11-6-2008

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Recommended Citation

Abraha, A.B. and Whalen, M.M. (2009), The role of p44/42 activation in tributyltin-induced inhibition of human natural killer cells: effects of MEK inhibitors. J. Appl. Toxicol., 29: 165-173. https://doi.org/10.1002/jat.1397

Published in final edited form as:
J Appl Toxicol. 2009 March ; 29(2): 165–173. doi:10.1002/jat.1397.

The role of p44/42 activation in tributyltin- induced inhibition of human natural killer cells: Effects of MEK inhibitors

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Abstract

Destruction of tumor cells is a key function of NK cells. Previous studies have shown that tributyltin (TBT) can significantly reduce the lytic function of the human NK cells with accompanying increases in the phosphorylation (activation) states of the mitogen activated protein kinases (MAPKs), p44/42. The current studies examine the role of p44/42 activation in the TBT-induced reduction of NK-lytic function, by using MAPK kinase (MEK) inhibitors, PD98059 and U0126. A 1 h treatment with PD98059 or U0126 or both decreased the ability of NK cells to lyse K562 tumor cells. PD98059, U0126 or a combination of both inhibitors were able to completely block TBT-induced activation of p44/42. However, when p44/42 activation was blocked by the presence of PD98059, U0126, or the combination, subsequent exposure to TBT was still able to decrease the lytic function of NK cells. These results indicate that TBT-induced activation of p44/42 occurs via the activation of its upstream activator, MEK, and not by a TBT-induced inhibition of p44/42 phosphatase activity. Additionally, as lytic function was never completely blocked by MEK inhibitors, the results indicate that activation of p44/42 pathway is not solely responsible for the activation of lytic function of freshly isolated human NK cells. Finally, the results showed that TBT-induced activation of p44/42 is not solely responsible for the loss of lytic function.

INTRODUCTION

Organotin compounds are widely used in various industrial and agricultural settings (Kannan et al., 1998; Karpiak et al., 2001). Tributyltin (TBT) is a butyltin (BT) and is known to significantly contaminant the environment (Kimbrough, 1976; Laughlin and Linden, 1985; Tanabe et al., 1998; Loganathan et al., 2000). TBT is the most toxic of BTs and was mainly in used in wood preservation, marine antifouling paints, disinfection of circulating industrial cooling waters, and slime control in paper mills (Kimbrough, 1976; Roper, 1992 and Yamada et al., 1993). TBT has been detected in human food, such as fish (Kannan and Falandyz, 1997; Kannan et al., 1995a,b,c). It is also found in various household products such as siliconized-paper baking parchments and shower curtains (Yamada et al., 1993). In animals, TBT causes irritation of the eye and the skin, together with inflammation of the respiratory tract (Snoeij et al., 1987; Kupper, 1989; WHO, 1990; Corsini et al., 1996). Studies using human intestinal Caco-2 cells have revealed that exposure to TBT may disorder the intestinal barrier functions (Tsukazaki et al., 2004). In humans, TBT residue has been detected in blood (Kannan et al., 1999; Whalen et al., 1999). The ingestion of contaminated food may act as a route of entry into the human body. Additional routes of entry may include absorption through the skin (Baaijens, 1987) and possibly inhalation for those who are occupationally exposed (WHO/FAO, 1984).

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Natural killer (NK) cells are lymphocytes in the non-adaptive immune system that can lyse tumor cells, virally infected cells, and antibody-coated cells (Vivier et al., 2004; Wu and Lanier, 2003) without the need for in vitro or in vivo activation (Moretta et al., 2002). Target cells become susceptible to lysis by NK cells when they lose or down-regulate major histocompatibility complex class I expression, which protects target cells in which it is expressed (Tajima et al., 2004). NK cells are primarily restricted to bone marrow, spleen, liver and peripheral blood (Moretta et al., 2002) and represent a cell subset accounting for approximately 10−20% of peripheral blood lymphocytes that do not express clonally distributed receptors for antigens typical of T cells or B cells (Moretta et al., 2002; Cooper et al., 2001).

TBT in blood could suppress immune cells' function, including NK cells. In vivo studies of mice fed TBT showed suppression of NK activity (Ghoneum et al., 1990). Not only has TBT been detected in blood (Kannan et al., 1999; Whalen et al., 1999), but further studies have found that TBT causes a decrease in the ability of human NK cells to destroy their target cells (Whalen et. al., 1999; Whalen et al., 2002a,b). It follows that chemicals such as TBT that compromise the function of NK cells will reduce the immune system's ability to fight viral infection and tumors.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that are part of the signal transduction pathways, which connect extracellular signals to intracellular responses (Chang and Karin, 2001). Both p44 and p42 MAP kinases (ERK 1 and ERK 2) function in a protein kinase cascade that plays a critical role in the regulation of cell growth and differentiation (Marshall et al., 1995; Hunter et al., 1995; Hill et al., 1995; Cowley et al., 1994). Activation of MAP kinases occurs through phosphorylation of threonine and tyrosine by an upstream MAP kinase kinase (MEK) (Sturgill et al., 1988; Payne et al., 1991). Once activated by the upstream kinases, MAPKs are rapidly inactivated by a family of protein phosphatases such as MAPK phosphatase-1 (MKP-1), an inducible dual specificity phosphatase (Camps et al., 2000; Farooq et al., 2004; Keyse et al., 2000). MKP-1 has been shown to dephosphorylate p44/42, p38 and JNK (Sun et al., 1993; Imasato et al., 2002 and Lasa et al., 2002).

Previous studies have found that activation of p44/42 occurs very rapidly in response to TBT exposure (Aluoch and Whalen, 2005; Aluoch, et al., 2006). This activation occurs at concentrations of TBT as low as 25 nM. A role for p44/42 activation in the tumor cell destroying (lytic) signaling process of the NK cell has been shown in NK cells lines and stimulated NK cells (Chan et al., 2001; Chini et al., 2000; Trotta et al., 1998; Trotta et al., 2000; Wei et al., 1998). However, the role of p44/42 activation in the lytic process of freshly isolated peripheral-blood human NK cells has not been established. One aim of the current study is to determine whether and to what extent p44/42 activation is required in the activation of the lytic process in freshly isolated NK cells. A further aim of this study is to determine the role that TBT-induced activation of p44/42 may have in the TBT-induced loss of lytic function. Since p44/42 activation appears to be involved in the lysis of tumor cells by NK cells, then activation of p44/42 by TBT exposure may leave the cell unable to further activate p44/42 in response to a subsequent interaction with a tumor cell. This would then result in the observed decrease in lytic function seen with TBT exposures. Therefore, the present study evaluated the role of p44/42 activation in the lytic function of normal human NK cells, as well as in the TBT-induced loss of lytic function, using the MEK inhibitors, PD98059 and U0126 (Pang et al., 1995; Favata et al., 1998).

MATERIALS AND METHODS

Preparation of human NK cells

Peripheral blood from healthy adult (male and female) volunteer donors was used for this study (Red Cross, Portland, OR). Highly purified NK cells were obtained using a rosetting procedure. Buffy coats were mixed with 1 ml of RosetteSep human NK cell enrichment antibody cocktail (StemCell Technologies, Vancouver, BC, Canada) per 30 ml of buffy coat. The mixture was incubated for 1 h at room temperature (25 C) with periodic mixing (approximately every 20 min). Following the incubation, 5 ml of the mixture was layered onto 4 ml of Ficoll-Hypaque (1.077 g/ml) (Sigma) and centrifuged at 1200 g for 30 min. The cell layer was collected and washed twice with PBS and stored in complete media (RPMI-1640 supplemented with 10% heat-activated BCS, 2 m*M L*-glutamine and 50 U penicillin G with 50 μg streptomycin/ml) at 1 million cells/ml. The resulting cell preparation was >95% CD16+, 0% CD3+ by fluorescence microscopy and flow cytotometry (Whalen et al., 2002a).

Radioactive Chromium Release Assay

NK lytic function was measured using a ^{51}Cr release assay (Whalen et al., 1999). The target cell in all lytic assays was the NK-susceptible K562 (Human Chronic Myelogenous Leukemia) cell line (ATCC, Manassas, VA). K562 cells (3 million) were incubated with 50 μCi ^{51}Cr in 0.3−0.5 ml of BCS for 1.5 h at 37 °C in 19:1 air/CO_2. Following this incubation, the target cells were washed twice with gelatin medium (RPMI 1640 supplemented with 0.5% gelatin (porcine skin, Type A), 2 mM *L*-glutamine and 50 U penicillin G with 50 μg streptomycin/mL). NK cells were exposed to the various conditions to be tested (described in figure legends). Following the various exposures, NK (effector) cells (1.2×10^5/100 μL for 12:1 ratio with target cells) were added to the wells of round-bottom microwell plates. The effectors were diluted to 6:1 ratio (0.6×10^5/100 μL) and 3:1 ratio (0.3×10^5/100 μL); each ratio was tested in triplicate. Targets were added (1×10^4/100 μL) to each well, and the plate was centrifuged at 300 × *g* for 3 min and incubated for 2 h at 37 °C (air/CO_2, 19:1). After incubation, a 0.1-mL aliquot of the supernatant was collected and counted for radioactivity for 60 s in a Packard COBRA gamma counter (Packard Instrument Co., Meriden, CT). Specific lysis was calculated as follows: [1-[100 × [(test c.p.m. − spontaneous c.p.m.)/(maximum c.p.m. − spontaneous c.p.m.)]]. Maximum release was produced by adding 100 μl of 10% Triton X-100.

Chemical preparation

Tributyltin was purchased from Aldrich Chemical Co. (Milwaukee, WI). TBT was suspended in double de-ionized H_2O to give a 1 mM solution. This TBT solution was diluted in gelatin media (0.5% gelatin replaced the calf serum in complete medium) to achieve final concentrations. The concentration of TBT used in treating the cells ranged from 100 to 300 nM, based on previous studies (Whalen et al., 1999; Whalen et al., 2002a). The concentration of TBT used in the experiments is not far greater than the highest concentration that was detected in human blood (as high as 260 nM) (Kannan et al., 1999; Whalen et al., 1999). MEK inhibitors, PD98059 and U0126, were purchased from EMD Biosciences, Inc. (La Jolla, CA). These MEK inhibitors were dissolved initially in DMSO to make a 50 mM stock. These stock solutions were diluted in gelatin media to achieve final concentrations of 50 μM and 100 μM.

Cell viability

Cell viability was determined by trypan blue exclusion prior to and following each exposure period (Wilson et al., 2004). Cell numbers and their viability did not vary among experimental conditions. Cell viability was normally greater than 90% for both control and TBT-treated cells.

Cell lysates

Cell lysates for western blot were made using: (1) NK cells exposed to media (control); (2) NK cells exposed to 100 μM PD98059 and/or U0126 for 1hr, followed by media for 10 min; (3) NK cells exposed to media for 1 h followed by exposure to 200 nM TBT for 10 min; (4) NK cells exposed to media for 1 h followed by exposure to 100 nM TBT for 10 min; (5) NK cells exposed to 100 μM PD98059and/or U0126 for 1hr followed by exposure to 200 nM TBT for 10 min (6) NK cells exposed to 100 μM PD98059 and/or U0126 for 1h followed by exposure to 100 nM TBT for 10 min. Following the above treatments, the cells were centrifuged and the cell pellets were lysed using 500 μL of lysis buffer (Active motif, Carlsbad, CA) per 10 million cells. The cell lysates were stored frozen at −80 °C up to the point when they were run on SDS-PAGE. Control and treated cells for a given experimental setup (described above) were from an individual donor. Each of the experimental setups (1−6) was repeated a minimum of three times using cells from different donors.

To prepare lysates for MAPK phosphatases, NK cells were treated with 300−25 nM TBT or control for 10 min. Following these treatments, the cells were centrifuged and washed with 1 mL of PBS (without phosphatase inhibitor) the cell pellets were then lysed with 500 μL/10 million cells of lysis buffer. The cell lysates were stored frozen at −80 °C up to the point when they were used to assay phosphatase activity. Control and TBT-exposed cells for a given experimental setup (described above) were from an individual donor.

Western blot

Cell lysates were run on 10% SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) and transferred to PVDF (polyvinylidene Difluoride) membrane. The PVDF was immunoblotted with anti-phospho-p44/42 (Thr202/Tyr204), anti-p44/42 and anti-β-actin antibodies (Cell Signaling Technologies, Beverly, MA). Antibodies were visualized using ECL chemiluminescent detection system (Amersham, Piscataway, NJ) and Kodak Image Station (Kodak, Rochester, NY). The density of each protein band was determined by densitometric analysis using the Kodak Image Station analysis software. The settings on the image station were optimized to detect the largest possible signal range and to prevent saturation of the system. A given experimental setup (as described in the cell lysate section) always had its own internal control. Thus, differences and changes in protein expression are determined relative to the internal control. This determination provides relative quantitation by evaluating whether a given treatment changed expression of phospho-p44/42, or p44/42 relative to untreated cells. β-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 β-actin to correct for small differences in protein loading among the lanes.

Phosphatase Assay

A serine/threonine phosphatase assay kit was purchased from AnaSpec, Inc. (San Jose, CA). Total ser/thr phosphatase activity in control and TBT-exposed NK cells was measured by the addition of 50 μL pNPP reaction mixture containing (12.5 μL of pNPP stock solution (100 ×), 1.24 mL of assay buffer and 3.75 μL of 1M DTT) to the appropriate dilutions of the cell lysates (50 μL) in the wells of a 96-well plate. The plate was incubated at room temperature and absorbance was measured at 405 nm in a microplate reader every 5 min for 40 min.

Statistical analysis

Analysis of variance (ANOVA) followed by pair-wise comparison of data was carried out for all studies. A significant ANOVA was followed by pair wise comparisons of control versus exposed data using a *t*-test. A minimum of three separate experiments were carried out for measurement of lytic function ($n \geq 9$). A minimum of three separate determinations were

carried out for each Western blot experimental set-up ($n \geq 3$) and statistical significance was noted at $p < 0.05$.

RESULTS

Effect of MEK inhibitors on lytic function NK cells

Figure 1 shows the effects of PD98059, U0126 and a combination of both MEK inhibitors on the ability of NK cells to lyse K562 tumor cells. NK cells exposed to PD98059 showed a statistically significant decrease in their lytic function. Decreases in lytic function of 38 and 37% were seen with 50 and 100 μM PD98059, respectively ($p<0.01$) (Fig 1). NK cells exposed to U0126 also showed a statistically significant decrease in lytic function. There was a concentration dependent decrease in lytic function, with 50 and 100 μM U0126 causing 37 and 69% decrease in lytic function, respectively ($p<0.01$) (Fig 1). The decreases in lytic function of NK cells exposed to both 100 μM PD98059 and 100 μM U0126 were not greater than those seen with U0126 alone (Fig 1).

Effect of MEK inhibitors on TBT-induced decrease in NK lytic function at 1 h

To examine the effect of MEK inhibitors on TBT-induced decrease in NK lytic function, NK cells were treated as described in the legend of Figure 2. Decreases in lytic function of 26, 69 and 78 % were seen with 100 μM PD98059 alone, 300 nM TBT alone, and NK cells pretreated with 100 μM PD98059 followed by 300 nM TBT for 1 h respectively (Fig 2) ($p<0.05$). Decreases in lytic function of 60, 69 and 89 % were also observed with 100 μM U0126 alone, 300 nM TBT alone and NK cells pretreated with 100 μM U0126 followed by 300 nM TBT for 1 h respectively (Fig 2) ($p<0.05$).

Effect of MEK inhibitors on TBT-induced decrease in NK lytic function in a 24 h period following a 1 h exposure to TBT

We also investigated the effect of MEK inhibitors on TBT-induced decreases in NK lytic function in a 24 h period following a 1 h exposure to TBT. To address this, NK cells were treated as described in the legend for Figure 3. There were significant decreases in lytic function when NK cells were exposed to 200 or 100 nM TBT alone followed by 24 h in TBT-free media of 62 and 26 % (Fig. 3). Decreases in lytic function of 23, 63 and 39 % were seen with 100 μM PD98059 alone, 100 μM PD98059 followed by 200 or 100 nM TBT respectively (Fig. 3) ($p<0.05$).

Similar results were observed with 100 μM U0126 alone, 100 μM U0126 followed by 200 or 100 nM TBT, producing 28, 92 and 71 % decreases in lytic function, respectively (Fig. 3) ($p<0.01$).

Effect of the MEK inhibitor, PD98059, on the TBT-induced phosphorylation of p44/42

Treatment of NK cells with 100 μM PD98059 for 1 h did not alter the phosphorylation of p44/42 as compared to control cells (Fig. 4A) ($p< 0.05$). NK cells treated with media for 1 h followed by 200 or 100 nM TBT alone for 10 min showed a significant increase in phospho-p44/42 compared to control (Fig. 4A) ($p< 0.05$). The average increases in phosphorylation of p44/42 level in NK cells exposed to 200 nM TBT for 10 min or 100 nM TBT for 10 min were 6.2 and 3.1, respectively (Fig 4A). NK cells treated with 100 μM PD98059 for 1 h prior to exposure to 200 or 100 nM TBT for 10 min showed no significant increases in phosphorylation of p44/42 as compared control (Fig. 4A) ($p>0.05$). Thus, TBT-induced phosphorylations were completely blocked when NK cells were exposed to either 100 μM PD98059 for 1 h followed by 200 nM TBT for 10 min or 100 μM PD98059 for 1 hr followed by 100 nM TBT for 10 min.

There was no significant difference in the total p44/42 levels among any of the treatment conditions (Fig. 4A) ($p>0.05$). Figure 4B shows data from a representative experiment.

Effect of MEK inhibitor, U0126, on the TBT-induced phosphorylation of p44/42

NK cells treated with 100 μM U0126 showed no significant change in the phosphorylation of p44/42 as compared to control cells (Fig. 5A) but NK cells treated with media for 1 h followed by 200 or 100 nM TBT for 10 min showed a significant increase in phospho-p44/42 compared to control (Fig. 5A) ($p< 0.05$). When NK cells were treated with U0126 followed by exposure to TBT (Fig. 5A), there were no significant TBT-induced increases in phosphorylation of p44/42 (Fig. 5A). There were no significant differences in total p44/42 level among all treatments conditions, as with PD98059. A representative experiment is shown in Fig 5B.

Effect of the combination of MEK inhibitors on the TBT-induced phosphorylation of p44/42

The combination of both MEK inhibitors (100 μM PD98059 + 100 μM U0126) gave the same results as those seen with each inhibitor alone (Fig. 6A). A representative experiment (western blot) showing the effects of the combined MEK inhibitors on TBT-induced phosphorylations of p44/42 is shown in Fig 6B.

Effect of 25−300 nM TBT on MAPK phosphatase activity

Lysates from NK cells treated with a range of TBT concentrations or control for 10 min at 37° C were used to measure the activity of ser/thr phosphatase activity. MAPK phosphatases are ser/thr phosphatases so this assay indicated whether TBT exposures were able to decrease MAPK phosphatase activity which could have also accounted for the observed increases in p44/42 phosphorylation. The results indicate that there was no significant difference among the treatments as compared to control (data not shown). Thus, these results indicate that TBT exposure is not inhibiting MAPK phosphatase activity.

DISCUSSION

Previous studies have shown that TBT has the ability to decrease human NK cell lytic function (Whalen et al., 1999; Whalen et al., 2002a,b). Those studies showed that NK cells lose their ability to lyse tumor target cells following exposure to a range of TBT concentrations. Other studies have established a role for the activation of p44/42 MAPK in stimulating the tumor lysing function of NK cell lines and stimulated NK cells (Wei et al., 1998; Wei et al., 2000; Trotta et al, 1998). Our laboratory has previously shown that TBT exposure can activate p44/42 in freshly isolated human NK cells, and thus, this TBT-induced activation could potentially leave the NK cell unable to respond to a subsequent encounter with a tumor cell. The goal of the present study was to further examine the role of p44/42 signaling pathway in the lytic function of fresh NK cells as well as to examine the role of p44/42 activation in TBT-induced loss of lytic function

Pharmacological inhibition of MEK is one means to examine the role of p44/42 MAPK in NK function. When freshly isolated NK cells were exposed to the MEK inhibitor PD98059 for 1 h they lost between 30−40% of their ability to lyse tumor cells. Western blot analysis of the activation of p44/42 using the same concentration of PD98059 showed that the activation of p44/42 was completely blocked by this treatment, thus the 60−70% of lytic function that was unaffected by the inhibitor had to be relying on an alternative pathway for the activation of tumor lysis. Studies using the MEK inhibitor PD98059 in the YT cell line (an NK-like oncogenically transformed cell line) showed a much greater dependence of lytic function on p44/42 activation (Wei et al., 1998; Wei et al., 2000) than we see when using normal resting NK cells. However, another study using NK cells obtained from a ten day co-culture of peripheral blood lymphocytes with irradiated RPMI 8866 cell, also showed an incomplete loss

of lytic function when the MEK inhibitor was present (Trotta et al., 1998) cells. The freshly isolated NK cells used in the current study are the least altered cells (no stimulation or oncogenic transformation) in which p44/42 activation in NK lytic function has been studied. The conclusion from the current study is that p44/42 activation in part determines whether normal resting NK cells are able to lyse tumor targets, but is not the only way to achieve this function. Future studies are needed to examine alternative routes in freshly isolated highly enriched NK cells for achieving tumor lysis.

Another goal of these studies was to examine the role of p44/42 activation in TBT-induced decreases of lytic function. Thus, NK cells where p44/42 activation was prevented by the presence of one or both of the MEK inhibitors were treated with varying concentrations and lengths of exposure to TBT. The results of these studies showed that the negative effects of TBT on NK- lytic function were not prevented even though the TBT was not able to activate p44/42 (as confirmed in the Western blot studies). This was true whether the exposure to TBT was at 300 nM for 1 h or at 200 or 100 nM for 1h followed by 24 h in TBT-free media. Past studies (Aluoch and Whalen, 2005; Aluoch et al. 2006) as well as the current study have confirmed that substantial TBT-induced activation of p44/42 is occurring at each of these concentrations and lengths of exposure. Thus, the current results indicate that while p44/42 is activated by TBT that this activation may be a consequence rather than the immediate cause of the loss of lytic function that is seen upon TBT exposure. Although it is possible that TBT-induced activation of p44/42 may be a partial cause of the loss of lytic function it cannot account for the full extent of the loss.

In addition to determining that activation of p44/42 by TBT is not the sole cause of the loss of lytic function, the current study also established that TBT-induced p44/42 activation is occurring through activation of MEK and not through inhibition of MAPK phosphatases. This was seen both through the western blot studies, which showed that MEK inhibitors completely block TBT-induced activation of p44/42 as well as through direct phosphatase assays. Although prior studies had shown that there was activation of MEK in response to TBT (Aluoch et al., 2006), there had been no previous examination of the role of phosphatase inhibition in increasing the levels of phosphorylated p44/42.

In summary, the results from this study indicate that : (1) exposure of NK cells to MEK inhibitors, PD98059 or U0126 or both, produced an incomplete loss of lytic function indicating that while p44/42 activation can stimulate lytic function of NK cells it is not the only way to stimulate lysis of target cells; (2) TBT-induced activation of p44/42 is not sole mechanism by which TBT decreases the lytic function of human NK cells; (3) TBT-induced activation of p44/42 occurs via the activation of its upstream activator, MEK, and not by inhibiting MAPK phosphatase activity.

Acknowledgement

This research was supported by Grant 2S06GM-08092-32 from the National Institutes of Health.

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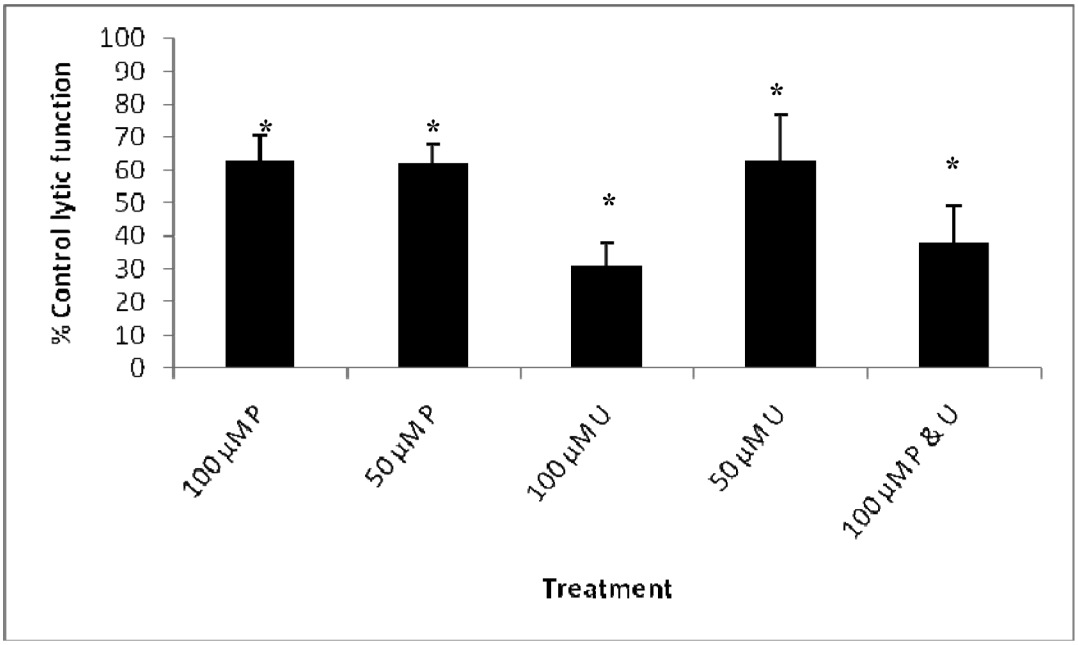


Figure 1. Effect of PD98059 or U0126 or both on K562 lysis of tumor cells

Starting from the left Bar, NK cells were treated with the indicated concentrations of PD98059 (P) or U0126 (U) or both for 1 h. Lysis of the target cells was measured by using a ^{51}Cr release assay. X-axis represents the indicated concentrations of MEK inhibitors. Y-axis represents the % control lysis activity. * indicates significant difference as compared to control, $p<0.05$.

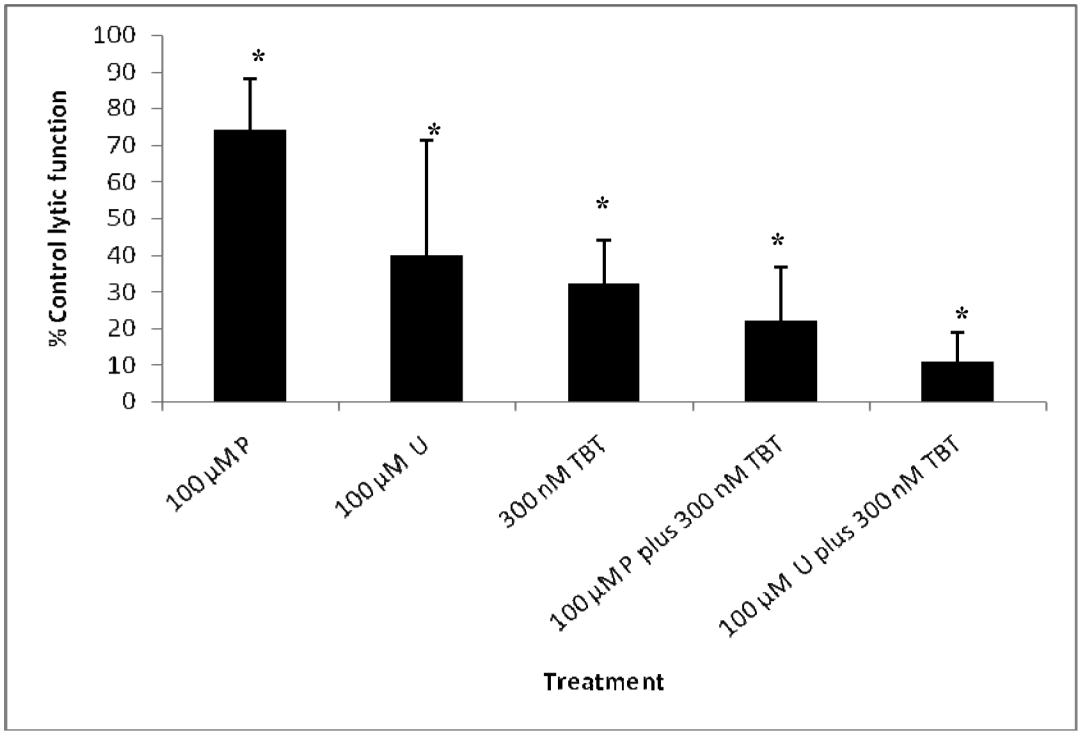


Figure 2. Effect of MEK inhibition on TBT-induced inhibition of NK lytic function

Starting from the left Bar 1= NK cells were treated with 100 µM PD98059 (P) followed by exposure to control media for 1 h; Bar 2 = NK cells were treated with 100 µM U0126 (U) followed by exposure to control media for 1 h; Bar 3 = NK cells were treated with control media for 1 h followed by exposure to 300 nM TBT for 1 h ; Bar 4 = NK cells were treated with 100 µM PD98059 (P) followed by exposure to 300 nM TBT for 1 h; and Bar 5 = NK cells were treated with 100 µM U0126 (U) followed by exposure to 300 nM TBT for 1 h. The entire treatment time was 2 h, with the initial treatment remaining during the second treatment. * indicates significant difference as compared to control, $p<0.05$.

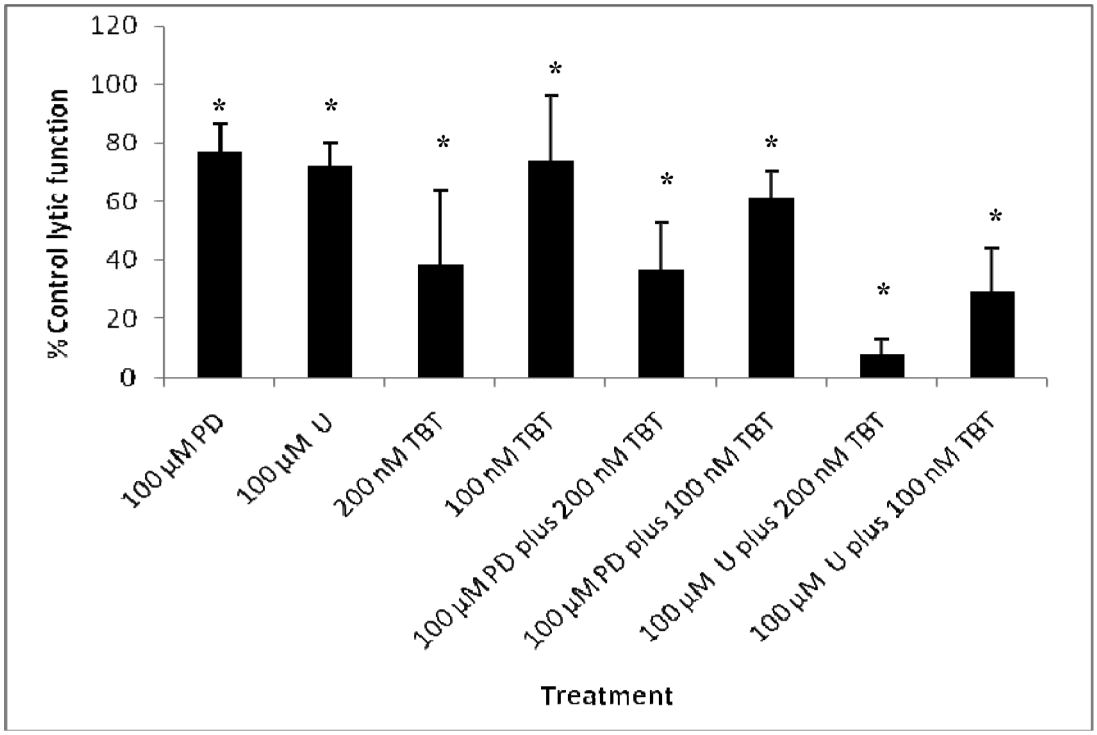
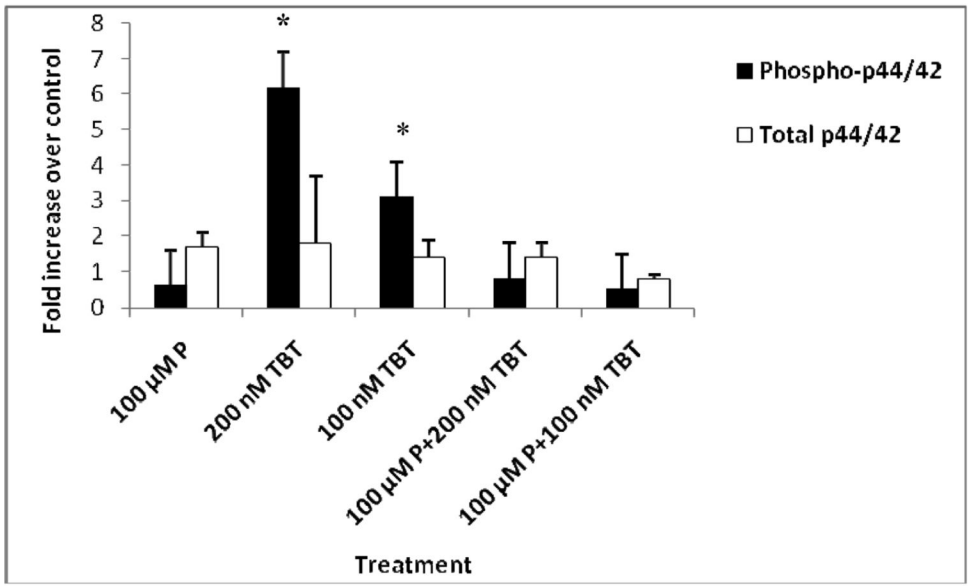


Figure 3. Effect of MEK inhibition on TBT-induced decreases in NK lytic function in 24 h period following a 1 h exposure TBT

Starting from the left Bar 1 = NK cells were treated with 100 μM PD98059 (P) followed by control media for 1 h, Bar 2 = NK cells were treated with 100 μM U0126 (U) followed by control media for 1 h, Bar 3 = NK cells were treated with control media for 1 h followed by exposure to 200 nM TBT for 1 h, Bar 4 = NK cells were treated with control media for 1 h followed by exposure to 100 nM TBT for 1 h, Bar 5 = NK cells were treated with 100 μM PD98059 (P) for 1 h followed by exposure to 200 nM TBT for 1 h, Bar 6 = NK cells were treated with 100 μM PD98059 (P) for 1 h followed by exposure to 100 nM TBT for 1 h, and Bar 7 = NK cells were treated with 100 μM U0126 (U) for 1 h followed by exposure to 200 nM TBT for 1 h, Bar 8 = NK cells were treated with 100 μM U0126 (U) for 1 h followed by exposure to 100 nM TBT for 1 h. The entire treatment time was 2 h, with the initial treatment remaining during the second treatment. Following the treatments the cells were washed twice with gel media and were incubated for 24 hrs in compound-free media prior to assaying for lytic funciton. * indicates significant difference as compared to control, $p<0.05$.

A



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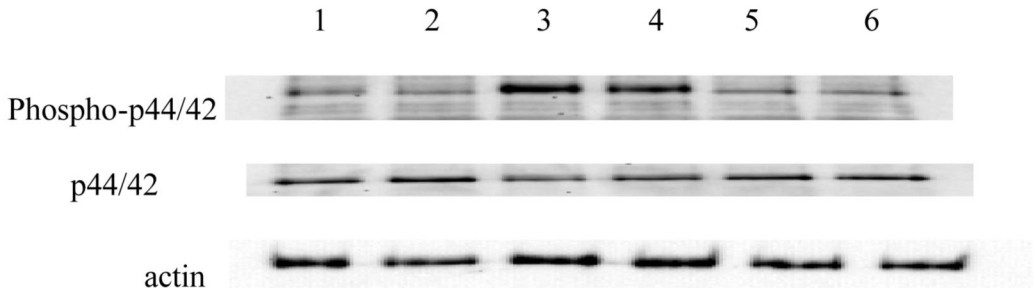

Figure 4. Effect of the MEK inhibitor, PD98059, on the TBT-induced phosphorylation of p44/42 and total p44/42

A.) levels of phospho-p44/42 and total p44/42 compared to control in pure NK cells exposed to 200 or 100 nM TBT for 10 min +/− a 1 h pre-incubation with 100 μM PD98059 (P). The band densities for both control and treated cells were first divided by corresponding β-actin density to correct for very small loading differences. The treatments were then normalized to control. The same procedure was followed for all the experiments. Values are mean ± S.D. from at least three separate experiments using cells from different donors. * indicates significant difference as compared to control, $p<0.05$. B.) Representative experiment (1) NK cells exposed to media for 1 h followed by exposure to media for 10 min (control) , (2) NK cells exposed to 100 μM PD98059 for 1h followed by exposure to media for 10 min, (3) NK cells exposed to media for 1 h followed by exposure to 200 nM TBT for 10 min (4) NK cells

exposed to media for 1h followed by exposure to 100 nM TBT for 10 min, (5) NK cells exposed to 100 μM PD98059 for 1h followed by exposure to 200 nM TBT for 10 min, (6) NK cells exposed to 100 μM PD98059 for 1h followed by exposure to 100 nM TBT for 10 min. The entire treatment time was 1 h and 10 min, with the initial treatment remaining during the second treatment.

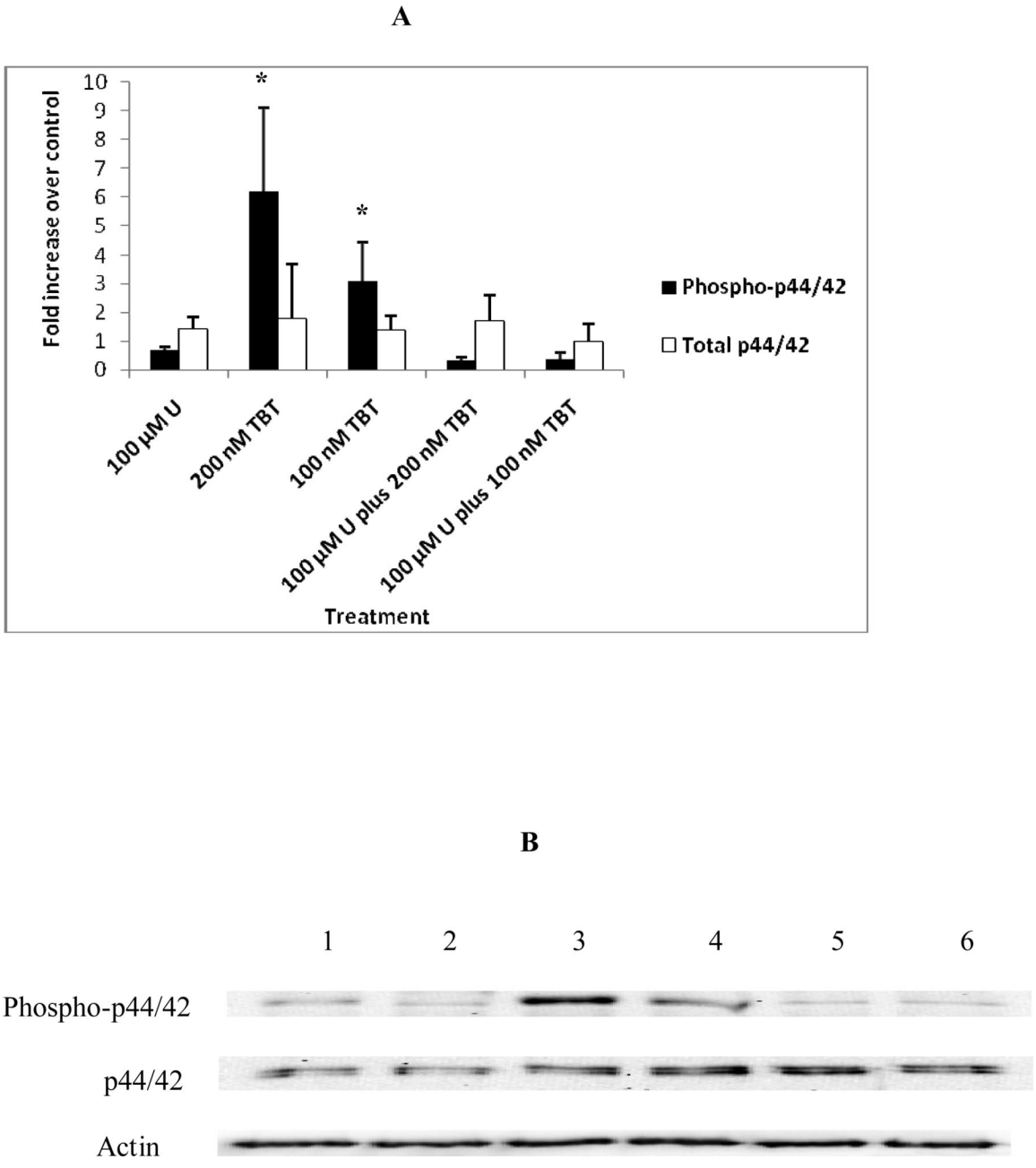

Figure 5. Effect of the MEK inhibitor, U0126, on the TBT-induced phosphorylation of p44/42 total p44/42

A.) levels of phospho-p44/42 and total p44/42 compared to control in pure NK cells exposed to 200 or 100 nM TBT for 10 min +/− a 1 h pre-incubation with 100 μM U0126 (U). The band densities for both control and treated cells were first divided by corresponding β-actin density to correct for very small loading differences. The treatments were then normalized to control. The same procedure was followed for all the experiments. Values are mean ± S.D. from at least three separate experiments using cells from different donors. * indicates significant difference as compared to control, $p<0.05$. B.) Representative experiment (1) NK cells exposed to media for 1 h followed by exposure to media for 10 min (control) , (2) NK cells exposed to 100 μM U0126 for 1h followed by exposure to media for 10 min, (3) NK cells exposed to media for 1 h followed by exposure to 200 nM TBT for 10 min (4) NK cells exposed to media for 1h

followed by exposure to 100 nM TBT for 10 min, (5) NK cells exposed to 100 μM U0126 for 1h followed by exposure to 200 nM TBT for 10 min, (6) NK cells exposed to 100 μM U0126 for 1h followed by exposure to 100 nM TBT for 10 min. The entire treatment time was 1 h and 10 min, with the initial treatment remaining during the second treatment.

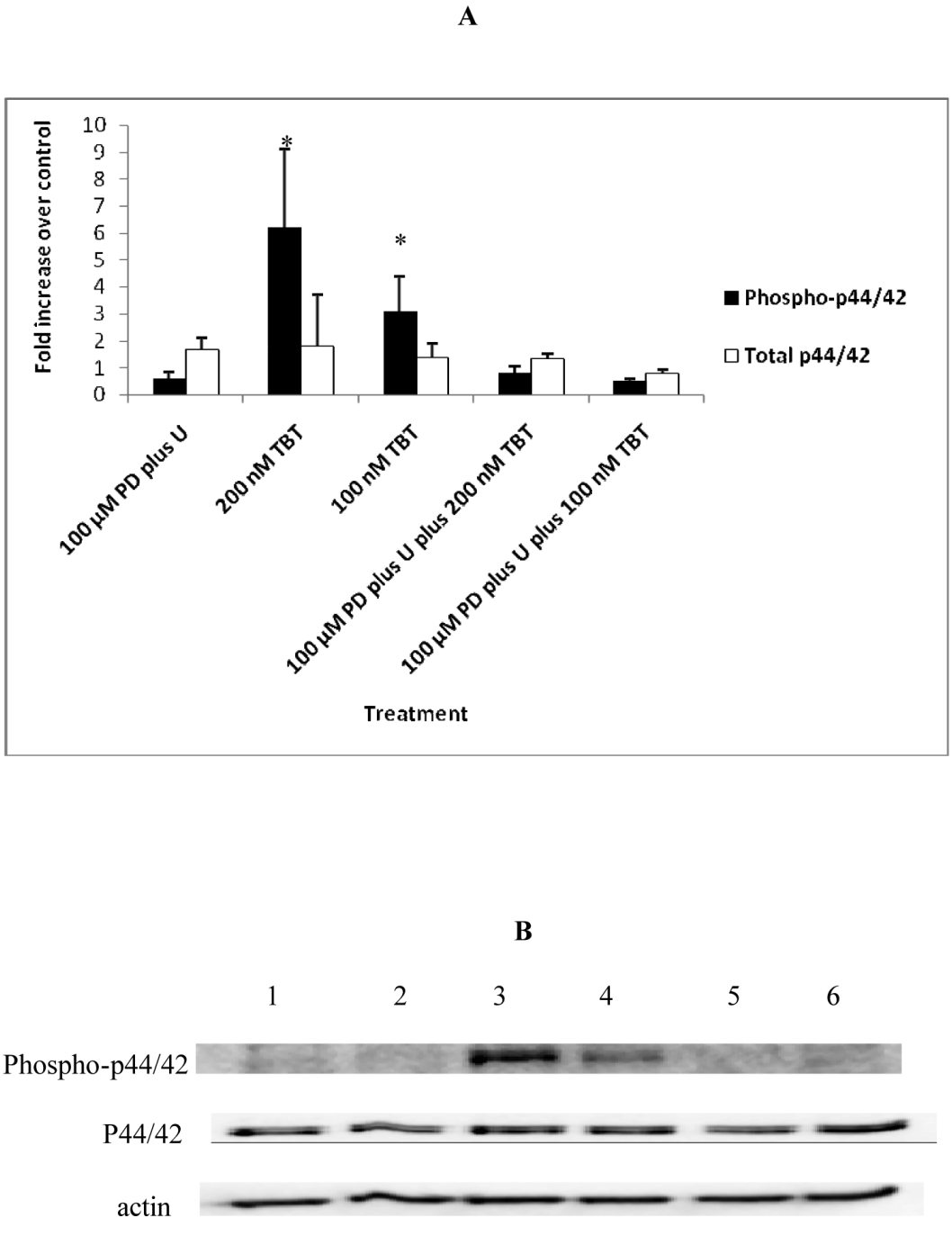

Figure 6. Effect of MEK inhibitors, PD98059 and U0126, on the TBT-induced phosphorylation of p44/42 and total p44/42

A.) levels of phospho-p44/42 and total p44/42 compared to control in pure NK cells exposed to 200 or 100 nM TBT for 10 min +/− a 1 h pre-incubation with 100 μM PD98059 (P) and 100 μM U0126 (U). The band densities for both control and treated cells were first divided by corresponding β-actin density to correct for very small loading differences. The treatments were then normalized to control. The same procedure was followed for all the experiments. Values are mean ± S.D. from at least three separate experiments using cells from different donors. * indicates significant difference as compared to control, $p<0.05$. B.) Representative experiment (1) NK cells exposed to media for 1 hr followed by exposure to media for 10 min

(control) , (2) NK cells exposed to 100 μM P and 100 μM U for 1h followed by exposure to media for 10 min, (3) NK cells exposed to media for 1 h followed by exposure to 200 nM TBT for 10 min (4) NK cells exposed to media for 1h followed by exposure to 100 nM TBT for 10 min, (5) NK cells exposed to 100 μM P and 100 μM U for 1h followed by exposure to 200 nM TBT for 10 min, (6) NK cells exposed to 100 μM P and 100 μM U for 1h followed by exposure to 100 nM TBT for 10 min. The entire treatment time was 1 h and 10 min, with the initial treatment remaining during the second treatment.