



# Synthesis of interleukin 1 beta and interleukin 6 in human lymphocytes is stimulated by tributyltin

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## Abstract

Tributyltin (TBT) is a widespread environmental contaminant that is present in human blood and other tissues. It has been shown to disrupt the immune function of human natural killer (NK) cells and to alter the secretion of a number of pro-inflammatory cytokines from immune cells. Secretion of both interleukin 1 $\beta$  (IL-1 $\beta$ ) and interleukin 6 (IL-6) from human lymphocytes can be increased dependent upon the level of TBT exposure. This study shows that the TBT-induced increases in secretion of both cytokines are due to TBT-induced increases in the synthesis of these proteins and not simply because of the release of pre-existing cytokine. Furthermore, the data indicate that these TBT-induced increases in IL-1 $\beta$  and IL-6 synthesis require MAP kinase signaling pathways. Additionally, elevated synthesis of IL-1 $\beta$  and IL-6 seen at the highest exposures to TBT (200, 200, 50 nM) were accompanied by increases in the mRNA for these cytokines. TBT-induced increases in IL-1 $\beta$  and IL-6 mRNAs were also shown to be dependent on MAP kinase signaling. The study suggests that TBT has the capacity to increase immune cell production of these 2 important pro-inflammatory cytokines and that this increase is in part explained by increased mRNA for the cytokines.

**Keywords** MD-PBMCs · Tributyltin · Interleukin 1 beta · Interleukin 6

## Introduction

Due to its multitude of uses as a biocide, tributyltin (TBT) widely contaminates the environment and has been found in human tissues including blood (as high as 261 nM in blood) (Antizar-Ladislao 2008; Kannan et al. 1995, 1999; Whalen et al. 1999). It was used as an antifouling agent in paints applied to the hulls of ships (Loganathan et al. 2001; Loganathan 2016). Its use was banned by the International Convention on the Control of Harmful Antifouling Systems on Ships in 2008 (Gipperth 2009), but it is still used by countries that are not part of the convention. It is also found in some household goods such as siliconized-paper baking parchments and shower curtains (Yamada et al. 1993). TBT exposure can cause increased incidences of tumors and thymic atrophy in mammals (Wester et al. 1990; Snoeij et al.

1988) and decrease the ability of human natural killer (NK) lymphocytes to destroy tumor cells (Dudimah et al. 2007). Additionally, TBT has been shown to alter the secretion of several important pro-inflammatory cytokines from human lymphocytes. These include interferon gamma (IFN $\gamma$ ), tumor necrosis factor alpha (TNF $\alpha$ ), IL-1 $\beta$ , and IL-6 (Hurt et al. 2013; Lawrence et al. 2015; Brown and Whalen 2015; Brown et al. 2018).

IL-1 $\beta$  regulates the inflammatory response and promotes cellular growth, and tissue repair (Apte and Voronov 2002; Arend 2002; Dinarello 1996, 2005, 2009). It is produced by monocytes, macrophages, T cells, NK cells, neutrophils, keratinocytes, and fibroblasts (Guma et al. 2009; Burger and Dayer 2002; Dinarello 2005; Apte and Voronov 2002; Voronov et al. 2002). IL-1 $\beta$  can act as a stimulator of the production of other pro-inflammatory cytokines, including IFN $\gamma$  and IL-6 (Tosato and Jones 1990; Cooper et al. 2001; Luo et al. 2003). When IL-1 $\beta$  levels are elevated inappropriately it contributes to chronic inflammation (Dinarello 2011). Elevated IL-1 $\beta$  is associated with a number of diseases including multiple sclerosis, rheumatoid arthritis, and cancer (Rossi et al. 2012; Choy and Panayi 2001; Arlt et al. 2002; Elaraj et al. 2006; Jin et al. 1997; Lewis and Varghese

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2006; Muerkoster et al. 2006). A drug that decreases IL-1 $\beta$  levels is an approved treatment for rheumatoid arthritis (Dinarello 2011).

Like IL-1 $\beta$ , IL-6 is a pro-inflammatory cytokine (22–27 kDA) (Kishimoto 1989) produced by a variety of cells types including lymphocytes, monocytes/macrophages, and granulocytes (Akira et al. 1993; Li and He 2006; Hall et al. 2010; Riedemann et al. 2004; Zimmermann et al. 2015, 2016). As with IL-1 $\beta$ , inappropriately elevated levels of IL-6 can lead to chronic inflammation (Tanaka et al. 2014) and increased IL-6 activity contributes to chronic inflammatory diseases such as colon cancer, colitis, rheumatoid arthritis, systemic lupus erythematosus, psoriasis, and Crohn's disease (Gabay 2006; Atreya et al. 2000; Hurst et al. 2001). An antibody to the IL-6 receptor, tocilizumab, has been approved to treat rheumatoid arthritis (Tanaka et al. 2014).

TBT has been shown to alter the secretion of both IL-1 $\beta$  and IL-6 from human lymphocytes (Brown and Whalen 2015; Brown et al. 2018). The current study examines whether these alterations in secretion are due to changes in the lymphocytes' synthesis of these cytokines or if it is simply due to changes in the secretion of pre-existing cytokine. To address this, both secreted and intracellular levels of each cytokine were measured from the same cells in response to exposures to TBT. TBT concentrations that caused increases in cytokine synthesis were then examined to determine if this reflected an increase in the mRNA for the cytokine. Additionally, signaling pathways known to be utilized in cellular production of each of the cytokines were examined for potential roles in any TBT-induced increases in either cytokine. These included, mitogen activated protein kinases (MAPKs) and nuclear factor kappa B (NF $\kappa$ B) (Gaestel et al. 2009).

## Materials and methods

### Preparation of MD-PBMCs

PBMCs were isolated from Leukocyte filters (PALL- RCPL or RC2D) obtained from the Red Cross Blood Bank Facility (Nashville, TN, USA) as described in Meyer et al. 2005. Leukocytes were retrieved from the filters by back-flushing them with sterile PBS containing 5 mM disodium EDTA and 2.5% [w/v] sucrose and collecting the eluent. The eluent was layered onto Ficoll-Hypaque (1.077 g/mL) and centrifuged at 1200g for 30–50 min. Mononuclear cells were collected and washed with PBS (500 g, 10 min). Following washing, the cells were layered on bovine calf serum for platelet removal. Monocyte-depleted PBMCs were prepared by incubating the cells in glass Petri dishes (150 $\times$ 15 mm) at 37 °C and air/CO<sub>2</sub>, 19:1 for 1.5 h. This cell preparation is referred to as MD-PBMCs cells.

### Chemical preparation

Tributyltin chloride (TBT) was purchased from Sigma-Aldrich (96%) (St. Louis, MO). 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.

### Inhibitor preparation

Enzyme inhibitors were purchased from Fisher Scientific (Pittsburgh, PA). The stock solution for each inhibitor was a 50 mM solution in dimethylsulfoxide (DMSO). Caspase-1 inhibitor II (50  $\mu$ M), MEK 1/2 pathway inhibitor (PD98059) (50  $\mu$ M), p38 inhibitor (SB202190) (25  $\mu$ M), JNK pathway inhibitor (JNK X BI78D3) (0.05  $\mu$ M), and NF $\kappa$ B inhibitor (BAY11-7085) (0.3125–1.25  $\mu$ M) were prepared by dilution of the stock solution into cell culture media.

### Cell treatments

MD-PBMCs were treated with TBT and appropriate control(s). Following the incubations, the cells were pelleted and the supernatants were collected and frozen at – 70 °C until assay. Cell pellets were lysed and stored at – 70 °C for Western blot analysis.

### IL-1 $\beta$ and IL-6 secretion assays

IL-1  $\beta$  and IL-6 levels were measured using the BD OptEIA™ Human enzyme-linked immunosorbent assay (ELISA) kits (BD-Pharmingen, San Diego, CA, USA).

### Cell lysis

MD-PBMCs (at a concentration of 4–6 million cells/mL) were treated with 2.5–200 nM TBT or control for 10, 30 min, 6, or 24 h. Following the treatments, the cells were centrifuged, supernatants collected, and the cell pellets were lysed using 133  $\mu$ L of lysis buffer (Active motif, Carlsbad, CA). For inhibitor studies, MD-PBMCs (at a concentration of 3 million cells/mL) were either treated with 5, 10, 25 nM TBT (IL-1 $\beta$  studies) or 50, 100, 200 nM TBT (IL-6 studies). The cell lysates were stored frozen at – 80 °C.

### Immunoblotting analysis

Cell lysates were run on 10% SDS-PAGE (sodium dodecyl-sulfate polyacrylamide gel electrophoresis) and transferred to a PVDF (polyvinylidene difluoride) membrane. The PVDF was immunoblotted with specific primary antibodies:

anti-IL-1 $\beta$ , anti-IL-6, and anti- $\beta$  actin (Cell Signaling Technologies, Beverly, MA). Antibodies were visualized using an ECL chemiluminescent detection system (Amersham, Piscataway, NJ, USA) and UVP Imager.  $\beta$ -actin levels were determined for each condition to verify that equal amounts of protein were loaded. In addition, the density of each protein band was normalized to  $\beta$ -actin to correct for slight differences in protein loading among the lanes.

### RNA isolation and RT-qPCR

RNA from MD-PBMCs (at a concentration of 4–6 million cells/mL) was extracted with RNeasy Mini Kit (Qiagen). PCR primers were designed using Primer Express 2.0 (Applied Biosystems). All RT-qPCR assays were conducted using QuantiTect SYBR Green RT-PCR kit (Qiagen). Reaction was done in 20  $\mu$ L containing 50 ng of total RNA and 0.4  $\mu$ M of each primer.

Gene	Forward sequence (5')	Reverse sequence (3')
IL-1 $\beta$	GCACGATGCACCTGT ACGAT	AGACATACCAAGCTTTT TTGCT
IL-6	CCGGAACGAAAGAG AAGCT	GCGCTTGTTGAGAAG GAGTT

### Statistical analysis

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

## Results

### Effects of TBT exposures on the synthesis (secretion + intracellular levels) of IL-1 $\beta$

#### 10 min exposures to TBT

The effects of 10 min exposures to TBT on intracellular and secreted levels of IL-1 $\beta$  were examined in MD-PBMCs from 3 separate donors. There were no increases in the synthesis of IL-1 $\beta$  (secretion + intracellular levels) after 10 min of exposure to TBT (data not shown).

#### 30 min exposures to TBT

Figure 1a summarizes the effects of 30 min exposure to TBT on intracellular and secreted IL-1 $\beta$  in MD-PBMCs from 4

separate donors. Cells from each of the 4 donors exhibited slight increases in synthesis of IL-1 $\beta$  (the combination of fold increase in intracellular + fold increase in secreted IL-1 $\beta$  > 1) at one or more TBT concentrations. For example, cells from donor F219 showed increases of 1.5, 1.2, 1.4, and 1.8 fold in IL-1 $\beta$  synthesis with exposures to 200, 10, 5, and 2.5 nM TBT.

#### 6 h exposures to TBT

The effects of 6 h exposures to TBT on IL-1 $\beta$  synthesis are shown in Fig. 1b. As was seen at 30 min, cells from all donors exhibited increased production (a combined intracellular fold increase + secreted fold increase of greater than 1). However, the extent of the increases was somewhat greater than that seen after 30 min and it occurred at 3 or more exposure concentrations.

#### 24 h exposures to TBT

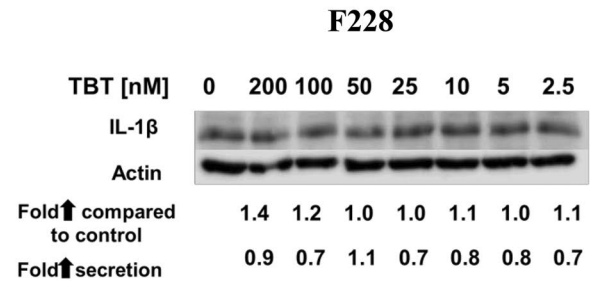
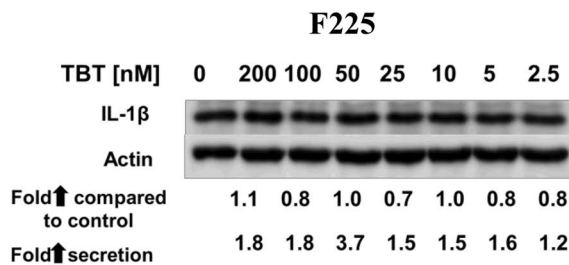
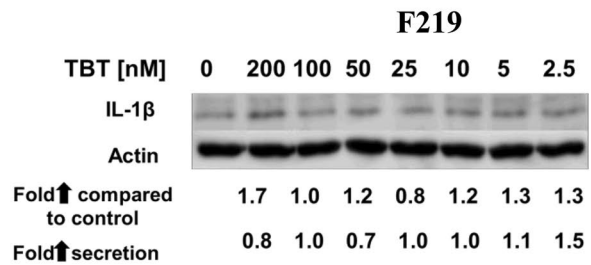
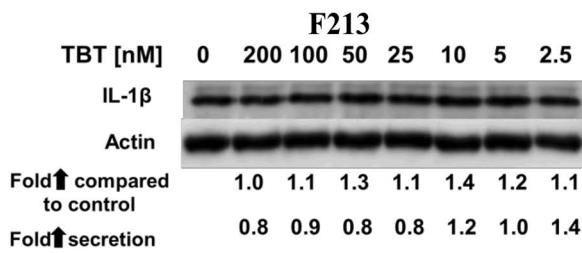
The changes in intracellular and secreted IL-1 $\beta$  after 24 h exposures to TBT are shown in Fig. 1c. When the effects on secretion + intracellular levels of IL-1 $\beta$  were examined, there was a notable increase in its production in the cells from each of the 4 donors. Every TBT exposure caused an increase in production many of which were quite large (ranging from 1.1 to 26.4 fold) indicating that after 24 h of exposure TBT stimulates very consistent and quite substantial increases in IL-1 $\beta$  production in MD-PBMCs.

### Effects of exposures to TBT on the synthesis of IL-1 $\beta$ by MD-PBMCs treated with signaling pathway inhibitors

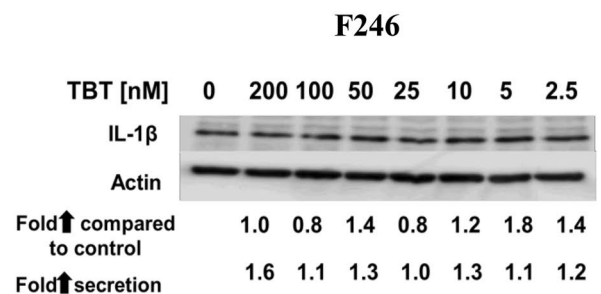
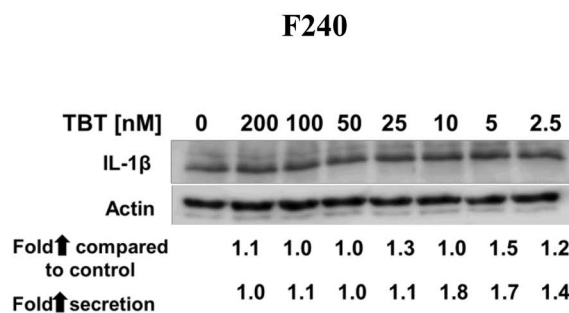
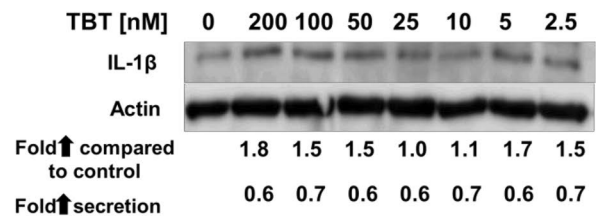
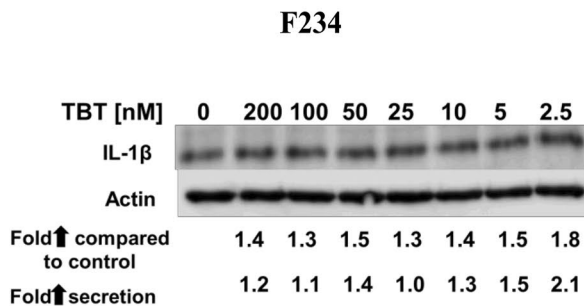
#### Mitogen activated protein kinase kinase (MAP2K), MEK, inhibitor (PD98059)

The effects of exposures to 5, 10, and 25 nM TBT synthesis (intracellular + secreted levels) of IL-1 $\beta$  from MD-PBMCs where p44/42 MAPK function had been inhibited with PD98059 are shown in Fig. 2a. Cells exposed to TBT when the inhibitor was present showed diminished TBT-stimulated synthesis of IL-1 $\beta$ . For instance, when cells from donor F300 were exposed to 5, 10, and 25 nM TBT in the absence of PD98059 inhibitor there were 4.2, 4.1, and 8.4 fold increases in IL-1 $\beta$  production. When the inhibitor was present, those same TBT exposures caused no increase in synthesis at the 5 and 10 nM exposures, and only a twofold increase in IL-1 $\beta$  production at the 25 nM exposure. This same effect was seen in the cells from 3 other donors tested. Overall, the results indicate that the p44/42 MAPK pathway is being utilized to some extent by TBT to lead to increases in IL-1 $\beta$  synthesis in the cells from the majority of donors.

## A 30 min



## B 6 h



**Fig. 1** Effects of exposures to TBT on IL-1  $\beta$  synthesis in monocyte-depleted PBMCs. **a** 30 min exposure to 0–200 nM TBT. The blots with accompanying secretion data are shown for the cells from 4 different donors. An increase in secretion or in intracellular level is a number greater than 1; a decrease in secretion compared to the control is a number less than 1. The control is arbitrarily set at 1. **A**

combined fold increase (secretion+intracellular) greater than 1 indicates an increase in synthesis. **b** 6 h exposure to 0–200 nM TBT. The blots with accompanying secretion data are shown for the cells from 4 different donors. **c** 24 h exposure to 0–200 nM TBT. The blots with accompanying secretion data are shown for the cells from 5 different donors

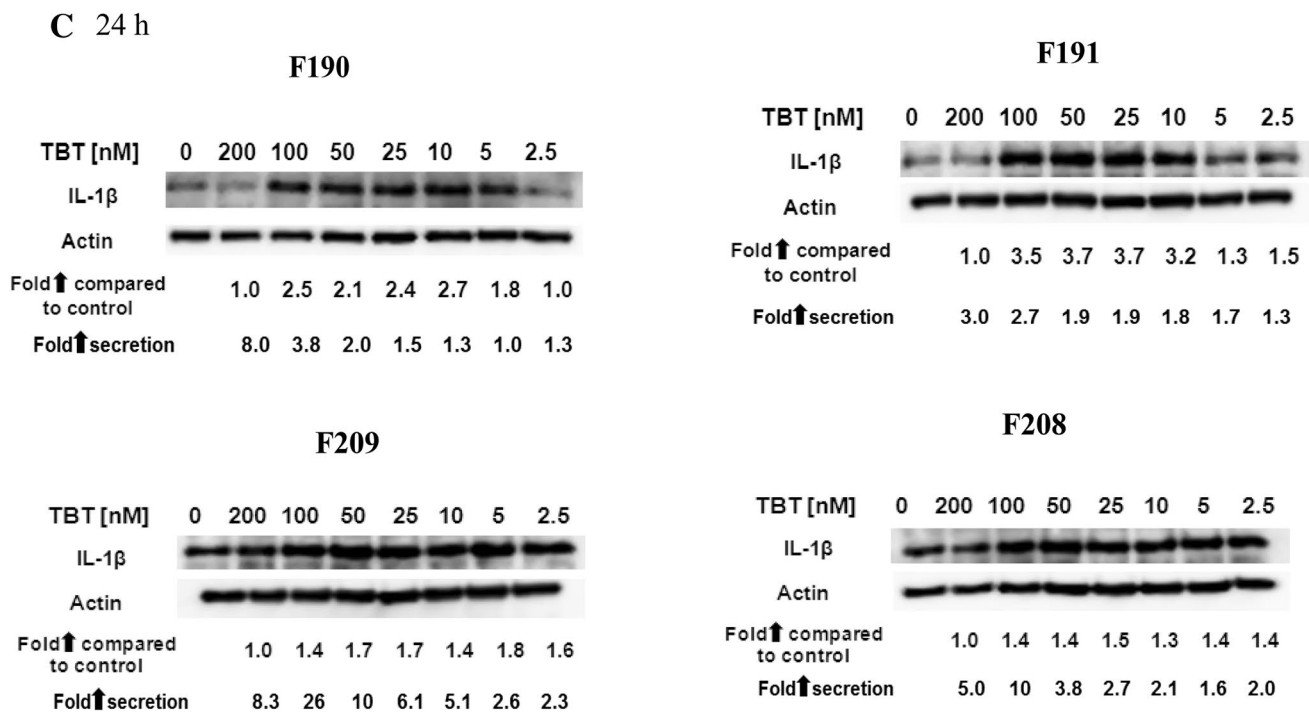


Fig. 1 (continued)

### p38 inhibitor (SB202190)

The effect of p38 MAPK pathway inhibition on TBT-induced increases in IL-1 $\beta$  synthesis from MD-PBMCs is shown in Fig. 2b. All donors consistently showed lowered TBT-induced IL-1 $\beta$  synthesis in the presence of SB202190. For example, donor F325 showed 2.3, 3.0, and 4.9 fold increases when MD-PBMCs were exposed to 5, 10, and 25 nM TBT in the absence of the p38 inhibitor. When the inhibitor was present, those same TBT exposures caused 1.5, 2.1, and 1.9 fold change. This indicates that the p38 pathway is being utilized by TBT to stimulate IL-1 $\beta$  production.

### Nuclear factor kappa B (NF $\kappa$ B) inhibitor (BAY 11-7085) and JNK inhibitor (JNK X BI78D3)

In contrast to the results seen with MEK and p38, inhibition of either the NF $\kappa$ B pathway or the JNK pathway caused no consistent loss of TBT-induced increases in IL-1 $\beta$  synthesis (data not shown). This indicated that neither of these pathways are consistently needed for TBT to increase IL-1 $\beta$  synthesis.

### Effects of exposures to TBT on IL-1 $\beta$ mRNA levels in MD-PBMCs

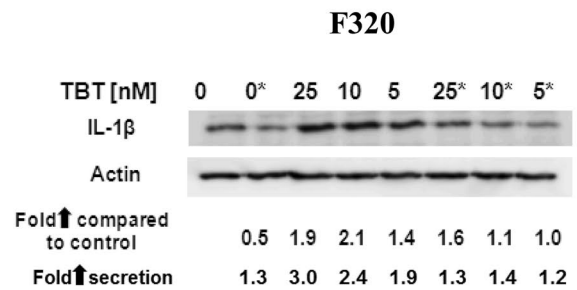
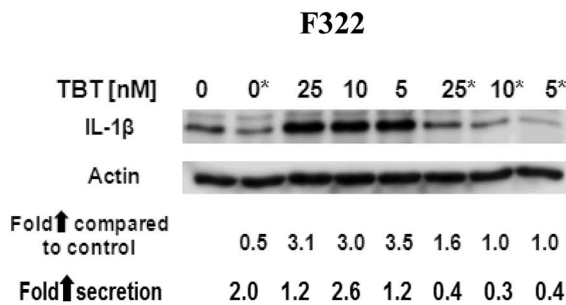
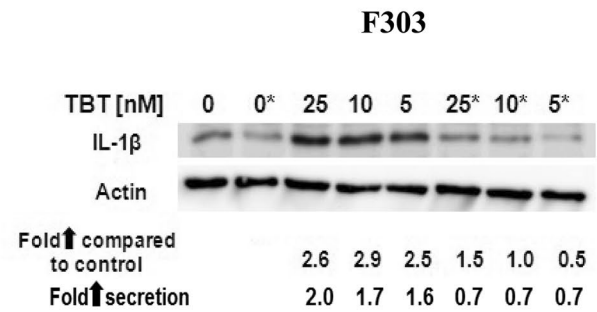
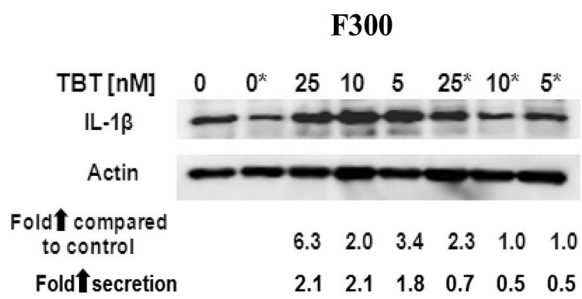
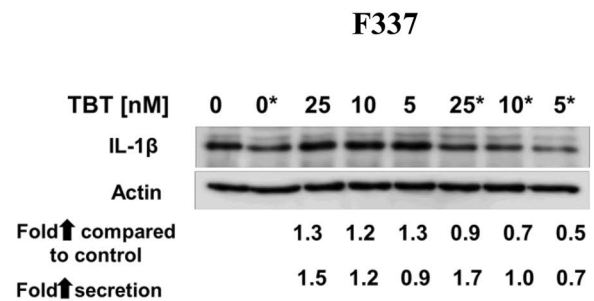
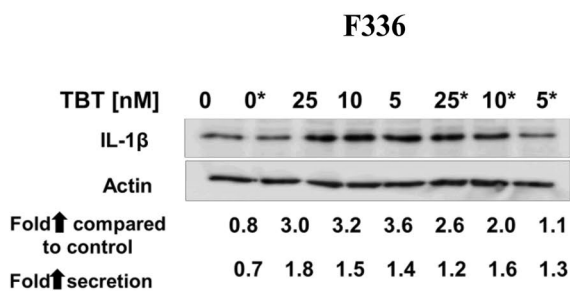
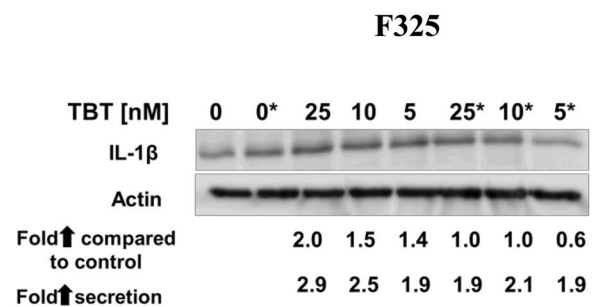
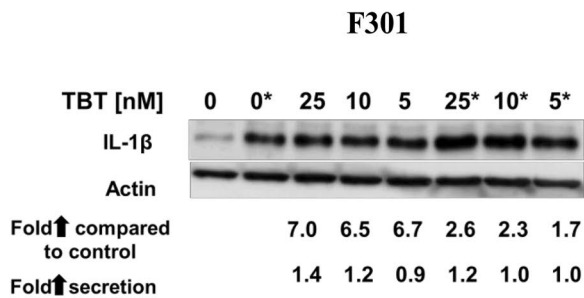
Table 1 shows IL-1 $\beta$  mRNA expression tested in 4 separate donors from MD-PBMCs with TBT concentrations ranging

from 2.5 to 200 nM. No significant increases were seen after 6 h for 3 of the 4 donors.

The effects of 24 h exposures to TBT on IL-1 $\beta$  mRNA levels in MD-PBMCs are also shown in Table 1. Higher concentrations of TBT (in the range of 25–200 nM) caused increased mRNA expression compared to the control for all donors at two or more TBT concentrations with fold increases ranging from 1.3 to 5.3. These results suggest that the TBT-induced increases in IL-1 $\beta$  synthesis are due to increases in IL-1 $\beta$  mRNA at levels of TBT of 25 nM or more.

### Effects of TBT exposures on IL-1 $\beta$ mRNA levels in MD-PBMCs treated with p44/42 (PD98059) and p38 (SB 202,190) pathway inhibitors

Based on its role in TBT-induced synthesis of IL-1 $\beta$ , we examined if the p44/42 MAPK pathway was needed for TBT-induced increases in IL-1 $\beta$  mRNA. Results indicated that p44/42 pathway is not consistently utilized by TBT to lead to increased levels of IL-1 $\beta$  mRNA (data not shown). However, when the p38 MAPK pathway was inhibited by SB202190, TBT-stimulated increases in IL-1 $\beta$  mRNA levels were blocked (Fig. 3). The ability of TBT to increase IL-1 $\beta$  mRNA was diminished in cells from all donors. There were 1.2, 1.9, and 1.7 fold increases in IL-1 $\beta$  mRNA when cells were exposed to 50, 100, and 200 nM TBT in the absence of inhibitor. There were no TBT-induced

**A** MEK inhibitor**B** p38 inhibitor

**Fig. 2** Effects of the inhibition of selected signaling pathways on the synthesis (secretion + intracellular levels) of IL-1 $\beta$  from human MD-PBMCs after 24 h exposure to TBT. **a** MEK inhibitor (PD98059); **b** p38 inhibitor (SB202190). An increase in secretion or in intracellular level is a number greater than 1 a decrease in secretion compared to

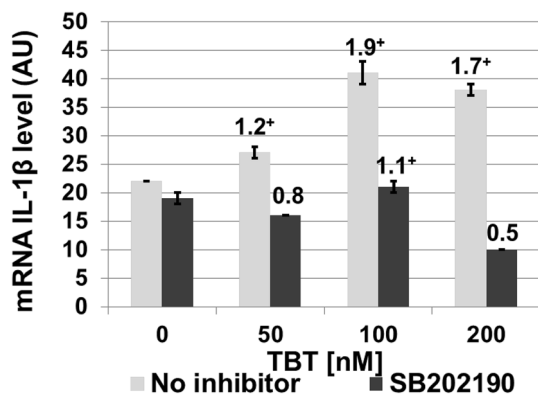
the control is a number less than 1. The control is arbitrarily set at 1. Cells treated with inhibitor are compared to control cells treated with inhibitor. A combined fold increase (secretion + intracellular) greater than 1 indicates an increase in synthesis

**Table 1** Effects of exposures to TBT on IL-1 $\beta$  mRNA levels in MD-PBMCs

6 h		Interleukin 1 beta mRNA expression (AU) (mean $\pm$ SD)			
[TBT] nM	F340	F374	F393	F397	
0	15 $\pm$ 1	19 $\pm$ 5	32 $\pm$ 8	23 $\pm$ 0	
2.5	18 $\pm$ 2	28 $\pm$ 12	35 $\pm$ 24	32 $\pm$ 25	
5	18 $\pm$ 1 <sup>+</sup>	24 $\pm$ 4	23 $\pm$ 2	41 $\pm$ 0	
0	18 $\pm$ 0 <sup>+</sup>	20 $\pm$ 1	14 $\pm$ 5*	39 $\pm$ 24	
25	22 $\pm$ 2 <sup>+</sup>	22 $\pm$ 4	14 $\pm$ 6*	50 $\pm$ 36	
50	22 $\pm$ 5	44 $\pm$ 20	27 $\pm$ 7	44 $\pm$ 10	
100	23 $\pm$ 3 <sup>+</sup>	34 $\pm$ 13	28 $\pm$ 14	26 $\pm$ 5	
200	22 $\pm$ 3 <sup>+</sup>	20 $\pm$ 3	1 $\pm$ 0*	23 $\pm$ 10	
24 h		Interleukin 1 beta mRNA expression (AU) (mean $\pm$ SD)			
[TBT] nM	F266	F277	F302	F322	
0	11 $\pm$ 1	15 $\pm$ 1	22 $\pm$ 1	9 $\pm$ 1	
2.5	14 $\pm$ 0 <sup>+</sup>	16 $\pm$ 1	28 $\pm$ 5	12 $\pm$ 3	
5	14 $\pm$ 1 <sup>+</sup>	17 $\pm$ 2	23 $\pm$ 1	13 $\pm$ 2	
10	14 $\pm$ 2	17 $\pm$ 2	23 $\pm$ 1	16 $\pm$ 4	
25	15 $\pm$ 2 <sup>+</sup>	20 $\pm$ 2 <sup>+</sup>	22 $\pm$ 2	19 $\pm$ 1 <sup>+</sup>	
50	17 $\pm$ 2 <sup>+</sup>	29 $\pm$ 3 <sup>+</sup>	24 $\pm$ 1 <sup>+</sup>	21 $\pm$ 6	
100	26 $\pm$ 3 <sup>+</sup>	36 $\pm$ 5 <sup>+</sup>	31 $\pm$ 2 <sup>+</sup>	30 $\pm$ 2 <sup>+</sup>	
200	20 $\pm$ 2 <sup>+</sup>	29 $\pm$ 7	16 $\pm$ 5	48 $\pm$ 9 <sup>+</sup>	

Values are mean  $\pm$  SD of triplicate determinations

<sup>+</sup>Indicates a significant increase in secretion and \* indicates a significant decrease compared to control cells,  $p < 0.05$



**Fig. 3** Effects of 24 h TBT exposure to 50, 100, and 200 nM on IL-1 $\beta$  mRNA from monocyte-depleted PBMCs treated with MAPK pathway inhibitors. **a** MEK Inhibitor (PD98059) Donor F465; **b** MEK Inhibitor (PD98059) donor F479; **c** p38 inhibitor (SB202190) Donor F479. Fold changes in mRNA levels compared to the appropriate control are shown above the bars. (+) indicates a significant increase ( $p < 0.05$ ) above the appropriate control and (\*) indicates a significant decrease ( $p < 0.05$ ) compared to the appropriate control

increases in IL-1 $\beta$  mRNA expression when the inhibitor was present at 50 and 200 nM TBT exposures and the increase at 100 nM went from 1.9 fold to 1.1 fold (Fig. 3). These results were replicated in cells from 3 additional

donors. This indicates that p38 pathway is needed to cause TBT-induced increases of IL-1 $\beta$  mRNA levels.

### Effects of TBT exposures on the synthesis (secretion + intracellular levels) of IL-6

#### 10 min exposures to TBT

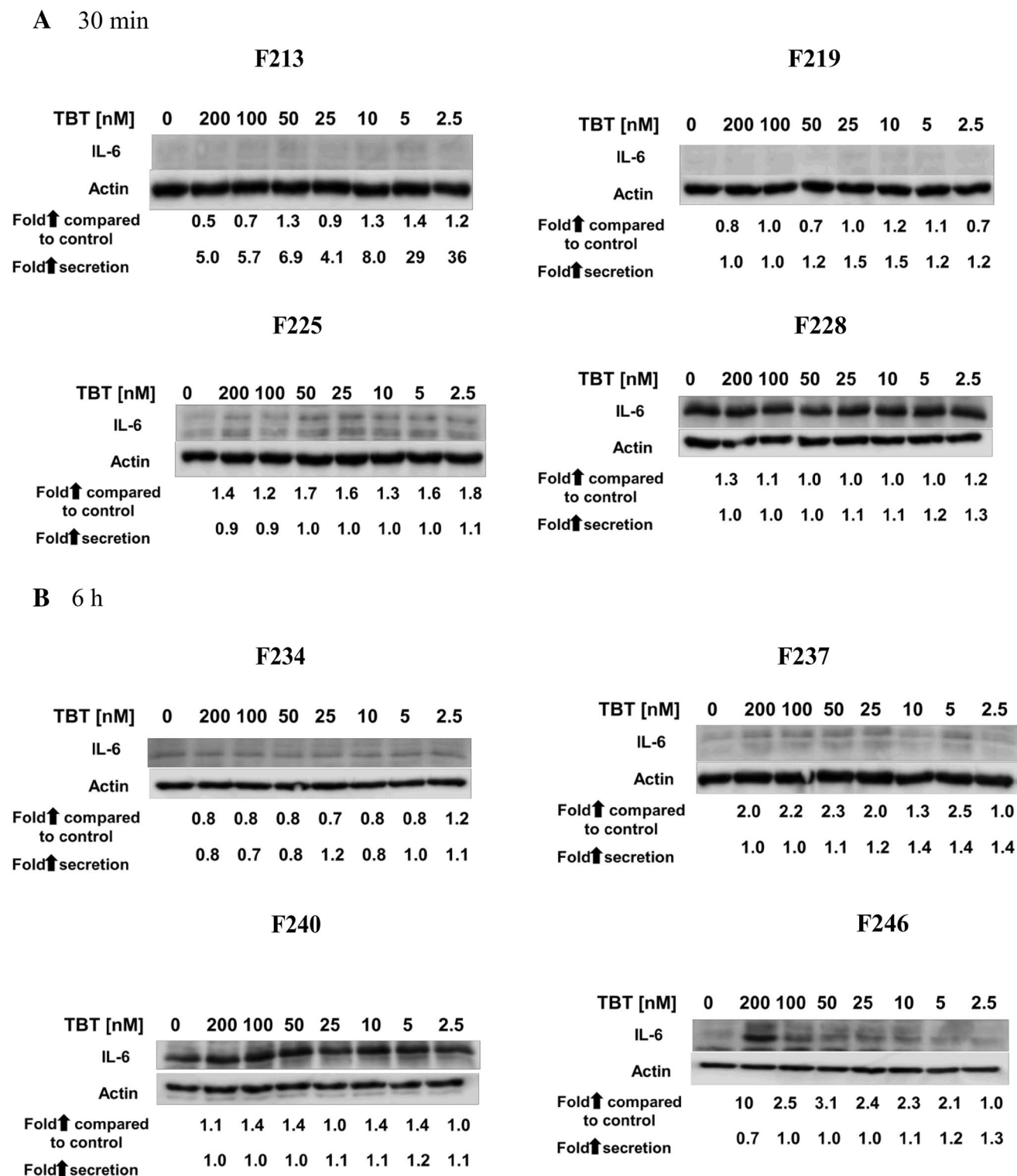
As was seen with TBT's effect on IL-1 $\beta$  production, there were no consistent increases in IL-6 synthesis seen after a 10 min exposure to TBT (data not shown).

#### 30 min exposures to TBT

After a 30 min exposure to TBT, cells from all donors showed increases in IL-6 production at a minimum of 3 concentrations. The changes in intracellular levels paired with the changes in fold secretion are shown in Fig. 4a. These results indicate that TBT induces generally small but consistent increases in IL-6 synthesis after 30 min of exposure to TBT.

#### 6 h exposures to TBT

When MD-PBMCs from 4 distinct donors were exposed to TBT for 6 h there was also typically a small but consistent



**Fig. 4** Effects of exposures to TBT on IL-6 synthesis in monocyte-depleted PBMCs. **a** 30 min exposure to 0–200 nM TBT. The blots with accompanying secretion data are shown for the cells from 4 different donors. An increase in secretion or in intracellular level is a number greater than 1 a decrease in secretion compared to the control is a number less than 1. The control is arbitrarily set at 1. **A**

combined fold increase (secretion+intracellular) greater than 1 indicates an increase in synthesis. **b** 6 h exposure to 0–200 nM TBT. The blots with accompanying secretion data are shown for the cells from 4 different donors. **c** 24 h exposure to 0–200 nM TBT. The blots with accompanying secretion data are shown for the cells from 5 different donors



## C 24 h

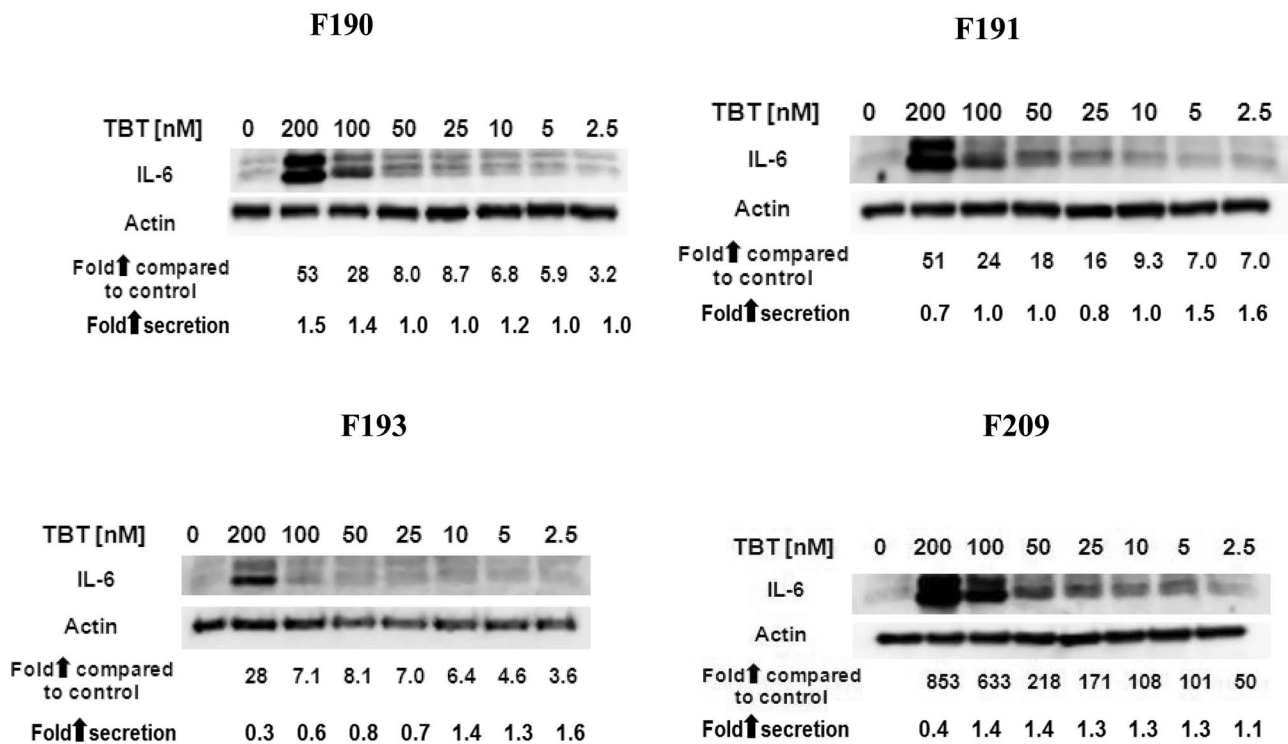


Fig. 4 (continued)

increase in IL-6 synthesis at 1 or more exposure (Fig. 4b). This was evidenced by the combined fold increases in intracellular and secreted levels being greater than 1. Increases ranged from 1.3 fold for cells from F234 at the 2.5 nM exposure to 9.7 fold for cells from F246 at the 200 nM concentration.

### 24 h exposures to TBT

Figure 4c shows the effects of 24 h exposure to TBT on synthesis of IL-6. Dramatic increases were seen in IL-6 synthesis at the higher TBT concentrations of 50, 100, and 200 nM. Fold increases ranged from 28 to 852 (200 nM TBT). There was not a commensurate increase in the secretion of IL-6 from these cells. This indicates that while TBT is able to increase the production of IL-6, it must also be to some extent blocking its secretion.

### Effects of exposures to TBT on the synthesis of IL-6 by MD-PBMCs treated with signaling pathway inhibitors

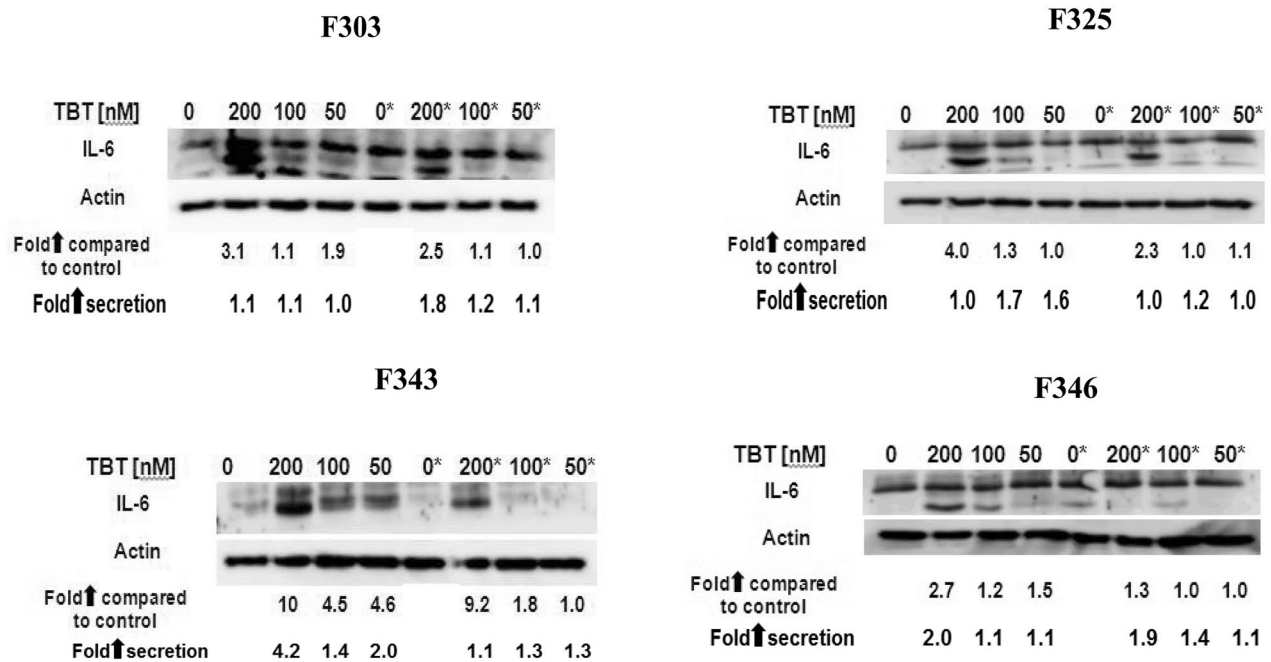
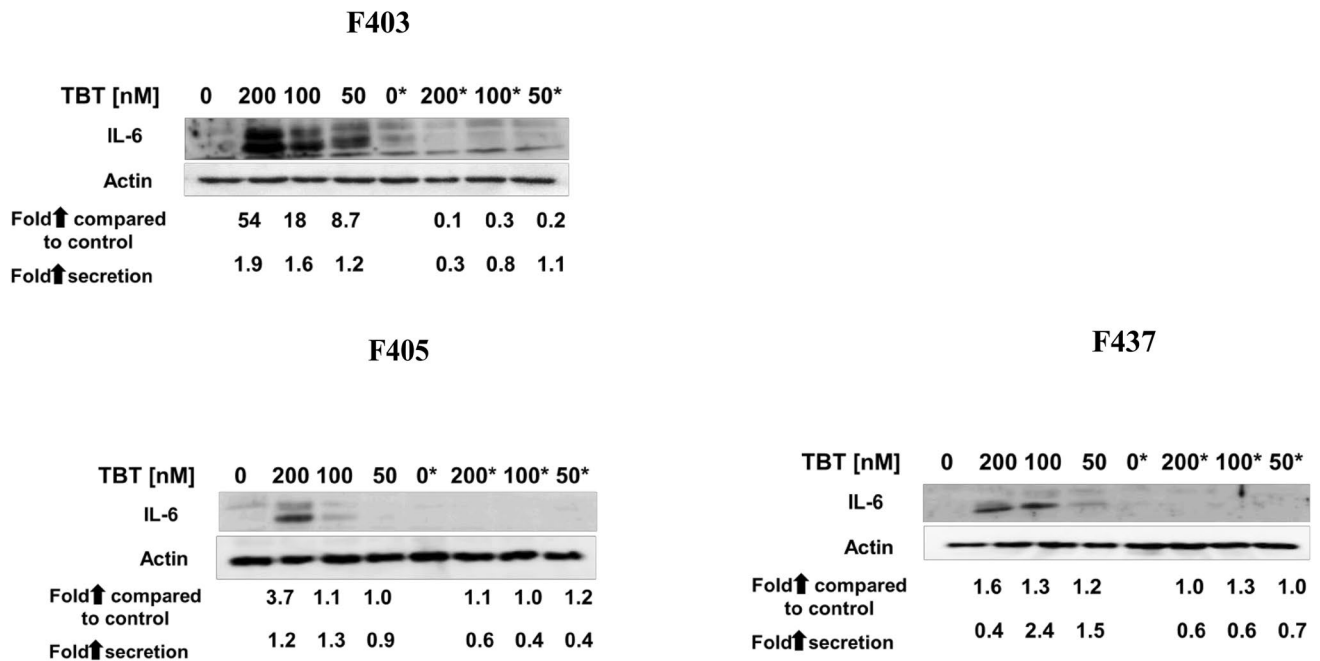
#### MEK, inhibitor (PD98059)

The effects of inhibiting the p44/42 mitogen activated protein kinase (MAPK) pathway with PD98059 (50  $\mu$ M)

on TBT-induced increases in IL-6 synthesis are shown in Fig. 5a (shows fold increases in intracellular and secreted levels). When this pathway was inhibited, the ability of TBT to stimulate IL-6 synthesis was decreased at one or more TBT concentration in cells from all 4 donors. For example, cells from donor F325 exposed to 50, 100, and 200 nM TBT in the absence of the inhibitor showed 1.6, 2.0, and 4.0 fold increases in IL-6 production. In the presence of inhibitor these same cells showed reduced TBT-induced production of IL-6 of 1.1, 1.2, and 2.3 fold at these same concentrations. This indicates that the p44/42 MAPK pathway is being utilized to some extent by TBT to increase IL-6 production.

#### p38 inhibitor (SB202190)

Figure 5b shows the effects of exposures to 50, 100, and 200 nM TBT on intracellular/secreted levels of IL-6 from MD-PBMCs where p38 MAPK function had been inhibited with SB202190. When the inhibitor was present, TBT-stimulated synthesis of IL-6 was completely blocked in MD-PBMCs from all donors tested. For instance, when cells from donor F405 were exposed to 50, 100, and 200 nM TBT in the absence of the inhibitor, there were 0.9, 1.4, and 3.9 fold increases in IL-6 production and no TBT-induced increases in IL-6 production when the inhibitor was present.

**A** MEK Inhibitor (PD98059)**B** p38 Inhibitor (SB202190)

**Fig. 5** Effects of the inhibition of selected signaling pathways on the production (secretion + intracellular levels) of IL-6 from human MD-PBMCs after 24 h exposure to TBT. **a** MEK inhibitor (PD98059); **b** p38 inhibitor (SB202190). An increase in secretion or in intracellular level is a number greater than 1, a decrease in secretion compared to

the control is a number less than 1. The control is arbitrarily set at 1. Cells treated with inhibitor are compared to control cells treated with inhibitor. A combined fold increase (secretion + intracellular) greater than 1 indicates an increase in synthesis

These results indicate that the p38 MAPK pathway is being utilized by TBT to lead to increases in IL-6 production.

### NFκB inhibitor (BAY 11-7085) and JNK inhibitor (JNK X BI78D3)

When either the NFκB pathway or JNK pathway was inhibited in MD-PBMCs with their respective inhibitors, the ability of TBT to increase IL-6 synthesis was not consistently decreased. Thus, it appears that as was seen with IL-1β, neither the NFκB pathway nor the JNK pathway was consistently used by TBT to induce increases in IL-6 synthesis.

### Effects of exposures to TBT on IL-6 mRNA levels in MD-PBMCs

The effects of 6 h exposures to TBT on IL-6 mRNA expression in MD-PBMCs are shown in Table 2. Tested in cells from 4 separate donors, no statistically significant increases were seen at any of the TBT exposures after 6 h. Table 2 also shows the effects of 24 h exposures to TBT on IL-6 mRNA levels. Higher concentrations of TBT (100 and 200 nM) showed increased mRNA expression compared to the control for 3 of the 4 donors. Fold increases

at the highest TBT concentration were 4.0 fold (F277), 7.5 fold (F266), and 54.7 fold (F322). These data indicate that TBT may be increasing IL-6 synthesis, at least in part, by increasing the mRNA for IL-6.

### Effects of TBT exposures on IL-6 mRNA levels in MD-PBMCs treated with p44/42 (PD98059) and p38 (SB 202190) pathway inhibitors

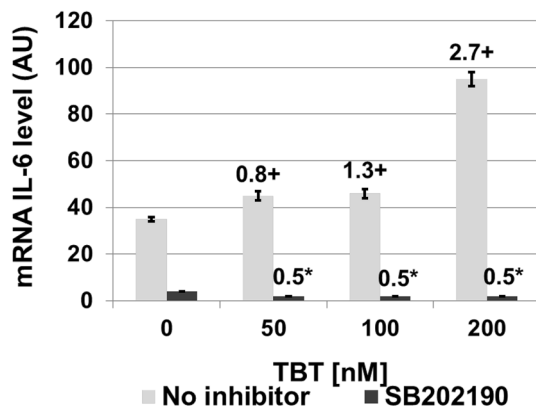
Figure 6 shows the effects of p38 MAPK pathway inhibition on TBT-stimulated increases in IL-6 mRNA levels. When this pathway was inhibited, the ability of TBT to increase IL-6 mRNA was blocked. Cells showed 1.3, 1.3, and 2.7 fold increases in IL-6 mRNA when exposed to 50, 100, and 200 nM TBT in the absence of inhibitor. There were no TBT-induced increases in IL-6 mRNA expression when the inhibitor was present (Fig. 6). This result was replicated in cells from 3 additional donors. Thus, TBT-induced increases in IL-6 mRNA appear to be dependent on the p38 pathway. As was seen with TBT-induced increases in IL-1β mRNA, inhibition of the p44/42 pathway with the MEK inhibitor (PD98059) did not effect TBT's ability to increase IL-6 mRNA levels in MD-PBMCs (data not shown).

**Table 2** Effects of exposures to TBT on IL-6 mRNA levels in MD-PBMCs

6 h [TBT] nM	Interleukin 6 mRNA expression (AU) (mean ± SD)			
	F340	F374	F393	F397
0	33 ± 6	35 ± 7	26 ± 5	21 ± 3
2.5	36 ± 3	41 ± 26	39 ± 20	32 ± 9
5	35 ± 10	34 ± 23	30 ± 4	28 ± 13
10	28 ± 2	32 ± 14	27 ± 2	31 ± 13
25	34 ± 2	41 ± 18	19 ± 6	32 ± 16
50	20 ± 5*	44 ± 30	31 ± 7	18 ± 10
100	29 ± 3	32 ± 4	31 ± 9	30 ± 5
200	26 ± 5	10 ± 3*	1 ± 0*	26 ± 3
24 h [TBT] nM	Interleukin 6 mRNA expression (AU) (mean ± SD)			
	F266	F277	F302	F322
0	8 ± 0	23 ± 2	21 ± 3	3 ± 0
2.5	10 ± 1 <sup>+</sup>	28 ± 6	21 ± 6	4 ± 1
5	10 ± 1 <sup>+</sup>	18 ± 2*	19 ± 1	3 ± 1
10	11 ± 1 <sup>+</sup>	20 ± 1	18 ± 2	4 ± 1
25	12 ± 1 <sup>+</sup>	22 ± 3	15 ± 1	6 ± 0 <sup>+</sup>
50	16 ± 1 <sup>+</sup>	33 ± 7	24 ± 3	3 ± 3
100	50 ± 6 <sup>+</sup>	36 ± 3 <sup>+</sup>	33 ± 4 <sup>+</sup>	19 ± 3 <sup>+</sup>
200	60 ± 17 <sup>+</sup>	90 ± 4 <sup>+</sup>	21 ± 9	164 ± 29 <sup>+</sup>

Values are mean ± SD of triplicate determinations

<sup>+</sup>Indicates a significant increase in secretion and \* indicates a significant decrease compared to control cells, *p* < 0.05



**Fig. 6** Effects of 24 h TBT exposure to 50, 100, and 200 nM on IL-6 mRNA from monocyte-depleted PBMCs treated with MAPK pathway inhibitors. p38 inhibitor (SB202190) donor F480. Fold changes in mRNA levels compared to the appropriate control are shown above the bars. (+) indicates a significant increase ( $p < 0.05$ ) above the appropriate control and (\*) indicates a significant decrease ( $p < 0.05$ ) compared to the appropriate control

## Discussion

TBT has been shown to increase the secretion of IL-1 $\beta$  and IL-6 within 24 h of exposure (Brown and Whalen 2015; Brown et al. 2018). However, it was not clear if TBT-induced increases in secretion of IL-1 $\beta$  or IL-6 were due to increased synthesis of the cytokines or simply release of pre-existing IL-1 $\beta$  or IL-6. Due to its ability to increase secretion of these 2 important pro-inflammatory proteins, TBT has the capacity to produce “sterile inflammation” (inflammation in the absence of microorganisms, Chen and Nunez 2010) which could then contribute to chronic inflammation. Chronic inflammation is a known promoter of a number of disease states including rheumatoid arthritis, Crohn’s disease, multiple sclerosis, and cancer (Choy and Panayi 2001; Dinarello 2011; Rossi et al. 2012; Arlt et al. 2002; Elaraj et al. 2006; Jin et al. 1997; Lewis and Varghese 2006; Muerkoster et al. 2006; Voronov et al. 2002; Chirivi et al. 1996; Vidal-Vanaclocha et al. 1996; Gabay 2006; Atreya et al. 2000; Hurst et al. 2001; Culig et al. 2005; Nilsson et al. 2005). If TBT is increasing the secretion of existent cytokine in the absence of increased cellular synthesis, then increased levels of these cytokines would be somewhat self-limiting. However, if TBT is also stimulating immune cells to produce these potent inflammatory proteins, then very significant “sterile inflammation” could result in response to TBT exposures.

Thus, intracellular levels and accompanying secretion of IL-1 $\beta$  and IL-6 were examined at 10, 30 min, 6, and 24 h. The measurement of both secreted and intracellular levels of the cytokine from the same cells made it possible to determine if TBT affected synthesis of these 2 cytokines

in immune cells. The results indicated that TBT at several exposure levels caused small but consistent increases in IL-1 $\beta$  production within 6 h of exposure and these changes were very robust after 24 h. Results after 24 h showed that TBT increased IL-1 $\beta$  synthesis at every concentration.

The effects of TBT exposures on IL-6 synthesis were similar to those seen with IL-1 $\beta$  in that increased IL-6 production was noticeable after 6 h and most clearly seen after 24 h with substantial increases seen at every TBT concentration. In contrast to the effect of TBT on IL-1 $\beta$  production, the increased production of IL-6 stimulated by TBT was not accompanied by increased secretion of the cytokine. This indicates that TBT while greatly activating the synthesis of IL-6 (at higher concentration) is simultaneously having an inhibitory effect on its secretion.

Synthesis of both IL-6 and IL-1 $\beta$  is regulated by MAP kinase pathways (Gaestel et al. 2009). The current studies indicate that TBT is utilizing both the p44/42 and p38 pathways to lead to increased IL-1 $\beta$  production, with a greater dependence on the p38 pathway. The pathway dependence of TBT-induced increases in IL-6 at 50, 100, and 200 nM was also examined. As was seen with IL-1 $\beta$ , both the p44/42 and p38 are utilized by TBT to lead to increased IL-6 production. The p38 pathway was necessary for TBT-induced increases in IL-6 and the p44/42 pathway contributed to TBT-induced increases but was not as crucial as p38, similar to what was seen with IL-1 $\beta$ .

The increases that were seen in IL-1 $\beta$  and IL-6 synthesis in response to TBT exposures could be due to increased transcription, stabilization of mRNA, increased translation, or a combination of these factors. Results indicated that increased mRNA levels of both cytokines were generally seen after 24 h at TBT concentrations of 50, 100, and 200 nM. However, increased protein levels were seen at all TBT concentrations after 24 h exposures. This suggests that an increase in mRNA is at least in part responsible for the TBT-induced increases in these 2 cytokines seen at higher TBT concentrations. However, TBT-induced increases in the mRNA of either IL-1 $\beta$  or IL-6 do not appear to explain the TBT-induced increases in IL-1 $\beta$  and IL-6 synthesis seen at the lower concentrations of TBT. It is possible that these increases are due to TBT effects on the translational process. TBT utilizes the MAPK pathways for increased production and it is known that MAPKs can lead to increased translation due to activation of the eukaryotic initiation factor 4E (eIF4E) (Shveygert et al. 2010).

As both p44/42 and p38 appear to have a role in the TBT-induced increases in IL-1 $\beta$  and IL-6 production, we investigated whether these signaling pathways were required for TBT-induced increases in IL-1 $\beta$  and IL-6 mRNA expression. The results suggest that p38 pathway is needed for TBT-induced increases of IL-1 $\beta$  and IL-6 mRNA levels.

In summary, the current study indicates that TBT induces increases in the cellular synthesis of both IL-1 $\beta$  and IL-6. Furthermore, it shows that elevated synthesis is detectable within 6 h of exposure. TBT causes synthesis of IL-1 $\beta$ , which then leads to increased secretion. In contrast, TBT-stimulated synthesis of IL-6 but did not increase secretion, indicating that TBT causes at least a partial inhibition of IL-6 secretion, which was not seen with IL-1 $\beta$ . TBT-induced synthesis of each of these cytokines is primarily dependent on the p38 MAPK pathway and to a lesser extent the p44/42 pathway. The highest levels of TBT caused increased mRNA for both IL-1 $\beta$  and IL-6, which could account for the TBT-elevated synthesis. However, lower concentrations of TBT did not increase the mRNAs for these cytokines even though they stimulated the synthesis of the proteins.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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