function in previous studies (Whalen *et al.*, 1999) and no effects at levels measured in human blood (Kannan *et al.*, 1999). The levels of TBT and DBT found in human blood samples are in the range of TBT and DBT concentrations that elevated IL-6 levels. The fact that both of these compounds are able to alter IL-6 secretion suggests that at higher concentrations they may decrease immune competency. Lower concentrations of either TBT or DBT may lead to increased levels of IL-6 which could produce chronic inflammation. Increased inflammation in the absence of a microorganism is referred to as “sterile inflammation” (Chen and Nunez, 2010). Compound such as TBT and DBT may have the capacity to induce sterile inflammation by their ability to increase the production of pro-inflammatory cytokines such as IL-6 and other pro-inflammatory cytokines (Hurt *et al.*, 2013; Brown and Whalen, 2015; Lawrence *et al.*, 2015; Brown *et al.*, 2017). The results of this study, as well as previous studies, indicate that these two compounds may be more of a problem in terms of causing chronic inflammation rather than loss of immune competence in exposed individuals due to the fact that a large percentage of the individuals whose blood levels were measured had levels in the range where effects on IL-6 secretion were seen *ex vivo* (Whalen *et al.*, 1999; Kannan *et al.*, 1999). Interestingly, a rise in Rheumatoid arthritis (Myasoedova *et al.*, 2010) (one of the several diseases where IL-6 is elevated) has been noted over the period of 1955–2007 as the use of compounds such as TBT and DBT with their concomitant contamination of the human system has been increasing.

In summary, both TBT and DBT are able to alter the secretion of IL-6 in a range of human immune cell preparations. Both compounds decrease the secretion of IL-6 at the highest concentration (TBT=200 nM, DBT= 5 and 2.5 μ M) from NK cells, MD-PBMCs, PBMCs, PBMCs+granulocytes, and granulocytes alone. TBT (50–2.5 nM) and DBT (0.5–0.05 μ M) are also able to increase the secretion of IL-6 at lower exposure levels and this effect is most consistent when either T lymphocytes or monocytes are present. The cell type that is most sensitive to TBT-induced increases in IL-6 are granulocytes. Cell preparations containing T lymphocytes and/or monocytes were the most sensitive to DBT-induced elevation of IL-6 secretion.

Acknowledgments

Grants U54CA163066 and 4T34GM007663 from the National Institutes of Health

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Figure 1.

Effects of 24 h, 48 h and 6 day exposures to TBT on IL-6 secretion from highly purified human NK cells, monocyte-depleted PBMCs, PBMCs, PBMCs plus granulocytes, and granulocytes in individual donors. A) NK cells exposed to 0–200 nM TBT (donor KB169). B) Monocyte-depleted PBMCs exposed to 0–200 nM TBT (donor F156). C) PBMCs exposed to 0–200 nM TBT (donor F261). D) PBMCs plus granulocytes exposed to 0–200 nM TBT (donor F261). E) Granulocytes exposed to 0–200 nM TBT (donor F261). * Indicates a significant decrease in secretion and + indicates a significant increase in secretion compared to control cells (cells treated with vehicle alone).

Figure 2.

Effects of 24 h, 48 h and 6 day exposures to DBT on IL-6 secretion from highly purified human NK cells, monocyte-depleted PBMCs, PBMCs, PBMCs plus granulocytes, and granulocytes in individual donors. A) NK cells exposed to 0–5 μM DBT (donor KB169). B) Monocyte-depleted PBMCs exposed to 0–5 μM DBT (donor F152). C) PBMCs exposed to 0–5 μM DBT (donor F285). D) PBMCs plus granulocytes exposed to 0–5 μM DBT (donor F285). E) Granulocytes exposed to 0–5 μM DBT (donor F285). * Indicates a significant decrease in secretion and + indicates a significant increase in secretion compared to control cells (cells treated with vehicle alone).

Figure 3.

Effects of TNF α and IL-1 β on secretion of IL-6. A) Cells exposed to 10,000 and 1000 pg/mL TNF α for 24 h. B) Cells exposed to 1000 and 100 pg/mL IL-1 β for 24 h. C) TNF α antibody (TAb), IL-1 β antibody (IAb), and an isotype control antibody (CAb) were added to cells 1 h prior to exposure to 0.25 μ M DBT for 24 h. Data are from representative experiment and results were repeated in cells from additional donors. ⁺ Indicates a significant increase in secretion compared to control cells (cells treated with vehicle alone).

Table 1

Percent viability of NK cells, Monocyte-depleted PBMCs, PBMCs, and Granulocytes exposed to TBT for 24 h, 48 h, and 6 days.

24 h exposure	Percent Viable Cells					
	[TBT] nM	NK	monocyte-depleted PBMCs	PBMCs with Granulocytes	Granulocytes	
48h exposure	0	95±3	97±2	98±1	97±2	98±1
	2.5	99±1	96±1	98±2	96±4	95±2
	5	98±1	97±2	95±2	96±1	97±1
	10	97±0	97±2	93±3	94±3	95±2
	25	98±1	95±4	95±3	97±2	95±1 *
	50	96±2	94±5	86±5 *	95±3	94±1 *
	100	96±3	97±2	80±4 *	98±0	96±2
	200	89±2 *	89±1 *	61±3 *	91±8	91±5
	0	93±3	95±3	95±2	97±2	98±3
	2.5	96±2	96±2	95±4	97±3	96±4
6 day exposure	5	96±4	97±3	97±3	92±5	95±4
	10	96±1	97±2	92±3	97±4	97±3
	25	95±1	96±3	93±1	95±5	95±3
	50	94±4	93±7	89±7	95±3	96±4
	100	94±5	90±10	76±7 *	92±5	91±5
	200	72±18	79±5 *	49±5 *	82±13	79±7 *
	0	68±15	80±8	89±6	75±8	92±3
	2.5	75±3	87±7	89±8	76±13	89±7
	5	75±6	87±8	83±14	76±2	91±2
	10	70±15	83±8	85±5	82±6	91±4
	25	68±5	85±12	88±7	84±8	93±3
	50	64±9	76±16	69±10	73±14	92±4

Percent Viable Cells						
24 h exposure	[TBT] nM	NK	monocyte-depleted PBMCs	PBMCs	PBMCs with Granulocytes	Granulocytes
	100	47±10	67±15	48±11 *	59±12	78±13
	200	34±6 *	44±20 *	50±9 *	48±20 *	62±2 *

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

* Indicates a significant decrease in viability compared to control cells (cells treated with vehicle alone), $p<0.05$

Table 2

Percent viability of NK cells, Monocyte-depleted PBMCs, PBMCs combined with Granulocytes, and Granulocytes exposed to DBT for 24 h, 48 h, and 6 days.

24 h exposure	Percent Viable Cells					Granulocytes
	[DBT] μ M	NK	monocyte-depleted PBMCs	PBMCs	PBMCs with Granulocytes	
48 h exposure	0	92 $\pm$ 6	94 $\pm$ 3	98 $\pm$ 1	98 $\pm$ 1	97 $\pm$ 2
	0.05	97 $\pm$ 3	98 $\pm$ 2	95 $\pm$ 4	99 $\pm$ 2	97 $\pm$ 2
	0.1	98 $\pm$ 2	98 $\pm$ 1	97 $\pm$ 3	98 $\pm$ 2	96 $\pm$ 4
	0.25	98 $\pm$ 1	97 $\pm$ 1	97 $\pm$ 1	99 $\pm$ 1	96 $\pm$ 3
	0.5	95 $\pm$ 4	91 $\pm$ 8	97 $\pm$ 4	98 $\pm$ 1	93 $\pm$ 8
	1	92 $\pm$ 5	92 $\pm$ 4	90 $\pm$ 10	94 $\pm$ 7	95 $\pm$ 4
	2.5	88 $\pm$ 1	74 $\pm$ 15 *	80 $\pm$ 17	88 $\pm$ 10	93 $\pm$ 5
	5	80 $\pm$ 7	80 $\pm$ 9 *	77 $\pm$ 8 *	93 $\pm$ 7	93 $\pm$ 5
	0	97 $\pm$ 1	94 $\pm$ 2	98 $\pm$ 2	98 $\pm$ 2	95 $\pm$ 3
	0.05	97 $\pm$ 0	96 $\pm$ 2	95 $\pm$ 4	96 $\pm$ 4	94 $\pm$ 6
6 day exposure	0.1	96 $\pm$ 1	97 $\pm$ 1 *	97 $\pm$ 4	96 $\pm$ 3	93 $\pm$ 7
	0.25	93 $\pm$ 0 *	96 $\pm$ 2	97 $\pm$ 2	95 $\pm$ 5	93 $\pm$ 6
	0.5	90 $\pm$ 1 *	94 $\pm$ 3	96 $\pm$ 3	95 $\pm$ 5	89 $\pm$ 9
	1	79 $\pm$ 12	80 $\pm$ 11 *	86 $\pm$ 3 *	92 $\pm$ 7	92 $\pm$ 3
	2.5	61 $\pm$ 6 *	56 $\pm$ 20 *	74 $\pm$ 20	81 $\pm$ 15	91 $\pm$ 6
	5	64 $\pm$ 8 *	55 $\pm$ 10 *	85 $\pm$ 21	85 $\pm$ 13	87 $\pm$ 10
	0	70 $\pm$ 15	87 $\pm$ 8	88 $\pm$ 14	76 $\pm$ 19	64 $\pm$ 33
	0.05	75 $\pm$ 8	89 $\pm$ 8	84 $\pm$ 15	71 $\pm$ 24	62 $\pm$ 40
	0.1	78 $\pm$ 7	86 $\pm$ 7	81 $\pm$ 21	77 $\pm$ 21	64 $\pm$ 36
	0.25	74 $\pm$ 9	81 $\pm$ 13	78 $\pm$ 14	69 $\pm$ 26	62 $\pm$ 37
	0.5	68 $\pm$ 9	67 $\pm$ 13 *	74 $\pm$ 25	61 $\pm$ 27	50 $\pm$ 22

Percent Viable Cells						
24 h exposure	[DBT] μ M	NK	monocyte-depleted PBMCs	PBMCs	PBMCs with Granulocytes	Granulocytes
	1	69 $\pm$ 3	52 $\pm$ 11 *	70 $\pm$ 25	60 $\pm$ 23	53 $\pm$ 31
	2.5	58 $\pm$ 7	47 $\pm$ 9 *	69 $\pm$ 23	47 $\pm$ 30	54 $\pm$ 28
	5	49 $\pm$ 19	52 $\pm$ 8 *	71 $\pm$ 22	56 $\pm$ 22	47 $\pm$ 23

Values are mean $\pm$ S.D. of triplicate determinations.

* Indicates a significant decrease in viability compared to control cells (cells treated with vehicle alone), $p < 0.05$

Table 3

Effects of 24 h, 48 h, 6 day exposures to TBT on IL-6 secretion from highly purified human NKs.

24 h Interleukin 6 secreted in pg/mL (mean±S.D.)				
[TBT] nM	KB166	KB167	KB168	KB169
0	4685±174	1618±31	12391±260	7374±250
2.5	5236±302	1375±109 *	8175±78 *	8893±785
5	4930±229	1141±31 *	8138±263 *	8004±688
10	4764±4	990±16 *	7777±277 *	8008±1380
25	4700±148	646±32 *	6996±206 *	5795±402 *
50	4405±124	419±24 *	5052±97 *	4393±278 *
100	2935±89 *	ND *	135±42 *	170±63 *
200	84±241 *	ND *	ND *	ND *

48 h Interleukin 6 secreted in pg/mL (mean±S.D.)				
[TBT] nM	KB166	KB167	KB168	KB169
0	4424±92	2139±61	16394±501	24580±941
2.5	4616±982	3934±204 +	13359±88 *	23221±1332
5	3991±464	3635±611 +	12967±466 *	20260±589 *
10	3745±260 *	1092±136	12166±341	23077±201 *
25	3527±190 *	1199±128 *	9540±156 *	11293±437 *
50	3682±329 *	1438±108 *	6342±149 *	345±34 *
100	2432±63 *	45±64 *	1201±56 *	ND *
200	29±10 *	357±171 *	96±44 *	ND *

6 day Interleukin 6 secreted in pg/mL (mean±S.D.)				
[TBT] nM	KB166	KB167	KB168	KB169
0	4030±200	1635±35	15482±575	13027±592
2.5	8341±396 +	2194±140 +	1710±132 *	12893±1138 *
5	7310±138 +	3036±177 +	14510±2402	10243±509 *
10	8299±179 +	1541±201	19291±920 +	9154±516 *
25	4780±17 +	2160±65 +	8625±231 *	8232±1225 *
50	3791±63	672±82 *	1920±99 *	5871±269 *
100	3291±76 *	ND *	1710±33 *	477±33 *
200	ND *	ND *	15463±297	ND *

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

* Indicates a significant decrease in secretion and

+ indicates a significant increase in secretion compared to control cells (cells treated with vehicle alone), p<0.05 ND = not detectable.

Table 4

Effects of 24 h, 48 h, 6 day exposures to TBT on IL-6 secretion from monocyte-depleted PBMCs.

24 h Interleukin 6 secreted in pg/mL (mean±S.D.)				
[TBT] nM	F137	F142	F145	F156
0	1495±113	64607±3102	8647±309	36151±711
2.5	1171±57 *	39411±9167 *	6675±42 *	42362±2282 ⁺
5	1105±72 *	38274±8450 *	6763±474 *	39758±2410
10	1072±49 *	47239±4826 *	6814±164 *	45813±1123 ⁺
25	1011±70 *	55389±2496 *	6689±508 *	39288±1090 ⁺
50	809±57 *	45285±2781 *	5680±313 *	41484±1238 ⁺
100	758±86 *	47883±1614 *	4897±157 *	40276±777 ⁺
200	241±168 *	2285±72 *	ND *	6958±796 *

48 h Interleukin 6 secreted in pg/mL (mean±S.D.)				
[TBT] nM	F137	F142	F145	F156
0	7059±349	94610±2681	11822±509	56627±1086
2.5	9471±661 ⁺	96509±397	7847±87 *	54410±1896
5	6646±447	82913±2441 *	6827±205 *	67550±3147 ⁺
10	7533±1649	80267±3758 *	7673±615 *	51100±1749 *
25	6844±506	77903±424 *	7021±201 *	55759±2608
50	6386±239	72954±2077 *	7026±46 *	71502±6127 ⁺
100	3567±436 *	68994±1794 *	7200±313 *	53157±7680
200	ND *	3337±455 *	ND *	11518±501 *

6 day Interleukin 6 secreted in pg/mL (mean±S.D.)				
[TBT] nM	F137	F142	F145	F156
0	223±6	46608±1494	9920±489	20362±315
2.5	165±17 *	57987±1806 ⁺	8187±347 *	22137±1585
5	116±23 *	59399±7568	7781±305 *	21855±1738
10	102±8 *	57118±493 ⁺	6417±445 *	25086±288 ⁺
25	106±17 *	57137±272 ⁺	7035±200 *	22047±361 ⁺
50	84±3 *	55719±981 ⁺	6472±421 *	24310±479 ⁺
100	32±11 *	49993±753 ⁺	4787±248 *	19560±792
200	ND *	6379±131 *	ND *	2515±240 *

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

* Indicates a significant decrease in secretion and

⁺ indicates a significant increase in secretion compared to control cells (cells treated with vehicle alone), p<0.05 ND = not detectable.

Table 5

a. Effects of 24 h, 48 h, 6 day exposures to TBT on IL-6 secretion from PBMCs.				
24 h Interleukin 6 secreted in pg/mL (mean±S.D.)				
[TBT] nM	F252	F257	F261	F264
0	107735±4735	123182±4413	108921±458	46005±308
2.5	119503±1019 ⁺	157545±2460 ⁺	156436±1916 ⁺	109857±1151 ⁺
5	106508±3257	160273±656 ⁺	130376±688 ⁺	90252±1561 ⁺
10	89450±4474 [*]	158333±1001 ⁺	102073±6556	80770±2100 ⁺
25	1365±220 [*]	150697±105 ⁺	119042±2900 ⁺	86894±964 ⁺
50	100201±8444	172636±10351 ⁺	106618±315 [*]	83240±4790 ⁺
100	12540±1176 [*]	120030±1211	55527±793 [*]	90844±901 ⁺
200	1386±477 [*]	ND [*]	ND [*]	ND [*]
48 h Interleukin 6 secreted in pg/mL (mean±S.D.)				
[TBT] nM	F252	F257	F261	F264
0	86054±3721	159936±402	189796±3587	141±370
2.5	112033±8360 ⁺	177617±1486 ⁺	118288±5352 [*]	104303±210 ⁺
5	112825±3112 ⁺	199994±2634 ⁺	200393±321 ⁺	98768±3031 ⁺
10	114158±1589 ⁺	198545±1328 ⁺	192884±1895	94040±1152 ⁺
25	79846±6771	219299±7449 ⁺	211481±5805 ⁺	99091±1283 ⁺
50	10596±1016 ⁺	233965±1424 ⁺	205656±8477	159333±1266 ⁺
100	ND [*]	155183±1086 ⁺	98358±3466 [*]	147333±1748 ⁺
200	ND [*]	2371±822 [*]	ND [*]	52990±573 ⁺
6 day Interleukin 6 secreted in pg/mL (mean±S.D.)				
[TBT] nM	F252	F257	F261	F264
0	143040±5912	164368±443	170226±2050	66006±1860
2.5	124754±5201 [*]	182069±2296 ⁺	165820±2790	77252±1936 ⁺
5	131230±4148 [*]	149609±939 [*]	199386±1616 ⁺	81020±2811 ⁺
10	150126±4018	197011±2704 ⁺	170226±11833	107049±1158 ⁺
25	156564±12441	212598±522 ⁺	172835±9901	97831±611 ⁺
50	150583±1126	217287±1795 ⁺	173241±8388	131513±1254 ⁺
100	94735±5760 [*]	154345±1202 [*]	38864±2611 [*]	228035±1313 ⁺
200	2011±695 [*]	7816±80 [*]	ND [*]	11223±100 [*]

b. Effects of 24 h, 48 h, 6 day exposures to TBT on IL-6 secretion from PBMCs plus Granulocytes.

24 h	Interleukin 6 secreted in pg/mL (mean±S.D.)			
[TBT] nM	F252	F257	F261	F264
0	24852±1323	37539±1932	26205±352	3124±3
2.5	30787±1395 ⁺	44261±1248 ⁺	18660±906 [*]	3107±16
5	38369±748 ⁺	46178±1355 ⁺	24221±203 [*]	3317±22 ⁺
10	37506±157 ⁺	55233±1673 ⁺	26693±593	3155±17
25	47101±460 ⁺	59678±966 ⁺	24774±982	3191±36
50	48068±1024 ⁺	66622±3007 ⁺	26985±1150	3514±69 ⁺
100	68199±958 ⁺	77344±2115 ⁺	40481±2245 ⁺	4951±47 ⁺
200	20591±432 [*]	22733±300 [*]	29652±1043 ⁺	4079±18 ⁺

48 h	Interleukin 6 secreted in pg/mL (mean±S.D.)			
[TBT] nM	F252	F257	F261	F264
0	8475±122	17469±302	3058±96	1085±18
2.5	9798±245 ⁺	23335±325 ⁺	3991±109 ⁺	1112±28
5	13232±1047 ⁺	24021±1180 ⁺	3800±15 ⁺	1183±30 ⁺
10	10586±367 ⁺	35240±198 ⁺	3823±81 ⁺	1216±7 ⁺
25	17818±316 ⁺	23488±540 ⁺	3980±122 ⁺	1385±45 ⁺
50	17101±364 ⁺	42078±994 ⁺	4429±48 ⁺	1642±40 ⁺
100	28636±786 ⁺	52935±293 ⁺	4577±166 ⁺	3264±32 ⁺
200	8182±121 [*]	15926±198 [*]	2188±62 [*]	2387±5 ⁺

6 day	Interleukin 6 secreted in pg/mL (mean±S.D.)			
[TBT] nM	F252	F257	F261	F264
0	280±34	1063±21	13±0	86±3
2.5	219±4	1080±187	16±1 ⁺	345±23 ⁺
5	375±14 ⁺	1159±7 ⁺	16±2	94±12
10	278±51	1782±18 ⁺	26±0 ⁺	64±5 [*]
25	531±4 ⁺	2380±7 ⁺	30±0 ⁺	117±12 ⁺
50	924±27 ⁺	3718±15 ⁺	35±2 ⁺	650±3 ⁺
100	3318±51 ⁺	7225±28 ⁺	97±2 ⁺	1581±31 ⁺
200	355±37	429±37 [*]	65±2 ⁺	347±16 ⁺

c. Effects of 24 h, 48 h, 6 day exposures to TBT on IL-6 secretion from Granulocytes.

24 h	Interleukin 6 secreted in pg/mL (mean±S.D.)			
[TBT] nM	F252	F257	F261	F264
0	122±2	137±11	785±53	137±13
2.5	882±41 ⁺	507±3 ⁺	1214±11 ⁺	460±9 ⁺

c. Effects of 24 h, 48 h, 6 day exposures to TBT on IL-6 secretion from Granulocytes.

24 h	Interleukin 6 secreted in pg/mL (mean±S.D.)			
[TBT] nM	F252	F257	F261	F264
5	1044±13 ⁺	300±5 ⁺	1238±42 ⁺	326±25 ⁺
10	963±30 ⁺	313±20 ⁺	1515±25 ⁺	309±23 ⁺
25	315±20 ⁺	219±34 ⁺	948±10 ⁺	209±2 ⁺
50	299±35 ⁺	161±1	1304±21 ⁺	516±19 ⁺
100	229±23 ⁺	162±6 ⁺	1306±17 ⁺	339±16 ⁺
200	ND [*]	ND [*]	206±22 [*]	ND [*]

48 h	Interleukin 6 secreted in pg/mL (mean±S.D.)			
[TBT] nM	F252	F257	F261	F264
0	6±6	110±15	87±14	1292±25
2.5	733±20 ⁺	949±12 ⁺	382±0 ⁺	1329±38
5	1635±15 ⁺	885±35 ⁺	305±27 ⁺	1428±42 ⁺
10	1380±15 ⁺	1260±12 ⁺	297±13 ⁺	1474±9 ⁺
25	365±6 ⁺	1110±51 ⁺	201±8 ⁺	1710±62 ⁺
50	394±19 ⁺	635±25 ⁺	203±2 ⁺	2068±55 ⁺
100	274±5 ⁺	163±13 ⁺	80±8 [*]	4328±45 ⁺
200	ND [*]	ND [*]	ND [*]	3106±6 ⁺

6 day	Interleukin 6 secreted in pg/mL (mean±S.D.)			
[TBT] nM	F252	F257	F261	F264
0	9±0	5±1	6±1	4±1
2.5	25±1 ⁺	48±1 ⁺	29±1 ⁺	14± ⁺
5	22±1 ⁺	53±1 ⁺	9±2	7±1 ⁺
10	12±1 ⁺	18±1 ⁺	9±1 ⁺	9±2 ⁺
25	10±0 ⁺	8±0 ⁺	8±1	8±1 ⁺
50	10±1	9±1 ⁺	7±2	8±1 ⁺
100	9±1	8±1 ⁺	10±1 ⁺	6±1
200	8±0 [*]	4±0	8±1 ⁺	4±1

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

* Indicates a significant decrease in secretion and

⁺ indicates a significant increase in secretion compared to control cells (cells treated with vehicle alone), p<0.05 ND = not detectable.

Table 6

Effects of 24 h, 48 h, 6 day exposures to DBT on IL-6 secretion from highly purified human NKs.

24 h Interleukin 6 secreted in pg/mL (mean±S.D.)			
[DBT] μ M	KB130	KB155	KB169
0	8077±166	3220±118	10146±309
0.05	9055±322 ⁺	3867±286 ⁺	9124±267 [*]
0.1	7864±480	3973±210 ⁺	9234±196 [*]
0.25	4963±596 [*]	3811±216 ⁺	6985±209 [*]
0.5	ND [*]	1569±139 [*]	2155±103 [*]
1	ND [*]	63±0 [*]	66±5 [*]
2.5	ND [*]	ND [*]	ND [*]
5	ND [*]	ND [*]	ND [*]

48 h Interleukin 6 secreted in pg/mL (mean±S.D.)			
[DBT] μ M	KB130	KB155	KB169
0	8422±204	10064±680	7338±72
0.05	12286±1696 ⁺	11037±510	871±518 [*]
0.1	9106±218 ⁺	10011±245	8203±377 ⁺
0.25	6710±136 [*]	8517±759 [*]	8768±9 [*]
0.5	1467±83 [*]	2908±100 [*]	3754±206 [*]
1	719±97 [*]	544±71 [*]	318±24 [*]
2.5	629±87 [*]	ND [*]	36±14 [*]
5	764±68 [*]	ND [*]	28±8 [*]

6 day Interleukin 6 secreted in pg/mL (mean±S.D.)			
[DBT] μ M	KB130	KB155	KB169
0	8834±530	13743±209	24580±941
0.05	8161±1807	11926±877	23221±1332
0.1	7559±908	12697±904	20260±589 [*]
0.25	5030±30 [*]	10789±538 [*]	23077±201
0.5	416±119 [*]	2697±199 [*]	11293±437 [*]
1	ND [*]	ND [*]	345±34 [*]
2.5	ND [*]	ND [*]	ND [*]
5	ND [*]	ND [*]	ND [*]

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

^{*} Indicates a significant decrease in secretion and⁺ indicates a significant increase in secretion compared to control cells (cells treated with vehicle alone), p<0.05 ND = not detectable.

Table 7

Effects of 24 h, 48 h, 6 day exposures to DBT on IL-6 secretion from monocyte-depleted PBMCs.

24 h Interleukin 6 secreted in pg/mL (mean±S.D.)						
[DBT] μ M	F142	F145	F152	F163	F166	
0	6583±1729	10955±346	23788±920	58347±2724	113749±2245	
0.05	62944±2481	11337±911	26616±1374 ⁺	59569±654 ⁺	128203±6793 ⁺	
0.1	63811±3934	9296±298 [*]	27626±1290 ⁺	84036±4023 ⁺	113633±4815	
0.25	69633±481 [*]	9150±149 [*]	33545±833 ⁺	111013±8251 ⁺	14444±581 [*]	
0.5	40967±4574 [*]	3369±173 [*]	26525±779 ⁺	111013±8251 ⁺	11971±1006 [*]	
1	ND [*]	ND [*]	11758±219 [*]	114524±839 ⁺	8609±1011 [*]	
2.5	ND [*]	ND [*]	727±52 [*]	ND [*]	ND [*]	
5	ND [*]	ND [*]	30±52 [*]	ND [*]	ND [*]	
48 h Interleukin 6 secreted in pg/mL (mean±S.D.)						
[DBT] μ M	F142	F145	F152	F163	F166	
0	112226±7067	11665±282	44353±1914	122785±7126	190821±7442	
0.05	66314±3381 [*]	13018±1151	41839±2357	76845±4077 [*]	242779±16720 ⁺	
0.1	74717±3591 [*]	11422±433	42564±1418	92633±1151 [*]	244363±6224 ⁺	
0.25	78792±8955 [*]	11089±115 [*]	50704±1374 ⁺	84330±2257 [*]	247029±3178 ⁺	
0.5	65384±1586 [*]	2412±298 [*]	50295±1771 ⁺	90148±4587 [*]	178404±13542	
1	6591±218 [*]	ND [*]	23605±1095 [*]	630±1261 [*]	39904±2032 [*]	
2.5	ND [*]	ND [*]	1570±54 [*]	ND [*]	9988±661 [*]	
5	ND [*]	ND [*]	1020±54 [*]	ND [*]	8863±781 [*]	
6 day Interleukin 6 secreted in pg/mL (mean±S.D.)						
[DBT] μ M	F142	F145	F152	F163	F166	
0	90029±2082	8847±73	107282±1965	26669±1165	119022±6314	
0.05	105705±27762	87938±4504 ⁺	115333±9391	29565±251 ⁺	136489±6076 [*]	

6 day	Interleukin 6 secreted in pg/mL (mean±S.D.)				
[DBT] μ M	F142	F145	F152	F163	F166
0.1	107114±17400	58278±3297 ⁺	116487±4223 ⁺	26367±801	129711±3272
0.25	91495±3511	40981±1125 ⁺	105590±4165	22966±2170	12467±677 [*]
0.5	81686±16334	3841±128 [*]	104897±22387	492±54 [*]	130867±10975
1	8790±380 [*]	4156±145 [*]	ND [*]	25857±602	11222±252 [*]
2.5	ND [*]	4047±378 [*]	ND [*]	3242±289 [*]	ND [*]
5	238±436 [*]	411±238 [*]	ND [*]	497±117 [*]	ND [*]

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

^{*}

Indicates a significant decrease in secretion and

⁺

indicates a significant increase in secretion compared to control cells (cells treated with vehicle alone), p<0.05 ND = not detectable.

Table 8

a. Effects of 24 h, 48 h, 6 day exposures to DBT on IL-6 secretion from PBMCs.

24 h	Interleukin 6 secreted in pg/mL (mean±S.D.)			
[DBT] μM	F285	F290	F294	F299
0	34255±142	14998±986	32382±357	95826±2410
0.05	63720±1078 ⁺	61442±2081 ⁺	92678±862 ⁺	112870±348 ⁺
0.1	60002±523 ⁺	65886±1367 ⁺	53085±3654 ⁺	118145±724 ⁺
0.25	76453±765 ⁺	69788±4877 ⁺	63289±641 ⁺	119362±362 ⁺
0.5	68152±745 ⁺	53195±3027 ⁺	61937±861 ⁺	61681±3093 [*]
1	14593±438 [*]	16405±1398	13622±735 [*]	17275±703 [*]
2.5	ND [*]	ND [*]	ND [*]	ND [*]
5	ND [*]	ND [*]	ND [*]	ND [*]

48 h	Interleukin 6 secreted in ng/mL (mean±S.D.)			
[DBT] μM	F285	F290	F294	F299
0	42735±177	53716±1836	30850±33	97222±962
0.05	61846±122 ⁺	76212±1266 ⁺	45764±344 ⁺	148278±631 ⁺
0.1	70898±912 ⁺	87289±884 ⁺	43992±772 ⁺	157389±1549 ⁺
0.25	88325±798 ⁺	108349±711 ⁺	63154±343 ⁺	124000±2333 ⁺
0.5	91577±266 ⁺	91289±1299 ⁺	52811±348 ⁺	83444±1678 [*]
1	15717±1110 [*]	49716±872 [*]	27078±788 [*]	23944±1798 [*]
2.5	ND [*]	ND [*]	50±216 [*]	ND [*]
5	ND [*]	ND [*]	ND [*]	ND [*]

6 day	Interleukin 6 secreted in ng/mL (mean±S.D.)			
[DBT] μM	F285	F290	F294	F299
0	3922±9	11020±543	35576±77	115832±599
0.05	14426±4 ⁺	18824±471 ⁺	50986±239 ⁺	152672±2344 ⁺
0.1	19597±8 ⁺	24039±245 ⁺	58209±77 ⁺	168474±3388 ⁺
0.25	41418±6 ⁺	37294±1387 ⁺	46682±674 ⁺	139042±905 ⁺
0.5	32052±1 ⁺	42980±1682 ⁺	30716±1216 [*]	89264±1864 [*]
1	ND [*]	15294±204 ⁺	7323±155 [*]	21314±226 [*]
2.5	ND [*]	ND [*]	116±51 [*]	ND [*]
5	ND [*]	ND [*]	ND [*]	ND [*]

b. Effects of 24 h, 48 h, 6 day exposures to DBT on IL-6 secretion from PBMCs plus Granulocytes.

24 h	Interleukin 6 secreted in pg/mL (mean±S.D.)			
[DBT] μ M	F285	F290	F294	F299
0	8587±89	8593±60	12411±381	15883±675
0.05	8138±302 *	10178±136 ⁺	15173±84 ⁺	18437±737 ⁺
0.1	12487±328 ⁺	9162±324	14586±300 ⁺	18727±658 ⁺
0.25	16162±363 ⁺	11058±195 ⁺	14788±307 ⁺	20621±407 ⁺
0.5	17618±430 ⁺	10477±130 ⁺	15558±28 ⁺	21298±388 ⁺
1	9364±102 ⁺	7475±34 *	7244±146 *	17080±404
2.5	265±25 *	520±17 *	0±18 *	2804±60 *
5	ND *	ND *	ND *	267±92 *

48 h	Interleukin 6 secreted in pg/mL (mean±S.D.)			
[DBT] μ M	F285	F290	F294	F299
0	5797±38	6346±13	11944±111	11795±238
0.05	6270±58 ⁺	9284±64 ⁺	15322±317 ⁺	13838±531 ⁺
0.1	6792±88 ⁺	7465±315 ⁺	14925±466 ⁺	15217±419 ⁺
0.25	9783±17 ⁺	11645±78 ⁺	17219±17 ⁺	19809±226 ⁺
0.5	10257±307 ⁺	11280±107 ⁺	15552±110 ⁺	20011±851 ⁺
1	7253±34 ⁺	9277±30 ⁺	8777±93 *	16104±2294
2.5	394±11 *	95±16 *	ND *	1150±264 *
5	215±45 *	ND *	ND *	ND *

6 day	Interleukin 6 secreted in pg/mL (mean±S.D.)			
[DBT] μ M	F285	F290	F294	F299
0	21±10	30±10	1843±18	49±4
0.05	161±4 ⁺	498±12 ⁺	1845±49	348±244
0.1	159±13 ⁺	531±9 ⁺	1991±36	656±166 ⁺
0.25	359±15 ⁺	260±6 ⁺	2699±16 ⁺	456±37 ⁺
0.5	550±15 ⁺	316±18 ⁺	2638±22 ⁺	321±26 ⁺
1	99±10 ⁺	108±5 ⁺	1020±35 *	107±8 ⁺
2.5	ND *	ND *	ND *	37±5 *
5	ND *	ND *	ND *	26±54

c. Effects of 24 h, 48 h, 6 day exposures to DBT on IL-6 secretion from Granulocytes.

24 h	Interleukin 6 secreted in pg/mL (mean±S.D.)			
[DBT] μ M	F285	F290	F294	F299
0	54±2	46±2	16±1	148±0
0.05	89±4 ⁺	71±10 ⁺	16±4	197±8 ⁺

c. Effects of 24 h, 48 h, 6 day exposures to DBT on IL-6 secretion from Granulocytes.

24 h		Interleukin 6 secreted in pg/mL (mean±S.D.)			
[DBT] μ M		F285	F290	F294	F299
0.1		72±3 ⁺	65±8 ⁺	10±1 [*]	254±9 ⁺
0.25		92±2 ⁺	93±25	17±3	22±1 [*]
0.5		79±1 ⁺	65±2 ⁺	1±2 [*]	252±3 ⁺
1		19±1 [*]	15±1 [*]	0±1 [*]	162±4 ⁺
2.5		ND [*]	ND [*]	ND [*]	17±0 [*]
5		ND [*]	ND [*]	ND [*]	8±0 [*]

48 h		Interleukin 6 secreted in pg/mL (mean±S.D.)			
[DBT] μ M		F285	F290	F294	F299
0		35±10	58±2	36±1	149±4
0.05		34±3	57±9	34±0 [*]	143±1
0.1		33±5	59±4	31±2 [*]	136±2 [*]
0.25		44±3 ⁺	78±6 ⁺	45±3 ⁺	221±8 ⁺
0.5		32±1 [*]	70±7	30±3 [*]	231±3 ⁺
1		5±2 [*]	20±0 [*]	6±1 [*]	131±9 [*]
2.5		ND [*]	ND [*]	ND [*]	ND [*]
5		ND [*]	ND [*]	ND [*]	ND [*]

6 day		Interleukin 6 secreted in pg/mL (mean±S.D.)			
[DBT] μ M		F285	F290	F294	F299
0		ND	6±0	ND	4±0
0.05		ND	22±1 ⁺	ND	9±1 ⁺
0.1		ND	23±4 ⁺	ND	5±1
0.25		ND	18±4 ⁺	ND	6±1
0.5		ND	10±3	ND	4±0
1		14±2 ⁺	7±3	ND	7±1
2.5		ND	4±2	ND	7±1 ⁺
5		ND	ND [*]	ND	6±1 ⁺

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

* Indicates a significant decrease in secretion and

⁺ indicates a significant increase in secretion compared to control cells (cells treated with vehicle alone), p<0.05 ND = not detectable.