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## Secretion of Interferon gamma (IFN $\gamma$ ) from Human Immune Cells is Altered by Exposure to Tributyltin (TBT) and Dibutyltin (DBT)

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### Abstract

Tributyltin (TBT) and dibutyltin (DBT) are widespread environmental contaminants found in food, beverages, and human blood samples. Both of these butyltins (BTs) interfere with the ability of human natural killer (NK) cells to lyse target cells and also alter secretion of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF $\alpha$ ) from human immune cells *in vitro*. The capacity of BTs to interfere with secretion of other pro-inflammatory cytokines has not been examined. Interferon gamma (IFN $\gamma$ ) is a modulator of adaptive and innate immune responses, playing an important role in overall immune competence. This study shows that both TBT and DBT alter secretion of IFN $\gamma$  from human immune cells. Peripheral blood cell preparations that were increasingly reconstituted were used to determine if exposures to either TBT or DBT affected IFN $\gamma$  secretion and how the makeup of the cell preparation influenced that effect. IFN $\gamma$  secretion was examined after 24 h, 48 h and 6 day exposures to TBT (200- 2.5 nM) and DBT (5- 0.05  $\mu$ M) in highly enriched human NK cells, a monocyte-depleted preparation of PBMCs, and monocyte-containing PBMCs. Both BTs altered IFN $\gamma$  secretion from NK cells at most of the conditions tested (either increasing or decreasing secretion). However, there was significant variability among donors as to the concentrations and time points that showed changes as well as the baseline secretion of IFN $\gamma$ . The majority of donors showed an increase in IFN $\gamma$  secretion in response to at least one concentration of TBT or DBT at a minimum of one length of exposure.

### Keywords

NK cells; PBMCs; Tributyltin; Dibutyltin; Interferon gamma

### INTRODUCTION

The use of butyltins (BTs) in various applications has led to very significant contamination of the environment by these compounds (Kimbrough, 1976; Tanabe et al. 1998; Loganathan et al., 2000; Roper, 1992; Gipperth, 2009; Takahashi et al., 1999; Kannan et al., 1995; Forsyth et al., 1992; 1997; Sadiki et al., 1996). Tributyltin (TBT) has been used as an ingredient in antifouling paints as well as an antifungal agent and biocide in textiles, paper production, industrial cooling waters, and wood preservation (Kimbrough, 1976; Laughlin

and Linden, 1985; Roper, 1992). Its use in antifouling paints has been banned since 2008 but it will continue to contaminate the environment for many years due to its chemical stability and other uses (Gipperth, 2009). TBT is found in food, especially fish, (Kannan et al., 1995; Takahashi et al., 1999) and also in human blood. Blood levels range as high as 261 nM (85 ng/mL) (Whalen et al., 1999; Kannan et al., 1999). Studies show that the exposure of mammals to TBT causes increased incidences of tumors (Wester et al., 1990), decreased NK cell function (Ghoneum et al., 1990), thymic atrophy and thymus dependent immunosuppression (Snoeij *et al.*, 1987, 1989; Vos et al. 1990). Dibutyltin (DBT) is used as a stabilizer in plastics (Roper, 1976) and as an anti-worming agent in some poultry (Epstein et al. 1991). DBT is also found in drinking water and other beverages due to leaching from PVC plastics used in their production and/or storage (Forsyth et al., 1992, 1997; Sadiki et al., 1996). Levels of DBT in human blood are as high as 300 nM (94 ng/mL) (Whalen et al., 1999; Kannan et al., 1999). Both TBT and DBT decrease lytic function, target-binding function, cell-surface protein expression, and cytolytic protein expression in human natural killer (NK) cells at levels that are in the range found in human blood (Whalen et al., 1999; Dudimah et al., 2007a, b; Whalen et al., 2002; Odman-Ghazi et al., 2003; Thomas et al., 2004; Catlin et al., 2005). These changes are accompanied by a rapid activation of a portion of the signaling pathway(s) that regulates NK lytic function (beginning with protein kinase C through mitogen-activated protein kinases (MAPKs) (Aluoch and Whalen, 2005; Aluoch et al., 2006; Aluoch et al., 2007; Odman-Ghazi et al., 2010; Abraha et al., 2010). Recently, we have shown that exposure to these BTs alters the secretion of the cytokine tumor necrosis factor alpha (TNF $\alpha$ ) from human immune cells including NK cells (Hurt et al. 2013).

The functions of both innate and adaptive immune cells are regulated by the cytokine interferon gamma (IFN $\gamma$ ). It causes increased antigen presentation on macrophages by increasing their expression of MHC class I molecules and regulates T cell immune response through Th1 cells. It is also involved in the recruitment of innate immune cells to sites of infection or tumor (Zaidi and Merlino, 2011). IFN $\gamma$  is an approximately 17kD protein, which is dimerized in its physiologically active form (Billiau and Matthys, 2009). It is secreted by T cells, NK cells, and to a lesser extent by myeloid lineage cells such as macrophages (Billiau and Matthys, 2009; Darwich et al., 2008). IFN $\gamma$  is a pro-inflammatory cytokine which has the ability to cause chronic inflammation, which has been shown to facilitate the development of certain cancers such as gastrointestinal cancers (Macarthur et al., 2004). Careful regulation of IFN $\gamma$  is crucial in order to avoid loss of immune competency or the increased risks associated with chronic inflammation. IFN $\gamma$  may behave both in a pro-tumorigenic as well as in an antitumor manner depending on the specific circumstances. Under circumstances where IFN $\gamma$  increases tumor cell death by macrophages, T cells, and NK cells it will prevent cancer development. However, the stimulation of myeloid derived suppressor cell development by IFN $\gamma$  may lead to cancer (Zaidi and Merlino, 2011).

Natural killer (NK) cells are capable of secreting IFN $\gamma$  (Andoniou et al., 2008; Girart et al., 2007) and we have found that both TBT and DBT have dramatic effects on the ability of human NK cells to destroy tumor cells (Dudimah et al., 2007a, b) as well as their ability to secrete the pro-inflammatory cytokine TNF $\alpha$  (Hurt et al., 2013). In addition we showed that both BTs interfered with the secretion of TNF $\alpha$  from a preparation of peripheral blood

mononuclear cells (PBMCs) that had been depleted of monocytes (Hurt et al., 2013). Thus, there is interest in investigating whether either TBT or DBT has the ability to alter immune cell secretion of other pro-inflammatory cytokines including IFN $\gamma$ .

The current study examined the effects of exposures to TBT or DBT on secretion of IFN $\gamma$  from highly purified NK cells, monocyte-depleted PBMCs, and PBMCs. Examination of the effects of these compound on IFN $\gamma$  secretion from increasingly reconstituted systems allowed us to determine whether there were differences in the effects of the BTs between the purified NK system and the more complex systems of immune cells, thus more closely approximating the physiological setting.

## MATERIALS AND METHODS

### Preparation of NK cells

NK cells were prepared from buffy coats (source leukocytes 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 25 min at room temperature (~25°C). Seven (7)–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  $\mu$ g streptomycin/ml) at 1 million cells/mL at 37 °C and air/CO<sub>2</sub> , 19:1.

### Preparation of monocyte- depleted PBMCs and PBMCs

Preparations of PBMCs were isolated from Leukocyte filters (PALL- 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 elution medium (PBS containing 5 mM disodium EDTA and 2.5% [w/v] sucrose) and collecting the eluent. Eluent contained the leukocytes with red cell contamination. Eluent was layered onto Ficoll-Hypaque (1.077g/mL) and centrifuged as described above. Granulocytes and red cells pelleted at the bottom of the tube while the PBMCs floated on the Ficoll-Hypaque. PBMCs were collected and washed (250 g, 10 min.) with PBS. Cells were then suspended in complete medium which consisted of RPMI-1640 supplemented with 10% heat-inactivated BCS, 2 mM *L*-glutamine and 50 U penicillin G with 50  $\mu$ g streptomycin/mL. This preparation constituted PBMCs. Monocyte-depleted PBMCs (10–20% CD16<sup>+</sup>, 10–20 % CD56<sup>+</sup>, 70–80% CD3<sup>+</sup>, 3–5% CD19<sup>+</sup>, 2–20% CD14<sup>+</sup>) were prepared by incubating the cells in glass Petri dishes (150 X 15 mm) at 37 °C and air/CO<sub>2</sub> , 19:1 for 1 h .

### Chemical preparation

TBT and DBT were purchased from (Sigma-Aldrich, St. Louis, MO). TBT was a neat standard, dissolved initially in deionized water to give a 1 mM solution. A DBT stock solution was prepared by dissolving DBT in dimethylsulfoxide (DMSO). Desired

concentrations of either TBT or DBT were prepared by dilution of the stock into complete media. The final concentration of DMSO for DBT exposures did not exceed 0.01%. Appropriate DMSO controls were run.

### Cell treatments

NK cells, monocyte-depleted PBMCs, or PBMCs (at a concentration of 1.5 million cells/mL) were treated with TBT with appropriate control at concentrations of 2.5–200 nM for 24 h, 48 h, or 6 days. Cells were treated with DBT with appropriate control at concentrations of 0.05–5  $\mu$ M for the same lengths of incubation as used with TBT. Following the incubations the cells were pelleted and the supernatants were collected and frozen at  $-70^{\circ}\text{C}$  until assay.

### 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. Results are shown in Tables 1 and 2.

### IFN $\gamma$ secretion assay

IFN $\gamma$  levels were assessed using the OptEIA™ enzyme-linked immunosorbent assay (ELISA) human IFN $\gamma$  kit (BD-Pharmingen, San Diego, CA). Capture antibody diluted in coating buffer was applied to the wells of a 96 well flat-bottom microwell plate specifically designed for ELISA (Fisher, St. Louis MO). The plate was incubated overnight at  $4^{\circ}\text{C}$ . Following the incubation, capture antibody was removed by washing the plate three times with wash buffer (PBS with 0.05% Tween-20). The wells were then treated with blocking buffer to prevent non-specific binding and the plate was sealed and incubated at room temperature for 1 h. Blocking buffer was removed with three washes, and cell supernatants and IFN $\gamma$  standards were added to the plate, which was sealed and incubated at room temperature for 2 h. The plate was washed five times following the incubation with samples and standards and detection antibody was added then incubated for 1 h at room temperature. Detection antibody was removed by washing 7 times and a substrate solution was added to each well and incubated for 30 min at room temperature. The incubation with 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 carried out utilizing 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, monocyte-depleted PBMCs, and PBMCs exposed to TBT

Table 1 shows the effects of TBT exposures (2.5–200 nM) on the viability of highly purified NK cells, monocyte-depleted PBMCs, and PBMCs. Exposure of NK cells to 2.5–200 nM TBT for 24 h had no effect on their viability as compared to the control. Exposure to these same concentrations for 48 h diminished viability by a small amount at the highest concentration of TBT (200 nM). NK cells exposed to 2.5–200 nM TBT for 6 days showed no change in viability as compared to controls. Like NK cells, monocyte-depleted PBMCs and PBMCs both showed no negative effects of a 24 h exposure to TBT at any concentration, but were slightly affected by the 200 nM TBT after 48 h and more significantly after 6 days. This indicated that there was not a generalized toxicity of the compound on cells due to TBT exposures.

### Viability of NK cells, monocyte-depleted PBMCs, and PBMCs exposed to DBT

The effects of DBT exposures (0.05–5  $\mu$ M) on the viability of highly purified NK cells, monocyte-depleted PBMCs, and PBMCs are shown in Table 2. NK cells exposed to 2.5 and 5  $\mu$ M DBT for 24 h showed small but significant decreases in viability as compared to the control. All other DBT concentrations had no effect on viability at the 24 h exposure. 48 h exposures to 1, 2.5 and 5  $\mu$ M DBT decreased NK viability by 18%, 30%, and 32%, respectively. Monocyte-depleted PBMCs and PBMCs also showed significant decreases in viability with exposures to the highest concentrations of DBT. But as with TBT, there were decreases in viability only at the highest concentrations, which increased slightly with length of exposure.

### IFN $\gamma$ secretion from NK cells exposed to TBT

Table 3 shows the effects of exposing highly purified NK cells to 0, 2.5, 5, 10, 25, 50, 100 and 200 nM TBT for 24 h, 48 h, and 6 days on IFN $\gamma$  secretion from each of the donors tested (KB=Key Biologic buffy coat). There was very wide variation in the baseline secretion of IFN $\gamma$  from one donor to the next at each of the time points tested. 24 h exposures were carried out with cells prepared from 7 individual donors and 6 of the 7 donors showed significant decreases in IFN $\gamma$  when exposed to 200 nM TBT. Cells from 5 of the 7 donors showed an increase in IFN $\gamma$  secretion with exposure to at least one TBT concentration. The concentrations that caused increases varied from one donor to the next. For instance, the cells from donor KB114 treated with 5, 10, 25, 50 and 100 nM TBT showed significant increases of 2.8, 2.6, 3.9, 4.3, and 1.4 fold respectively, while the cells from donor KB113 showed an increase of 5.5 fold only at the 25 nM exposure. When NK cells from 4 individual donors were exposed to TBT for 48 h there was a significant decrease in IFN $\gamma$ , as compared to control in cells from 3 of the 4 donors at the 200 and 100 nM concentrations. Increases in IFN $\gamma$  secretion were seen in 2 of the 4 donors, while the other 2 donors only showed decreases (or no change) when exposed to TBT. NK cells exposed to TBT for 6 days all showed a significant decrease in IFN $\gamma$  secretion at the 200 nM concentration. Cells from each of the 4 donors secreted higher levels of IFN $\gamma$  when exposed to a minimum of one TBT concentration. The concentrations at which increases were seen again varied depending on the donor. Likewise the intensity of the increase varied from one

donor to the next as it did at 24 h and 48 h. Comparing donor KB146 to donor KB147, the 5, 10, 25, 50, and 100 nM concentrations of TBT each caused very significant increases (4, 8.5, 6.2, 13.8, and 4.5 fold, respectively) in secretion in cells from KB146, while only the 50 nM exposure to TBT caused an increase in cells from KB147 (4 fold). Figure 1A shows the effects at each of the time points for an individual donor (KB147).

### **IFN $\gamma$ secretion from monocyte-depleted PBMCs exposed to TBT**

The effects of exposures to TBT on secretion of IFN $\gamma$  from monocyte-depleted PBMCs after 24 h, 48 h, and 6 d from each donor (F=filter obtained from the Red Cross) are shown in Table 4. As with the enriched NK cell preparation, there was wide variation in the baseline secretion of IFN $\gamma$  from monocyte-depleted PBMCs. This preparation is largely NK cells and T cells. When monocyte-depleted PBMCs were exposed to TBT for 24 h there were statistically significant increases in IFN $\gamma$  secretion induced by TBT for every donor at the 25 and 50 nM concentrations. Exposure to 200 nM TBT caused nearly complete inhibition of secretion in every donor. Figure 1B shows the effects of TBT exposures at each of the lengths of exposure for an individual donor (F142). The same pattern of increases in IFN $\gamma$  secretion at 25 and 50 nM and a nearly complete block of secretion at the 200 nM exposure was seen at each of the other lengths of exposure for all donors.

### **IFN $\gamma$ secretion from PBMCs exposed to TBT**

The effects of exposing PBMCs from 4 individual donors to 0–200 nM TBT for 24 h, 48 h, and 6 d on the secretion of IFN $\gamma$  are shown in Table 5 (F=filter obtained from the Red Cross). Again, there was variation among donors in the baseline secretion of IFN $\gamma$  (donor F116 was at the high end and donor F120 at the low end). PBMCs from all 4 donors showed significant decreases in secretion after exposure to 200 nM TBT for 24 h. Cells from 2 of the 4 donors (F116 and F117) were either unchanged or showed decreased secretion of IFN $\gamma$  when exposed to TBT at any length of exposure. PBMCs from donors F119 and F120 produced increased amount of IFN $\gamma$  when exposed to certain concentrations of TBT at every length of exposure. For example, F120 PBMCs produced higher levels of IFN $\gamma$  than control cells after each length of exposure, but the concentration at which the increases occurred varied: 5–100 nM TBT caused increased secretion after 24h (ranging as high as 2.6 fold at 100 nM); only 50 and 100 nM TBT increased secretion after 48 h (with 100 nM causing an 8 fold increase); after 6 d of exposure to 100 nM TBT the increase in secretion was 19 fold. The effects of TBT exposures on PBMCs from donor F120 at each of the time points are shown in Figure 1C.

### **IFN $\gamma$ secretion from NK cells exposed to DBT**

Effects of exposing highly purified NK cells to 0, 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5  $\mu$ M DBT for 24 h, 48 h, and 6 days on IFN $\gamma$  secretion from several individual donors are shown in Table 6. As with the TBT exposed cells there is notable variation in the baseline secretion of IFN $\gamma$  from individual donors. NK cells prepared from 6 donors were tested for the effect of 24 h exposures to DBT. Secretion of IFN $\gamma$  by NK cells from all 6 donors was essentially completely blocked by exposures to 0.5, 1, 2.5, and 5  $\mu$ M DBT for 24 h. However, 4 of the 6 donors showed increased secretion with exposures to 0.1  $\mu$ M DBT for 24 h. Increases were also seen at the 0.05  $\mu$ M in 3 of the 6 donors. 48 h exposures to DBT caused essentially a



complete block of IFN $\gamma$  secretion at the same concentrations that blocked secretion after 24 h. However, NK cells from all donors had increased secretion of IFN $\gamma$  at the lowest level of DBT exposure (0.05  $\mu$ M). Cells that did not show any increase in IFN $\gamma$  after 24 h exposures to DBT exhibited increased secretion after 48 h (KB130 and KB139). Following a 6 d exposure to 1, 2.5, and 5  $\mu$ M DBT, there was decreased secretion from cells from all 3 donors tested. Cells from KB130 and KB155 showed increased secretion in response to 6 day exposures to several DBT concentrations (peak increases of 1.5 fold and 2.8 fold at 0.05  $\mu$ M for KB130 and KB155, respectively). Data from donor KB155 is plotted for all time points in Figure 1D.

### IFN $\gamma$ secretion from monocyte-depleted PBMCs exposed to DBT

Results of exposing monocyte-depleted PBMCs isolated from different donors to 0–5  $\mu$ M DBT for 24 h, 48 h, and 6 days on IFN $\gamma$  secretion are shown in Table 7. When cells isolated from 6 donors were exposed to DBT for 24 h, secretion of IFN $\gamma$  was significantly decreased at the 0.5, 1, 2.5, and 5  $\mu$ M concentrations in 5 of the 6 donors. Cells from 5 of the 6 donors also showed increased secretion with exposures to DBT for 24 h. The concentrations at which DBT caused increased secretion varied among donors. Exposures to 1, 2.5, and 5  $\mu$ M DBT for 48 h almost completely blocked the secretion of IFN $\gamma$  from PBMCs regardless of donor. Likewise, PBMCs from all donors had increased secretion of IFN $\gamma$  at exposure to DBT at lower concentrations (which varied among donors). The intensity of the increase also varied depending on the donor. This same pattern persisted out to 6 days of exposure. Figure 1E shows the effects of 0–200 nM exposure for 24 h, 48 h, and 6d for cells from donor F142.

### IFN $\gamma$ secretion from PBMCs exposed to DBT

Alterations in IFN $\gamma$  secretion caused by exposing PBMCs isolated from different donors to 0–5  $\mu$ M DBT for 24 h, 48 h, and 6 days are shown in Table 8. As was seen with NK cells and monocyte-depleted PBMCs, there is a consistent decrease in IFN $\gamma$  secretion at the highest DBT concentrations (0.5–5  $\mu$ M) after 24 h. In contrast to the less reconstituted cell preparations, PBMCs did not show a strong pattern of increased secretion at the lower concentration of DBT with a 24 h exposure. While the highest concentrations of DBT (5 and 2.5) continued to cause decreased IFN $\gamma$  secretion after 48 h and 6d, there were significant increases in IFN $\gamma$  secretion in 4 of 6 donors after 48 h and 3 of 4 donors after 6 d. All but one donor (F117) showed an increase in IFN $\gamma$  after at least one exposure length (concentration at which this occurred varied among the donors). Data for 24 h, 48 h, and 6 d exposure of PBMCs from donor F120 are plotted in Figure 1F.

## DISCUSSION

IFN $\gamma$  is an important regulator of immune responsiveness that is produced primarily by T and NK lymphocytes and to some extent by myeloid cells (Zaidi and Merlino, 2011, Billiau and Matthys, 2009; Darwich et al., 2008). It is important to determine the effects of environmental contaminants such as the BTs (which have been found in human blood) (Whalen et al., 1999; Kannan et al., 1999) on the secretion of this potent regulator. Compound-induced decreases in IFN $\gamma$  secretion could lead to loss of appropriate Th-1 cell



development and macrophage stimulation, both of which are crucial in establishing an immune response to pathogens (Schroder et al., 2004). Alternatively, if a compound were to increase IFN $\gamma$  levels, this could lead to out of control inflammatory response, which is known to be a factor in a number of diseases including cancer (Macarthur et al., 2004). In this study we examined the effects of both TBT and DBT on secretion of IFN $\gamma$  by immune system cells.

The effects of TBT and DBT on IFN $\gamma$  secretion were examined in three distinct cell preparations (highly enriched NK cells, monocyte-depleted PBMCs and PBMCs). This type of study is important in order to determine if increasing the complexity of the cell population altered the effects of exposures to the compounds. It is not possible to carry out studies of these compounds in humans. Thus, studies carried out in more reconstituted systems such as the ones examined here will more closely approximate the effects that might be seen *in vivo*.

When highly purified NK cells were exposed to 200 nM TBT there was a substantial decrease in secretion of IFN $\gamma$ . This effect remained as the cell preparation became more complex, with both monocyte-depleted PBMCs and PBMCs having the same patterns of response to TBT exposures as did the highly enriched NK cells. A notable difference was that monocyte-depleted PBMCs (which are primarily T cells and NK cells) had a consistent increase at the lower TBT concentrations at more time points than were seen with either NK cells alone or with PBMCs. Additionally, stimulation of IFN $\gamma$  secretion with TBT was less common in the PBMCs than in NK cells or monocyte-depleted NK cells. This suggests that the presence of monocytes diminished the increases in secretion seen at low TBT concentrations (usually in the 50 nM to 2.5 nM range) that were quite consistently seen in the non-monocyte containing preparation. As monocytes are not considered to be significant secretors of IFN $\gamma$ , their presence may simply be dampening the ability to see the TBT-induced stimulations. Alternatively, it may be that monocytes are secreting a factor(s) that interferes with TBT-induced secretion of IFN $\gamma$ . The fact that increases are seen but not as consistently or intensely could be explained by either scenario. TBT-induced decreases in IFN $\gamma$  secretion at the 200 and 100 nM TBT concentrations are quite reproducible among the 3 different cell preparations. Thus, TBT is altering secretion of IFN $\gamma$  by diminishing its secretion at the highest levels of exposure while stimulating secretion at concentrations of 50 nM and below. These changes are seen with even the most reconstituted cell system studied, indicating that the effect has significant potential to be occurring in a physiologically relevant setting.

Decreases induced by the higher TBT concentrations would prevent IFN $\gamma$  from carrying out its important role in regulating T-cell development and macrophage effector functions (Schroder et al. 2004). Conversely, TBT-induced increases in a pro-inflammatory cytokine such as IFN $\gamma$  can lead to a number of diseases that are caused or exacerbated by increased inflammation (Macarthur et al., 2004). Secretion of IFN $\gamma$  by human NK cells has been shown to require mitogen activated protein kinase (MAPK) activation (Girart et al., 2007). The effects of TBT on IFN $\gamma$  secretion are occurring at levels that are found in human blood and because of the important role of this cytokine have significant implications for human health. TBT at these levels also decreases the lytic function of human NK cells (Dudimah et al., 2007a, Whalen et al., 2002). We have previously shown that TBT is able to activate

MAPKs in NK cells (Aluoch and Whalen, 2005; Aluoch et al., 2006; Aluoch et al., 2007). Future studies will investigate the role of MAPK activation in the TBT-induced changes in IFN $\gamma$  secretion (both decreases and increases).

DBT caused very significant alterations of IFN $\gamma$  secretion from each of the 3 types of cell preparations examined. In general, DBT at concentrations of 0.5–5  $\mu$ M significantly decreased IFN $\gamma$  secretion, with concentrations of 1–5  $\mu$ M causing a complete block of secretion in nearly every donor of each type of cell preparation. In highly purified NK cells of donor KB155 there was a 12 fold increase in IFN $\gamma$  secretion when the cells were exposed to 0.1 $\mu$ M DBT (100 nM) for 48 h, which is well below the highest levels that have been found in some human blood samples (300 nM) (Whalen et al., 1999; Kannan et al., 1999). In monocyte-depleted PBMCs, this same concentration of DBT after 48 h was able to cause a similar (8.5 fold) very large increase in IFN $\gamma$  secretion in cells from donor F105. The peak increase for that donor after 48 h was at the 0.25  $\mu$ M concentration and was 16.7 fold. PBMCs treated for 48 h at these same concentrations of DBT also had one donor (F120) that showed a very large stimulation of IFN $\gamma$  secretion. The majority of increases seen with DBT were more modest than those mentioned above but range in the 2–3 fold level. Thus, in contrast to what was seen with TBT, the effects of DBT did not seem to be different as the complexity of the cell population increased. Like TBT, DBT exposures also caused very large decreases in IFN $\gamma$  secretions at the highest concentrations and increased secretion at lower concentrations. The decreases of secretion cannot be accounted for simply by compound-induced killing of the cells, although concentrations of 1–5  $\mu$ M do cause some cell death as length of exposure increases. Stimulation of secretion occurs at concentrations that have no negative impact on cell viability. Thus, indicating that the compound is in some manner activating IFN $\gamma$  secretion and/or synthesis. As with TBT, DBT has been shown to decrease NK lytic function (Dudimah et al., 2007b, Whalen et al., 2002) and activate MAPKs (Odman-Ghazi et al., 2010) at some of these same concentrations that are affecting IFN $\gamma$  secretion.

It is clear from this study that there is significant variability among donors both in their response to compound exposures as well as the baseline levels of IFN $\gamma$  secretion. It is not clear what causes the variation in baseline IFN $\gamma$  secretion, but clearly individuals vary quite widely in their baseline secretion and in the exact concentrations and lengths of exposure to a compound that elicit a stimulatory effect on IFN $\gamma$  secretion. Interestingly, while there is some variation in baseline lytic function of highly purified NK cells among different donors it is less than what is seen with IFN $\gamma$  secretion. Additionally, the effects of TBT and DBT exposures on NK lytic function were also not as variable (Dudimah et al. 2007a, 2007b). The monocyte-depleted PBMCs and PBMCs also show some variability in lytic function from donor to donor and are far less susceptible to exposure to BTs and other contaminants than the highly purified NK cells (Whalen et al., 1999; Whalen et al., 2003; Reed et al., 2004; Taylor et al., 2005). While the lytic function of highly purified NK cells is blocked by exposures as low as 25 nM after 6 days of exposure only the highest concentrations of TBT (200 nM) caused decreases in lytic function of the monocyte-depleted PBMCs and PBMCs at any length of incubation including 6 days (Whalen et al., 1999; Dudimah et al., 2007a). Again, the response of lytic function to compound exposures was far less variable in any of the cell preparations than is the response of IFN $\gamma$  secretion. Additionally, the capacity of

both BTs to stimulate secretion of IFN $\gamma$  was seen at lower concentrations of BT exposure where no effects on lytic function were seen (especially in the more reconstituted cell systems).

A stimulatory effect on secretion is common to nearly every donor with both TBT and DBT exposures. This is true from the least to the most reconstituted cell preparations. A previous study examined effects of both TBT and DBT on secretion of TNF $\alpha$  from human white blood cells. That study showed that TBT caused decreased secretion of TNF $\alpha$  in NK cells. There was no TBT-induced stimulation of secretion at any concentration in purified NK cells. However TBT (at lower concentrations) did stimulate secretion of TNF $\alpha$  in a cell preparation that contained both T and NK cells (monocyte-depleted PBMCs). DBT, at higher concentrations, decreased TNF $\alpha$  secretion and increased secretion at lower concentrations in both NK cell and the monocyte-depleted cell preparations (Hurt et al., 2013). This is in contrast to the effects of these compounds on IFN $\gamma$ , where the composition of the cell preparation did not influence the effectiveness of DBT and had only a moderating influence with TBT.

In summary, the current study shows that secretion of IFN $\gamma$  in three distinct immune cell preparations is altered by BT exposures. Exposures to both TBT and DBT result in significant decreases in IFN $\gamma$  secretion at the highest exposure levels. Each compound is able to stimulate secretion of IFN $\gamma$  from each of the three cell preparations, but the magnitude of the increase as well as the concentration and length of exposure at which the stimulation occurred, varied from one donor to the next.

## Acknowledgments

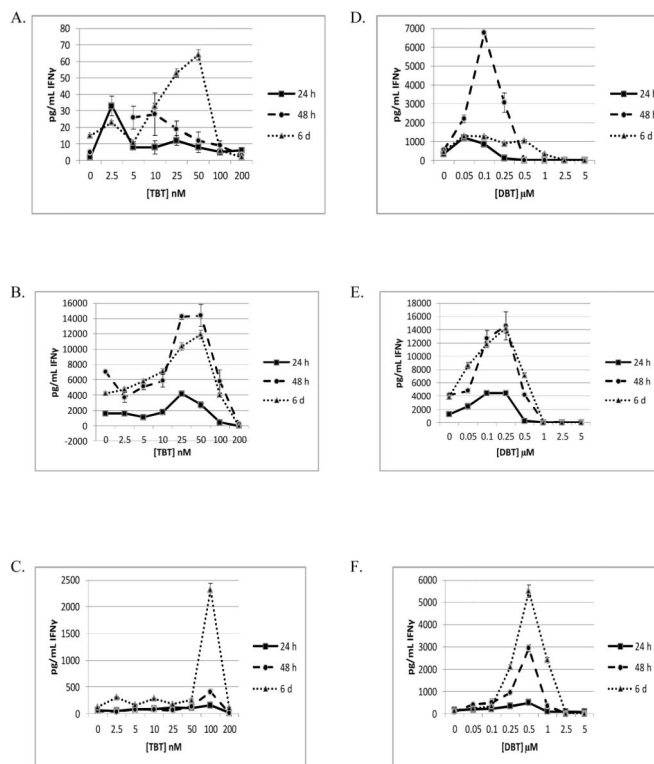
Grant U54CA163066-02 and 2T34GM007663-32 from the National Institutes of Health

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**Figure 1.**

Effects of 24 h, 48 h and 6 day exposures to TBT and DBT on IFN $\gamma$  secretion from highly purified human NK cells, monocyte-depleted PBMCs, and PBMCs in an individual donor. A) NK cells exposed to 0–200 nM TBT (donor KB147). B) Monocyte-depleted PBMCs exposed to 0–200 nM TBT (donor F142). C) PBMCs exposed to 0–200 nM TBT (donor F120). D) NK cells exposed to 0–5  $\mu$ M DBT (donor KB155). E) Monocyte-depleted PBMCs exposed to 0–5  $\mu$ M DBT (donor F142). F) PBMCs exposed to 0–5  $\mu$ M DBT (donor F120). The data point for a 48 h exposure of NK cells to 2.5 nM TBT ( $177 \pm 120$  pg/mL) was omitted in (A) as it was not significant and made it difficult to see the effects at other concentrations.

**Table 1**

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

|                |          | Percent Viable Cells |                         |       |
|----------------|----------|----------------------|-------------------------|-------|
| 24 h exposure  | [TBT] nM | NK                   | Monocyte-depleted PBMCs | PBMCs |
|                | 0        | 96±3                 | 97±3                    | 96±3  |
|                | 2.5      | 96±3                 | 98±1                    | 98±2  |
|                | 5        | 97±2                 | 99±1                    | 97±3  |
|                | 10       | 97±2                 | 98±2                    | 96±2  |
|                | 25       | 98±1                 | 98±2                    | 98±2  |
|                | 50       | 96±2                 | 96±6                    | 97±2  |
|                | 100      | 96±1                 | 98±2                    | 97±2  |
|                | 200      | 92±6                 | 92±4                    | 95±2  |
| 48 h exposure  | 0        | 96±1                 | 95±3                    | 96±3  |
|                | 2.5      | 96±2                 | 98±1                    | 94±5  |
|                | 5        | 97±1                 | 99±1                    | 96±3  |
|                | 10       | 97±1                 | 97±1                    | 97±7  |
|                | 25       | 95±3                 | 96±1                    | 95±4  |
|                | 50       | 96±2                 | 94±3                    | 96±4  |
|                | 100      | 94±3                 | 89±9                    | 98±1  |
|                | 200      | 82±5*                | 77±5*                   | 84±4* |
| 6 day exposure | 0        | 78±4                 | 84±11                   | 93±2  |
|                | 2.5      | 78±4                 | 87±4                    | 92±1  |
|                | 5        | 81±3                 | 88±10                   | 95±2  |
|                | 10       | 80±7                 | 90±10                   | 94±2  |
|                | 25       | 80±5                 | 85±13                   | 94±2  |
|                | 50       | 74±8                 | 74±17                   | 92±6  |
|                | 100      | 72±6                 | 64±12                   | 86±8  |
|                | 200      | 66±11                | 54±13*                  | 74±5* |

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

\* Indicates a significant decrease in viability compared to control cells,  $p < 0.05$



**Table 2**

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

|                |                     | Percent Viable Cells |                         |              |
|----------------|---------------------|----------------------|-------------------------|--------------|
| 24 h exposure  | [DBT] $\mu\text{M}$ | NK                   | Monocyte-depleted PBMCs | PBMCs        |
|                | 0                   | 95 $\pm$ 2           | 96 $\pm$ 2              | 95 $\pm$ 3   |
|                | 0.05                | 96 $\pm$ 3           | 97 $\pm$ 2              | 98 $\pm$ 2   |
|                | 0.1                 | 95 $\pm$ 4           | 98 $\pm$ 3              | 97 $\pm$ 2   |
|                | 0.25                | 94 $\pm$ 6           | 96 $\pm$ 2              | 96 $\pm$ 2   |
|                | 0.5                 | 94 $\pm$ 2           | 97 $\pm$ 3              | 95 $\pm$ 4   |
|                | 1                   | 87 $\pm$ 9           | 95 $\pm$ 4              | 93 $\pm$ 7   |
|                | 2.5                 | 88 $\pm$ 6*          | 84 $\pm$ 9*             | 90 $\pm$ 14  |
|                | 5                   | 81 $\pm$ 10*         | 83 $\pm$ 6*             | 87 $\pm$ 7*  |
| 48 h exposure  | 0                   | 93 $\pm$ 4           | 96 $\pm$ 2              | 96 $\pm$ 2   |
|                | 0.05                | 96 $\pm$ 4           | 98 $\pm$ 2              | 96 $\pm$ 1   |
|                | 0.1                 | 95 $\pm$ 3           | 99 $\pm$ 1              | 96 $\pm$ 3   |
|                | 0.25                | 93 $\pm$ 2           | 97 $\pm$ 1              | 96 $\pm$ 4   |
|                | 0.5                 | 89 $\pm$ 2           | 94 $\pm$ 6              | 95 $\pm$ 7   |
|                | 1                   | 75 $\pm$ 7*          | 86 $\pm$ 3*             | 84 $\pm$ 13  |
|                | 2.5                 | 63 $\pm$ 3*          | 71 $\pm$ 16*            | 76 $\pm$ 11* |
|                | 5                   | 61 $\pm$ 11*         | 69 $\pm$ 9*             | 73 $\pm$ 4*  |
| 6 day exposure | 0                   | 73 $\pm$ 15          | 87 $\pm$ 9              | 90 $\pm$ 7   |
|                | 0.05                | 74 $\pm$ 7           | 88 $\pm$ 7              | 89 $\pm$ 6   |
|                | 0.1                 | 77 $\pm$ 8           | 89 $\pm$ 8              | 91 $\pm$ 8   |
|                | 0.25                | 75 $\pm$ 8           | 79 $\pm$ 11             | 85 $\pm$ 14  |
|                | 0.5                 | 72 $\pm$ 2           | 67 $\pm$ 13*            | 80 $\pm$ 17  |
|                | 1                   | 67 $\pm$ 6           | 57 $\pm$ 10*            | 73 $\pm$ 11* |
|                | 2.5                 | 57 $\pm$ 10          | 53 $\pm$ 14*            | 69 $\pm$ 10* |
|                | 5                   | 57 $\pm$ 14          | 64 $\pm$ 5*             | 67 $\pm$ 1*  |

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

\* Indicates a significant decrease in viability compared to control cells,  $p < 0.05$

**Table 3**Effects of 24 h, 48 h and 6 day exposures to TBT on IFN $\gamma$  secretion from highly purified human NK cells.

| 24 h Interferon gamma secreted in pg/mL (mean $\pm$ S.D.) |               |                   |               |                   |             |               |               |  |  |  |
|---|---------------|-------------------|---------------|-------------------|-------------|---------------|---------------|--|--|--|
| [TBT] nM  | KB113         | KB114             | KB115         | KB121             | KB147       | KB149         | KB155         |  |  |  |
| 0   | 34 $\pm$ 10   | 5,213 $\pm$ 140   | 40 $\pm$ 14   | 9,453 $\pm$ 419   | 2 $\pm$ 1   | 658 $\pm$ 22  | 292 $\pm$ 8   |  |  |  |
| 2.5   | 20 $\pm$ 32   | 8,097 $\pm$ 2529  | 221 $\pm$ 52* | 7,129 $\pm$ 956   | 33 $\pm$ 6* | 605 $\pm$ 49  | 534 $\pm$ 28* |  |  |  |
| 5   | 0 $\pm$ 20    | 14,656 $\pm$ 125* | 98 $\pm$ 13*  | 6,517 $\pm$ 1499  | 8 $\pm$ 1*  | 605 $\pm$ 49  | 425 $\pm$ 10* |  |  |  |
| 10  | 62 $\pm$ 21   | 13,375 $\pm$ 835* | 102 $\pm$ 12* | 4,530 $\pm$ 1318* | 8 $\pm$ 4   | 640 $\pm$ 30  | 321 $\pm$ 1*  |  |  |  |
| 25  | 186 $\pm$ 56* | 20,158 $\pm$ 845* | 292 $\pm$ 16* | 6,747 $\pm$ 315*  | 12 $\pm$ 3* | 261 $\pm$ 21* | 329 $\pm$ 15* |  |  |  |
| 50  | 74 $\pm$ 21   | 22,519 $\pm$ 133* | 58 $\pm$ 4    | 5,653 $\pm$ 1852  | 8 $\pm$ 3   | 124 $\pm$ 11* | 181 $\pm$ 4*  |  |  |  |
| 100   | 0 $\pm$ 0*    | 7,467 $\pm$ 456*  | 100 $\pm$ 6*  | 3,289 $\pm$ 248*  | 5 $\pm$ 2   | 14 $\pm$ 37*  | 54 $\pm$ 2*   |  |  |  |
| 200   | 0 $\pm$ 2*    | 25 $\pm$ 35*      | 21 $\pm$ 12   | 0 $\pm$ 4*        | 6 $\pm$ 2   | 0 $\pm$ 4*    | 36 $\pm$ 14*  |  |  |  |

| 48 h Interferon gamma secreted in pg/mL (mean $\pm$ S.D.) |                    |                   |               |             |  |  |  |  |  |  |
|---|--------------------|-------------------|---------------|-------------|--|--|--|--|--|--|
| [TBT] nM  | KB114              | KB120             | KB147         | KB148       |  |  |  |  |  |  |
| 0   | 12,605 $\pm$ 990   | 39,468 $\pm$ 6507 | 5 $\pm$ 1     | 60 $\pm$ 8  |  |  |  |  |  |  |
| 2.5   | 21,752 $\pm$ 1631* | 46,178 $\pm$ 1008 | 177 $\pm$ 120 | 70 $\pm$ 4  |  |  |  |  |  |  |
| 5   | 12,412 $\pm$ 435   | 40,426 $\pm$ 5335 | 26 $\pm$ 7*   | 16 $\pm$ 1* |  |  |  |  |  |  |
| 10  | 16,765 $\pm$ 542*  | 42,306 $\pm$ 2340 | 28 $\pm$ 13   | 22 $\pm$ 9* |  |  |  |  |  |  |
| 25  | 26,972 $\pm$ 759*  | 41,545 $\pm$ 7501 | 19 $\pm$ 5*   | 11 $\pm$ 4* |  |  |  |  |  |  |
| 50  | 31,592 $\pm$ 947*  | 37,357 $\pm$ 3866 | 12 $\pm$ 5    | 8 $\pm$ 5*  |  |  |  |  |  |  |
| 100   | 499 $\pm$ 211*     | 622 $\pm$ 167*    | 9 $\pm$ 3     | 0 $\pm$ 1*  |  |  |  |  |  |  |
| 200   | 0 $\pm$ 0*         | 0 $\pm$ 0*        | 3 $\pm$ 2     | 0 $\pm$ 0*  |  |  |  |  |  |  |

| 6 day Interferon gamma secreted in pg/mL (mean $\pm$ S.D.) |             |            |               |                 |  |  |  |  |  |  |
|--|-------------|------------|---------------|-----------------|--|--|--|--|--|--|
| [TBT] nM   | KB146       | KB147      | KB148         | KB155           |  |  |  |  |  |  |
| 0  | 4 $\pm$ 3   | 15 $\pm$ 1 | 23 $\pm$ 5    | 864 $\pm$ 15    |  |  |  |  |  |  |
| 2.5  | 49 $\pm$ 32 | 23 $\pm$ 9 | 266 $\pm$ 168 | 2,145 $\pm$ 20* |  |  |  |  |  |  |

| 6 day    |       | Interferon gamma secreted in pg/mL (mean±S.D.) |         |           |  |  |
|----------|-------|--|---------|-----------|--|--|
| [TBT] nM | KB146 | KB147  | KB148   | KB155     |  |  |
| 5        | 16±5* | 11±3   | 208±50* | 1,709±28* |  |  |
| 10       | 34±5* | 33±11  | 71±10*  | 1,320±20* |  |  |
| 25       | 25±8* | 53±51  | 72±18*  | 1,498±61* |  |  |
| 50       | 55±7* | 64±4*  | 36±3*   | 642±7*    |  |  |
| 100      | 18±1* | 6±2*   | 9±4*    | 103±10*   |  |  |
| 200      | 1±1*  | 2±2*   | 5±6*    | 76±6*     |  |  |

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

\* Indicates a significant change in secretion compared to control cells,  $p < 0.05$

**Table 4**

Effects of 24 h, 48 h and 6 day exposures to TBT on IFN $\gamma$  secretion from monocyte-depleted PBMCs.

| 24 h Interferon gamma secreted in pg/mL (mean $\pm$ S.D.)  |                   |                  |                   |                    |                  |  |
|--|-------------------|------------------|-------------------|--------------------|------------------|--|
| [TBT] nM   | F93               | F96              | F97               | F126               | F142             |  |
| 0  | 1,037 $\pm$ 75    | 245 $\pm$ 18     | 13 $\pm$ 1        | 4,667 $\pm$ 130    | 1,585 $\pm$ 119  |  |
| 2.5  | 1,492 $\pm$ 193*  | 1,445 $\pm$ 100* | 55 $\pm$ 16*      | 4,473 $\pm$ 103    | 1,576 $\pm$ 53*  |  |
| 5  | 1,339 $\pm$ 184   | 811 $\pm$ 145*   | 84 $\pm$ 19*      | 3,247 $\pm$ 104*   | 1,100 $\pm$ 32*  |  |
| 10   | 3,430 $\pm$ 71*   | 1,773 $\pm$ 193* | 94 $\pm$ 20*      | 5,548 $\pm$ 427    | 1,761 $\pm$ 62   |  |
| 25   | 5,698 $\pm$ 473*  | 2,260 $\pm$ 278* | 178 $\pm$ 33*     | 20,473 $\pm$ 1520* | 4,182 $\pm$ 249* |  |
| 50   | 6,779 $\pm$ 420*  | 2,216 $\pm$ 328* | 177 $\pm$ 62*     | 7,484 $\pm$ 573*   | 2,745 $\pm$ 388* |  |
| 100  | 12,340 $\pm$ 659* | 138 $\pm$ 115    | 0 $\pm$ 3         | 774 $\pm$ 116*     | 423 $\pm$ 48*    |  |
| 200  | 0 $\pm$ 18*       | 0 $\pm$ 22*      | 0 $\pm$ 2*        | 882 $\pm$ 452*     | 0 $\pm$ 20*      |  |
| 48 h Interferon gamma secreted in pg/mL (mean $\pm$ S.D.)  |                   |                  |                   |                    |                  |  |
| [TBT] nM   | F96               | F97              | F126              | F142               |                  |  |
| 0  | 3,250 $\pm$ 325   | 152 $\pm$ 3      | 9,160 $\pm$ 935   | 7,045 $\pm$ 52     |                  |  |
| 2.5  | 6,555 $\pm$ 1859  | 135 $\pm$ 15     | 10,616 $\pm$ 287  | 3,707 $\pm$ 655*   |                  |  |
| 5  | 5,186 $\pm$ 359*  | 180 $\pm$ 1*     | 10,327 $\pm$ 393  | 5,113 $\pm$ 426*   |                  |  |
| 10   | 6,836 $\pm$ 650*  | 308 $\pm$ 33*    | 13,138 $\pm$ 592* | 5,872 $\pm$ 849    |                  |  |
| 25   | 9,293 $\pm$ 263*  | 364 $\pm$ 40*    | 21,938 $\pm$ 494* | 14,251 $\pm$ 383*  |                  |  |
| 50   | 9,319 $\pm$ 955*  | 531 $\pm$ 94*    | 31,804 $\pm$ 428* | 14,402 $\pm$ 1415* |                  |  |
| 100  | 2,652 $\pm$ 203   | 76 $\pm$ 11*     | 549 $\pm$ 271*    | 5,784 $\pm$ 1497   |                  |  |
| 200  | 0 $\pm$ 66*       | 0 $\pm$ 1*       | 127 $\pm$ 33*     | 184 $\pm$ 15*      |                  |  |
| 6 day Interferon gamma secreted in pg/mL (mean $\pm$ S.D.) |                   |                  |                   |                    |                  |  |
| [TBT] nM   | F96               | F126             | F142              |                    |                  |  |
| 0  | 2,057 $\pm$ 81    | 5,752 $\pm$ 264  | 4,234 $\pm$ 229   |                    |                  |  |
| 2.5  | 3,586 $\pm$ 213*  | 3,903 $\pm$ 151* | 4,772 $\pm$ 225*  |                    |                  |  |

| 6 day    |            | Interferon gamma secreted in pg/mL (mean±S.D.) |             |  |  |
|----------|------------|--|-------------|--|--|
| [TBT] nM | F96        | F126   | F142        |  |  |
| 5        | 3,382±120* | 10,721±157*                                    | 5,821±112*  |  |  |
| 10       | 3,873±127* | 21,479±262*                                    | 7,095±128*  |  |  |
| 25       | 4,171±171* | 41,550±852*                                    | 10,351±19*  |  |  |
| 50       | 3,875±239* | 39,620±1000*                                   | 11,933±270* |  |  |
| 100      | 2,458±79*  | 358±132*                                       | 4,011±78    |  |  |
| 200      | 0±0*       | 1,236±1759*                                    | 0±13*       |  |  |

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

\* Indicates a significant change in secretion compared to control cells,  $p < 0.05$

**Table 5**  
Effects of 24 h, 48 h and 6 day exposures to TBT on IFN $\gamma$  secretion from PBMCs.

| 24 h Interferon gamma secreted in pg/mL (mean $\pm$ S.D.) |                  |               |                 |               |  |  |
|---|------------------|---------------|-----------------|---------------|--|--|
| [TBT] nM  | F116             | F117          | F119            | F120          |  |  |
| 0   | 2,209 $\pm$ 154  | 658 $\pm$ 32  | 1,052 $\pm$ 44  | 60 $\pm$ 2    |  |  |
| 2.5   | 1,556 $\pm$ 359  | 454 $\pm$ 39* | 1,443 $\pm$ 32* | 53 $\pm$ 10   |  |  |
| 5   | 1,698 $\pm$ 107* | 190 $\pm$ 16* | 1,958 $\pm$ 5*  | 79 $\pm$ 6*   |  |  |
| 10  | 1,373 $\pm$ 130* | 149 $\pm$ 41* | 1,158 $\pm$ 118 | 84 $\pm$ 2*   |  |  |
| 25  | 1,103 $\pm$ 51*  | 162 $\pm$ 20* | 823 $\pm$ 12*   | 113 $\pm$ 12* |  |  |
| 50  | 789 $\pm$ 50*    | 173 $\pm$ 26* | 692 $\pm$ 7*    | 105 $\pm$ 3*  |  |  |
| 100   | 159 $\pm$ 25*    | 0 $\pm$ 11*   | 662 $\pm$ 15*   | 156 $\pm$ 14* |  |  |
| 200   | 31 $\pm$ 22*     | 0 $\pm$ 8*    | 0 $\pm$ 14*     | 13 $\pm$ 1*   |  |  |

| 48 h Interferon gamma secreted in pg/mL (mean $\pm$ S.D.) |                    |                 |                  |               |  |  |
|---|--------------------|-----------------|------------------|---------------|--|--|
| [TBT] nM  | F116               | F117            | F119             | F120          |  |  |
| 0   | 10,317 $\pm$ 827   | 2,193 $\pm$ 9   | 1,367 $\pm$ 14   | 51 $\pm$ 13   |  |  |
| 2.5   | 6,571 $\pm$ 423*   | 1,949 $\pm$ 400 | 2,765 $\pm$ 276* | 38 $\pm$ 4    |  |  |
| 5   | 5,419 $\pm$ 13*    | 1,501 $\pm$ 50* | 2,847 $\pm$ 24*  | 78 $\pm$ 17   |  |  |
| 10  | 7,803 $\pm$ 564*   | 1,659 $\pm$ 71* | 1,958 $\pm$ 81*  | 73 $\pm$ 33   |  |  |
| 25  | 8,462 $\pm$ 1,911  | 1,033 $\pm$ 53* | 1,395 $\pm$ 66   | 61 $\pm$ 38   |  |  |
| 50  | 10,658 $\pm$ 259   | 975 $\pm$ 36*   | 1,854 $\pm$ 24*  | 132 $\pm$ 2*  |  |  |
| 100   | 1,672 $\pm$ 1,010* | 182 $\pm$ 5*    | 1,461 $\pm$ 23*  | 410 $\pm$ 48* |  |  |
| 200   | 2,259 $\pm$ 724*   | 62 $\pm$ 34*    | 76 $\pm$ 5*      | 11 $\pm$ 3*   |  |  |

| 6 day Interferon gamma secreted in pg/mL (mean $\pm$ S.D.) |                  |                 |                |               |  |  |
|--|------------------|-----------------|----------------|---------------|--|--|
| [TBT] nM   | F116             | F117            | F119           | F120          |  |  |
| 0  | 9,362 $\pm$ 538  | 4,189 $\pm$ 378 | 1,631 $\pm$ 63 | 122 $\pm$ 2   |  |  |
| 2.5  | 5,926 $\pm$ 942* | 3,841 $\pm$ 248 | 1,811 $\pm$ 4* | 306 $\pm$ 25* |  |  |

| 6 day    |              | Interferon gamma secreted in pg/mL (mean±S.D.). |           |          |  |  |  |
|----------|--------------|---|-----------|----------|--|--|--|
| [TBT] mM | F116         | F117  | F119      | F120     |  |  |  |
| 5        | 5,285±924*   | 4,617±59  | 2,308±87* | 162±19   |  |  |  |
| 10       | 11,772±5,415 | 3,424±185                                       | 2,358±76* | 283±38*  |  |  |  |
| 25       | 8,656±1,252  | 3,546±174                                       | 2,014±56* | 178±5*   |  |  |  |
| 50       | 13,887±6,679 | 1,926±80*                                       | 1,591±51  | 256±7*   |  |  |  |
| 100      | 1,156±714*   | 396±95*   | 886±61*   | 2325±76* |  |  |  |
| 200      | 0±67*        | 72±40*  | 0±4*      | 110±22   |  |  |  |

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

\* Indicates a significant change in secretion compared to control cells,  $p < 0.05$



**Table 6**Effects of 24 h, 48 h and 6 day exposures to DBT on IFN $\gamma$  secretion from highly purified human NK cells.

| 24 h Interferon gamma secreted in pg/mL (mean $\pm$ S.D.) |               |                     |              |                  |               |                 |
|---|---------------|---------------------|--------------|------------------|---------------|-----------------|
| [DBT] $\mu$ M   | KB113         | KB114               | KB125        | KB130            | KB139         | KB155           |
| 0   | 172 $\pm$ 2   | 19,094 $\pm$ 545    | 15 $\pm$ 1   | 6,148 $\pm$ 60   | 638 $\pm$ 12  | 341 $\pm$ 17    |
| 0.05  | 121 $\pm$ 25* | 36,510 $\pm$ 1,193* | 99 $\pm$ 18* | 4,474 $\pm$ 111* | 658 $\pm$ 72  | 1,196 $\pm$ 29* |
| 0.1   | 408 $\pm$ 22* | 27,635 $\pm$ 525*   | 72 $\pm$ 13* | 2,296 $\pm$ 62*  | 664 $\pm$ 29  | 872 $\pm$ 27*   |
| 0.25  | 125 $\pm$ 2*  | 6,052 $\pm$ 776*    | 7 $\pm$ 1*   | 78 $\pm$ 3*      | 104 $\pm$ 14* | 122 $\pm$ 18*   |
| 0.5   | 4 $\pm$ 4*    | 0 $\pm$ 0*          | 0 $\pm$ 0*   | 0 $\pm$ 0*       | 0 $\pm$ 23*   | 0 $\pm$ 13*     |
| 1   | 7 $\pm$ 12*   | 0 $\pm$ 0*          | 0 $\pm$ 0*   | 0 $\pm$ 0*       | 0 $\pm$ 0*    | 0 $\pm$ 6*      |
| 2.5   | 3 $\pm$ 3*    | 0 $\pm$ 0*          | 0 $\pm$ 0*   | 0 $\pm$ 0*       | 0 $\pm$ 0*    | 0 $\pm$ 6*      |
| 5   | 11 $\pm$ 9*   | 0 $\pm$ 0*          | 0 $\pm$ 0*   | 0 $\pm$ 0*       | 0 $\pm$ 0*    | 0 $\pm$ 36*     |

| 48 h Interferon gamma secreted in pg/mL (mean $\pm$ S.D.) |               |                     |                     |                  |  |  |
|---|---------------|---------------------|---------------------|------------------|--|--|
| [DBT] $\mu$ M   | KB125         | KB130               | KB139               | KB155            |  |  |
| 0   | 58 $\pm$ 4    | 30,765 $\pm$ 934    | 4,035 $\pm$ 916     | 554 $\pm$ 118    |  |  |
| 0.05  | 450 $\pm$ 13* | 37,971 $\pm$ 1,579* | 5,469 $\pm$ 453*    | 2,214 $\pm$ 222* |  |  |
| 0.1   | 825 $\pm$ 18* | 23,575 $\pm$ 940*   | 9,343 $\pm$ 1,630*  | 6,766 $\pm$ 67*  |  |  |
| 0.25  | 877 $\pm$ 26* | 6,559 $\pm$ 1,104*  | 11,796 $\pm$ 2,500* | 3,065 $\pm$ 509* |  |  |
| 0.5   | 4 $\pm$ 1*    | 0 $\pm$ 0*          | 1,538 $\pm$ 823*    | 0 $\pm$ 20*      |  |  |
| 1   | 0 $\pm$ 0*    | 0 $\pm$ 0*          | 0 $\pm$ 0*          | 0 $\pm$ 0*       |  |  |
| 2.5   | 0 $\pm$ 0*    | 0 $\pm$ 0*          | 0 $\pm$ 0*          | 0 $\pm$ 0*       |  |  |
| 5   | 0 $\pm$ 0*    | 0 $\pm$ 0*          | 0 $\pm$ 0*          | 0 $\pm$ 0*       |  |  |

| 6 day Interferon gamma secreted (mean $\pm$ S.D.) in pg/mL |                     |                  |                |
|--|---------------------|------------------|----------------|
| [DBT] $\mu$ M  | KB130               | KB139            | KB155          |
| 0  | 26,050 $\pm$ 1,626  | 2,101 $\pm$ 390  | 472 $\pm$ 42   |
| 0.05   | 40,183 $\pm$ 3,125* | 1,052 $\pm$ 220* | 1,302 $\pm$ 8* |

| 9 day         | Interferon gamma secreted (mean±S.D.) in pg/mL |          |            |  |
|---------------|--|----------|------------|--|
| [DBT] $\mu$ M | KB130  | KB139    | KB155      |  |
| 0.1           | 35,283±12,021                                  | 2078±467 | 1,267±169* |  |
| 0.25          | 6,317±333*                                     | 416±122* | 904±47*    |  |
| 0.5           | 0±0*   | 0±0*     | 1,044±106* |  |
| 1             | 0±0*   | 0±0*     | 325±11*    |  |
| 2.5           | 0±0*   | 0±0*     | 0±0*       |  |
| 5             | 0±0*   | 0±0*     | 0±0*       |  |

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

\* Indicates a significant change in secretion compared to control cells,  $p < 0.05$

**Table 7**  
Effects of 24 h, 48 h, 6 day exposures to DBT on IFN $\gamma$  secretion from monocyte-depleted PBMCs.

| 24 h Interferon gamma secreted in pg/mL (mean $\pm$ S.D.) |                 |                  |             |                  |             |                  |                  |  |  |
|---|-----------------|------------------|-------------|------------------|-------------|------------------|------------------|--|--|
| [DBT] $\mu$ M   | F93             | F99              | F105        | F126             | F139        | F142             | F145             |  |  |
| 0   | 80 $\pm$ 4      | 650 $\pm$ 17     | 41 $\pm$ 2  | 1,247 $\pm$ 50   | 22 $\pm$ 2  | 1,259 $\pm$ 57   | 3,104 $\pm$ 64   |  |  |
| 0.05  | 272 $\pm$ 88    | 665 $\pm$ 185    | 42 $\pm$ 4  | 3,055 $\pm$ 77*  | 44 $\pm$ 19 | 2,462 $\pm$ 349* | 3,869 $\pm$ 140* |  |  |
| 0.1   | 309 $\pm$ 114   | 3,270 $\pm$ 1263 | 59 $\pm$ 4* | 7,448 $\pm$ 234* | 85 $\pm$ 1* | 4,426 $\pm$ 163* | 4,827 $\pm$ 175* |  |  |
| 0.25  | 353 $\pm$ 20*   | 623 $\pm$ 104    | 69 $\pm$ 8* | 1,888 $\pm$ 44*  | 18 $\pm$ 5  | 4,426 $\pm$ 241* | 2,447 $\pm$ 85*  |  |  |
| 0.5   | 542 $\pm$ 25*   | 185 $\pm$ 31*    | 11 $\pm$ 5* | 27 $\pm$ 18*     | 0 $\pm$ 1*  | 248 $\pm$ 46*    | 211 $\pm$ 73*    |  |  |
| 1   | 1,840 $\pm$ 30* | 201 $\pm$ 21*    | 3 $\pm$ 1*  | 0 $\pm$ 15*      | 0 $\pm$ 1*  | 0 $\pm$ 8*       | 60 $\pm$ 7*      |  |  |
| 2.5   | 58 $\pm$ 101    | 145 $\pm$ 0*     | 5 $\pm$ 2*  | 0 $\pm$ 4*       | 0 $\pm$ 8*  | 0 $\pm$ 42*      | 80 $\pm$ 64*     |  |  |
| 5   | 47 $\pm$ 60     | 192 $\pm$ 59*    | 1 $\pm$ 1*  | 4 $\pm$ 26*      | 0 $\pm$ 2*  | 0 $\pm$ 58*      | 144 $\pm$ 67*    |  |  |

| 48 h Interferon gamma secreted in pg/mL (mean $\pm$ S.D.) |                  |               |                   |                   |                    |                  |  |  |  |
|---|------------------|---------------|-------------------|-------------------|--------------------|------------------|--|--|--|
| [DBT] $\mu$ M   | F99              | F105          | F126              | F139              | F142               | F145             |  |  |  |
| 0   | 652 $\pm$ 25     | 41 $\pm$ 2    | 3,914 $\pm$ 506   | 1,147 $\pm$ 46    | 4,157 $\pm$ 220    | 2,660 $\pm$ 81   |  |  |  |
| 0.05  | 2,054 $\pm$ 30*  | 122 $\pm$ 9*  | 5,559 $\pm$ 433*  | 1,500 $\pm$ 390   | 4,770 $\pm$ 382    | 4,017 $\pm$ 118* |  |  |  |
| 0.1   | 2,233 $\pm$ 106* | 350 $\pm$ 6*  | 9,381 $\pm$ 335*  | 3,253 $\pm$ 185*  | 12,667 $\pm$ 1210* | 5,685 $\pm$ 85*  |  |  |  |
| 0.25  | 1,915 $\pm$ 28*  | 685 $\pm$ 63* | 8,309 $\pm$ 1212* | 16,667 $\pm$ 150* | 14,590 $\pm$ 2154* | 3,509 $\pm$ 210* |  |  |  |
| 0.5   | 162 $\pm$ 35*    | 369 $\pm$ 3*  | 1,104 $\pm$ 186*  | 347 $\pm$ 58*     | 4,210 $\pm$ 165    | 141 $\pm$ 30*    |  |  |  |
| 1   | 0 $\pm$ 12*      | 24 $\pm$ 2*   | 0 $\pm$ 8*        | 0 $\pm$ 81*       | 90 $\pm$ 17*       | 0 $\pm$ 26*      |  |  |  |
| 2.5   | 0 $\pm$ 7*       | 1 $\pm$ 1*    | 0 $\pm$ 18*       | 0 $\pm$ 64*       | 37 $\pm$ 30*       | 0 $\pm$ 24*      |  |  |  |
| 5   | 0 $\pm$ 15*      | 0 $\pm$ 1*    | 0 $\pm$ 18*       | 0 $\pm$ 114*      | 67 $\pm$ 111*      | 0 $\pm$ 10*      |  |  |  |

| 6 day Interferon gamma secreted in pg/mL (mean $\pm$ S.D.) |              |                   |                 |                  |                 |  |
|--|--------------|-------------------|-----------------|------------------|-----------------|--|
| [DBT] $\mu$ M  | F105         | F126              | F139            | F142             | F145            |  |
| 0  | 67 $\pm$ 2   | 4,029 $\pm$ 108   | 2,057 $\pm$ 198 | 3,893 $\pm$ 594  | 2828 $\pm$ 386  |  |
| 0.05   | 155 $\pm$ 2* | 12,468 $\pm$ 902* | 1,780 $\pm$ 110 | 8,648 $\pm$ 354* | 3791 $\pm$ 226* |  |

| 6 day Interferon gamma secreted in pg/mL (mean±S.D.) |         |             |            |             |          |  |  |
|--|---------|-------------|------------|-------------|----------|--|--|
| [DBT] $\mu$ M  | F105    | F126        | F139       | F142        | F145     |  |  |
| 0.1  | 336±21* | 15,390±524* | 2,027±90   | 11,836±497* | 4778±17* |  |  |
| 0.25   | 688±8*  | 14,590±104* | 4,223±555* | 14,203±322* | 3348±336 |  |  |
| 0.5  | 522±38* | 1,301±121*  | 2,243±100  | 7,128±187*  | 356±86*  |  |  |
| 1  | 42±2*   | 0±88*       | 480±89*    | 0±51*       | 0±23*    |  |  |
| 2.5  | 4±1*    | 0±151*      | 447±185*   | 0±6*        | 0±138*   |  |  |
| 5  | 7±1*    | 0±750*      | 523±157*   | 0±61*       | 0±222*   |  |  |

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

\* Indicates a significant change in secretion compared to control cells,  $p < 0.05$

**Table 8**Effects of 24 h, 48 h and 6 day exposures to DBT on IFN $\gamma$  secretion from PBMCs.

| 24 h Interferon gamma secreted in pg/mL (mean $\pm$ S.D.) |                 |               |                  |               |                  |      |
|---|-----------------|---------------|------------------|---------------|------------------|------|
| [DBT] $\mu$ M   | F116            | F117          | F119             | F120          | F150             | F150 |
| 0   | 2,009 $\pm$ 69  | 899 $\pm$ 11  | 2,948 $\pm$ 7    | 153 $\pm$ 23  | 2,470 $\pm$ 82   |      |
| 0.05  | 2,679 $\pm$ 92* | 555 $\pm$ 35* | 1,645 $\pm$ 1*   | 192 $\pm$ 23  | 1,390 $\pm$ 100* |      |
| 0.1   | 2,492 $\pm$ 42* | 354 $\pm$ 12* | 2,129 $\pm$ 47*  | 218 $\pm$ 25* | 2,260 $\pm$ 104  |      |
| 0.25  | 2,532 $\pm$ 35* | 195 $\pm$ 5*  | 2,597 $\pm$ 29*  | 338 $\pm$ 15  | 473 $\pm$ 15*    |      |
| 0.5   | 588 $\pm$ 11*   | 17 $\pm$ 4*   | 1,503 $\pm$ 132* | 492 $\pm$ 84* | 187 $\pm$ 132*   |      |
| 1   | 1 $\pm$ 11*     | 0 $\pm$ 8*    | 61 $\pm$ 5*      | 99 $\pm$ 26   | 126 $\pm$ 58*    |      |
| 2.5   | 0 $\pm$ 15*     | 0 $\pm$ 4*    | 14 $\pm$ 29*     | 88 $\pm$ 28*  | 77 $\pm$ 38*     |      |
| 5   | 0 $\pm$ 17*     | 0 $\pm$ 2*    | 0 $\pm$ 6*       | 76 $\pm$ 3*   | 120 $\pm$ 105*   |      |

| 48 h Interferon gamma secreted in pg/mL (mean $\pm$ S.D.) |                     |                 |                 |                  |                  |                  |
|---|---------------------|-----------------|-----------------|------------------|------------------|------------------|
| [DBT] $\mu$ M   | F116                | F117            | F119            | F120             | F125             | F150             |
| 0   | 7,257 $\pm$ 1,184   | 2,420 $\pm$ 91  | 3,244 $\pm$ 86  | 84 $\pm$ 64      | 840 $\pm$ 30     | 3,368 $\pm$ 48   |
| 0.05  | 11,604 $\pm$ 106*   | 1,673 $\pm$ 59* | 2,915 $\pm$ 171 | 403 $\pm$ 42*    | 1,691 $\pm$ 54*  | 2,321 $\pm$ 137* |
| 0.1   | 18,491 $\pm$ 966*   | 1,435 $\pm$ 8*  | 3,850 $\pm$ 399 | 489 $\pm$ 159*   | 4,373 $\pm$ 139* | 3,361 $\pm$ 255  |
| 0.25  | 30,877 $\pm$ 1,051* | 810 $\pm$ 32*   | 3,928 $\pm$ 63* | 942 $\pm$ 60*    | 6,359 $\pm$ 140* | 1,681 $\pm$ 205* |
| 0.5   | 6,650 $\pm$ 1,082   | 233 $\pm$ 3*    | 2,815 $\pm$ 239 | 2,943 $\pm$ 158* | 801 $\pm$ 40     | 38 $\pm$ 15*     |
| 1   | 0 $\pm$ 270*        | 0 $\pm$ 9*      | 724 $\pm$ 32*   | 343 $\pm$ 129    | 0 $\pm$ 25*      | 23 $\pm$ 30*     |
| 2.5   | 0 $\pm$ 197*        | 0 $\pm$ 0*      | 54 $\pm$ 38*    | 0 $\pm$ 0*       | 0 $\pm$ 3*       | 31 $\pm$ 45*     |
| 5   | 0 $\pm$ 170*        | 0 $\pm$ 3*      | 31 $\pm$ 13*    | 0 $\pm$ 4*       | 0 $\pm$ 20*      | 28 $\pm$ 18*     |

| 6 d Interferon gamma secreted in pg/mL (mean $\pm$ S.D.) |                 |                 |                  |
|--|-----------------|-----------------|------------------|
| [DBT] $\mu$ M  | F117            | F119            | F125             |
| 0  | 2,643 $\pm$ 90  | 3,258 $\pm$ 187 | 125 $\pm$ 2      |
| 0.05   | 2,084 $\pm$ 34* | 2,647 $\pm$ 69* | 233 $\pm$ 4*     |
|  |                 |                 | 1,951 $\pm$ 133* |

| 6 d Interferon gamma secreted in pg/mL (mean±S.D.) |           |            |            |            |  |  |
|--|-----------|------------|------------|------------|--|--|
| [DBT] $\mu$ M                                      | F117      | F119       | F120       | F125       |  |  |
| 0.1  | 2,107±88* | 4,270±143* | 337±26*    | 5,432±60*  |  |  |
| 0.25   | 2,108±31* | 6,075±103* | 2,096±119* | 7,162±303* |  |  |
| 0.5  | 1,026±17* | 2,616±149* | 5,510±143* | 1,625±100* |  |  |
| 1  | 0±10*     | 712±10*    | 2,406±94*  | 0±4*       |  |  |
| 2.5  | 0±3*      | 59±7*      | 9±6*       | 0±0*       |  |  |
| 5  | 0±1*      | 0±7*       | 0±9*       | 0±1*       |  |  |

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

\* Indicates a significant change in secretion compared to control cells,  $p < 0.05$