

The Nrf2 activator tBHQ inhibits the activation of primary murine natural killer cells

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ABSTRACT

Tert-butylhydroquinone (tBHQ) is a commonly used food preservative with known immunomodulatory activity; however, there is little information regarding its role on natural killer (NK) cell activation and function. tBHQ is a known activator of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), which results in induction of cytoprotective genes. Activation of Nrf2 has been shown to modulate immune responses in a number of different models. In addition, studies in our laboratory have shown that tBHQ inhibits numerous early events following T cell activation. In the current study, we investigated whether activated NK cells are impacted by tBHQ, since many signaling cascades that control NK cell effector function also contribute to T cell function. Splenocytes were isolated from female, wild-type C57Bl/6J mice and treated with 1 μ M or 5 μ M tBHQ. NK cell function was assessed after activation with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 24 h. Activation of NK cells in the presence of tBHQ decreased total NK cell percentage, production of intracellular interferon gamma (IFN γ), granzyme B, and perforin, and induction of the cell surface proteins CD25 and CD69, which are markers of NK cell activation. In addition to NK cell effector function, NK cell maturation was also altered in response to tBHQ. Notably, this is the first study to demonstrate that the Nrf2 activator, tBHQ, negatively impacts effector function and maturation of NK cells.

1. Introduction

Natural killer (NK) cells play a vital role in innate immunity and are necessary for a rapid response against infected and cancerous cells. Activating and inhibitory receptors allow NK cells to effectively monitor tissues for abnormal cells (Lanier, 2008). Upon infection, NK cells are able to recognize and directly kill infected cells, in part through secretion of perforin, granzyme, and proinflammatory cytokines, such as interferon gamma (IFN γ) (Biron et al., 1999; Henkart, 1994). Mechanisms for activation not only occur with recognition through activating receptors or lack of ligand binding inhibitory receptors, but also by cytokine stimulation. Effector functions are

acquired as NK cells mature and can be observed at distinct stages. NK cells are produced from lymphoid progenitors in the bone marrow and circulate in the periphery where they can continue to mature and gain function (Kim et al., 2002). Maturation in mice can be classified using surface markers, CD27 and CD11b, in four stages: CD27-CD11b-, CD27 + CD11b-, CD27 + CD11b+, and CD27-CD11b+ (Chiossone et al., 2009). CD27 is the first marker expressed followed by the appearance of CD11b, which is an indicator of cell effector function (Chiossone et al., 2009). Double positive NK cells, CD27 + CD11b+, have a lower activation threshold and therefore, are the most responsive phenotype with high cytolytic capacity and rapid cytokine production (Hayakawa and Smyth, 2006). NK cells that are terminally

Abbreviations: ADI, Acceptable daily intake; DMEM, Dulbecco's Modified Eagle Medium; FBS, Fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid solution; IFN, Interferon; IL, Interleukin; Keap1, Kelch ECH associating protein 1; MFI, Mean fluorescence intensity; NK cell, Natural killer cell; Nrf2, Nuclear factor erythroid 2-related factor 2; PMA, Phorbol 12-myristate 13-acetate; SE, Standard error; tBHQ, Tert-butylhydroquinone

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differentiated are identified by the loss of CD27 expression and are also capable of inducing effector functions. However, these cells are more tightly regulated and have a higher activation threshold due to increased expression of Ly-49, an inhibitory receptor (Hayakawa and Smyth, 2006; Kim et al., 2002; Yokoyama, 1998).

Studies from our group and others suggest that NK cell cytotoxicity and development are greatly influenced by dietary interventions. Our laboratory previously demonstrated alterations in NK cell maturation and impaired function in calorie-restricted mice (Clinthorne et al., 2013; Gardner et al., 2011; Ritz et al., 2008). Other studies involving vitamin supplementation, alcohol consumption, and excessive energy intake have also been shown to impact NK cell function and development (Heuser and Vojdani, 1997; Ritz et al., 2006; Smith et al., 2007; Zhang and Meadows, 2008).

Tert-butylhydroquinone (tBHQ) is widely used as a food additive to delay rancidification of fats, and it can be found in many foods including oils, crackers, and cereals, among others (Shahidi, 2000). The U.S. Food and Drug Administration regulates the amount of tBHQ in foods with a limit of 0.02% of the oil or fat content of the food (USFDA, 2018). Additionally, the acceptable daily intake (ADI) of tBHQ is 0.7 mg/kg body weight per day. Regardless of the regulations and recommendations, the amount of tBHQ consumed by an individual is difficult to determine. Estimates of tBHQ intake have been performed using several methods. Based on poundage, the estimates of tBHQ intake were below the ADI. Consistently, model diets measuring the intake of tBHQ found the average consumer below the ADI, yet individuals with high tBHQ consumption exceeded the ADI, up to 1100% of the ADI (WHO, 1999). Previous studies in humans have found serum concentrations in the high micromolar range after consuming 100–150 mg tBHQ, suggesting that the compound is readily absorbed following consumption (WHO, 1975).

tBHQ is a well characterized nuclear factor erythroid 2-related factor 2 (Nrf2) activator. Nrf2 is a transcription factor that acts as a sensor for cell stress by inducing expression of cytoprotective genes when activated. Under basal conditions, Nrf2 is tethered to Kelch ECH associating protein 1 (Keap1), a cytosolic repressor protein, and is polyubiquitinated for proteasomal degradation. Activation by cellular and environmental stresses leads to Nrf2 translocation to the nucleus where it binds to antioxidant response elements, causing upregulation of cytoprotective genes (Kensler et al., 2007). tBHQ is a potent activator of Nrf2 through modification of thiol groups of cysteines on the Keap1 protein, which prevents Nrf2 repression by Keap1, therefore allowing induction of antioxidant responses by Nrf2 (Li and Kong, 2009).

Much of the toxicology research on tBHQ has primarily focused on potential carcinogenic effects in humans, with the research indicating that tBHQ is not carcinogenic (Shahidi, 2000). However, research from our laboratory has shown that Nrf2 has numerous immunomodulatory effects in T cells. We previously showed tBHQ significantly reduces IFN γ secretion and skews differentiation of primary murine CD4⁺ T cells from T helper type 1 (Th1) to T helper type 2 (Th2) in a Nrf2-dependent manner (Rockwell et al., 2012). Additionally, in human Jurkat T cells, tBHQ inhibits interleukin (IL)-2 production and decreases expression of CD25 (Zagorski et al., 2018, 2013). More recently, treatment of primary human T cells with tBHQ causes decreased expression of CD25 and CD69 surface proteins and reduced production of IL-2 and IFN γ , suggesting that overall T cell activation may be inhibited (Turley et al., 2015). Based on these findings, we hypothesized that NK cells could also be impacted by tBHQ because NK cells and T cells possess many similarities, including robust IFN γ production and cytotoxic capabilities, and shared transcription factors and cell surface molecules (Sun, 2016). To our knowledge, the role of the food additive tBHQ has not been studied in isolated NK cells. The goal of the present study was to determine the effects of the Nrf2 activator, tBHQ, on NK cells in a mixed murine splenocyte preparation using flow cytometric analysis. Notably, this is the first study to demonstrate that the Nrf2 activator, tBHQ, inhibits activation, alters maturation and diminishes

effector function in NK cells.

2. Methods and materials

2.1. Materials

All materials were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise specified.

2.2. Mice

Female, wild-type C57Bl/6J mice (13 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were acclimated for a week and given food and water *ad libitum*. All animal studies were conducted in accordance with the Guide for the Care and Use of Animals as adopted by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University.

2.3. Cell preparation

Spleens were aseptically removed for lymphocyte isolation by grinding and filtering tissues through a 40 μ m strainer. Cells were subsequently washed, counted, and adjusted to a cell density of 5×10^6 c/mL. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid solution (HEPES), 10% fetal bovine serum (FBS), non-essential amino acid (1X final concentration from 100X), 100 U/mL penicillin, 100 U/mL streptomycin, and 1000 U/mL 2-mercaptoethanol (Thermo Fisher Scientific, Waltham, MA). Splenocytes were either treated with tBHQ or vehicle (VEH) at concentrations indicated in the figure legends 30 min prior to activation with 40 nM phorbol 12-myristate 13-acetate (PMA) and 0.5 μ M ionomycin. Up to 5 μ M tBHQ was used in the present studies; the concentrations were selected in part because they are known not to affect viability based on broad concentration responses previously conducted in our lab. Further, while information on the blood concentrations of tBHQ within the general public is not readily available, research has shown that a bolus dose of tBHQ provided orally to humans translates into a blood concentration reaching around 200 μ M (WHO, 1975), suggesting the concentrations used in this study are well within the range of potential human exposure. Immediately following the addition of PMA and ionomycin, 15 μ M monensin solution was used to block protein secretion for intracellular labeling (eBioscience, San Diego, CA). Splenocytes were then incubated for 24 h.

2.4. Flow cytometry

After 24 h of incubation, splenocytes were transferred to a 96-well V-bottom plate and stained with Zombie Aqua Fixable Viability Dye following the manufacturer protocol (Biolegend, San Diego, CA). Samples were treated with Fc-Block (antibody to CD16/32 [2.4G2], BD Bioscience, San Jose, CA) and labeled for surface markers using appropriate antibodies for 30 min. Subsequently, samples were fixed using BD Cytofix Fixation Buffer (BD Biosciences). For intracellular staining, splenocytes were permeabilized, labeled for intracellular markers, and fixed following previously published procedures with minor modifications (Babcock, 2004). Combinations of the following fluorochrome-conjugated antibodies (Biolegend, eBioscience or BD Biosciences) were used: CD3e (AlexaFluor488 [17A2] or AlexaFluor700 [eBio500A2]), NK1.1 (BrilliantViolet711 [PK136]), CD27 (PerCP/eFluor710 [LG.7F9]), CD11b (AlexaFluor647 [M1/70]), CD69 (AlexaFluor700 [H1.2F3]), CD25 (PE [PC61] or [3C7]), IFN γ (APC [XMG1.2]), granzyme B (FITC [GB11]), and perforin (PE [eBio0MAK-D]). Samples were acquired and analyzed on an Attune NxT Acoustic Focusing Cytometer from Life Technologies (Thermo Fisher Scientific, Waltham, MA).

2.5. Statistical analysis

Treatment groups are presented as mean $\pm$ standard error (SE). SigmaPlot 12.3 (Systat, Chicago, IL) was used to statistically analyze data. The Shapiro-Wilk Normality Test was performed to determine normal distribution. Normally-distributed data were analyzed by one-way ANOVA followed by a Dunnett's *post-hoc* test to determine statistical significance of $p < 0.05$ comparing all groups to the VEH treatment. Data not normally distributed were analyzed using Kruskal-Wallis one-way analysis of variance on ranks. A sample size of 5 or 10 mice was used for each experiment and is noted in the figure legends.

3. Results

3.1. The *Nrf2* activator, tBHQ, modestly decreases the percentage of NK cells in Spleen

Our laboratory has previously shown the immunomodulatory effects of *Nrf2* activation by tBHQ in primary murine CD4⁺ T cells, Jurkat T cells, and primary human CD4⁺ T cells (Rockwell et al., 2012; Turley et al., 2015; Zagorski et al., 2018, 2013); however, the effects of *Nrf2* activation by tBHQ in NK cells has yet to be characterized. To determine the effect of tBHQ on NK cells, we quantified the NK cell population by gating CD3-NK1.1⁺. tBHQ concentrations were selected based on concentrations used previously in our lab that did not demonstrate cytotoxicity in these experiments. Consistent with these previous studies, tBHQ did not decrease viability in the current studies (data not shown). Compared to the VEH group, splenocytes treated with 5 μ M tBHQ displayed a modest, though statistically significant, reduction in the percentage of NK cells within the splenocyte population (Fig. 1, VEH, 2.19 $\pm$ 0.08%; 5 μ M tBHQ, 1.79 $\pm$ 0.1%).

3.2. The *Nrf2* activator, tBHQ, decreases NK cell expression of CD25 and CD69

The activation of NK cells by PMA and ionomycin can be assessed in part via the induction of the cell surface molecules CD25 and CD69, which serve as markers of activation of lymphocytes. Previously, our laboratory demonstrated reduced induction of CD25 and CD69 in activated T cells with tBHQ treatment (Turley et al., 2015). This prompted us to quantify CD25 and CD69 expression in activated NK cells. The

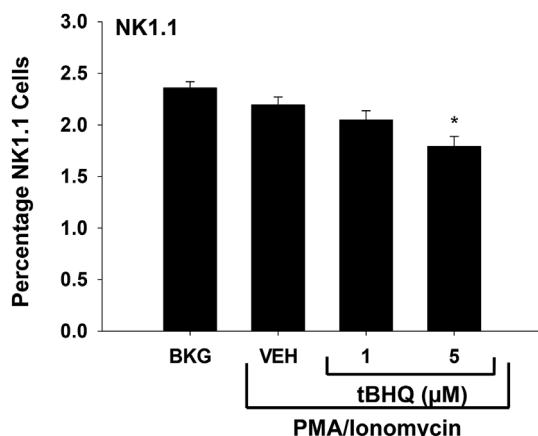


Fig. 1. tBHQ treatment modestly decreases the percentage of NK cells in spleen. Murine splenocytes were isolated and treated with tBHQ (1 μ M or 5 μ M), VEH (0.01% ethanol), or complete DMEM (BKG) for 30 min, then either unactivated (BKG) or activated by PMA and ionomycin (VEH, 1 μ M tBHQ, and 5 μ M tBHQ) for 24 h. Cells were then labeled with fluorescently conjugated antibodies against CD3 ϵ and NK1.1 for FACS analysis. NK cells were gated on viable cells and identified as CD3 negative and NK1.1 positive. $n = 10$

*indicates $p < 0.05$ compared to VEH group.

percentage of NK cells expressing CD25 was significantly reduced in groups treated with 1 μ M and 5 μ M tBHQ compared to the VEH group (Fig. 2A, VEH, 1.4 $\pm$ 0.15%; 1 μ M tBHQ, 0.85 $\pm$ 0.07%; 5 μ M tBHQ, 0.58 $\pm$ 0.13%). Likewise, NK cell expression of CD69 followed the same trend with significant decline in expression for both tBHQ-treated groups (Fig. 2B, VEH, 63.57 $\pm$ 1.39%; 1 μ M tBHQ, 53.39 $\pm$ 0.88%; 5 μ M tBHQ, 46.08 $\pm$ 1.01%). Activated NK cells in the presence of tBHQ markedly decreased induction of CD25 and CD69 to levels lower than that of unactivated NK cells (BKG) (Fig. 2, CD25 BKG, 1.1 $\pm$ 0.12%; CD69 BKG, 52.46 $\pm$ 1.07%). Taken together, the food preservative tBHQ inhibits expression of activation markers CD25 and CD69 in activated NK cells, which is consistent with the effects of tBHQ in T cells.

3.3. Treatment with the *Nrf2* activator, tBHQ, alters NK cell maturation *In vitro*

NK cell effector function is acquired through maturation and is defined by distinct stages (Sun, 2016). Therefore, we examined whether the *Nrf2* activator, tBHQ, altered NK cell maturation within the total NK cell population. As expected, NK cells expressing an immature phenotype, CD27-CD11b⁻, were significantly greater in the BKG group likely due to the absence of cell activation (Fig. 3A, VEH, 27.87 $\pm$ 2.73%; BKG, 37.91 $\pm$ 3.48%). A more mature, yet not fully functional, subset of NK cell maturation is defined by the expression of CD27. We found this NK cell subset, CD27⁺CD11b⁻, at significantly lower percentages in groups treated with 1 μ M (5.23 $\pm$ 0.41%) and 5 μ M tBHQ (3.21 $\pm$ 0.32%) in relation to the VEH group (7.19 $\pm$ 0.61%) (Fig. 3B). Following the progression of maturation, the most responsive NK cell subset, CD27⁺CD11b⁺, was highest in the VEH group, while the percentage of this subset was decreased in the tBHQ-treated groups. Though these results were not significant ($p = 0.052$), treatment with 5 μ M tBHQ (2.09 $\pm$ 0.37%) exhibited the greatest difference compared to the VEH group (4.6 $\pm$ 0.71%) in which 5 μ M tBHQ decreased the CD27⁺CD11b⁺ subset to a percentage lower than that of the (un-activated) BKG group (Fig. 3C). Interestingly, the most mature and terminally differentiated NK cell phenotype, CD27-CD11b⁺, was significantly increased with treatment of 5 μ M tBHQ (67.83 $\pm$ 2.67%) compared to the VEH group (60.33 $\pm$ 2.34%) (Fig. 3D). Collectively, these results indicate that the food preservative, tBHQ, has significant effects on NK cell maturation.

3.4. Treatment with the *Nrf2* activator, tBHQ, inhibits IFN γ production in NK cells

As NK cells mature and express CD11b, they acquire effector function and have increased capacity for producing cytokines such as IFN γ . A previous study from our laboratory showed tBHQ decreased production of IFN γ in CD4⁺ T cells (Turley et al., 2015). Because tBHQ altered NK cell maturation, we investigated the effects of tBHQ on IFN γ production by NK cells. The percentage of NK cells producing IFN γ was significantly reduced in groups treated with 1 μ M and 5 μ M tBHQ in a concentration-dependent manner (Fig. 4A, VEH, 53.38 $\pm$ 4.64%; 1 μ M tBHQ, 29.35 $\pm$ 2.28%; 5 μ M tBHQ, 12.62 $\pm$ 1.25%). In addition to a decrease in the percentage of IFN γ -producing NK cells, tBHQ also caused a reduction in mean fluorescence intensity (MFI) (Fig. 4B, VEH, 14567.2 $\pm$ 1876.06; 5 μ M tBHQ, 2261.2 $\pm$ 128.83), suggesting a decrease in IFN γ expression in individual NK cells.

3.5. The *Nrf2* activator, tBHQ, decreases production of perforin and granzyme B in NK cells

Due to a decreased production of IFN γ in NK cells treated with tBHQ, we decided to further investigate fundamental NK cell effector functions, including production of perforin and granzyme B. As concentrations of tBHQ increased, the percentage of NK cells producing

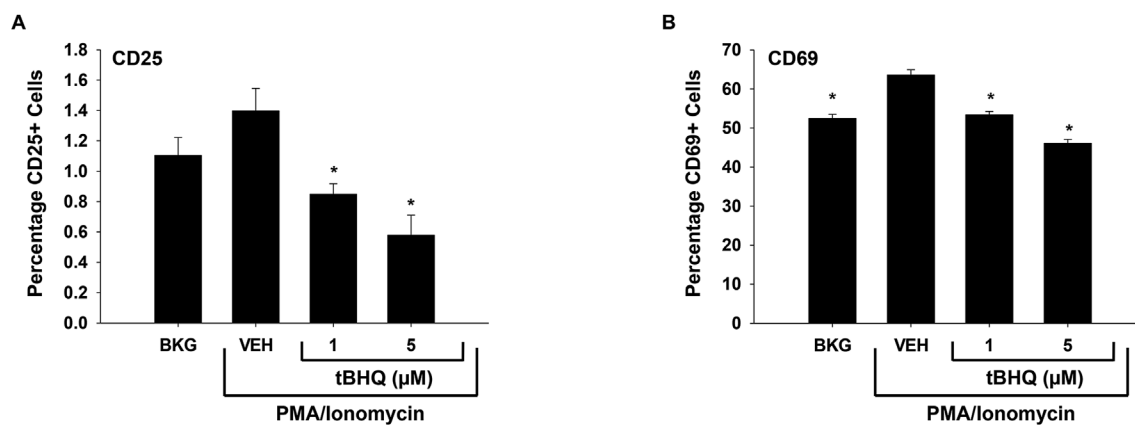


Fig. 2. tBHQ treatment inhibits expression of cell surface molecules CD25 and CD69 in NK cells.

Murine splenocytes were isolated and treated with tBHQ (1 μ M or 5 μ M), VEH (0.01% ethanol), or complete DMEM (BKG) for 30 min, then either unactivated (BKG) or activated by PMA and ionomycin (VEH, 1 μ M tBHQ, and 5 μ M tBHQ) for 24 h. Cells were labeled with fluorescently conjugated antibodies against CD3e, NK1.1, CD25, and CD69 for FACS analysis. NK cells were gated on viable cells and identified as CD3 negative and NK1.1 positive prior to analysis of (A) CD25 and (B) CD69 expression. $n = 5$

* indicates $p < 0.05$ compared to VEH group.

perforin was reduced in a concentration-dependent manner with significant reduction observed by 5 μ M tBHQ (Fig. 5A, VEH, $3.98 \pm 0.29\%$; 5 μ M tBHQ, $1.8 \pm 0.09\%$). Similarly, granzyme B production by activated NK cells was significantly decreased in both groups treated with tBHQ compared to the VEH group (Fig. 5B, VEH,

$31.74 \pm 2.21\%$; 1 μ M tBHQ; $20.37 \pm 2.46\%$; 5 μ M tBHQ, $14.9 \pm 1.67\%$). Collectively, these results demonstrate inhibitory effects of the food preservative, tBHQ, on vital NK cell cytolytic events.

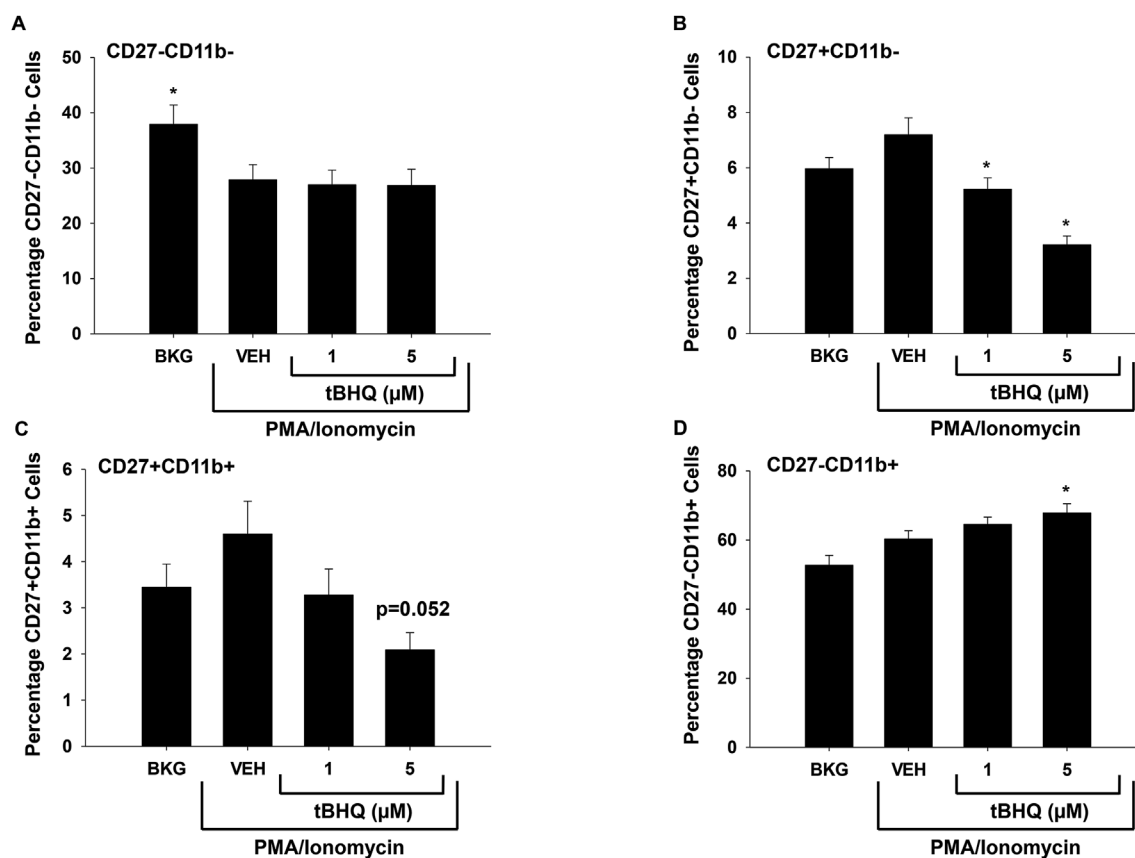


Fig. 3. tBHQ treatment alters NK cell maturation.

Murine splenocytes were isolated and treated with tBHQ (1 μ M or 5 μ M), VEH (0.01% ethanol), or complete DMEM (BKG) for 30 min, then either unactivated (BKG) or activated by PMA and ionomycin (VEH, 1 μ M tBHQ, and 5 μ M tBHQ) for 24 h. Cells were labeled with fluorescently conjugated antibodies against CD3e, NK1.1, CD27, and CD11b for FACS analysis. NK cells were gated on viable cells and identified as CD3 negative and NK1.1 positive prior to FACS analysis of maturation markers, CD27 and CD11b. Distinct stages of NK cell maturation occur in the following order: (A) CD27-CD11b-, (B) CD27 + CD11b-, (C) CD27 + CD11b+, (D) CD27-CD11b+. $n = 10$

*indicates $p < 0.05$ compared to VEH group.

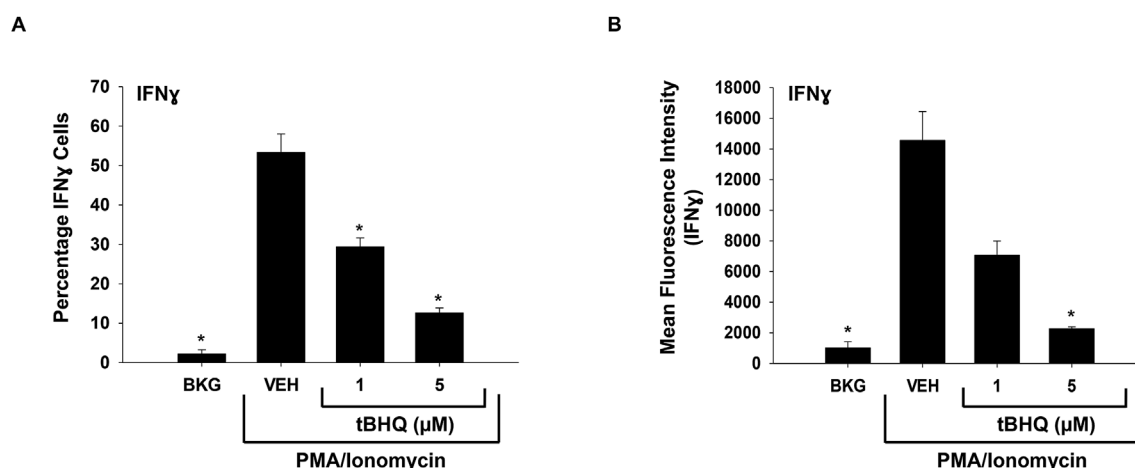


Fig. 4. tBHQ treatment inhibits production of the cytokine IFN γ in NK cells.

Murine splenocytes were isolated and treated with tBHQ (1 μ M or 5 μ M), VEH (0.01% ethanol), or complete DMEM (BKG) for 30 min, then either unactivated (BKG) or activated by PMA and ionomycin (VEH, 1 μ M tBHQ, and 5 μ M tBHQ) for 24 h. Cells were labeled with fluorescently conjugated antibodies against CD3 ϵ and NK1.1 followed by intracellular labeling of IFN γ with fluorescently conjugated antibodies for FACS analysis. NK cells were gated on viable cells and identified as CD3 negative and NK1.1 positive prior to analysis of IFN γ by (A) percentage and (B) mean fluorescence intensity (MFI). n = 5

*indicates $p < 0.05$ compared to VEH group.

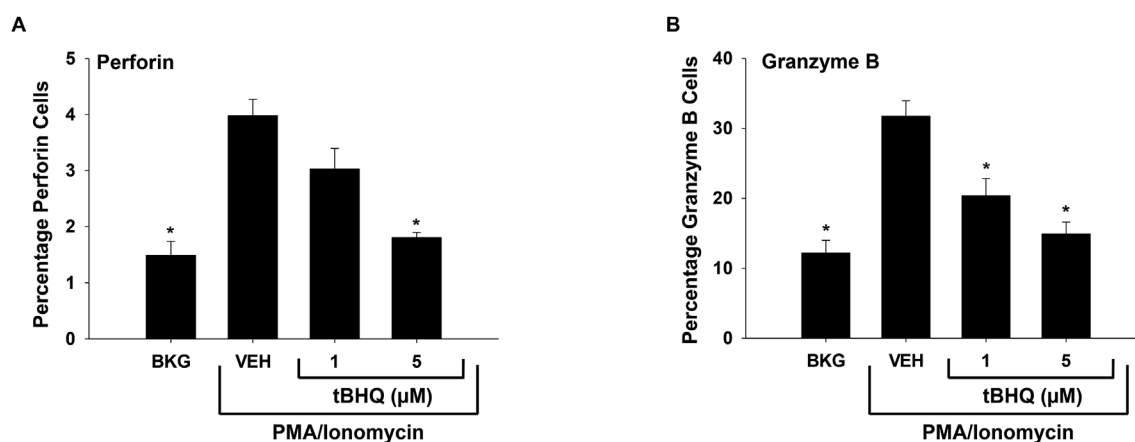


Fig. 5. tBHQ treatment inhibits the production of perforin and granzyme B in NK cells.

Murine splenocytes were isolated and treated with tBHQ (1 μ M or 5 μ M), VEH (0.01% ethanol), or complete DMEM (BKG) for 30 min, then either unactivated (BKG) or activated by PMA and ionomycin (VEH, 1 μ M tBHQ, and 5 μ M tBHQ) for 24 h. Cells were labeled with fluorescently conjugated antibodies against CD3 ϵ and NK1.1 followed by intracellular labeling of perforin and granzyme B with fluorescently conjugated antibodies for FACS analysis. NK cells were gated on viable cells and identified as CD3 negative and NK1.1 positive prior to analysis of (A) perforin and (B) granzyme B. n = 5

*indicates $p < 0.05$ compared to VEH group.

4. Discussion

The current study is the first to show inhibition of NK cell activation and effector functions in murine splenocytes with increasing concentrations of tBHQ. tBHQ at low micromolar concentrations diminished NK cell activation-induced CD25 and CD69 expression in accord with previous studies using primary human CD4⁺ T cells treated with tBHQ (Turley et al., 2015). Additionally, tBHQ induced NK cell maturation as indicated by the decreased percentage of the intermediate NK cell subsets, CD27 + CD11b⁻ and CD27 + CD11b⁺, and increased percentage of terminally differentiated NK cells, CD27-CD11b⁺. NK cell effector function was also found to be markedly reduced after supplementation of tBHQ. Notably, tBHQ significantly inhibited the production of IFN γ in NK cells in a concentration-dependent manner. Likewise, significant reductions in perforin and granzyme B production were observed with increasing concentrations of tBHQ, suggesting impairment of NK cell effector function after supplementation with tBHQ.

The cell surface proteins CD27 and CD11b are commonly used to identify specific stages of murine NK cell development (Chiossone et al.,

2009). NK cell function has correlated with developmental stages based on expression of CD27 and CD11b (Hayakawa and Smyth, 2006). In the spleen, NK cells represent a small fraction (< 2.5%) of the lymphocyte population with the majority of NK cells expressing CD27-CD11b⁺ (Gregoire et al., 2007; Hayakawa and Smyth, 2006). Consistent with our results, less than 2.5% of the splenic lymphocytes were identified as NK cells, and of those NK cells, CD27-CD11b⁺ was the predominant phenotype within all treatment groups (Figs. 1 and 3D). Interestingly, the percentage of CD27-CD11b⁺ NK cells was significantly greater when treated with 5 μ M tBHQ, while CD27 + CD11b⁺ NK cells were reduced in both tBHQ-treated groups (Fig. 3C and D). Prior studies have also demonstrated decreased cytolytic capacity and cytokine production in mature CD27-CD11b⁺ NK cells compared to CD27 + CD11b⁺ NK cells (Hayakawa and Smyth, 2006; Kim et al., 2002). The present study found a decreased percentage of CD27 + CD11b⁺ NK cells to be reflective of the diminished production of IFN γ , perforin, and granzyme B with tBHQ treatments (Figs. 3C, 4 and 5). The increase in CD27-CD11b⁺ NK cells treated with 5 μ M tBHQ (Fig. 3D) may provide relatively small amounts of IFN γ , perforin,

and granzyme B. Still, the majority of effector functions are elicited by the CD27 + CD11b + NK cell subset. NK cells identified as CD27 + CD11b were significantly reduced in the 1 μ M and 5 μ M tBHQ-treated groups (Fig. 3B). However, this subpopulation does not exhibit substantial cytolytic function or cytokine production, therefore does not contribute to the reduced effector functions seen with tBHQ treatment. Taken together, our results suggest that although tBHQ may cause an increase in the proportion of mature, fully-differentiated NK cells, it may also diminish the effector function of these cells.

Several studies have noted immunomodulatory effects of Nrf2 when activated by tBHQ. As mentioned earlier, our laboratory has demonstrated that tBHQ strongly activates Nrf2 and modulates T cells in primary mouse CD4⁺ T cells, Jurkat T cells, and primary human CD4⁺ T cells (Rockwell et al., 2012; Turley et al., 2015; Zagorski et al., 2018, 2013). Furthermore, dendritic cells were found to be affected by tBHQ through Nrf2 activation. Treatment of tBHQ in lipopolysaccharide-activated human dendritic cells potentially inhibited the secretion of the cytokine IL-12, which is involved in the activation NK cells and the differentiation of CD4⁺ T cells (Macocho et al., 2015; Vivier et al., 2008). Studies have also shown macrophages to be affected by Nrf2 activation. In particular, macrophages producing proinflammatory cytokines, tumor necrosis factor alpha (TNF α) and IL-6, were inhibited with tBHQ treatment in a concentration-dependent manner after lipopolysaccharide stimulation (Ma and Kinneer, 2002; Wei et al., 2018). Additionally, activation of Nrf2 by tBHQ was found to regulate macrophage polarization by promoting the anti-inflammatory M2 macrophages (Wei et al., 2018). Consistent with other immune cell types, our results also demonstrate the immunomodulatory effects of the food additive, tBHQ, on splenic murine NK cells, including reduced expression of activation markers CD25 and CD69, diminished production of IFN γ , perforin, and granzyme B, and altered maturation.

Collectively, these results suggest treatment of murine splenocytes with the Nrf2 activator, tBHQ, greatly impact activated NK cells. tBHQ inhibited the upregulation of CD25 and CD69 in NK cells upon activation with PMA and ionomycin in a concentration-dependent manner. Additionally, tBHQ treatment increased the percentage of NK cells displaying a more mature phenotype, CD27-CD11b⁺. Interestingly, markers that measure NK cell function, such as IFN γ , perforin, and granzyme B, were significantly decreased in groups treated with tBHQ. In conclusion, this study has shown that doses of tBHQ that are relevant to human exposure can significantly impair NK cell function *in vitro*. Therefore, future studies should determine if the effects of tBHQ on NK cell maturation and function will significantly impact human health and disease, particularly in the context of host defense and cancer.

Conflicts of interest

All authors declare no conflict of interest.

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Transparency document

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