THE EFFECTS OF *TERT*-BUTYLHYDROQUINONE ON THE MURINE NATURAL KILLER CELL ACTIVATION, EFFECTOR FUNCTION, AND PRIMARY RESPONSE TO INFLUENZA

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ABSTRACT

Seasonal influenza poses a significant threat to the public due to its ability to cause illness that ranges in severity and can lead to hospitalization and death. Research to identify potential contributing factors to the susceptibility and severity of influenza infection is necessary to decrease the burden of disease. Natural killer (NK) cells provide early protection during infection by providing an early source of cytokines and cytotoxicity of virally infected cells. Recently, an immunomodulatory role for the transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), has been shown in several immune cell types and is activated by oxidative stress and various exogenous compounds, such as the food additive, *tert*-butylhydroquinone (tBHQ). However, the role of Nrf2 and tBHQ in NK cells remains largely unknown. The overall goal of these studies is to understand the impact of tBHQ and Nrf2 on NK cell activity and to determine the effect of tBHQ on NK cell response to influenza. To assess this, we determined the effects of tBHQ on NK cell activation and effector function and whether these effects were dependent on Nrf2. Activation of NK cells was significantly decreased as shown by a reduction in early activation markers, CD25 and CD69, with 1 and 5 μM tBHQ compared to the control vehicle group (VEH). Additionally, tBHQ induced maturation as demonstrated by a decrease in the percentage of intermediate stage NK cells, CD27+CD11b+, and an increase in terminally differentiated NK cells (CD27-CD11b+). tBHQ also inhibited production of the antiviral cytokine, IFNγ, at 1 and 5 μM tBHQ compared to the VEH control. Furthermore, cytotoxic molecules, perforin and granzyme B, were also reduced with tBHQ in a concentration-dependent manner. To investigate the role of Nrf2, Nrf2-deficient mice were utilized to assess whether these effects were mediated by Nrf2. Maturation of NK cells had a pronounced genotype and tBHQ effect with a significant increase in

the percentage of immature NK cells (CD27-CD11b-) in activated splenocytes from Nrf2-null mice. In addition, tBHQ caused a Nrf2-dependent decrease in the expression of the activation markers, CD25 and CD69, in PMA/ionomycin-activated NK cells. Likewise, tBHQ decreased expression of fas ligand in IL-12/IL-18-activated NK cells in a Nrf2-dependent manner. In contrast, inhibition of IFNγ induction by tBHQ was only partially Nrf2-dependent. These findings demonstrate tBHQ negatively impacts NK cell activation and effector function *in vitro.* However, little is known regarding the effect of dietary tBHQ on NK cells *in vivo*, specifically in response to influenza infection. Mice were fed a diet containing either 0.0014% tBHQ or control diet 10 days prior to being infected with influenza A/PR/8/34 (H1N1). NK cell responses were assessed at days two and three post-infection. Mice exhibited similar weight loss throughout infection regardless of their respective diet. At day three, tBHQ modestly decreased the percentage of NK cells in the lung, and NK cells adopted a more immature phenotype. tBHQ significantly reduced expression of fas ligand and production of IFNγ in NK cells compared to those on a control diet at day two. NK cell cytotoxicity decreased with tBHQ at day three, as demonstrated by reduced CD107a expression. Induction of effector genes was decreased in the lungs of mice on a tBHQ diet at days two and three of infection. Collectively, these are the first studies show that the food additive, tBHQ, negatively impacts NK cell activation, effector function and early responses to influenza infection.

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
ADI	Allowable daily intake
AHCC	Active hexose correlated compound
AhR	Aryl hydrocarbon receptor
ARE	Antioxidant response element
bZIP	Basic leucine zipper
CCR	Chemokine receptor
CDDO-Im	2-Cyano-3,12 dioxooleana-1,9 diene-28-imidazolide
CLP	Common lymphoid progenitor
CMV	Cytomegalovirus
CNC	Cap'n'collar
COPD	Chronic obstructive pulmonary disease
Cul3	Cullin 3
CXCR	CXC-chemokine receptor
DC	Dendritic cell
DEM	Diethylmaleate
DMF	Dimethylfumarate
DNA	Deoxyribonucleic acid
Eomes	Eomesodermin
ER	Endoplasmic reticulum
FasL	Fas ligand

GSH	Glutathione
GST	Glutathione S-transferase
HA	Hemagglutinin
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HO-1	Heme oxygenase-1
HSC	Hematopoietic stem cells
HSV	Herpes simplex virus
IAV	Influenza A virus
ID2	Inhibitor of deoxyribonucleic acid (DNA) binding 2
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNK	Immature natural killer cell
Keap1	Kelch ECH-associated protein 1
M1	Matrix 1
M2	Matrix 2
MACRO	Macrophage receptor with collagenous structure
MHC	Major histone complex
NA	Neuraminidase
NOAEL	No observed adverse effect level

NQO-1	NAD(P)H oxidoreductase
NCR	Natural cytotoxicity receptor
Neh	Nuclear factor erythroid 2-related factor 2 (Nrf2)-ECHhomology
NFE212	Nuclear factor (erythroid-derived 2)-like 2
NFκB	Nuclear factor kappa B
NK	Natural killer
NKP	Natural killer cell precursor
NP	Nucleoprotein
Nrf2	Nuclear factor erythroid 2-related factor 2
NSP1	Non-structural protein 1
NSP2	Non-structural protein 2
OVA	Ovalbumin
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB1-F2	Polymerase basic protein 1- F2
PB2	Polymerase basic protein 2
PERK	Protein kinase ribonucleic acid (RNA)-like endoplasmic reticulum kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
preNKP	Pre natural killer cell progenitor
RNA	Ribonucleic acid
ROS	Reactive oxygen species

RSV	Respiratory syncytial virus
SFN	Sulforaphane
SLE	Systemic lupus erythematosus
T-bet	T-box expressed in T cells
tBBQ	*tert*-butyl benzoquinone
tBHQ	*tert*-butylhydroquinone
TCR	T cell receptor
TGFβ	Transforming growth factor β
Th	T helper
Tim-3	T cell immunoglobulin mucin-3
TLR	Toll-like receptor
TNFα	Tumor necrosis factor-α
vRNP	Viral ribonucleoproteins
WT	Wild-type
ZEB2	Zinc finger E-box- binding protein 2

CHAPTER 1

Literature Review

I. Influenza Virus

A. General

Influenza A virus (IAV) belongs to the Orthomyxoviridae family and is a negative-sense, single-stranded, enveloped ribonucleic acid (RNA) virus that contains a segmented genome, which encodes several proteins involved in viral entry and infection, replication, and viral release including: hemagglutinin (HA), neuraminidase (NA), matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP), non-structural protein 1 (NSP1), non-structural protein 2 (NSP2), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase basic protein 1- F2 (PB1-F2). Entry into the host cell is initiated by HA binding to sialic acid residues on the host cell's membrane. The virus enters the host via receptor-mediated endocytosis. The acidic environment of the endosome allows fusion of viral and host membranes and opens M2 channel, leading to acidification and release of viral ribonucleoproteins (vRNP) from M1, which is comprised of PB1, PB2, and PA, into the cytoplasm. vRNP enter the nucleus by nuclear localization signals. Replication occurs by synthesis of positive-sense complementary RNA (cRNA) followed by copying of cRNA into newly synthesized negative-sense viral RNA. Newly assembled viral RNA is transported to the plasma cell membrane and released by NA[1,2].

IAV can be categorized based on the virus structure of surface glycoproteins HA and NA. Currently, there are 18 HA and 11 NA subtypes, but the main subtypes that are infectious to humans include H1N1 and H3N2[2]. Although there are 4 types of seasonal influenza viruses, types A, B, C, and D, that can cause seasonal epidemics, only influenza

A virus is known to have caused pandemics, such as the pandemic in 2009.[3] Influenza continues to pose a significant threat to society with 8% of the United States population infected each year.[4] Globally, influenza infections are estimated at 5-10% in adults and 20-30% in children.[3,5] However, it should be noted that precise data on influenza morbidity and mortality are based on data from industrialized countries. Therefore, the impact of influenza on lower income countries are less known.[6,7]

Influenza virus burden of disease persists due to high mutation rates in antigenic sites. Influenza viral RNA polymerase lacks proofreading machinery which results in significant mutations during the viral replication at a rate of 10^{-3} to 10^{-4}. To evade immune responses, IAV evolve by mechanisms that include antigenic drift, antigenic shift, and recombination. Specifically, mutations in HA or NA proteins can escape host immune responses that have been acquired through immunization or previous infection.

Vaccination against influenza is the most effective way to prevent disease, and it has been estimated to prevent millions of illnesses, medical visits, hospitalization, and deaths each season.[8] Despite the advancements in vaccine effectiveness and coverage, the burden of disease remains high, affecting millions in the United States alone.[9] Individual factors, including demographic, genetic, and environmental, are known to influence immune responses, which provides an explanation for the wide variations in susceptibility and severity of infection.[10] Even though everyone can be affected by influenza infection, certain groups are at higher risk than others. High risk groups are defined as pregnant persons, children, elderly, and people with chronic medical

conditions. Additionally, certain occupations can increase exposure to influenza, such as health care workers.[3]

B. Innate Immunity Against IAV

Initial immune response to viral pathogens is essential to limit viral replication and bridges host immune responses from initial infection to adaptive immunity. Although adaptive immunity is vital in viral clearance and provides protection from reinfection, the variable nature of IAV limits long term protection. Additionally, innate immunity plays a key role in providing signals, accelerating adaptive immune responses, and augmenting vaccine responses.

1. Natural Killer Cell Overview

NK cells are effector innate lymphocytes with cytotoxicity and cytokine-producing effector functions against infected or transformed cells. Mechanisms used to differentiate between 'self' cells and target cells include a vast array of activating and inhibitory receptors, and engagement of receptors determine NK cell activation and target cell killing.[11,12] Ligands detected by NK cell activating receptors can include stress-induced self-ligands, infectious non self-ligands, and Toll-like receptor (TLR) ligands.[13,14] NK cell inhibitory receptors recognize constitutively expressed self-molecules, such as major histocompatibility complex (MHC) class I and non-MHC self-molecules (Clr-b, LLT-1, and CD48), that protect cells from NK cell-mediated killing.[15] However, loss of MHC class I allows for NK cell activation and cytotoxicity.[16] Robust NK cell responses depend on interactions with other immune cells, including dendritic cells (DCs), macrophages, and T cells, and cytokine signals.[11,17] Conversely, negative

regulation of NK cell function can be mediated by transforming growth factor (TGF)-β secreted from regulatory T cells.[18]

NK cells reach optimal functional status through the process of maturation from immature to mature NK cells. Defects in NK cell maturation have been shown to diminish effector capabilities, including decreased target killing, and reduce the overall NK cell population in the periphery.[19,20] Therefore, it is important to identify NK cell maturation status and the factors involved in the maturation process, such as cytokines and transcription factors. NK cell maturation occurs in a stepwise cell differentiation process. In the bone marrow, NK cells develop from hematopoietic stem cells (HSCs) and differentiate into common lymphoid progenitors (CLPs). At this stage, CLPs can develop into either pro-B cells, pro-T cells, innate lymphoid cell precursors or preNK cell progenitors (preNKPs). PreNKPs are early NK-committed precursors that can differentiate to NK cell precursors (NKPs). The above developmental processes are similar in murine and human NK cells. However, differences in development occur following the NKP stage in NK cell maturation. In mice, final commitment of NK cells happens after the acquisition of NK1.1 and natural cytotoxicity receptor (NCR) and are classified as immature NK cells (iNK). Following this stage, NK cells continue to mature in the bone marrow and periphery where they acquire functional capacity and can be identified by changes in expression of the phenotype markers CD27 and CD11b. This late-stage maturation is defined by the upregulation of CD11b and downregulation of CD27. During this process, maturing NK cells acquire cytotoxic capacity and gradually lose proliferative potential towards reaching terminal differentiation. Late-stage

maturation progresses in the following order from immature to terminally mature NK cells: CD27-CD11b-, CD27+CD11b-, CD27+CD11b+, and CD27-CD11b+.[21] In humans, expression of CD122 on NKPs is necessary for NK cell lineage commitment. Additionally, final NK cell maturation in humans is defined by the appearance of CD56, which can be further divided into subsets $CD56^{bright}$ and $CD56^{dim}$. NK cell maturation in humans has been demonstrated by the transition from a more immature $CD56^{bright}$ NK cells to terminally mature subset $CD56^{dim}$ NK cells with the acquisition of CD16.[22,23] The majority of circulating NK cells (up to 90%) are cytotoxic $CD56^{dim}$CD16+ cells, whereas $CD56^{bright}$CD16- produce more proinflammatory cytokines, such as IFNγ.[24,25]

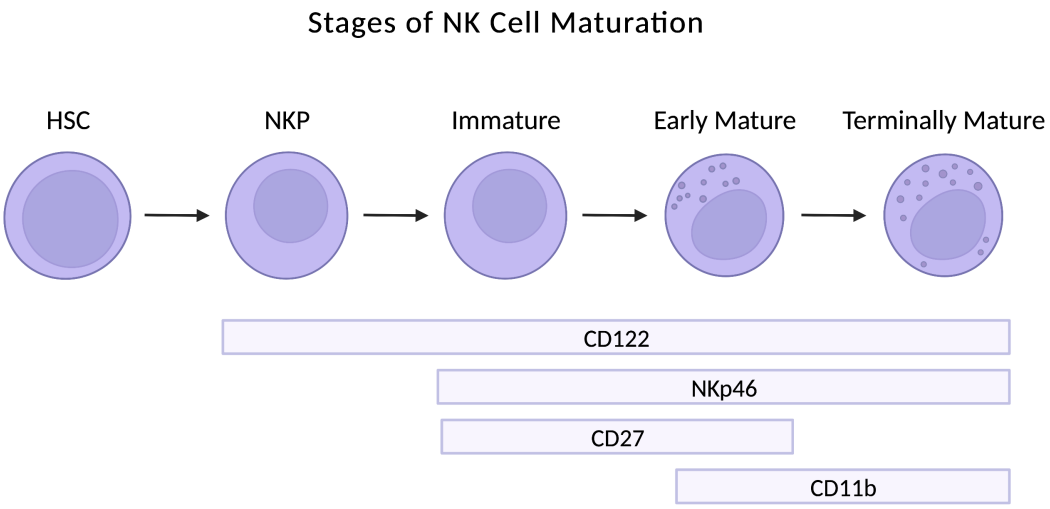

Figure 1: Stages of NK cell maturation. A schematic illustrating stages of NK cell maturation and associated surface markers. Created with Biorender.

Transcription factors required for NK cell maturation include ID2 (inhibitor of DNA binding 2), T-bet (T-box expressed in T cells), Eomes (eomesodermin), and ZEB2 (zinc finger E-box- binding protein 2). ID2 is expressed at lineage commitment of NK cells and throughout subsequent maturation stages. Experimental evidence has shown

ID2 deficiency to reduce NK cell numbers and decrease CD11b and granzyme expression.[26] Additionally, ID2 is needed for IL-15 receptor signaling, which is essential for mature NK cell survival and expansion.[27–29] T-bet and Eomes are highly homologous T-box transcription factors that have been shown to play a key role in maturation. In T-bet-/- mice, NK cell numbers were decreased in peripheral tissues but increased in the bone marrow, suggesting the presence of T-bet is essential for NK cell egression from bone marrow. Additionally, T-bet deficiency was shown to reduce expression of CD11b.[19] In addition, a study had found temporal depletion of T-bet and Eomes in mature NK cells downregulates NK1.1 and NKp46. Loss of Eomes alone resulted in the loss of maturity markers, which ultimately caused the cells to express a more immature phenotype identified as TRAIL+ and DX5-.[30] T-bet also synergizes with the transcription factor Zeb2, which regulates terminal maturation downstream of T-bet. Furthermore, induction of T-bet was found to upregulate expression of Zeb2. In addition, loss of ZEB2 in mice resulted in retention of NK cells in the bone marrow, whereas overexpression of ZEB2 increased NK cell numbers in peripheral tissues while decreasing the NK cell population in bone marrow, suggesting ZEB2 is essential for NK cell homeostasis.[20]

There is increasing evidence regarding the role of NK cells in negatively regulating cellular and humoral immune responses.[31–34] For instance, NK cells can lyse immature dendritic cells that lack inhibitory receptors, KIRs (human)/Ly49 (mouse), whereas mature DCs are protected from lysis due to the upregulation of MHC class I.[35–37] Microglia cells have also been shown to be susceptible to NK cell-mediated cytotoxicity via NKG2D and NKp46 engagement. However, upregulation of MHC class I

molecules inhibited NK cell lysis of microglia cells.[38] Studies have also shown NK cells to influence T helper (Th) cell polarization and B cell activation and isotype switching.[39–41] NK cells rapidly secrete tumor necrosis factor-α (TNFα) upon direct contact with mature DCs, which promotes polarization of activated T cells towards the Th1 phenotype. However, activated T cells upregulate ligands for the NK cell activating receptor, NKG2D, making them susceptible to NK cell-mediated lysis. Conversely, simultaneous expression of ligands specific to NK cell inhibitory receptor, NKG2A, can inhibit NK cell activity.[35,42] Although NK cells are potent producers of Th1-associated cytokines, such as IFNγ, studies have shown prolonged activation leads to augmented production of the regulatory cytokine, IL-10.[43] Furthermore, studies of autoimmunity have suggested a protective role for NK cells through the secretion of Th2-associated cytokines, IL-13 and IL-5.[44–47] NK cells in mucosal tissues have been shown to produce IL-22, a cytokine involved in tissue repair.[48,49] Together, NK cells can function to limit or exacerbate immune responses, which is dependent on cell interactions and the cytokine microenvironments.

2. Natural Killer Cell Responses to Primary Infection

NK cells are large granular lymphocytes that can rapidly produce cytokines and directly kill virally infected cells without prior exposure to IAV. They play an essential role in limiting viral replication and potentiating the adaptive immune response.[50] Unlike the adaptive immune response, NK cells elicit effector functions without priming or prior sensitization to pathogens. Instead, NK cells rapidly respond to transformed or infected cells via mechanisms involving a host of activating and inhibitory receptor

signals, cytokine stimulation, and antibody-dependent cellular cytotoxicity (ADCC).[11] The importance of NK cell antiviral activity was first noted when a patient lacking NK cells was diagnosed with reoccurring viral infections.[51] After this key finding, numerous studies were conducted to elucidate the role of NK cells in viral infection, further emphasizing the significance of NK cells in host immune responses.

Resident NK cells in the lung make up approximately 10% of the lymphocyte population under homeostatic conditions.[52] During initial hours to days of infection, NK cells are recruited to the lung by CXC-chemokine receptor 3 (CXCR3) and CC- chemokine receptor 5 (CCR5), which have been found to be the main contributor for increased NK cell accumulation in the lung while proliferation of resident NK cells is minimal[53] NK cell responses during viral infection are regulated by a wide array of germline-encoded activating and inhibitory receptors. Normal healthy cells express MHC class I molecules that are recognized by NK cell inhibitory receptors. However, in virally infected cells, MHC class I is downregulated or lost thereby withholding inhibitory signals that would prevent target cell lysis.[54] Virus-infected cells upregulate self-encoded molecules and stress ligands which bind to natural cytotoxicity receptors (NCRs), NKp30, NKp44, and NK46, and C-type lectin-like receptors, such as NKG2D.[13,55,56] Of relevance to influenza infection, NK cell activating receptor, NKp46, can interact with influenza HA protein via the α-2,6-linked terminal sialic acid and become activated.[57] NK cell receptor binding to corresponding ligands induces signaling cascades modulating NK cell function, which include release of cytotoxic granules containing granzymes and perforin, cytokine production, and Fas ligand (FasL)- mediated apoptosis.[24,58] Additionally, NK cells can

execute cell-to-cell cytolysis of virus-infected cells coated with immunoglobulin (Ig)G antibodies via ADCC upon ligation of the CD16 receptor.[59] Activation of NK cells through contact-dependent and -independent mechanisms has also been demonstrated with virus-infected macrophages.[60] Contact- independent mechanisms include cytokine signals secreted by antigen-presenting cells and virus-infected cells which activate NK cells during infection. Cytokines, including, but not limited to IL-2, IL-12, IL-15, IL-18, and type 1 interferons, are important in NK cell survival, proliferation, and activation.[61,62]

Influenza is a respiratory virus that mainly infects lung epithelial cells. Like many viruses, infection may lead to severe inflammation and tissue damage. The role of NK cells in response to influenza virus remains complex as studies have shown differing outcomes in relation to the magnitude of NK cell response. Defects in activation or depletion of NK cells have been shown to impair viral clearance and increase morbidity and mortality.[63–68] Conversely, studies have found NK cells to cause immunopathology in mice when infected with influenza virus. For example, mice depleted of NK cells infected with a high viral dose of influenza A/PR/8/34 virus had increased survival rates compared to mice depleted of NK cells receiving a low dose of virus.[69] Additionally, NK cells adoptively transferred from mice infected with a high dose of virus resulted in increased morbidity and mortality. Whereas NK1.1+ cell-depleted mice found to have minimal morbidity and no effect on viral clearance when infected with a lethal dose of virus.[70]

3. Nutritional Interventions and IAV Outcomes

Alterations in NK cell development, activation, and effector function have resulted in dampened responses to influenza infection. Our group has shown differences in NK cell activity in both aged and calorie-restricted mice following primary influenza infection. Compared to young mice, aged mice exhibited a reduction in the percentage of NK cells in the lungs and decreased NK cell production of IFNγ after infection.[63,71] Additionally, calorie restriction in mice increased mortality and decreased NK cell cytotoxicity following influenza infection.[63] Furthermore, in aged mice, calorie restriction further diminished the response to influenza resulting in decreased survival, increased virus titers, and reduced NK cell activity compared to aged mice fed ad libitum and calorie restricted, young mice.[72,73] Others have found diet-induced obesity in mice decreased NK cell cytotoxicity and the expression anti-viral cytokines.[74] In contrast, supplementation of active hexose correlated compound (AHCC) increased NK cell activity and NK cell number in the lungs following influenza infection.[75] Other interventions have been shown to alter NK cell activity and effector function in the absence of infection, including vitamin supplementation and alcohol consumption.[76,77] The following studies suggest that NK cells are highly influenced by a variety of environmental factors, and impairment of NK cells during influenza infection may lead to decreased viral clearance and survival.

II. Nrf2

A. Discovery

Nuclear factor erythroid 2-related factor 2 (Nrf2) was discovered in 1994 as a stress-induced transcription factor with a basic leucine zipper (bZIP) DNA binding domain that belongs to the cap'n'collar (CNC) family and is encoded by the gene NFE212 (nuclear factor (erythroid-derived 2)-like 2).[78] Nrf2 is highly expressed among various tissues and is structurally and functionally conserved across species.[78–80] Canonically, Nrf2 binds to the *cis*-acting DNA sequence termed antioxidant response element (ARE) as a heterodimer with small Maf proteins and regulates gene transcription of phase II detoxifying enzymes, such as glutathione S-transferases, NAD(P)H oxidoreductase (NQO-1), and γ-glutamyl cysteine synthase.[81–85] Nrf2 has also been found to associate with c-Jun to upregulate ARE-mediated expression of NQO-1 gene.[86] To determine the function of Nfe2l2 (Nrf2) gene, Nrf2-deficient mice were generated. Although Nrf2 is constitutively expressed, the Nfe2l2 gene function was found not necessary for mouse development, growth, and fertility.[87] The development of Nrf2-deficient mice were found to have increased susceptibility to oxidative and xenobiotic stressors, which resulted in various pathological characteristics.[88–91] In humans, Nrf2 polymorphisms have been identified in the regulatory region of the gene. However, Nrf2 polymorphisms was not associated with increased risk for systemic lupus erythematosus and chronic obstructive pulmonary disease.[92]

B. Structure and Regulation

Nrf2 is divided into six highly conserved domains, Neh1 to Neh6 (Nrf2-ECHhomology). A considerable number of studies identifying functional activity of Nrf2 have been implemented, including in-depth analysis of the Nrf2 domains. Nrf2 is regulated by its repressor protein, Kelch ECH-associated protein 1 (Keap1), which is localized in the cytoplasm. Keap1 mediates Nrf2 repression by binding specifically to Neh2 domain, which is one of the six highly conserved regions of Nrf2. Keap1 regulates the cellular level of Nrf2 protein through proteasomal-mediated degradation via the 26S proteasome.[93] This process involves the direct interaction between the intervening-region (IVR) domain of Keap1 with the N-terminal region of Cullin 3 (Cul3), a subunit of the E3 ligase complex .[93–98] In the presence of oxidative stress or electrophiles, Nrf2/Keap1 complex is disrupted allowing Nrf2 translocation to the nucleus, heterodimerization with small maf proteins, and transcriptionally activation of ARE-dependent genes.[99] In addition, several exogenous activators of Nrf2 interact with highly reactive cysteine residues of Keap1, which act as primary cellular sensors.[100] Modifications can alter the configuration of the Nrf2/Keap1 complex inhibiting Nrf2 release and degradation, which allows Nrf2 accumulation via *de novo* synthesis. Another mechanism involves Nr2 dissociation from Keap1 resulting in Nrf2 translocation to the nucleus.[101,102] Additionally, dissociation of the E3 ligase complex from the Nrf2/Keap1 complex has been proposed, which allows newly synthesized Nrf2 to evade degradation.[103] A noncanonical pathway for Nrf2 activation has also been demonstrated through the upregulation of p62 by autophagy deficiency in which p62

sequesters Keap1 into aggregates. Aggregation of Keap1 hinders Nrf2 ubiquitination by E3 ligase complex thereby inhibiting Nrf2 degradation.[104–106]

Post-translational modifications involving phosphorylation of Nrf2 are known to regulate Nrf2 activation and induction of ARE-mediated gene expression. Several cytosolic kinases have been shown to play a role in the activation of Nrf2 signaling. Phosphorylation at serine 40 residue (S40) of Nrf2 by protein kinase C (PKC) induced nuclear translocation and upregulation of ARE-mediated genes.[107,108] Additionally, casein kinase 2 (CK2) was found to phosphorylate transcription activation domains Neh4 and Neh5 of Nrf2, and in the presence of tBHQ, phosphorylation increased nuclear translocation. [109,110] Others have shown Nrf2 phosphorylation at the Neh2 domain by protein kinase RNA-like endoplasmic reticulum kinase (PERK) after exposure to endoplasmic reticulum (ER) stress.[111,112] Acetylation of Neh1 domain of Nrf2 has been found to enhance Nrf2 binding to ARE.[111]

C. Nrf2 Activators

Nrf2/Keap1 responds to a wide range of chemical compounds using various distinct mechanisms including Nrf2 stabilization, oxidative stress, and Keap1 inhibition. These chemical compounds include endogenous compounds, such as reactive oxygen species, reactive nitrogen species, lipid aldehydes, 15-deoxy-$D^{12,14}$- prostaglandin J_2, and exogenous compounds, including heavy metals and electrophilic compounds.[113,114] Several studies have established specific mechanisms for the activation of Nrf2, which include electrophilic reactions with cysteines of Keap1 and phosphorylation of Nrf2. For example, *tert*-butylhydroquinone (tBHQ), dimethylfumarate (DMF), sulforaphane (SFN),

ebselen, diethylmaleate (DEM), and 2-cyano-3,12 dioxooleana-1,9 diene-28-imidazolide (CDDO-Im) are Nrf2 inducers that react with cysteine residue, Cys151, of Keap1 to trigger the Keap1-Nrf2 response. Cys151 was found to be critical for tBHQ, DEM, and DEF.[115] However, Cys151-independent Nrf2 inducers have been recognized. Cadmium chloride ($CdCl_2$) has been shown to modulate zinc binding capacity of Keap1, and studies have found Cys151 is not required for activation in this case.[114,116] tBHQ and SFN have been shown to stabilize Nrf2 by inhibiting Keap1-mediated ubiquitination. Conversely, mechanism for activation of Nrf2 by arsenic trioxide (As(III)) is through augmented association of Keap1 and Cul3, which ultimately prevents continual degradation of Nrf2 by Keap1.[117] However, the studies on inorganic arsenic compounds overall demonstrate that multiple mechanisms are involved in Nrf2 activation. In contrast to arsenic trioxide, sodium arsenite increased expression of Nrf2 and accumulation of Nrf2 in the nucleus, which was enhanced by production of H_2O_2, while simultaneously increasing Keap1 expression.[118] Other studies found Cys151, Cys273, and Cys288 in Keap1 were required for Nrf2 activation by inorganic arsenic.[119] Interestingly, copper-mediated oxidation of *para*- or *ortho*- hydroquinone compounds was necessary for subsequent interaction with cysteine residues of Keap1.[120] In contrast, tBHQ-mediated Nrf2 activation has been shown to occur through the induction of mitochondrial oxidative stress, at least in some model systems.[121]

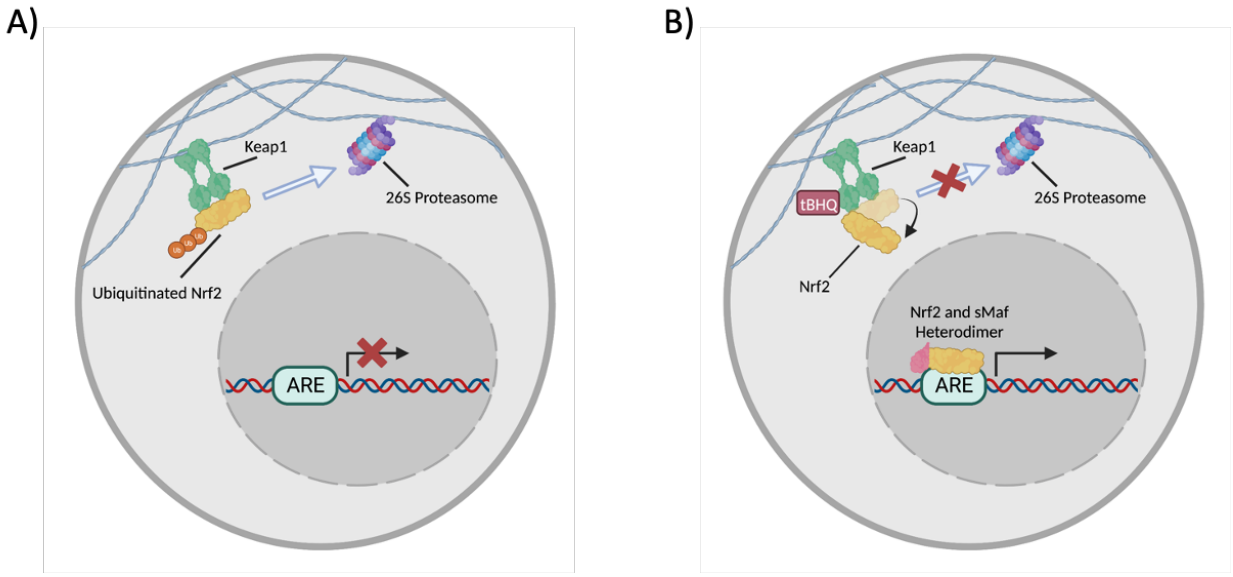

Figure 2: Nrf2 activation. A representation illustrating A) unactivated Nrf2 and B) activated Nrf2 by tBHQ interaction with Keap1. Created with Biorender.

D. Immune Cells

1. Macrophages

Nrf2 responses are often recognized as anti-inflammatory. Studies have shown Nrf2 directly limits induction of inflammatory cytokine genes, IL-6, and IL-1β, in M1 macrophages.[122] Additionally, activation of Nrf2 in macrophages skewed polarization towards an M2-phenotype while downregulating IL-1 and IL-6. Knocking down Keap1 in macrophages increased M2- macrophages polarization by cancer cells. Consistently, the Nrf2 activator, DEM, upregulated Nrf2 activation in macrophages therefore promoting cancer cell-induced M2 macrophages.[123] Several studies have also identified heme oxygenase-1 (HO-1), a cytoprotective gene induced by Nrf2 activation, to influence M2 macrophage polarization.[124–126] In patients with chronic obstructive pulmonary disease (COPD), alveolar macrophages treated with SFN enhanced bacterial phagocytosis by increasing expression of scavenger receptor, macrophage receptor with collagenous

structure (MACRO), with Nrf2 activation.[127] In Nrf2-deficient mice, hyperoxia induced oxidative stress, which was accompanied by an increase in inflammatory cytokine gene expression of IL-6 and IL-1β in alveolar macrophages. Additionally, macrophages from Nrf2-deficient mice were defective in bacterial killing compared to wild-type macrophages.[128] Others have found macrophage-mediated phagocytosis and clearance of *Listeria monocytogenes* to be inhibited by Nrf2-regulated expression of CD36 and HO-1 and T cell immunoglobulin mucin-3 (Tim-3).[129]

2. Dendritic Cells

Bone marrow derived DCs from Nrf2-deficient mice show increased oxidative stress. Additionally, Nrf2-deficient DCs have higher cell surface expression of MHC class II and costimulatory molecules suggesting a heightened proinflammatory state. [130] Similarly, bone marrow-derived Nrf2-deficient DCs were found to have impaired phagocytosis, increased DC maturation, and increased capacity to induce CD8 T cell proliferation and activation.[131] Another study assessing the adjuvant effects of ultrafine particles (UFP) on allergic sensitization to ovalbumin (OVA) in mice found Nrf2-deficient DCs to possess a cytokine profile favoring Th2 polarization with low expression of IL-12p70 and increased expression of IL-6 when compared to wild-type DCs.[132] In bone marrow derived DCs from Nrf2-deficient mice, increased amounts of IL-6 and TNF-α were secreted compared to wild-type counterparts.[133] In activated DCs, treatment with either Nrf2 activators, arsenic or tBHQ, significantly inhibited IL-12 expression at the transcriptional and protein level. However, arsenic-induced reduction of IL-12 was only exhibited in DCs from wild-type mice and not Nrf2-deficient mice.[134]

3. Natural Killer Cells and Natural Killer T Cells

The role of Nrf2 in NK cells and NKT cells remains largely unknown. In tumor-bearing mice, mice exposed to topical tBHQ delayed tumor growth in Rag2-/- mice but not Rag2-/- γc-/- suggesting NK cell involvement in the mediation of tumor rejection. Nrf2 was found to directly induce IL-17D expression, whereas knockdown of IL-17D or Nrf2 within tumor cells abolished any NK cell increase that was mediated by tBHQ.[135] In mice infected with influenza A/PR/8/34 H1N1 virus, IL-27-induced increase in NK cell function was mediated by activating receptor, NKG2D. Additionally, Mafs and Nrf2 transcriptional pathways were partly dependent in mediating IL-27 signaling.[136] In contrast, our lab has demonstrated impaired NK cell activation and effector function with tBHQ in murine splenocytes.[137] In Keap1-deficient mice, NKT cells had elevated apoptosis and reduced peripheral NKT cell numbers due to defects in development. However, Nrf2 deletion restored NKT cell defects observed in Keap1-deficient mice.[138]

4. B Cells

While many differences in immunoglobulin levels have been described between wild-type and Nrf2-null mice, there is limited evidence regarding a direct role for Nrf2 in modulating B cell response. Our group has demonstrated a Nrf2-dependent decrease in B cell activation and increase in IgM production with tBHQ in activated murine B cells.[139] Additionally, in mice infected with *Haemophilus influenzae*, Nrf2-deficient mice had increased lung infiltration and higher immunoglobulin-secreting cells compared to wild-type mice.[140]

5. T Cells

There has been increasing evidence regarding the immunomodulatory role of Nrf2 in T cells. In the development of pulmonary fibrosis, Nrf2 was found to regulate Th1 and Th2 immune responses. Specifically, Nrf2-deficient mice were more susceptible to bleomycin-induced pulmonary fibrosis and had increased levels of TNF-α and nuclear factor Kappa B (NFκB) activation compared to wild-type mice. Regarding Th1/Th2 balance, Nrf2-deficient mice exhibited, IFNγ-producing CD4+ and CD8+ T cells was significantly lower while IL-4-producing CD4+ and CD8+ T cells as enhanced when compared to wild-type mice. Furthermore, assessment of Th1 and Th2 master regulators, T-bet and GATA3 respectively, showed an increase in GATA3 and decrease in T-bet levels in Nrf2-deficient mice suggesting absence of Nrf2 skews Th1/Th2 balance toward Th2 phenotype in the pulmonary fibrosis disease model.[141] Conversely, our group has shown Nrf2 activation skews CD4+ T cells toward Th2 differentiation by demonstrating increased IFNγ-expression by anti-CD3/anti-CD28-activated CD4 T cells derived from Nrf2-deficient mice relative to wild-type. Likewise, tBHQ inhibited IFNγ induction by activated wild-type, but not Nrf2-null, CD4 T cells. Additionally, Nrf2-deficient mice had decreased secretion of the Th2 cytokines, IL-4, IL-5, and IL-13, by activated CD4+ T cells. The transcription factors, GATA-3 and T-bet, DNA-binding was also assessed, which showed enhanced binding of T-bet, which promotes Th1 polarization, and diminished binding of GATA-3, which promotes Th2 polarization, in CD4 T cells derived from Nrf2-null mice.[142] Consistent with these effects in primary murine CD4 T cells, in primary human CD4+ T cells, tBHQ treatment induced expression

of Nrf2-related genes, while decreasing production of IL-2 and IFNγ and early CD4+ T cell activation markers.[143]

In mice genetically modified to express high levels of Nrf2 in T cells, mice induced with ischemia reperfusion (IR) exhibited increased regulatory T cells and decreased levels of CD4+ T cell cytokines, TNFα, IFNγ, and IL-17. Additionally, high expression of Nrf2 protected mice from IR injury and increased survival.[144] The Nrf2- activator, CDDO-Im, was shown to inhibit IFNγ-secretion by activated T cells derived from wild-type mice in a Nrf2-dependent manner.[145] In Jurkat T cells, treatment of tBHQ demonstrated decreased IL-2 production and CD25 expression, which was associated with reduced NFκB transcriptional activity.[146] To assess the role of Nrf2 in Jurkat T cells, Nrf2 was deleted using CRISPR/Cas9 gene editing. These studies showed tBHQ-mediated suppression of IL-2 was largely independent of Nrf2 while inhibition of CD25 (IL-2 receptor) was partially Nrf2- dependent in Jurkat T cells. In contrast to tBHQ, IL-2 suppression by CDDO-Im in activated Jurkat T cells was Nrf2-dependent.[147]

In addition to effects on cytokines and cytokine receptors, another study showed that Keap1 deletion in T cells increased antioxidant potential which was dependent on Nrf2 expression. Additionally, primary human T cells with Keap1 deletion in vitro had higher frequency of CD4+ T cells and lower frequency of CD8+ T cells relative to controls. CD25 expression was also significantly increased while production of IL-17 was reduced with Keap1 deletion. More specifically, Keap1-edited regulatory T cells had significantly increased CD69 and IL-10 expression, suggesting Nrf2 involvement in promoting immunosuppressive T cells in this model.[148] Interestingly, and in contrast to

this study, in a *cre-flox* mouse model, mice heterozygous mice with Keap1 deletion in FoxP3+ cells had increased CD4+ T cell activation and effector memory T cells compared to wild-type mice. Homozygous mice for Keap1 deletion in FoxP3+ cells had increase IFNγ- producing T cells and liver and lung inflammation, suggesting constitutive Nrf2 activation results in diminished regulatory T cell-mediated immune tolerance.[149]

E. Nrf2 Autoimmunity

Characterization of Nrf2-deficient mice lead to the discovery of Nrf2 as a potential candidate in determining susceptibility to autoimmune disease. In aged, Nrf2-null female mice, characteristics similar to human lupus nephritis were observed, which lead to the utilization of Nrf2-deficient mice as a model for lupus-like autoimmune nephritis.[92] Similarly, Nrf2-deficient female mice on a different genetic background, developed an autoimmune disease resembling systemic lupus erythematosus (SLE) as demonstrated by splenomegaly, vasculitis, glomerulonephritis, hepatitis, and myocarditis. In addition, increased oxidative stress and apoptosis in the absence of Nrf2 expression strongly suggests Nrf2 involvement in the prevention of SLE.[150] In addition to multiorgan disease manifestation, CD4+ T cells from Nrf2-deficient mice exhibited hyperproliferation upon T cell receptor (TCR) activation resulting in a potential mechanism for Nrf2 mediation of oxidative tissue damage and T proliferation.[151] Likewise, the Nrf2 activator, SFN, ameliorated renal injury in pristine-induced lupus nephritis in wild-type, but not Nrf2-null mice, by negatively regulating NFκB and TGFβ1 signaling pathways. In the same study, elevated expression of Nrf2-target genes was observed in human lupus nephritis patients, but not in normal kidney tissue.[152] Nrf2 has

also been implicated in psoriasis, multiple sclerosis, rheumatoid arthritis, Type 1 diabetes, graft versus host disease, among other autoimmune diseases.[153–162]

F. Nrf2 in Infectious Disease

Infectious agents include bacteria, fungi, viruses, and parasites that can produce infection and/or infectious disease. Infection by these organisms often leads to ROS production which induces activation of the Nrf2/Keap1 signaling pathway. In addition, an alternate ROS-independent mechanism for Nrf2 activation involves Toll-like receptor (TLR) signaling. Emerging evidence suggest an immunomodulatory role for Nrf2 in host immune responses in the context of various pathogens, including viruses.

Respiratory syncytial virus (RSV) causes acute viral respiratory tract infections and hospitalizations, particularly in children and infants. In Nrf2-null mice, RSV infection resulted in increased viral titers, inflammation, and lung injury compared to wild-type mice.[163] Additionally, there was significant induction of Nrf2-target genes in RSV-infected wild-type mice, which was not observed in Nrf2-null mice.[164] Conversely, RSV was also shown to induce Nrf2 degradation via Keap1-independent mechanisms. However, treatment with Nrf2 inducers was shown to rescue Nrf2-dependent gene expression in the context of RSV infection in epithelial cells and in mice.[165,166]

Influenza virus is also a persistent threat to society as it causes millions of infections and hundreds of thousands of hospitalizations and deaths yearly.[7] It is well established that Nrf2 modulates virus-associated inflammation. However, the relationship between Nrf2 activation and virus susceptibility and clearance has yet to be explained. Notably, suppression of Nrf2 was shown to increase viral entry of influenza

in epithelial cells *in vitro*, while overexpression of Nrf2 blocked entry. Additionally, pre-treatment of epithelial cells with Nrf2 activators significantly decreased viral entry.[167] In humans, nasal lavage fluids and biopsies were collected from smokers after receiving live attenuated influenza virus vaccine to assess Nrf2-dependent enzyme expression in the context of influenza A infection. Short term ingestion of broccoli sprout homogenates, which are a source for the Nrf2 activator sulforaphane, increased NAD(P)H.[168] In Nrf2-deficient mice exposed to cigarette smoke and infected with influenza, macrophages showed increased NFκB activation and inflammatory gene expression in macrophages when compared to macrophages from wild-type mice. Moreover, cigarette smoke exposure in Nrf2-deficient mice caused greater lung pathology and mortality rates following influenza infection compared to virus-infected wild-type mice.[169] When the Nrf2 activator, carbocisteine, was given to cigarette smoke-exposed wild-type mice during infection, neutrophil counts in the BALF were significantly reduced compared to controls. Treatment with carbocisteine also decreased inflammation and mucus hypersecretion in infected wild-type mice exposed to cigarette smoke.[170]

A role for Nrf2 activation was also discovered in mosquito-borne viral diseases. With Zika virus infection, Nrf2 activation and upregulation of HO-1 by its natural physiological substrate hemin impaired viral replication.[171] In dengue virus-exposed macrophages, genome-wide transcriptome studies found Nrf2 to upregulate antioxidant pathways in response to ROS production. Silencing of Nrf2 with Nrf2-specific siRNA decreased antioxidant genes and increased ROS production in human monocyte-derived

dendritic cells infected with dengue virus.[172] Dengue virus was shown to induce ER stress-PERK activation of Nrf2 leading to the upregulation of C-type lectin domain family 5, member A and enhanced TNFα production.[173] In dengue-infected patients, Nox-2 (NADPH oxidase) and Nrf2 expression were down regulated upon hospital admittance in comparison to other febrile illness. Interestingly, between time of hospital admittance to release, Nox-2 expression increased in non-severe and severe dengue cases, whereas increased Nrf2 expression was only observed in non-severe dengue cases.[174]

Human immunodeficiency virus-1 infection (HIV) is enhanced with NFκB activation, whereas activated Nrf2 has been shown to inhibit NFκB activation. Consistent with this, treatment of HIV-infected monocyte-derived macrophages with the Nrf2-activator, dimethyl fumarate, diminished HIV replication, inhibited NFκB nuclear entry, and suppressed TNFα production while enhancing Nrf2 and NQO-1 expression.[175] Similarly, sulforaphane inhibited HIV infection in primary macrophages, and siRNA-mediated depletion of Nrf2 increased susceptibility to HIV.[176] HIV-1 Tat, a protein necessary for viral replication, was shown to enhance Nrf2 at both transcriptional and protein levels.[177]

In hepatitis B virus (HBV), ARE-gene expression was upregulated, and specific viral proteins for hepatitis C virus were shown to activate the Nrf2/ARE pathway by various mechanisms, including ROS-dependent and -independent phosphorylation.[178] Nrf2 knockdown in a hepatitis C virus (HCV)-persistently-infected cell line demonstrated a critical role for Nrf2 in lipid droplet formation.[179] Others have shown treatment with

the Nrf2 activator bardoxolone methyl (BARD) inhibited replication of HBV and HCV through both Nrf2-dependent and -independent mechanisms.[180]

Nrf2 activation in Herpes simplex virus (HSV)-1 was implicated in resistance to infection, and addition of the Nrf2 activators, sulforaphane or bardoxolone methyl, impaired virus replication.[181] In Nrf2-deficient mice, type I IFNs were significantly increased and HSV replication decreased compared to wild-type mice following HSV challenge. Furthermore, Keap1 deletion promoted HSV replication, suggesting Nrf2 dampens innate antiviral immune responses and increased susceptibility to HSV.[182]

Cytomegalovirus (CMV) infection in primary human fibroblasts was shown to activate Nrf2, and upregulation of HO-1 gene expression in infected cells was dependent on Nrf2.[183] Nrf2-deficient mice infected with mouse CMV had decreased survival, increased viral burden, and impaired immune cell recruitment to the peritoneum compared to wild-type mice. Also, injection of the Nrf2 activator, tBHQ, decreased weight loss and viral burden against mouse CMV infection in Nrf2-deficient mice. However, the protective effects of tBHQ were greater in CMV-infected wild-type mice as compared to Nrf2-null mice.[184]

G. Nrf2 Activator, *tert*-Butylhydroquinone

tBHQ is a widely used food additive used in a variety of foods to prevent oxidation and rancidification of fats. Foods containing tBHQ include cooking oils, cereals, and crackers, among others. Initial toxicology studies in rats, mice and dogs concluded that TBHQ was not carcinogenic.[185] The World Health Organization's and United Nations' Food and Agriculture Organization's Joint Expert Committee of Food

Additives (JECFA) has set the allowable daily intake (ADI) of tBHQ at 0.7 mg/kg/day, which was based on a no-observed-adverse-effect-level (NOAEL) of 72 mg/kg/bw per day in dogs with a 100-fold safety factor.[186] Dietary tBHQ exposure in humans is difficult to quantify. Therefore, experts have estimated model diets based on countries, such as United States, Australia, and China, and found high consumers of fats, oils, fat emulsions, and frozen fish products are capable of exceeding the recommended ADI for tBHQ.[186] In addition to its commercial use in the food industry, tBHQ is also used experimentally as a well-characterized Nrf2 activator.[187]

The metabolic fate of tBHQ has been evaluated in several species.[188–192] In rats, dogs, and humans, more than 90% of tBHQ is absorbed in the gastrointestinal tract and distributed via serum albumin proteins. During phase I metabolism in the liver, tBHQ is oxidized to *tert*-butyl benzoquinone (tBBQ) by either cytochrome P450 enzymes or autoxidation and/or Cu^{2+} ion-catalyzed redox cycling. In phase II, glutathione (GSH) conjugation of tBBQ is facilitated by metabolizing enzymes, such as NQO-1, glutathione S-transferase (GST), UDP-glucuronosyltransferase, and epoxide hydrolase.[193] Furthermore, studies in rats and dogs have shown tBHQ to be primarily excreted as *O*-sulphate and *O*-glucuronide conjugates in the urine within the first 24 hr.[188,194] However, an increase in tBHQ intake (from 100 mg/kg to 400 mg/kg) was found to decrease urinary elimination with the remaining amounts recovered in feces.[188] In addition, tBHQ accumulation in tissues was assessed in short-term and long-term exposure studies in rats, which found insignificant levels of tBHQ residues in liver, brain, and kidney. In humans, tBHQ in the serum and metabolites of tBHQ in the urine have

been examined following the ingestion of 100- 150 mg of tBHQ. Similar to rats and dogs, tBHQ was excreted as *O*-sulphate and *O*-glucuronide conjugates at a ratio of approximately 3:1 in the urine within the first 24 h. Unchanged tBHQ was not detected in the urine of humans, while minor amounts of tBHQ were excreted in feces (estimated between 2.4- 3.7 %). It should be noted, the urinary recoveries of tBHQ were shown to be dependent on the vehicle. For example, a vehicle containing 30% corn oil had a greater recovery of tBHQ in the urine compared to a vehicle containing 10% corn oil, suggesting tBHQ consumed in a high fat vehicle increases absorption compared to a low-fat vehicle.[188]

III. Rationale for the Presented Studies

Evidence regarding the immunomodulatory role of Nrf2 in disease and on T and B cell immune responses has increased, with previous studies from our lab demonstrating Nrf2 activation by tBHQ modulates both T and B cell activity.[139,142,143,145–147] In particular, tBHQ significantly reduced IFNγ secretion in activated primary human and primary murine T cells.[142,143] Additional studies from our lab have shown impaired NK cell response to primary influenza infection and increased influenza severity in calorie-restricted mice.[63,71] Given the importance of NK cell responses during primary influenza infection, the widespread use of tBHQ in food and the unknown effects of tBHQ on NK cells, the following studies described aim to determine whether tBHQ impairs NK cell activation in a Nrf2-dependent manner and whether dietary tBHQ at concentrations relevant to human exposure diminishes NK cell responses to influenza infection *in vivo.*

CHAPTER 2

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

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 μM or 5 μM 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 (IFNγ), 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.

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.[195] 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 (IFNγ).[196,197] 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 [198]. Maturation in mice can be classified using surface markers, CD27 and CD11b, in four stages: CD27-CD11b-, CD27+CD11b-, CD27+CD11b+, and CD27-CD11b+.[199] CD27 is the first marker expressed followed by the appearance of CD11b, which is an indicator of cell effector function.[199] 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.[200] NK cells that are terminally 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.[198,200,201]

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.[202–204] Other

studies involving vitamin supplementation, alcohol consumption, and excessive energy intake have also been shown to impact NK cell function and development [205–208].

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.[209] 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.[210] 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.[186] 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.[191]

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.[211] 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.[212]

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.[209] However, research from our laboratory has shown that Nrf2 has numerous immunomodulatory effects in T cells. We previously showed tBHQ significantly reduces IFNγ 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.[213] Additionally, in human Jurkat T cells, tBHQ inhibits interleukin (IL)-2 production and decreases expression of CD25.[214,215] 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 IFNγ, suggesting that overall T cell activation may be inhibited.[216] 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 IFNγ production and cytotoxic capabilities, and shared transcription factors and cell surface molecules.[217] 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.

Materials and Methods

Materials

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

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.

Cell Preparation

Spleens were aseptically removed for lymphocyte isolation by grinding and filtering tissues through a 40 μm strainer. Cells were subsequently washed, counted, and adjusted to a cell density of 5 x 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 μM ionomycin. Up to 5μM 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 200μM, suggesting the concentrations used in this study are well within the range of potential human exposure.[191] Immediately following the addition of PMA and ionomycin, 15 μM monensin solution was used to block protein secretion for intracellular labeling (eBioscience, San Diego, CA). Splenocytes were then incubated for 24 h.

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.[218] Combinations of the following fluorochrome-conjugated antibodies (Biolegend, eBioscience or BD Biosciences) were used: CD3ε (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]), IFNγ (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).

Statistical Analysis

Treatment groups are presented as mean ± 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.

Results

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; however, the effects of Nrf2 activation by tBHQ in NK cells has yet to be characterized.[213–216] 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 μM tBHQ displayed a modest, though statistically significant, reduction in the percentage of NK cells within the splenocyte population (**Figure 3**, VEH, 2.19 ± 0.08%; 5 μM tBHQ, 1.79 ± 0.1%).

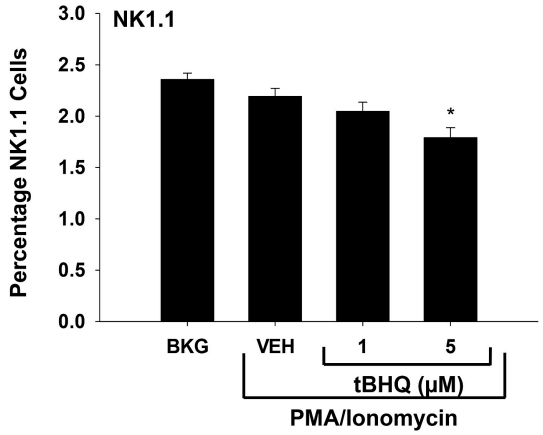

Figure 3: tBHQ treatment modestly decreases the percentage of NK cells in spleen. Murine splenocytes were isolated and treated with tBHQ (1 μM or 5 μM), VEH (0.01% ethanol), or complete DMEM (BKG) for 30 min, then either unactivated (BKG) or activated by PMA and ionomycin (VEH, 1 μM tBHQ, and 5 μM tBHQ) for 24 h. Cells were then labeled with fluorescently conjugated antibodies against CD3ε 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.

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.[216] This prompted us to quantify CD25 and CD69 expression in activated NK cells. The percentage of NK cells expressing CD25 was significantly reduced in groups treated with 1 μM and 5 μM tBHQ compared to the VEH group (**Figure 4A**, VEH, $1.4 \pm 0.15\%$; 1 μM tBHQ, $0.85 \pm 0.07\%$; 5 μM 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 (**Figure 4B**, VEH, $63.57 \pm 1.39\%$; 1 μM tBHQ, $53.39 \pm 0.88\%$; 5 μM 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) (**Figure 4**, CD25 BKG, 1.1

± 0.12%; CD69 BKG, 52.46 ± 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.

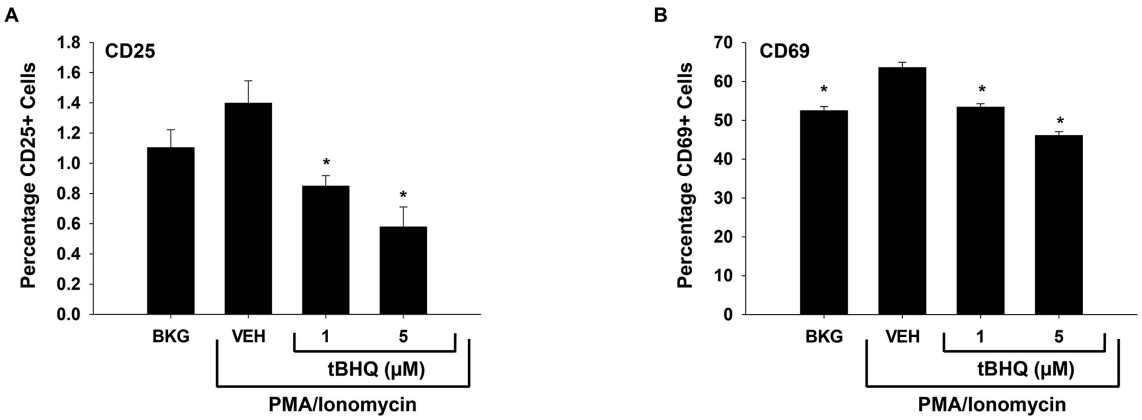

Figure 4: tBHQ treatment inhibits expression of cell surface molecules CD25 and CD69 in NK cells. Murine splenocytes were isolated and treated with tBHQ (1 μM or 5 μM), VEH (0.01% ethanol), or complete DMEM (BKG) for 30 min, then either unactivated (BKG) or activated by PMA and ionomycin (VEH, 1 μM tBHQ, and 5 μM tBHQ) for 24 h. Cells were labeled with fluorescently conjugated antibodies against CD3ε, 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.

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.[217] 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 (**Figure 5A**, VEH, 27.87 ± 2.73%; BKG, 37.91 ± 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 μM (5.23 ± 0.41%) and 5 μM tBHQ (3.21 ± 0.32%) in relation to the VEH group (7.19 ± 0.61%)

(**Figure 5B**). 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 μM tBHQ ($2.09 \pm 0.37\%$) exhibited the greatest difference compared to the VEH group ($4.6 \pm 0.71\%$) in which 5 μM tBHQ decreased the CD27+CD11b+ subset to a percentage lower than that of the (unactivated) BKG group (**Figure 5C**). Interestingly, the most mature and terminally differentiated NK cell phenotype, CD27-CD11b+, was significantly increased with treatment of 5 μM tBHQ ($67.83 \pm 2.67\%$) compared to the VEH group ($60.33 \pm 2.34\%$) (**Figure 5D**). Collectively, these results indicate that the food preservative, tBHQ, has significant effects on NK cell maturation.

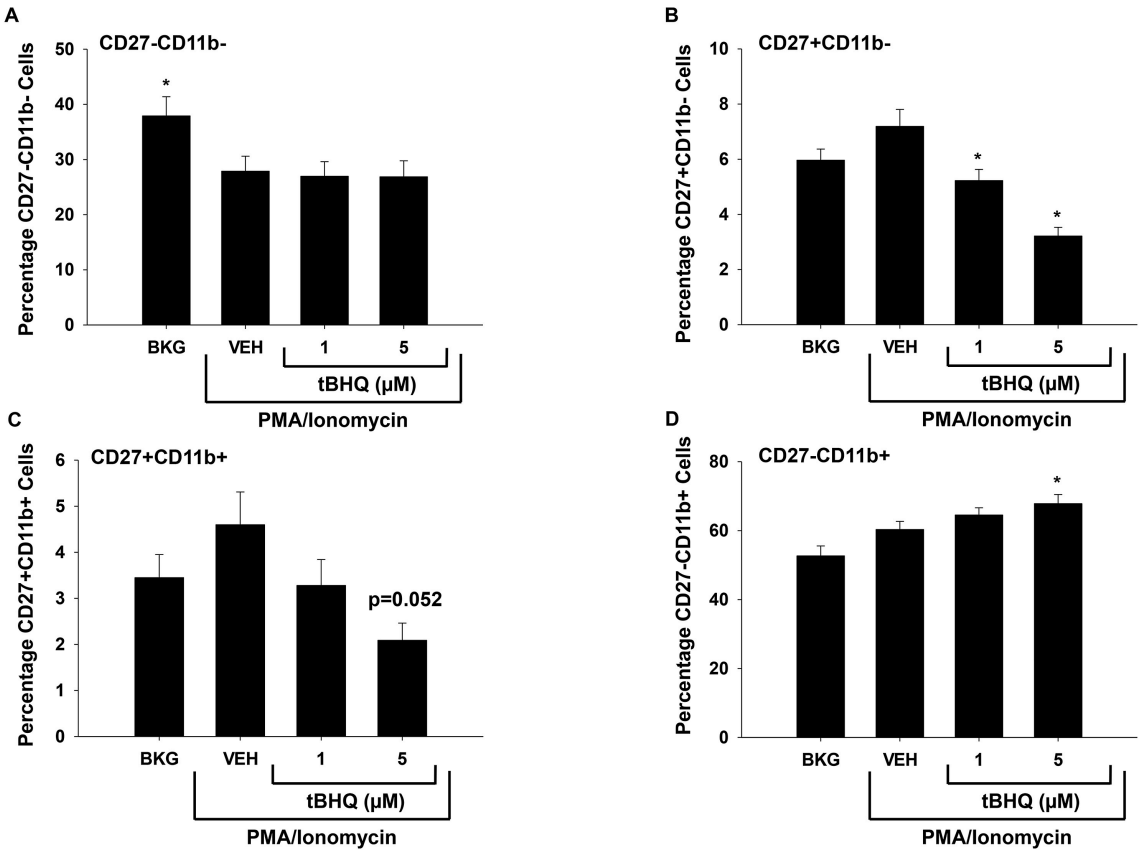

Figure 5: tBHQ treatment alters NK cell maturation. Murine splenocytes were isolated and treated with tBHQ (1 μM or 5 μM), VEH (0.01% ethanol), or complete DMEM (BKG) for 30 min, then either unactivated (BKG) or activated by PMA and ionomycin (VEH, 1 μM tBHQ, and 5 μM tBHQ) for 24 h. Cells were labeled with fluorescently conjugated antibodies against CD3ε, 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.

Treatment with the Nrf2 Activator, tBHQ, Inhibits IFNγ Production in NK Cells

As NK cells mature and express CD11b, they acquire effector function and have increased capacity for producing cytokines such as IFNγ. A previous study from our laboratory showed tBHQ decreased production of IFNγ in CD4+ T cells.[216] Because tBHQ altered NK cell maturation, we investigated the effects of tBHQ on IFNγ production by NK cells. The

percentage of NK cells producing IFNγ was significantly reduced in groups treated with 1 μM and 5 μM tBHQ in a concentration-dependent manner (**Figure 6A**, VEH, 53.38 ± 4.64%; 1 μM tBHQ, 29.35 ± 2.28%; 5 μM tBHQ, 12.62 ± 1.25%). In addition to a decrease in the percentage of IFNγ-producing NK cells, tBHQ also caused a reduction in mean fluorescence intensity (MFI) (**Figure 6B**, VEH, 14567.2 ± 1876.06; 5 μM tBHQ, 2261.2 ± 128.83), suggesting a decrease in IFNγ expression in individual NK cells.

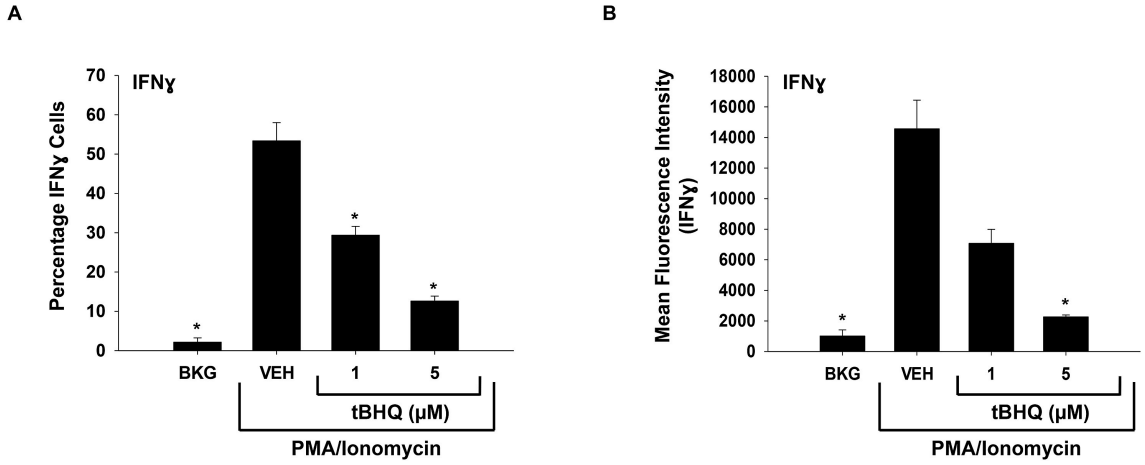

Figure 6: tBHQ treatment inhibits production of the cytokine IFNγ in NK cells. Murine splenocytes were isolated and treated with tBHQ (1 μM or 5 μM), VEH (0.01% ethanol), or complete DMEM (BKG) for 30 min, then either unactivated (BKG) or activated by PMA and ionomycin (VEH, 1 μM tBHQ, and 5 μM tBHQ) for 24 h. Cells were labeled with fluorescently conjugated antibodies against CD3ε and NK1.1 followed by intracellular labeling of IFNγ 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 IFNγ by (A) percentage and (B) mean fluorescence intensity (MFI). n = 5
*Indicates $p < 0.05$ compared to VEH group.

The Nrf2 Activator, tBHQ, Decreases Production of Perforin and Granzyme B in NK Cells

Due to a decreased production of IFNγ 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 perforin was reduced in a concentration-dependent manner with significant reduction

observed by 5 μM tBHQ (**Figure 7A**, VEH, 3.98 ± 0.29%; 5 μM tBHQ, 1.8 ± 0.09%). Similarly, granzyme B production by activated NK cells was significantly decreased in both groups treated with tBHQ compared to the VEH group (**Figure 7B**, VEH, 31.74 ± 2.21%; 1 μM tBHQ; 20.37 ± 2.46%; 5 μM tBHQ, 14.9 ± 1.67%). Collectively, these results demonstrate inhibitory effects of the food preservative, tBHQ, on vital NK cell cytolytic events.

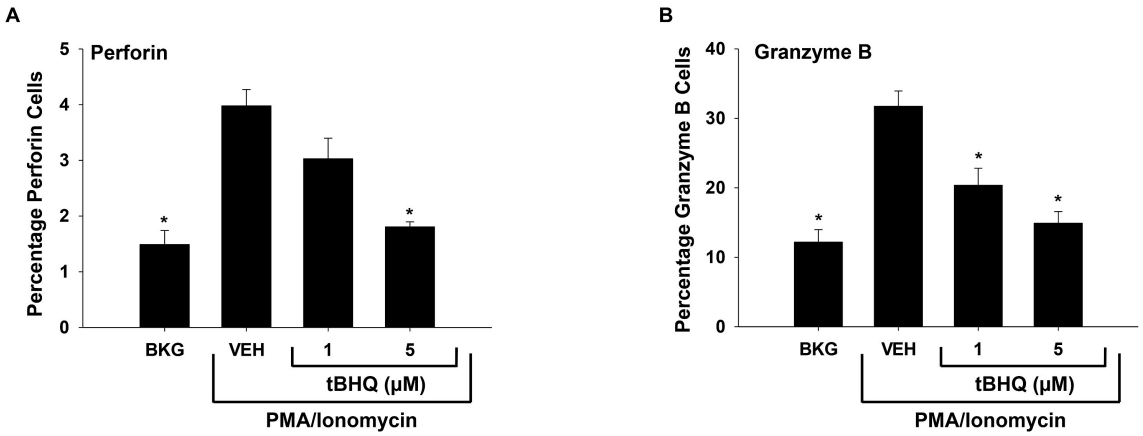

Figure 7: tBHQ treatment inhibits the production of perforin and granzyme B in NK cells. Murine splenocytes were isolated and treated with tBHQ (1 μM or 5 μM), VEH (0.01% ethanol), or complete DMEM (BKG) for 30 min, then either unactivated (BKG) or activated by PMA and ionomycin (VEH, 1 μM tBHQ, and 5 μM tBHQ) for 24 h. Cells were labeled with fluorescently conjugated antibodies against CD3ε 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.

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.[216] 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 IFNγ 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.[199] NK cell function has correlated with developmental stages based on expression of CD27 and CD11b.[200] In the spleen, NK cells represent a small fraction (< 2.5%) of the lymphocyte population with the majority of NK cells expressing CD27-CD11b+.[200,219] 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 (**Figure 1** and **Figure 5D**). Interestingly, the percentage of CD27-CD11b+ NK cells was significantly greater when treated with 5 μM tBHQ, while CD27+CD11b+ NK cells were reduced in both tBHQ-treated groups (**Figure 5C** and **5D**). Prior studies have also demonstrated decreased cytolytic capacity and cytokine production in mature CD27-CD11b+ NK cells compared to CD27+CD11b+ NK cells.[198,200] The present study found a decreased percentage of CD27+CD11b+ NK cells to be reflective of the diminished production of IFNγ, perforin, and granzyme B with tBHQ treatments (**Figure 5C**, **Figure 6** and **Figure 7**). The increase in CD27-CD11b+ NK cells treated with 5 μM tBHQ (**Figure 5D**) may provide relatively small amounts of IFNγ, 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 μM and 5 μM tBHQ-treated groups (**Figure 5B**). 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.[213–216] Furthermore, dendritic cells were found to be affected by tBHQ through Nrf2 activation. Treatment of tBHQ in lipopolysaccharide-activated human dendritic cells potently inhibited the secretion of the cytokine IL-12, which is involved in the activation NK cells and the differentiation of CD4+ T cells.[220,221] Studies have also shown macrophages to be affected by Nrf2 activation. In particular, macrophages producing proinflammatory cytokines, tumor necrosis factor alpha (TNFα) and IL-6, were inhibited with tBHQ treatment in a concentration-dependent manner after lipopolysaccharide stimulation.[222,223] Additionally, activation of Nrf2 by tBHQ was found to regulate macrophage polarization by promoting the anti-inflammatory M2 macrophages.[223] 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 IFNγ, 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 IFNγ, 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.

CHAPTER 3

Activation of Nrf2 by tBHQ promotes maturation, while concurrently inhibiting activation and expression of effector molecules by activated NK cells

Abstract

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) upregulates antioxidant, detoxification, and cell stress genes when activated by oxidative stress or exogenous compounds. *tert*-butylhydroquinone (tBHQ) is a potent activator of Nrf2 and a food preservative. Previously, we have found tBHQ negatively impacted NK cell activation and effector function and altered NK cell maturation. In the current study, we examined the role of Nrf2 in these effects. Splenocytes were isolated from wild-type (WT) C57Bl/6J mice or Nrf2-deficient mice and treated with varying concentrations of tBHQ (0.1 μM- 5 μM tBHQ). Following treatment, NK cells were activated for 24 hours with either phorbol 12-myristate 13-acetate (PMA) and ionomycin or a specific NK cell activator, IL-12/IL-18 cytokines prior to flow cytometry analysis. There were pronounced genotype and tBHQ effects on NK cell maturation. Specifically, there was a significant increase in the percentage of immature NK cells (CD27-CD11b-) in activated splenocytes derived from Nrf2-knockout mice. tBHQ increased the percentage of terminally differentiated NK cells derived from wild-type, but not Nrf2-null, mice. In addition, tBHQ caused a Nrf2-dependent decrease in expression of the activation markers, CD25 and CD69, in PMA/ionomycin-activated NK cells. Likewise, tBHQ decreased expression of Fas ligand in IL-12/IL-18-activated NK cells in a Nrf2-dependent manner. In contrast, inhibition of IFNγ induction by tBHQ appeared to be only partially Nrf2-dependent. Taken together, the data suggest that activation of Nrf2 by tBHQ promotes NK cell terminal differentiation, while concurrently inhibiting activation and effector function, which could negatively impact NK cell-mediated host defense.

Introduction

The nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that regulates the expression of cytoprotective genes in response to endogenous and exogenous cellular stresses. Under quiescent conditions, Nrf2 is retained in the cytoplasm by its repressor protein, Kelch ECH associating protein 1 (Keap1), and is rapidly degraded through the ubiquitin-proteasome pathway.[224] However, in the presence of oxidative and electrophilic stimuli, critical cysteine residues in Keap1 that serve as stress sensors undergo modification resulting in conformational changes, thereby disrupting the ubiquitination and subsequent proteasomal degradation of Nrf2. Nrf2 accumulates in the cytoplasm and translocates to the nucleus where it binds to antioxidant response elements, leading to the upregulation of cytoprotective genes that enhance cell survival.[81,225–227]

Tert-butylhydroquinone (tBHQ) is a well-characterized inducer of Nrf2 and is commonly used as a food preservative to prevent rancidification and oxidation of lipids.[185,187] Previous studies in humans have shown serum levels of tBHQ reaching the high micromolar range following ingestion of 150 mg tBHQ.[191] Additionally, assessments of tBHQ intake based on model diets suggest high consumers of fats, oils, and frozen fish products are likely to exceed the recommended acceptable daily intake (ADI) of tBHQ, which is set at 0.7 mg/kg body weight.[186] The current study used concentrations of tBHQ in the low micromolar range of 0.1- 5 μM tBHQ, which our lab has previously shown to activate Nrf2 without causing cytotoxicity in T cells.[142]

Increasing evidence shows involvement of Nrf2 in mediating anti-inflammatory responses in various disease models, including allergy, autoimmune diseases, and inflammatory

diseases.[150,151,228–234] Studies have also identified Nrf2-mediated effects in several immune cell types when treated with tBHQ. Our lab demonstrated Nrf2 activation by tBHQ promotes Th2 differentiation, whereas absence of Nrf2 skews CD4+ T cells toward Th1 differentiation.[142] Additional studies found the Nrf2 activator, tBHQ, inhibited early events following T cell activation in human Jurkat T cells, primary human CD4+ T cells, and primary murine splenocytes.[142,143,145–147] In B cells, we showed a Nrf2-dependent increase in IgM production by tBHQ. Others showed tBHQ potently inhibited IL-12 expression in LPS-stimulated dendritic cells.[235] In macrophages, tBHQ attenuated LPS-induced production of pro-inflammatory markers and promoted M2 polarization, which was further confirmed by the finding that Nrf2 interferes with the transcriptional upregulation of proinflammatory cytokines induced by LPS.[236–238]

Importantly, the role of Nrf2 in NK cells remains largely unknown. Previously, our lab investigated the effects of tBHQ on activated NK cells and found tBHQ induced maturation and inhibited activation and effector function.[137] Since tBHQ is a potent activator of Nrf2, the current study examines whether these effects are mediated by Nrf2.

Materials and Methods

Materials

tBHQ and all other materials were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated.

Animals and Housing

Nrf2-null mice on a mixed C57BL/6 and background were generated and received from Dr. Jefferson Chan at the University of California San Francisco, San Francisco, CA.[239] Mice were

backcrossed on the C57BL/6 background for eight generations and were 99% congenic (analysis performed by Jackson Laboratory, Bay Harbor, ME). Wild-type (Jackson Laboratories, Bar Harbor, ME) and Nrf2-null female mice, ages 9-22 weeks old, were used for this study. Age-matched mice were given food and water *ad libitum.* Animal studies were performed following the Guide for Care and Use of Animals adopted by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University.

Cell Preparation

Single-cell suspensions from spleens were obtained via grinding and filtering of tissue through a 40 μm strainer. Subsequently, cells were washed and depleted of red blood cells by using ACK lysis buffer following manufacturer's protocol. Cells adjusted to a concentration of $5x10^6$ cells/mL and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 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 100 U/mL 2-mercaptoethanol (Thermo Fisher Scientific, Waltham, MA). Cells were left alone (background, BKG) or treated with tBHQ at various concentrations ranging from 0.1- 5 μM tBHQ or 0.005% ethanol (vehicle, VEH). Concentrations of tBHQ are consistent with previous studies conducted in our lab and are relevant to human exposure, as research has shown a bolus dose of tBHQ taken orally equates to an estimated blood concentration of 200 μM.[191] After 30 min, cells were activated with either 10 ng/mL of IL-12/IL-18 or 40 nM phorbol 12-myristate 13-acetate (PMA)/0.5 μM Ionomycin for 24 h. Cells in the BKG treatment group did not receive either activator. Monensin (1X) was added to cells 5 h

prior to cell collection (19 h post treatment) to inhibit cytokine secretion for intracellular labeling (eBioscience, San Diego, CA).

Flow Cytometry

After 24 h of incubation, cells were transferred to a 96-well V-bottom plate and washed with dPBS prior to staining with Zombie Aqua Fixable Viability Dye following manufacturer's protocol (Biolegend, San Diego, CA). Cells were washed with FACS buffer (1% FBS in dPBS) and treated with Fc-Block (CD16/32 [2.4G2], BD Bioscience, San Jose, CA) prior to surface marker staining with fluorescently conjugated antibodies (**Table 1**). After 30 min, samples were permeabilized using FoxP3/Transcription Buffer Staining Kit (Invitrogen, Waltham, MA) following the manufacturer's protocol and stained with the intracellular antibodies listed in **Table 1** for 30 minutes. Cells were subsequently washed and immediately analyzed by flow cytometry using an Attune NxT Flow Cytometer (Thermo Fisher, Waltham, MA). Following the manufacturer's protocol, compensation was performed using Ultra-Comp eBeads (Invitrogen, Waltham, MA).

Table 1: List of Antibodies

Antibody	Fluorochrome	Clone	Supplier
Live/Dead	Zombie Aqua Dye		Biolegend
CD3	Alexa Fluor 488	17A2	Biolegend
NK1.1	Brilliant Violet 711	PK136	Biolegend
CD27	PerCP-eFluor 710	LG.7F9	eBioscience
CD11b	Alexa Fluor 647	M1/70	Biolegend
CD25	Pacific Blue	PC61	Invitrogen

Table 1 (cont'd): List of Antibodies

CD69	Alexa Fluor 700	H1.2F3	Biolegend
CD107a	PE-Cy7	1D4B	BD Biosciences
CD178 (FasL)	PE	MFL3	Biolegend
Granzyme B	Pacific Blue	QA16A02	Biolegend
IFN-γ	APC	XMG1.2	Biolegend
Perforin	PE	S16009B	Biolegend

IFNγ ELISA

Supernatants from samples activated with IL-12/IL-18 were collected 24 h after treatment for the analysis of IFNγ protein by ELISA. Mouse IFNγ ELISA MAX Standard Kit (Biolegend, San Diego, CA) was used to detect IFNγ and quantified at an absorbance of 450 nm by Tecan Infinite M1000 Pro Microplate Reader (Tecan; San Jose, CA).

Statistical Analysis

The mean ± standard error (SE) was determined for each treatment group. Normally distributed data were analyzed by two-way ANOVA followed by a Dunnett's post hoc test to compare treatment groups to vehicle control. Analyses were performed using GraphPad PRISM software version 8.4.2 (GraphPad Software, La Jolla, CA).

Results

Nrf2 Modestly Increases NK Cells with IL-12/IL-18 Activation.

Our previous studies showed a potent effect of the food additive tBHQ on PMA/ionomycin-activated NK cells in vitro.[137] To determine the role of Nrf2 in these effects, we

used splenocytes from wild-type and Nrf2-null mice. We found no difference in the percent of NK cells between Nrf2-null and wild-type mice, which is consistent with a previous report that found no significant difference in the numbers of splenic NK cells between wild-type and Nrf2-null mice.[240] We next wanted to identify the effects of tBHQ on NK cells following activation with IL-12/IL-18 or PMA/Ionomycin (where PMA/ionomycin was the NK cell activator we used in our previous study). We did not observe a difference between treatment groups in the percentage of NK cells regardless of activation, however we observed a trend toward a modest increase in percentage of NK cells in the 5 μM tBHQ + IL-12/IL-18 group that was not statistically significant (**Figure 8A**). Overall, the results suggest that neither tBHQ nor Nrf2 affects the percentage of splenic NK cells following activation with either PMA/Ionomycin or IL-12/IL-18.

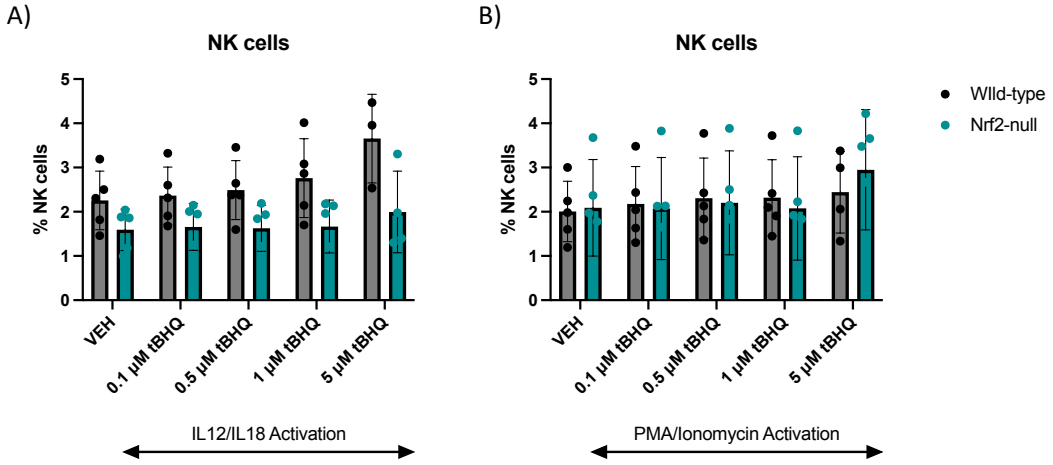

Figure 8: Nrf2 modestly increases NK cells with IL-12/IL-18 activation. Splenocytes were isolated from wild-type or Nrf2-null C57Bl/6 mice. Cells were either left untreated (BKG) or treated with tBHQ (0.1- 5 μM) or 0.005% ethanol (VEH) for 30 min prior to addition of activators (A) IL-12/IL-18 or (B) PMA/Ionomycin. After 24 h, cells were stained with viability dye and fluorescently conjugated antibodies against CD3ε and NK1.1 for analysis by flow cytometry. NK cells were identified as viable CD3-NK1.1+ cells.
* P ≤ 0.05; ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001 compared to vehicle of respective genotype

Figure 8 (cont'd):
# P ≤ 0.05, ## P ≤ 0.01, ### P ≤ 0.001, #### P ≤ 0.0001 compared to wild-type vs Nrf2-null of respective treatment
n= 5

Nrf2 Increases Maturation of NK Cells.

NK cell maturation is defined by differential surface expression of CD27 and CD11b with four distinct stages of maturation which progress in the following order: CD27-CD11b-, CD27+CD11b-, CD27+CD11b+, and CD27-CD11b+. During the maturation process, NK cells gradually acquire cytotoxic capabilities.[21] Unexpectedly, we found significant genotype differences in NK cell maturation in the untreated groups with an increase in percentage of immature NK cells in the spleens from Nrf2-null mice, whereas wild-type splenic NK cells displayed a more mature phenotype (**Figure 9A-D**). tBHQ treatment did not affect maturation with IL-12/IL-18 activation. However, in PMA/Ionomycin activated cells, 5 μM tBHQ increased the percentage of terminally mature NK cells from wild-type, but not Nrf2-null, mice (Figure 2H). Overall, these data indicate that Nrf2 plays a role in the progression of maturation of NK cells.

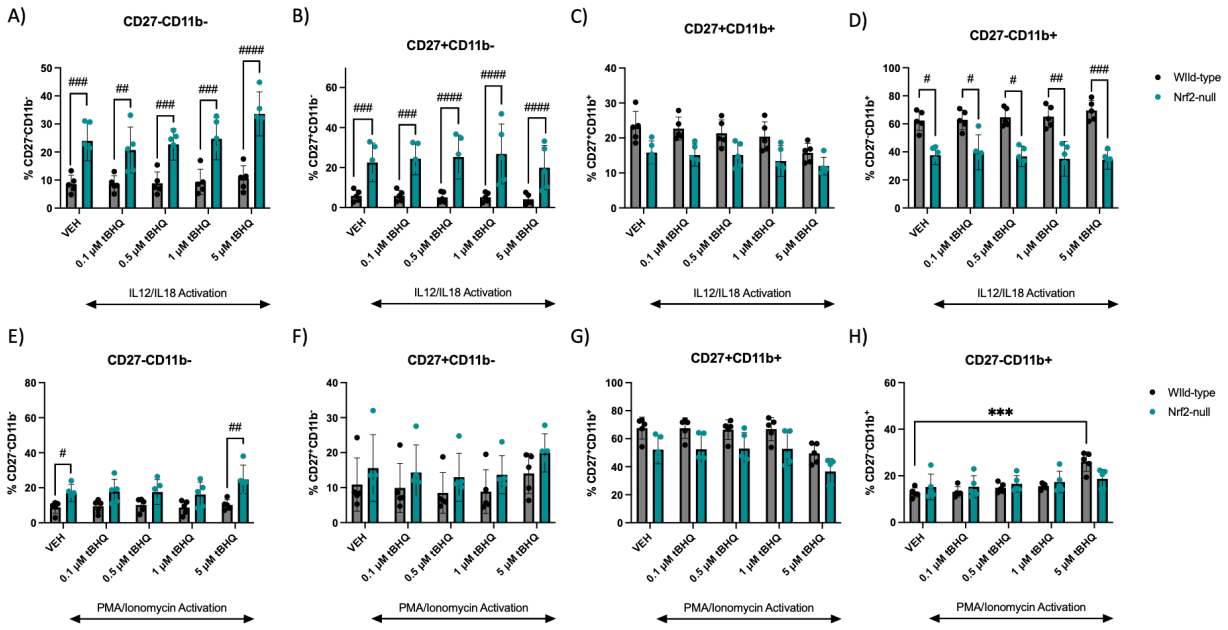

Figure 9: Nrf2 increases maturation of NK cells. Splenocytes were isolated from wild-type or Nrf2-null C57Bl/6 mice. Cells were either left untreated (BKG) or treated with tBHQ (0.1- 5 μM) or 0.005% ethanol (VEH) for 30 min prior to addition of activators (A-D) IL-12/IL-18 or (E-H) PMA/Ionomycin. After 24 h, cells were stained with viability dye and fluorescently conjugated antibodies against CD3ε, NK1.1, CD27, and CD11b for analysis by flow cytometry. NK cells were identified as viable CD3-NK1.1+ cells prior to FACS analysis of maturation markers, CD27 and CD11b. Maturation occurs in the following order from Immature to mature NK cells: (A, E) CD27-CD11b-, (B, F) CD27+CD11b-, (C, G) CD27+CD11b+, (D, H) CD27-CD11b+.
* P ≤ 0.05; ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001 compared to vehicle of respective genotype
# P ≤ 0.05, ## P ≤ 0.01, ### P ≤ 0.001, #### P ≤ 0.0001 compared to wild-type vs Nrf2-null of respective treatment
n= 5

tBHQ Treatment Decreases Activation of NK Cells Independently of Nrf2.

To evaluate activation by IL-12/IL-18 and PMA/ionomycin, surface expression of CD69 and CD25 were assessed on NK cells. Interestingly, early activation markers significantly decreased with 5 μM tBHQ in IL-12/IL-18- activated NK cells. This decrease was not dependent on Nrf2, as a reduction in CD69+CD25+ expression occurred in both wildtype- and Nrf2-null NK cells (**Figure 10A**). However, in PMA/ionomycin-activated NK cells, CD69+CD25+ expression

was inhibited with 5 μM tBHQ solely in wildtype cells (**Figure 10B**). Overall, the data suggest that tBHQ inhibits early activation markers in NK cells regardless of the mode of activation. The inhibition is largely Nrf2-dependent when the cells are activated with PMA/ionomycin, but interestingly, independent of Nrf2 when activated with IL-12/IL-18.

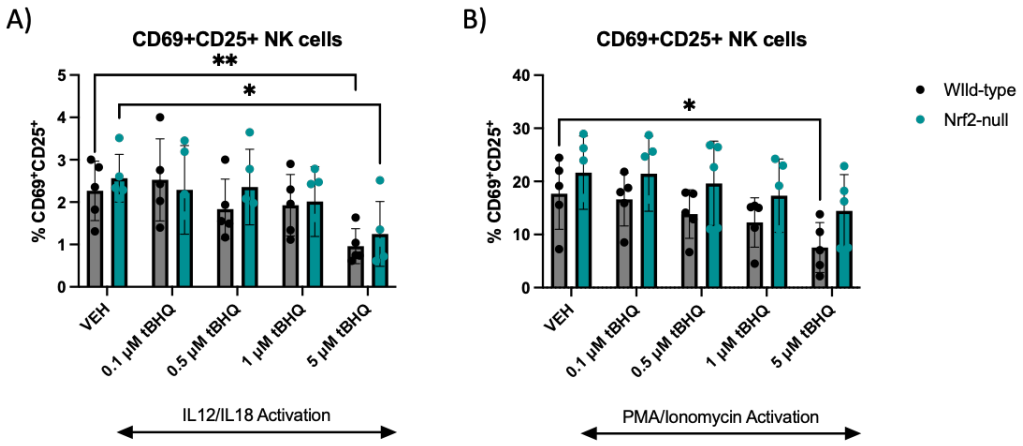

Figure 10: tBHQ decreases activation of NK cells independently of Nrf2. Splenocytes were isolated from wild-type or Nrf2-null C57Bl/6 mice. Cells were either left untreated (BKG) or treated with tBHQ (0.1- 5 μM) or 0.005% ethanol (VEH) for 30 min prior to addition of activators (A) IL-12/IL-18 or (B) PMA/Ionomycin. After 24 h, cells were stained with viability dye and fluorescently conjugated antibodies against CD3ε, NK1.1, CD69, and CD25 for analysis by flow cytometry. NK cells were identified as viable CD3-NK1.1+ cells prior to FACS analysis of activation markers, CD69 and CD25.
* P ≤ 0.05; ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001 compared to vehicle of respective genotype
# P ≤ 0.05, ## P ≤ 0.01, ### P ≤ 0.001, #### P ≤ 0.0001 compared to wild-type vs Nrf2-null of respective treatment
n= 5

5 μM tBHQ Decreases FasL Expression in NK Cells in a Nrf2-Dependent Manner with IL-12/IL-18 Activation.

FasL is expressed on activated NK cells, and upon ligation to target cells, the apoptotic signaling cascade is activated which leads to the activation of caspase 8 and 10 and ultimately induction of apoptosis.[241] Here we show a Nrf2-dependent decrease in FasL expression in NK

cells activated with IL-12/IL-18, as demonstrated by a decrease in FasL at 5 μM tBHQ in NK cells from wild-type, but not Nrf2-null, mice (**Figure 11A**). Conversely, FasL was decreased in both genotypes at 5 μM tBHQ when activated with PMA/Ionomycin. However, there is a non-statistically significant trend toward greater inhibition in the WT group, suggesting both Nrf2-dependent and Nrf2-independent mechanisms may play a role.

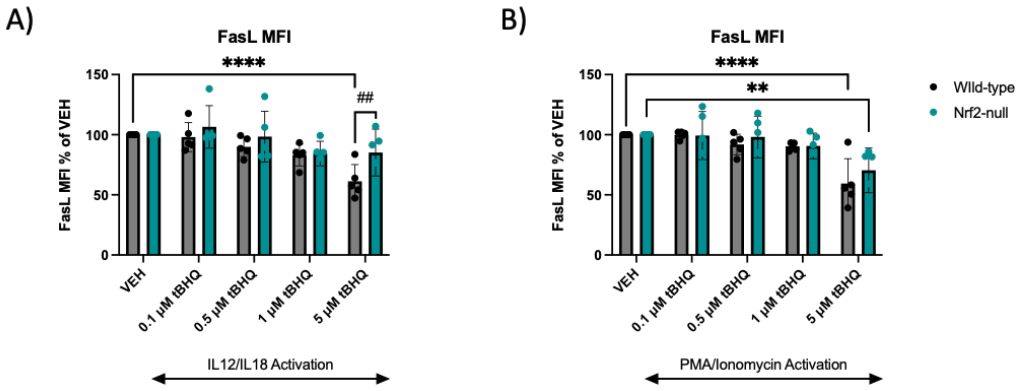

Figure 11: tBHQ decreases FasL expression in a Nrf2-dependent manner with IL-12/IL-18 activation. Splenocytes were isolated from wild-type or Nrf2-null C57Bl/6 mice. Cells were either left untreated (BKG) or treated with tBHQ (0.1- 5 μM) or 0.005% ethanol (VEH) for 30 min prior to addition of activators (A) IL-12/IL-18 or (B) PMA/Ionomycin. After 24 h, cells were stained with viability dye and fluorescently conjugated antibodies against CD3ε, NK1.1 and FasL for analysis by flow cytometry. NK cells were identified as viable CD3-NK1.1+ cells prior to FACS analysis of FasL.
* P ≤ 0.05; ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001 compared to vehicle of respective genotype
# P ≤ 0.05, ## P ≤ 0.01, ### P ≤ 0.001, #### P ≤ 0.0001 compared to wild-type vs Nrf2-null of respective treatment
n= 5

Nrf2 Promotes NK Cell Cytotoxicity.

Previously, we found tBHQ decreases NK cell production of perforin and granzyme following activation with PMA/Ionomycin.[137] Similarly, we found 5 μM of tBHQ to significantly decrease granzyme B in wild-type NK cells with PMA/Ionomycin (**Figure 12C**). However, while we did not see statistically significant differences with tBHQ treatments in Nrf2-null NK cells,

there was a trend toward decreased granzyme B in the Nrf2-null group, suggesting that a Nrf2-independent mechanism may be involved. While there was considerable variability in the granzyme B data sets, there was a consistent trend toward increased granzyme B induction in the NK cells derived from Nrf2-null mice. Likewise, perforin displayed significant differences between genotypes with wild-type NK cells producing significantly more perforin compared to Nrf2-null NK cells with both activators (**Figure 12B** and **12D**). Additionally, treatment with 5 μM tBHQ significantly decreased perforin in wild-type NK cells but not Nrf2-null NK cells (**Figure 12B** and **12D**). Taken together, the data suggest Nrf2 promotes perforin, and possibly granzyme B, induction and this effect does not require exogenous Nrf2 activators.

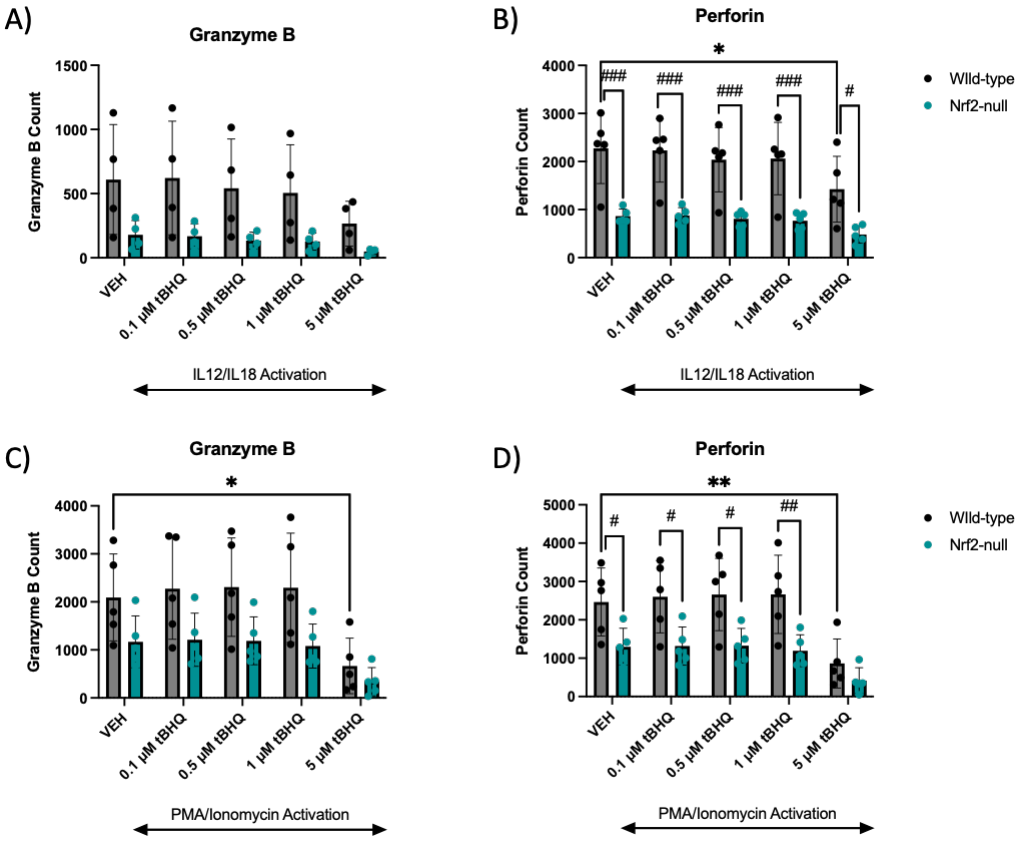

Figure 12: Nrf2 promotes perforin induction in NK cells. Splenocytes were isolated from wild-type or Nrf2-null C57Bl/6 mice. Cells were either left untreated (BKG) or treated with tBHQ

Figure 12 (cont'd):
(0.1- 5 μM) or 0.005% ethanol (VEH) for 30 min prior to addition of activators (A-B) IL-12/IL-18 or (C-D) PMA/Ionomycin. Monensin was added to samples 5 h prior to flow cytometry analysis (19 h post treatment). After 24 h, cells were stained with viability dye and fluorescently conjugated antibodies against CD3ε, NK1.1, granzyme B, and perforin for analysis by flow cytometry. NK cells were identified as viable CD3-NK1.1+ cells prior to FACS analysis of of intracellular markers, (A, C) granzyme B and (B, D) perforin.
* P ≤ 0.05; ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001 compared to vehicle of respective genotype
# P ≤ 0.05, ## P ≤ 0.01, ### P ≤ 0.001, #### P ≤ 0.0001 compared to wild-type vs Nrf2-null of respective treatment
n= 5

Reduction of IFNγ by Nrf2 Activator tBHQ.

IFNγ is an important antiviral cytokine that is rapidly secreted upon activation. Our lab has shown tBHQ to significantly reduce NK cell expression of IFNγ with PMA/Ionomycin activation.[137] Consistently, IFNγ significantly decreased with 1 and 5 μM tBHQ in wild-type NK cells with PMA/Ionomycin activation (**Figure 13B**). However, in Nrf2-null NK cells, IFNγ reduction was only demonstrated with 5 μM tBHQ (**Figure 13B**). With IL-12/IL-18 activation, 5 μM tBHQ-treated cells decreased NK cell expression of IFNγ in both genotypes (**Figure 13A**), suggesting a Nrf2-independent decrease in NK cell expression of IFNγ with tBHQ. Unexpectedly, however, when we measured secreted IFNγ in supernatants of cells activated with IL-12/IL-18, 5 μM tBHQ significantly decreased IFNγ in wild-type splenocytes, while tBHQ treatment did not affect IFNγ secretion in Nrf2-null splenocytes, which is not consistent with a fully Nrf2-independent mechanism (**Figure 14**). The contradiction between the two data sets may be due to kinetics as MFI is a "snapshot" of IFNγ expression, whereas the ELISA values indicate IFNγ accumulation over time. Collectively, these data indicate that tBHQ inhibits IFNγ through a partially Nrf2-dependent mechanism at 1, but not 5 μM, tBHQ, when looking at

expression at 24 h via MFI. In addition, the tBHQ-mediated decrease in IFNγ accumulation over 24 h appears to occur through a partially Nrf2-dependent mechanism.

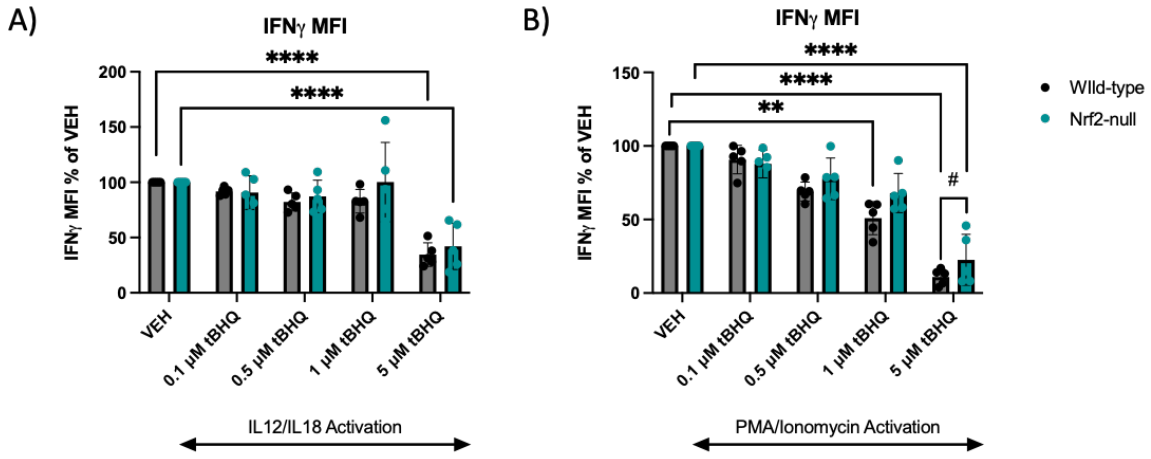

Figure 13: Reduction of IFNγ expression with tBHQ is independent of Nrf2 in NK cells.
Splenocytes were isolated from wild-type or Nrf2-null C57Bl/6 mice. Cells were either left untreated (BKG) or treated with tBHQ (0.1- 5 μM) or 0.005% ethanol (VEH) for 30 min prior to addition of activators (A) IL-12/IL-18 or (B) PMA/Ionomycin. Monensin was added to samples 5 h prior to flow cytometry analysis (19 h post treatment). After 24 h, cells were stained with viability dye and fluorescently conjugated antibodies against CD3ε, NK1.1, and IFNγ for analysis by flow cytometry. NK cells were identified as viable CD3-NK1.1+ cells prior to FACS analysis of intracellular marker, IFNγ.
* P ≤ 0.05; ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001 compared to vehicle of respective genotype
# P ≤ 0.05, ## P ≤ 0.01, ### P ≤ 0.001, #### P ≤ 0.0001 compared to wild-type vs Nrf2-null of respective treatment
n= 5

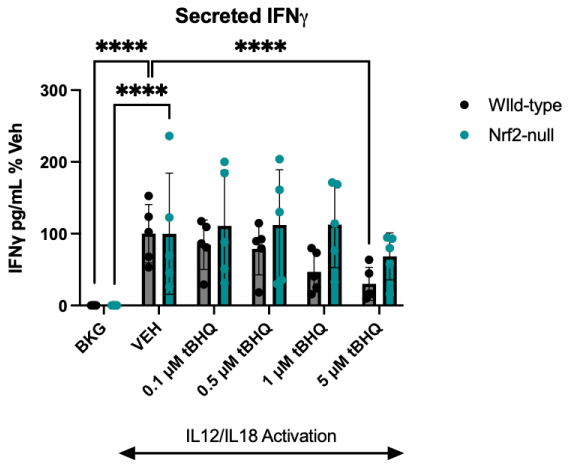

Figure 14: Secreted IFNγ protein accumulation is inhibited by tBHQ in a partially Nrf2-dependent manner. Splenocytes were isolated from wild-type or Nrf2-null C57Bl/6 mice. Cells were either left untreated (BKG) or treated with tBHQ (0.1- 5 μM) or 0.005% ethanol (VEH) for 30 min prior to addition of activator, IL-12/IL-18; BKG did not receive IL-12/IL-18. After 24 h, supernatants were collected for analysis of secreted IFNγ protein by ELISA.
* P ≤ 0.05; ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001 compared to vehicle of respective genotype
# P ≤ 0.05, ## P ≤ 0.01, ### P ≤ 0.001, #### P ≤ 0.0001 compared to wild-type vs Nrf2-null of respective treatment
n= 5

Discussion

Collectively, these results suggest that activation of Nrf2 by tBHQ alters NK cell maturation, decreases NK cell activation, and negatively impacts effector function. Notably, the presence of Nrf2 promoted terminal differentiation in NK cells, while tBHQ further increased the percent of mature NK cells with PMA/ionomycin activation. tBHQ treatment inhibited the upregulation of CD25 and CD69 through a partially Nrf2-dependent mechanism when the NK cells were activated with PMA/ionomycin, but not IL-12/IL-18. Similarly, tBHQ decreased FasL expression in NK cells activated with IL-12/IL-18 in a Nrf2-dependent manner. IFNγ expression in NK cells was also inhibited by tBHQ through a partially Nrf2-dependent mechanism at 1 μM, but not 5 μM, tBHQ. However, secreted IFNγ protein accumulation was reduced by tBHQ in a

partially Nrf2-dependent manner in the 5 μM tBHQ group. This study demonstrates a potential role of Nrf2 activation by tBHQ on NK cell maturation, activation, and effector function.

In the current study, we used two different NK cell activators, including PMA/ionomycin and the cytokines IL-12 and IL-18. IL-12 and IL-18 are known NK cell stimulatory cytokines that elicit IFNγ production and cytotoxicity.[242] While PMA/ionomycin can activate numerous immune cell types by activation of protein kinase C by PMA and robust calcium influx by ionomycin (a calcium ionophore), which leads to the activation of several intracellular signaling pathways.[243] Differences in genotype and tBHQ treatment effects between PMA/ionomycin and IL-12/IL-18 activated NK cells are likely due to the different modes of activation. Additionally, other cell types activated by PMA/ionomycin may influence NK cell responses, whereas IL-12/IL-18 activation is more specific to NK cell activation and may have less outside influence from other cell types.

Notably, there was a striking difference in maturation between wildtype and Nrf2-null NK cells. Wildtype NK cells displayed a more mature phenotype (**Figure 9A-D**), which is important because as NK cells mature, they acquire effector function. This may also help explain the genotype differences in NK cell effector functions, such as granzyme B and perforin (**Figure 12**). Maturation of NK cells are defined by their expression of CD27 and CD11b surface markers. CD27+CD11b- and CD27+CD11b+ NK cells have a higher capacity for secreting cytokines, such as IFNγ.[244] In contrast to perforin and granzyme B, IFNγ and FasL expression in NK cells did not show significant genotype effects, which suggests that factors separate from or in addition to NK cell maturation may modulate expression of these effector molecules. Interestingly, and in contrast to NK cells, IFNγ expression is increased in activated T cells derived

from Nrf2-null mice as compared to wild-type.[142] Taken together, the data suggest that while the role of Nrf2 in modulating expression of perforin and granzyme B expression may be tied to its effects on NK cell maturation, there is no genotype effect in the expression of IFNγ and FasL, suggesting that other (unidentified) factors may play a role. Although tBHQ is a potent Nrf2 activator, Nrf2-independent effects of tBHQ may be explained by alternative mechanisms. For example, tBHQ may impact reactive oxygen species, which are known to play an important role in the regulation of the redox-sensitive transcription factor, NFκB.[230] In particular, antioxidants structurally similar to tBHQ have been shown to inhibit NFκB.[245] Therefore, Nrf2-independent effects of tBHQ may be directly due to its antioxidant capacity. Additionally, tBHQ has been shown to cause a delay and a decrease in calcium influx in Jurkat T cells, which is relevant because intracellular calcium levels are critical in NK cell activation.[146] tBHQ has also been shown to activate aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that has many immunomodulatory effects.[246] Overall, there are a number of potential alternative mechanisms that could play a role in the Nrf2-independent effects of tBHQ. Together, tBHQ-mediated effects may be attributed to mechanisms independent of Nrf2.

Although Nrf2 is well known for its cytoprotective effects, the role for Nrf2 in NK cell activity remains uncertain. In models of inflammatory disease, studies have demonstrated an anti-inflammatory role for Nrf2. The current study is the first to elucidate the Nrf2-dependent and -independent effects of tBHQ on activated NK cells. Notably, this study suggest that Nrf2 represents a novel mechanism for the regulation of NK cell activation. Further studies will be needed to identify the mechanisms underlying the Nrf2-independent effects of tBHQ on NK cells.

CHAPTER 4

The Nrf2 activator, tBHQ, inhibits early NK cell responses to influenza

Abstract

Influenza virus is estimated to cause millions of illnesses and thousands of hospitalizations and deaths yearly in the United States, making influenza a major public health concern. It is well known that an early natural killer (NK) cell response against influenza is vital for effective viral clearance. Tert-butylhydroquinone (tBHQ) is a widely used food preservative with known immunomodulatory activity. Our lab has shown tBHQ to negatively impacts NK cell activation, effector function, and maturation *ex vivo*. However, little is known regarding the effects of tBHQ consumption on NK cells *in vivo*, specifically in response to influenza infection. In the current study, we examined whether the consumption of tBHQ impairs NK cell response two and three days after primary influenza virus infection. Female C57Bl/6J mice were fed a diet containing either 0.0014% tBHQ or control diet 10 days prior to infection. Mice were intranasally infected with influenza A/PR/8/34 (H1N1), and at day two and three post-infection, lungs were collected and processed for analysis by flow cytometry, qPCR, and ELISA. Mice exhibited similar weight loss throughout infection regardless of their respective diet. There were no statistical differences in the infiltration or maturation of NK cells in the lungs between the groups at day two of infection. At day three, tBHQ modestly decreased the percentage of NK cells in the lung, and NK cells adopted a more immature phenotype. Notably, tBHQ significantly reduced expression of FasL and production of IFNγ in NK cells compared to those on a control diet at day two. NK cell cytotoxicity decreased with tBHQ at day three, as demonstrated by reduced CD107a expression. Likewise, induction of antiviral effector genes was decreased in the lungs of mice on a tBHQ diet at days two and three of infection. Taken together, the data suggests the food additive, tBHQ, negatively impacts early NK cell responses

to influenza infection by inhibiting FasL, CD107a, and IFNγ expression. Furthermore, tBHQ impaired the induction of genes associated with NK cell effector function in influenza-infected mice.

Introduction

Influenza virus is a contagious respiratory illness that causes a persistent threat to society as it infects approximately 8% of the US population each year.[4] Although efforts in influenza vaccination coverage and effectiveness have increased, the burden of disease remains high, affecting millions in the United States alone.[247] Increased susceptibility to influenza infection and risk of developing severe influenza-related diseases are determined by a wide range of viral, host, and environmental factors. The CDC defines individuals with higher risk for severe influenza A virus-related health outcomes to include people of advanced age (>65 years old), young children (<5 years old), pregnant people, and people with chronic diseases. However, there are discrepancies between susceptibility of healthy individuals infected with influenza, which have been attributed to several factors, including climate, occupation, and geography, among others.[248] Therefore, the importance for identifying components that may contribute to the range in susceptibility and severity in infection is necessary, especially when vaccination efficacy is poor or unavailable.

Natural killer (NK) cells are large, granular innate lymphocytes that play a critical role in host antiviral responses by rapidly producing cytokines, such as IFNγ, and directly killing virally infected cells through the release of granzymes and perforin. Early on in infection, NK cells can limit viral replication while allowing time for the adaptive immune response to mount.[249,250] However, defects in NK cell responses have been linked to increased pathology and worsened outcomes. Our group has shown increased mortality and decreased NK cell cytotoxicity in mice on a calorie-restricted mice infected with influenza A.[73] Furthermore, in aged mice, calorie restriction further diminished the response to influenza resulting in decreased survival,

increased virus titers, and reduced NK cell activity compared to aged mice fed ad libitum and calorie restricted, young mice.[72] In diet-induced obese mice, influenza-infected mice had increased mortality while NK cells exhibited reduced cytotoxicity and antiviral cytokine production.[74] In contrast, supplementation of active hexose correlated compound increased NK cell activity and NK cell number in the lungs following infection.[75] Previous studies have shown altered NK cell activity in response to influenza infection after implementation of other nutritional interventions to the diet including, cocoa and fortified yogurt.[251,252] In the absence of influenza, studies have demonstrated NK cell activity and effector function to be influenced by vitamin supplementation and alcohol consumption.[76,77] Together, these studies suggest that NK cells are highly influenced by environmental factors, and impairment during influenza infection may lead to decreased viral clearance and survival.

Tert-butylhydroquinone (tBHQ) is a widely used food additive that is found in a variety of products to prevent the oxidation and rancidification of fats, such as cooking oils, cereals, and crackers, among others.[185] Our lab has also shown T cell modulation with tBHQ treatment in numerous models, including primary mouse CD4+ T cells, Jurkat T cells, and primary human CD4+ T cells.[142,143,145,147] Additionally, studies from our lab and others have demonstrated immunomodulatory effects of tBHQ in B cells, dendritic cells, and macrophages.[139,235–237] Of particular interest, our lab has demonstrated tBHQ greatly impacts primary NK cell maturation, activation, and effector function *in vitro*. Specifically, we found tBHQ reduced expression of early activation markers, CD69 and CD25, and induction of IFNγ and the cytotoxic molecules, granzyme B and perforin, by activated NK cells.[137] Therefore, the following study aims to

identify whether dietary tBHQ influences the early immune cell response to primary influenza A infection by NK cells.

Materials and Methods

Materials

Materials were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated.

Animals, Diets, and Virus

Influenza A/PR/8/34 (H1N1) was generously gifted by Dr. Kymberly Gowdy and East Carolina University in Greenville, North Carolina. The aliquot of virus was propagated and quantified following methods previously published.[253] Using specific pathogen-free, embryonated chicken eggs (Charles River Laboratories, Wilmington, MA), the virus was inserted into the allantoic fluid and incubated for 48 hours at 37°C. Afterwards, infected eggs were incubated for another 24 hours at 4°C. Supernatants from allantoic fluid were collected, distributed into single-use aliquots, and stored at -80°C. Tissue culture infectious dose 50 ($TCID_{50}$) and hemagglutinin assays were used to quantify propagated virus stock. The $TCID_{50}$ assay was performed by adding a serial dilution of virus sample to confluent monolayers of MDCK cells (ATCC, Manassas, VA) in a 96-well plate. Cells were inspected daily for cytopathic effect (CPE) or cell death, and the dilution, at which point 50% of wells show CPE, was used to calculate $TCID_{50}$ via the Reed-Muench method.[254] For hemagglutinin assay, virus stock was serially diluted across a 96-well plate containing 0.5% chicken red blood cells, followed by a 30-minute incubation at room temperature. After, wells were analyzed for agglutination, and the highest dilution of virus that caused complete hemagglutination was used to determine virus

titer. The virus stock used in this experiment was determined to be 2.5 x 10^5 $TCID_{50}$/mL and 7260 HAU/mL.

All animal studies were performed in the accordance with the Guide for Care and Use of Animals as adopted by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Michigan State University. Female C57BL/6 mice (12 weeks old) were purchased from Jackson Laboratories (Bay Harbor, ME) and housed in cages containing 3- 5 animals per cage. Upon arrival, mice were given AIN-93G purified rodent diet containing 0% or 0.0014% tBHQ and water *ad libitum*. Food was monitored daily, and diets were replaced every three days to avoid spoilage. After 2 weeks of acclimation on their respective diets, initial body weights were recorded, followed by anesthetization via intraperitoneal injection with 2,2,2-tribromoethanol, avertin (Alfa Aesar, Ward Hill, MA). Subsequently, mice were intranasally instilled with either 30 μL of sterile saline (CTRL) or influenza A/PR/8/34 (H1N1) (VIR) at a titer of 2.5 x 10^3 $TCID_{50}$/mL (72.6 HAU/mL), which resulted in a total amount of 75 $TCID_{50}$ or 2.18 HAU per mouse. After recovering from anesthesia, mice were returned to their respective cages. Daily health checks were performed and changes in food and body weights were recorded. Two separate experiments were completed to assess NK cells responses at early timepoints of infection. A schematic of the timeline for the following experiments is shown in **Figure 15**.

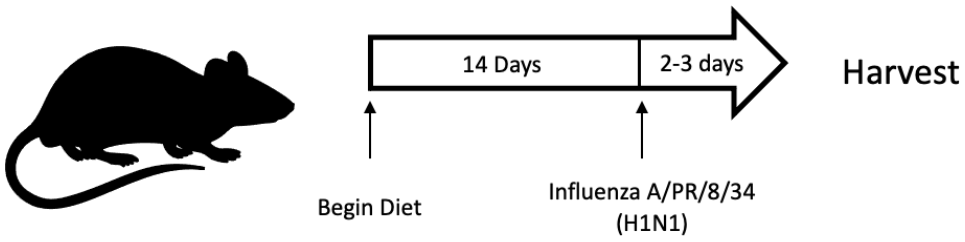

Figure 15: Schematic of Experimental Design. Mice arrived at Michigan State University from Jackson Laboratories and were placed on their respective diets for an acclimation period of 14 days. Mice were infected with influenza A/PR/8/34 (H1N1), and after 2- or 3-days post infection, tissues were collected for analysis.

Tissue Collection and Cell Isolation

At day two or three post-infection, mice were anesthetized with avertin and euthanized via exsanguination by severing the vena cava. Lungs were excised and placed in DMEM containing 1 mg/mL collagenase D (Roche, Indianapolis, IN) and dissociated twice using the gentleMacs dissociator (Miltenyi Biotec, Auburn, CA) with a 30 min incubation at 37°C between dissociations. Subsequently, 1 mL of dissociated lung was removed, centrifuged, and placed in TRIzol reagent for later RNA isolation. Remaining sample was washed with FACS buffer (1% FBS in dPBS) and filtered through a 40 μm strainer. Red blood cells from lung were lysed using ammonium chloride-potassium (ACK) lysis buffer (Lonza, Walkersville, MD). Single-cell suspensions were subsequently used for flow cytometry analysis.

Immunophenotyping

Single-cell suspensions from the lungs were added to a 96-well V-bottom plate and washed with dPBS prior to assessing cell viability by Zombie Aqua Fixable Viability Kit (Biolegend, San Diego, CA) following manufacturer's protocol. Afterward, cells were washed with FACS buffer and incubated for 10 minutes with anti-CD32/16 antibody (BD Pharmingen, San Diego, CA), to block non-specific binding. Cells were then labeled with fluorochrome

conjugated antibodies for 30 minutes in the dark at 4°C. The surface antibodies used are listed in **Table 2**. Cells were washed, fixed using BD Cytofix Fixation Buffer (BD Biosciences, Franklin Lakes, NJ) per manufacturer's protocol, and stored at 4°C in the dark until later FACs analysis by Attune Nxt (Thermo Scientific, Waltham, MA).

Ex Vivo Stimulation and Intracellular Labeling

Following lung cell isolation, single-cell suspensions were cultured in complete media, containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 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 100 U/mL 2-mercaptoethanol (Thermo Fisher Scientific, Waltham, MA). Cells were stimulated using 10 ng/mL of recombinant mouse IL-12 (P70) (Biolegend, San Diego, CA) for 5 hours in the presence of monensin (Biolegend, San Diego, CA). After, samples were transferred to a 96-well V-bottom plate and labeled with Zombie Aqua Fixable Viability Kit. Cells were then incubated with anti-CD32/CD16 and labeled with fluorochrome-conjugated antibodies for surface markers, NK1.1 and CD3. Following surface labeling, cells were permeabilized using FoxP3/Transcription Buffer Staining Kit (Invitrogen, Waltham, MA) per manufacturer's protocol and labeled with intracellular antibodies listed in **Table 2**. Cells were fixed with 1% formaldehyde fixative and stored in the dark at 4°C until FACS analysis on the Attune NxT.

Table 2: List of Antibodies

Antibody	Fluorochrome	Clone	Supplier
Live/Dead	Zombie Aqua Dye		Biolegend
CD3	Alexa Fluor 488	17A2	Biolegend
CD3	Alexa Fluor 700	eBio500A2	eBioscience
NK1.1	Brilliant Violet 711	PK136	Biolegend
CD27	PerCP-eFluor 710	LG.7F9	eBioscience
CD11b	Alexa Fluor 647	M1/70	Biolegend
CD25	Pacific Blue	PC61	Invitrogen
CD69	Alexa Fluor 700	H1.2F3	Biolegend
CD107a	PE-Cy7	1D4B	BD Biosciences
CD178 (FasL)	PE	MFL3	Biolegend
NKG2D	APC	CX5	Biolegend
Granzyme B	FITC	NGZB	Invitrogen
T-bet	PE	eBio4B10	Biolegend
IFN-γ	APC	XMG1.2	Biolegend
Perforin	PE	S16009B	Biolegend

RNA Isolation and Quantitative PCR

RNA was isolated from homogenized lung tissues using TRIzol reagent (Life Technologies, Grand Island, NY). RNA was quantified using the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and cDNA was synthesized by M-MLV Reverse Transcriptase (Promega, Madison, WI) following the manufacturer's protocol.

Real-time was performed with SYBR green PCR master mix using the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA). Expression values were normalized to expression of ribosomal protein L13A (RPL13A) and quantified by the ΔΔCt method. Gene-specific primers are listed in **Table 3**.

Table 3: Primer Sequences

Gene	Forward Primer Sequence	Reverse Primer Sequence
Influenza M1	CAAAGCGTCTACGCTGCAGTCC	AAGACCAATCCTGTCACCTCTGA
IFNγ	TGAGCTCATTGAATGCTTGG	ACAGCAAGGCGAAAAGGAT
Granzyme B	AGTGGGGCTTGACTTCATGT	AGTGGGGCTTGACTTCATGT
Perforin	TGGAGGTTTTTGTACCAGGC	TAGCCAATTTTGCAGCTGAG
T-bet	CAAGGGGGCTTCCAACAATG	GGCTCTCCATCATTCACCTCC
IL-18	TCCTTGAAGTTGACGCAAGA	TCCAGCATCAGGACAAAGAA
IL-1β	GGTCAAAGGTTTGGAAGCAG	TGTGAAATGCCACCTTTTGA
IFNα	TAGGAGGGTTGCATTCCAAG	CCAGCAGATCAAGAAGGCTC
RPL13a	GTTGATGCCTTCACAGCGTA	AGATGGCGGAGGTGCAG

Statistical Analysis

Treatment groups are presented as mean ± SEM for each individual experiment. In virus-infected groups, differences in treatment (CTRL vs tBHQ) were assessed using two-tailed T-tests with GraphPad PRISM software version 8.4.2 (GraphPad Software, La Jolla, CA). Data sets with unequal variances were analyzed using Welch's t-tests. Saline-instilled mice were not included in the statistical analysis, as their purpose for the current study was to serve as a

reference and to confirm that absence of immune response in uninfected mice. Animals which had no detectable viral RNA were excluded from statistical analysis.

Results

tBHQ Modestly Decreased Viral RNA but did not Affect Weight Loss.

Indication of infection in mice is often characterized by a decrease in appetite followed by weight loss. Upon arrival, mice were given diets containing either no tBHQ or 0.0014% tBHQ, and daily consumption was monitored for the duration of the study. Since mice were not singly housed, the average amount of diet consumed per mouse was estimated based upon consumption from the whole cage. Dietary tBHQ did not impact weight loss in mice infected with influenza with an average weight loss of 4.56% and 5.24% at day two (**Figure 16A**) and 9.53% and 9.07% at day three (**Figure 16B**) for CTRL and tBHQ groups, respectively. Interestingly, tBHQ had a significant decrease in viral RNA at day two of infection (**Figure 17A**) and trended downward at day 3 (**Figure 17B**), however this was not significant. Although viral RNA was decreased with dietary tBHQ, similar food consumption patterns and weight loss were observed between virus-infected tBHQ and CTRL groups throughout the infection.

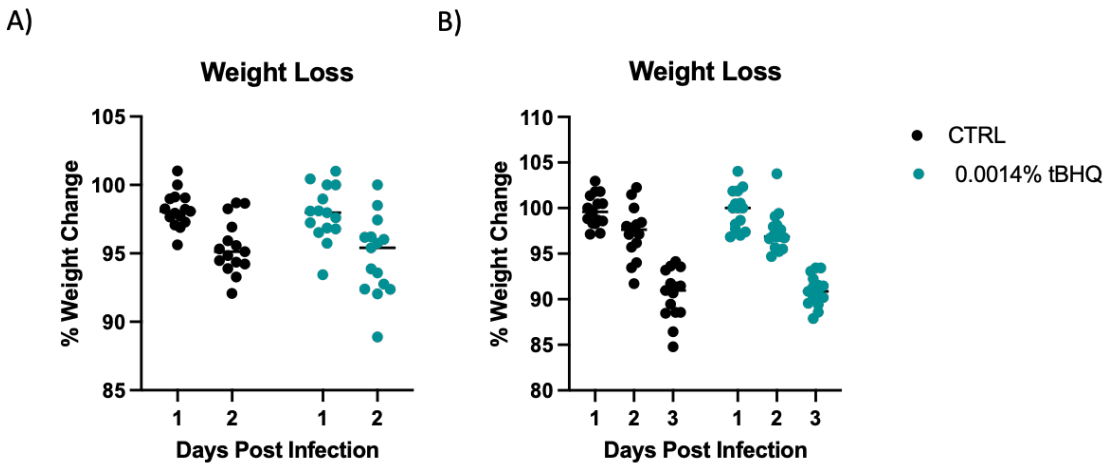

Figure 16: Dietary tBHQ did not affect weight change during early infection. Mice were placed on a diet either containing 0.0014% tBHQ or no tBHQ (CTRL) for two weeks prior to influenza A/PR/8/34 infection. Weights were recorded daily up to (A) 2- or (B) 3-days post infection, and percent weight change was calculated

Equation 1: Calculation of Daily tBHQ Intake per Mouse

$$\frac{Food\ eaten\ per\ cage\ (g)}{\#\ of\ mice\ per\ cage} x \frac{0.014\ mg\ tBHQ}{1\ g\ food} x \frac{1\ mouse}{Average\ weight\ of\ mice\ in\ cage\ (g)} x \frac{1000\ g}{1\ kg} = \frac{mg\ tBHQ}{kg\ bodyweight}$$

Table 4: tBHQ Consumption during 2-day Influenza Infection (mg/kg)

Primary Exposure with H1N1	Day 0	Day 1	Day 2
tBHQ Saline	1.47727273	1.35333333	1.52224053
tBHQ Virus	1.08229426	1.17223814	0.83815789

Table 5: tBHQ Consumption during 3-day Influenza Infection (mg/kg)

Primary Exposure with H1N1	Day 0	Day 1	Day 2	Day 3
tBHQ Saline	1.95081967	1.66993464	1.53548387	1.92283951
tBHQ Virus	1.54901326	1.50762245	0.85088011	0.05976521

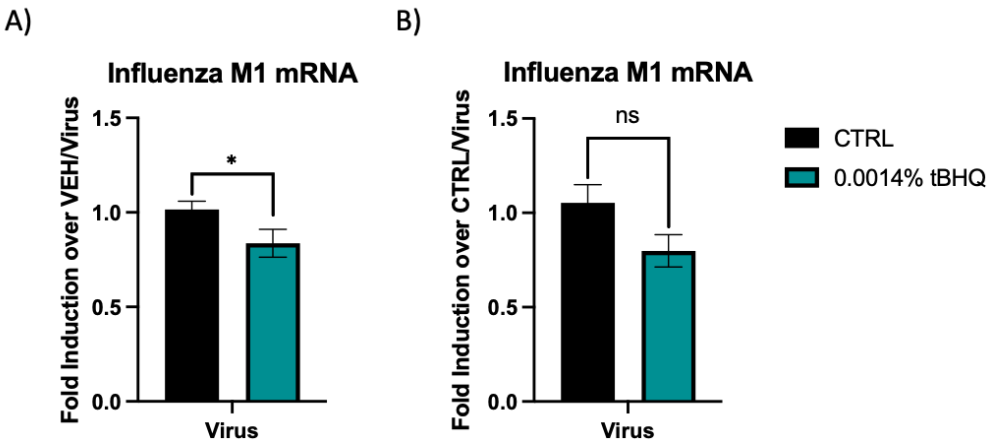

Figure 17: Dietary tBHQ decreased viral titer in lungs of infected mice. Mice were placed on a diet either containing 0.0014% tBHQ or no tBHQ (CTRL) for two weeks prior to intranasal instillation with influenza A/PR/8/34 infection. After (A) 2- to (B) 3-days post infection, lungs were collected and processed for viral RNA quantification by real-time PCR. Viral quantification was performed using viral matrix protein, M1, and was normalized using housekeeper gene, RPL13a. * $P \leq 0.05$; ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

Dietary tBHQ had Minimal Influence on NK cell Population in the Lungs of Infected Mice.

At days 2 and 3 post-infection, lungs were collected and processed for flow cytometry analysis. Single-cell suspensions were labeled with fluorochrome-conjugated antibodies against CD3 and NK1.1. NK cells were identified as viable CD3-NK1.1+ cells. In agreement with previous reports, the NK cell population ranged from 10-20% of lung lymphocytes (**Figure 18**).[255] Although the percent of NK cells in the lung at days two and three of infection displayed a modest decrease with tBHQ, the difference did not reach significance (**Figure 18B**).

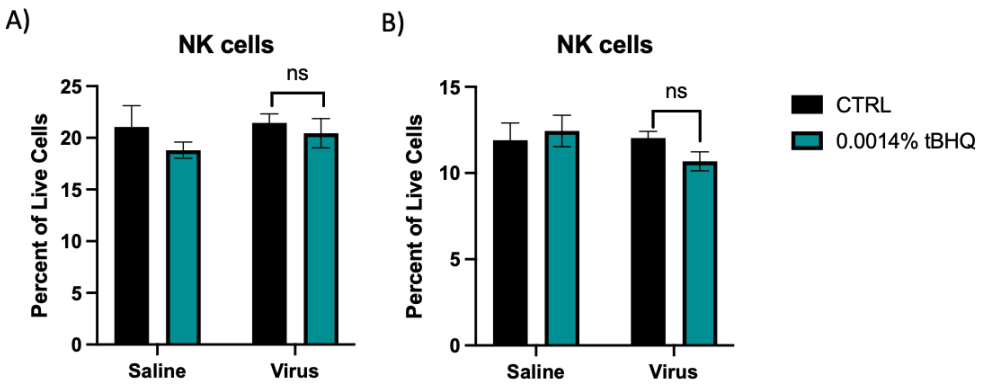

Figure 18: tBHQ had no effect on lung NK cell population at days 2 and 3 post-infection. Mice were placed on a diet either containing 0.0014% tBHQ or no tBHQ (CTRL) for two weeks prior to intranasal instillation with either saline or influenza A/PR/8/34 infection. After (A) 2- to (B) 3-days post infection, lungs were collected and processed for flow cytometry analysis. Single-cell suspensions were labeled with antibodies against CD3 and NK1.1 and quantified by flow cytometry. * $P \leq 0.05$; ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

tBHQ Decreased the Percentage of Terminally Mature NK cells at Day 3 of Influenza Infection.

Maturation of NK cells in the periphery are divided into 4 stages defined by the expression of CD27 and CD11b, starting with immature NK cells, CD27-CD11b-. As NK cells mature, they express CD27 and gain effector function capabilities. CD11b expression is later acquired signifying double positive, CD27+CD11b+, NK cells, which are highly cytotoxic and produce large amounts of cytokines. Terminally mature NK cells are identified by the loss of CD27 expression and are comparable in effector responses to CD27+CD11b+ NK cells.[21] In the current study, the majority of NK cells in the lung were terminally mature, as indicated by CD27-CD11b+ phenotype (**Figure 19D** and **19H**). There were no differences in maturation at day 2 post-infection. However, at day 3 of infection, tBHQ modestly, but significantly, decreased terminally mature NK cells (**Figure 19H**) while increasing CD27+CD11b- NK cells (**Figure 19F**). Potential explanations for altered maturation at day 3 but not day 2 of infection

include the increased proliferative capacity of CD27+CD11b- NK cells or potential differences in phenotype of migrating NK cells.[21]

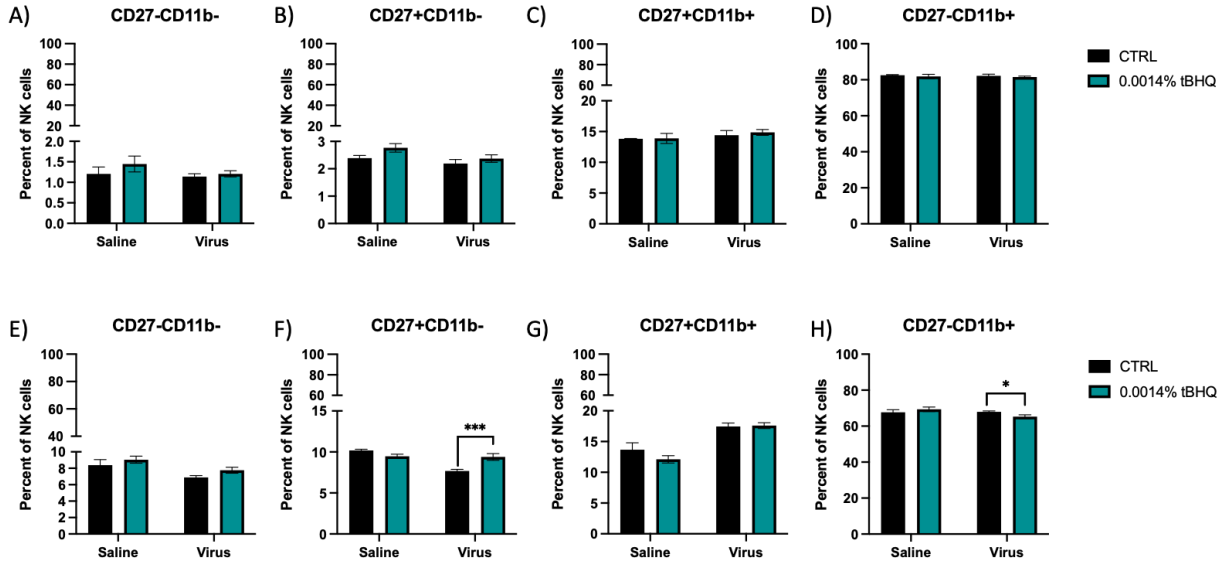

Figure 19: tBHQ caused a minor decrease in terminally mature NK cells at day 3 of infection. Mice were placed on a diet either containing 0.0014% tBHQ or no tBHQ (CTRL) for two weeks prior to intranasal instillation with either saline or influenza A/PR/8/34 infection. After (A-D) 2- to (E-H) 3-days post infection, lungs were collected and processed for flow cytometry analysis. Single-cell suspensions were labeled with antibodies against CD3, NK1.1, CD27, and CD11b and quantified by flow cytometry. NK cells were identified as NK1.1+CD3- prior to analysis of maturation using CD27 and CD11b. Maturation occurs in the following order: (A, E) CD27-CD11b-, (B, F) CD27+CD11b-, (C, G) CD27+CD11b+, (D, H) CD27-CD11b+. * $P \leq 0.05$; ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

Dietary tBHQ Impaired NK cell Effector Functions and Diminished Expression of Genes Associated with NK Cell Effector Function.

The ability for NK cells to elicit a sufficient response is crucial in limiting viral replication early on in infection. To assess NK cell effector functions, we analyzed the surface expression of CD107a and FasL on NK cells, as well as the intracellular expression of IFNγ, granzyme B, and perforin by flow cytometry. At day 2 of infection, FasL expression on NK cells was decreased with tBHQ exposure (**Figure 20B**). However, this decrease was insignificant by day 3, by which

time FasL expression was decreased as compared to day 2 (**Figure 20D**). NK cell cytotoxicity was assessed by CD107a expression, a marker for active degranulation, and production of granzyme and perforin in NK cells. CD107a was greater on day 3 as compared to day 2 following influenza infection. Dietary tBHQ significantly reduced CD107a at day three of infection (**Figure 20C**). Interestingly, intracellular expression of granzyme B were not affected by tBHQ at either timepoints (**Figure 21B** and **21E**), and perforin was slightly decreased at day 2 of influenza infection (**Figure 21C**). Notably, NK cell production of cytokine, IFNγ, was significantly diminished at day 2 of infection with dietary tBHQ but had recovered by day 3 (**Figure 21A** and **21D**). Coinciding with our flow cytometry results, several genes associated with NK cell effector functions were significantly reduced at day 2 and 3 of influenza infection (**Figure 22** and **23**). Specifically, at day 2 of infection tBHQ markedly diminished IFNγ mRNA expression, which was consistent with the decrease in IFNγ protein expression by NK cells at this time point. In contrast, tBHQ also decreased granzyme B mRNA expression, whereas granzyme B protein expression by NK cells was not affected by tBHQ at this time point. It is important to note, however, that whole lung tissue was used to quantify gene expression. Therefore, differences in gene expression cannot be attributed solely to NK cells. Collectively, the data suggest tBHQ decreases the expression of a number of important effector molecules by NK cells at early time points following influenza infection.

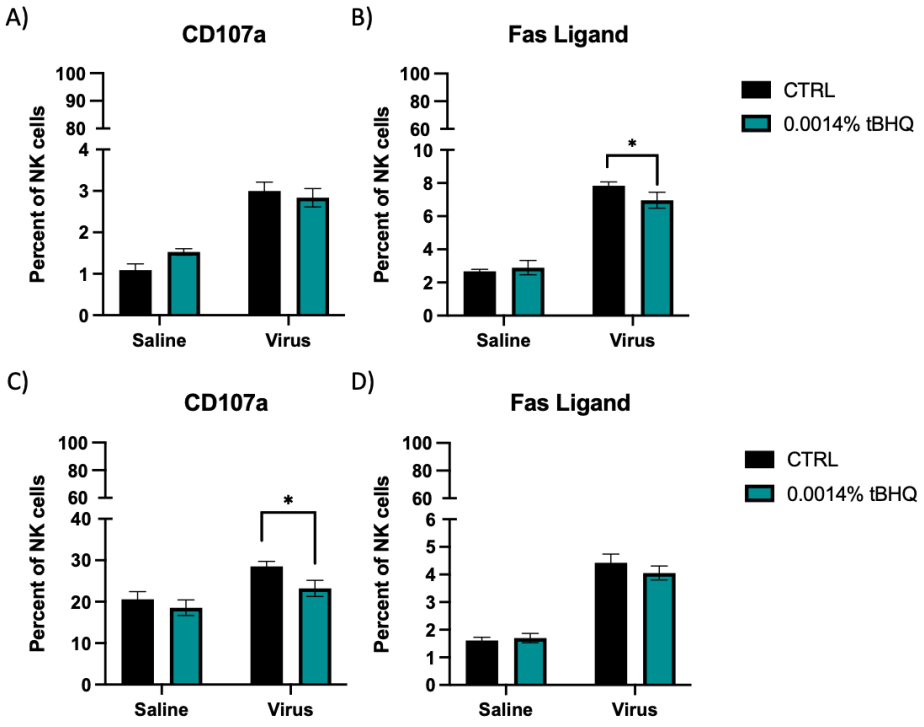

Figure 20: NK cell expression of FasL decreased at day 2 of infection while CD107a expression decreased at day 3 of infection with dietary tBHQ. Mice were placed on a diet either containing 0.0014% tBHQ or no tBHQ (CTRL) for two weeks prior to intranasal instillation with either saline or influenza A/PR/8/34 infection. After (A-B) 2- to (C-D) 3-days post infection, lungs were collected and processed for flow cytometry analysis. Single-cell suspensions were labeled with antibodies against CD3, NK1.1, CD107a, and FasL and quantified by flow cytometry. NK cells were identified as NK1.1+CD3- prior to analysis percent NK cells expressing (A, C) CD107a and (B, D) FasL. * $P \leq 0.05$; ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

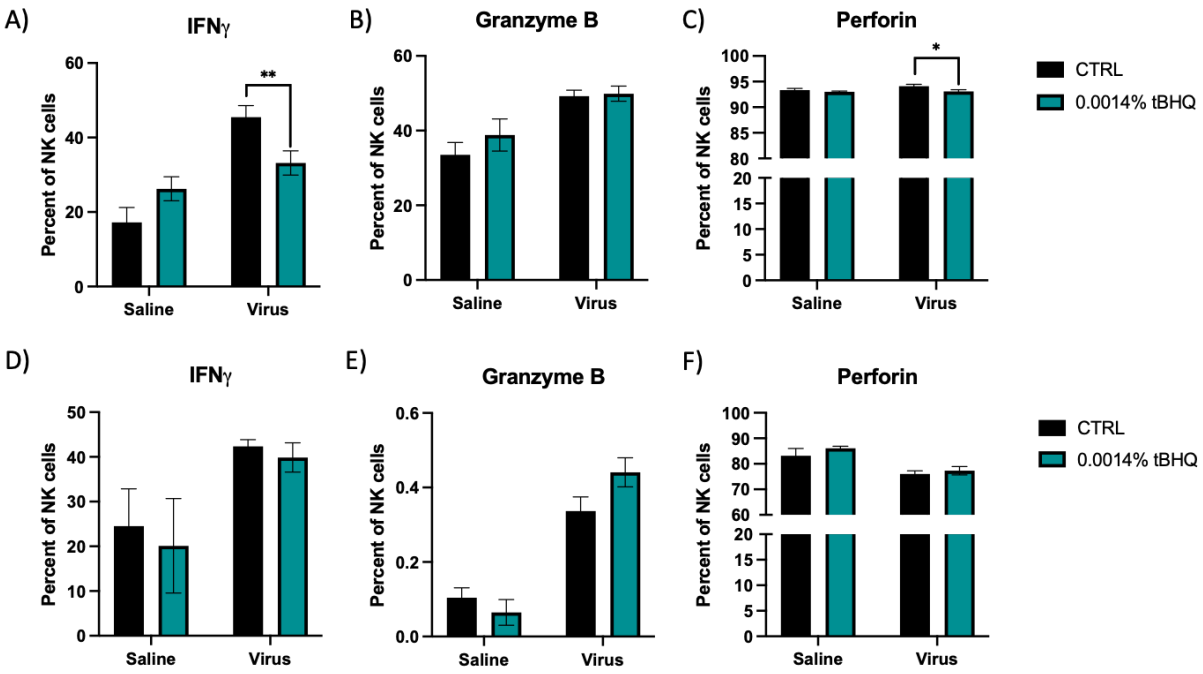

Figure 21: tBHQ decreased NK cell expression of IFNγ and perforin at day 2 post-infection. Mice were placed on a diet either containing 0.0014% tBHQ or no tBHQ (CTRL) for two weeks prior to intranasal instillation with either saline or influenza A/PR/8/34 infection. After (A-C) 2- to (D-F) 3-days post-infection, lungs were collected and processed for flow cytometry analysis. Single-cell suspensions were cultured with 10 ng/mL IL-12 in the presence of monensin for 5 hours. After, cells were labeled with antibodies against CD3, NK1.1, IFNγ, granzyme B, and perforin and quantified by flow cytometry. NK cells were identified as NK1.1+CD3- prior to analysis of percent NK cells expressing (A, D) IFNγ, (B, E) granzyme B, and (C, F) perforin. * $P \leq 0.05$; ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

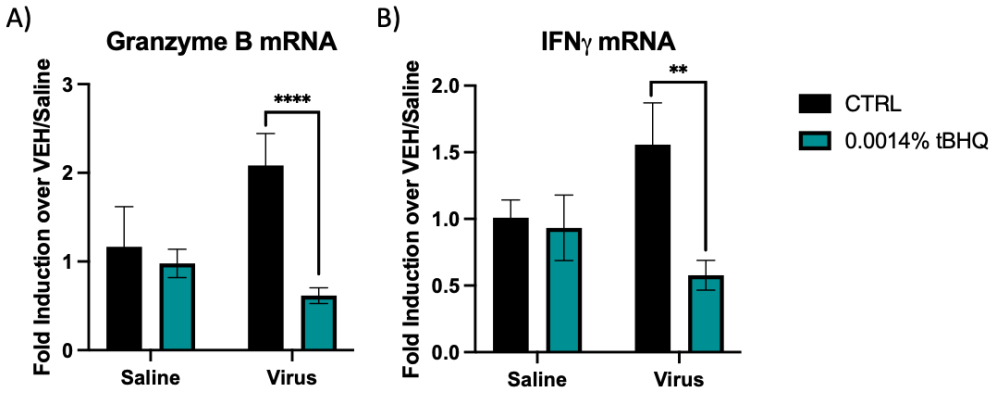

Figure 22: Low concentration of tBHQ in the diet decreased mRNA expression of granzyme B and IFNγ at day 2 post-infection. Mice were placed on a diet either containing 0.0014% tBHQ or no tBHQ (CTRL) for two weeks prior to intranasal instillation with either saline or influenza A/PR/8/34 infection. After 2-days post-infection, lungs were collected and processed for RNA

Figure 22 (cont'd):
quantification. Real-time PCR was done to quantify (A) granzyme B and (B) IFNγ mRNA. * P ≤ 0.05; ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001

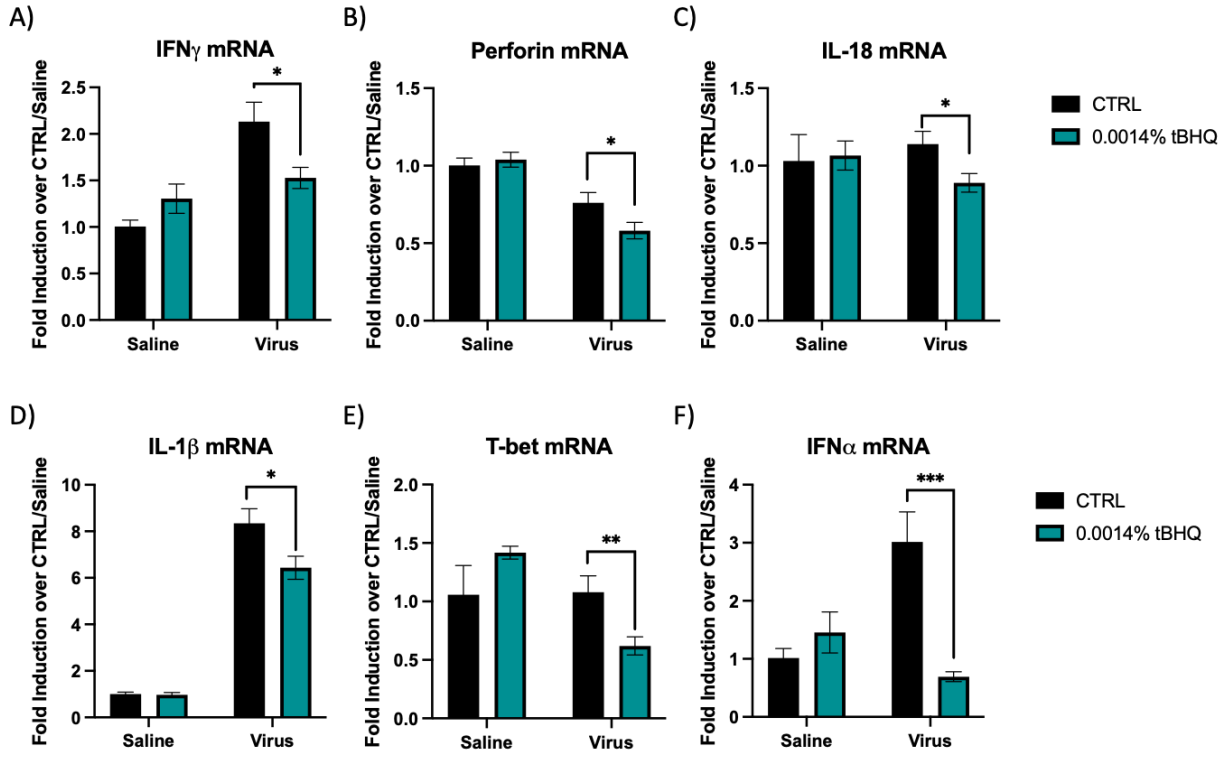

Figure 23: tBHQ in the diet decreased mRNA expression of genes associated with NK cell effector function at day 3 post-infection. Mice were placed on a diet either containing 0.0014% tBHQ or no tBHQ (CTRL) for two weeks prior to intranasal instillation with either saline or influenza A/PR/8/34 infection. After 3-days post-infection, lungs were collected and processed for RNA quantification. Real-time PCR was done to quantify (A) IFNγ, (B) perforin, (C) IL-18, (D) IL-1β, (E) T-bet, and (F) IFNα mRNA. * P ≤ 0.05; ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001

Discussion

Collectively, these results suggest consumption of tBHQ greatly influences the primary NK cell response to influenza at days two and three of infection. The percentage of NK cells trended toward a modest reduction in mice fed a diet containing tBHQ (**Figure 18**). In addition, tBHQ caused NK cells to adopt a more immature phenotype, which is known to be less functional at day three of infection (**Figure 19E-H**). At day two of infection, expression of FasL,

which is involved in initiating apoptosis, was significantly reduced in NK cells of mice on a tBHQ diet (**Figure 20B**). Additionally, tBHQ markedly reduced IFNγ production in NK cells at day two of infection (**Figure 21A**). IFNγ and granzyme B gene expression was also reduced at day two of infection with tBHQ (**Figure 22**). At day three, NK cell cytotoxicity decreased with tBHQ, as demonstrated by reduced CD107a expression (**Figure 20C**). Notably, at day three, tBHQ suppressed the expression of several genes important in antiviral immunity, including IFNα, IFNγ, T-bet, IL-1β, IL-18, and perforin (**Figure 23)**.

In murine NK cells, maturation in the periphery occurs in four stages depending on expression of CD27 and CD11b. Maturation occurs in the following order: CD27-CD11b-, CD27+CD11b-, CD27+CD11b+, and CD27-CD11b+. It is well known that NK cells with a phenotype of CD27+CD11b+ and CD27-CD11b+ are more functional, as they can rapidly release cytotoxic molecules and cytokines. In contrast, more immature NK cells, including CD27-CD11b- and CD27+CD11b-, have greater proliferative capacity compared to mature NK cells.[21,256,257] In the current study, dietary tBHQ modestly increased CD27+CD11b- NK cells and decreased CD27-CD11b+ NK cells at day 3 of infection (**Figure 19F** and **19H**), while no difference was found at day 2 (**Figure 19A-B**), which could be due to differential levels of proliferation and/or recruitment. Previously, a study found lung NK cell proliferation to peak by day 4 of influenza infection with 2% of NK cells in the lung proliferating, which is comparably low to other cell types at similar timepoints. However, the accumulation in lung NK cells was due to NK cell recruitment during influenza infection with CD27-CD11b+ subset having the greatest increase in the lung compared to other subsets.[53] Therefore, it is possible that tBHQ impairs NK cell recruitment to the lung at day 3 of influenza infection as demonstrated by a decrease in the

percent of terminally mature NK cells. Further studies elucidating the effects of tBHQ on NK cells at later timepoints are essential to understand the impact on NK cell recruitment.

Notably, the current study examined the effects of low dose tBHQ on NK cell responses to a relatively high concentration of virus at two early time points during infection. Others have suggested NK cells to exacerbate pathology of influenza virus when infected with lethal amount of virus. They found mice depleted of NK cells *in vivo* and infected with a high viral dose of influenza A/PR/8/34 virus to have increased survival rates.[69] Furthermore, adoptively transferred NK cells from mice infected with a high dose of virus had higher morbidity and mortality. Whereas NK1.1+ cell-depleted mice were protected from lethal influenza virus infection, as demonstrated by minimal morbidity.[70] While the results in the current study suggest that in particular cytokine response was strongly inhibited by tBHQ under these conditions, future studies are needed to investigate the effects of higher concentrations of tBHQ, which might serve as a model for high consumers of tBHQ. Additionally, the effects of tBHQ in NK cells following infection at a lower dose of influenza virus is necessary to model seasonal influenza virus infections rather than pandemic-like responses.

Of importance to human relevance, the amount of tBHQ consumed per animal in the current study equated to approximately 1-2 mg/kg/day (**Table 4** and **5**), which exceeds the recommended acceptable daily intake of 0.7 mg/kg/day for tBHQ. However, tBHQ intake was well below the set NOAEL of 72 mg/kg/day, which was used in establishing the current ADI. Additionally, model diets estimating tBHQ consumption have shown high consumers of tBHQ to substantially exceed the ADI with 7.7 mg/kg/day of tBHQ.[186] By day 3 of infection, tBHQ consumption was well below the ADI, suggesting the effects of dietary tBHQ persist with levels

below the ADI. Future work is necessary to understand whether tBHQ has long-term effects on NK cell responses, especially when tBHQ is removed from the diet prior to influenza infection.

Overall, this study demonstrates that low concentrations of the commonly used food additive tBHQ inhibits IFNγ, and to a lesser extent, FasL and CD107a, by NK cells. These data suggest that consumption of tBHQ may impact early innate immunity to influenza.

CHAPTER 5

Summary, Significance, and Future Directions

Summary of Findings

Previous studies from our lab have examined the role of tBHQ and Nrf2 activation on T cell responses. More specifically, these studies demonstrate that tBHQ inhibits CD4+ T cell activity via Nrf2-dependent and -independent mechanisms. However, investigation of the impact of the Nrf2 activator, tBHQ, remains limited in NK cells. The central hypothesis for the current studies is that the food preservative, tBHQ, impairs NK cell function through a mechanism involving the activation of the transcription factor, Nrf2. Initial studies assessed the impact of tBHQ on primary murine NK cells *in vitro.* Preliminary data showed inhibition of activation and effector function in NK cells following treatment of primary murine splenocytes with tBHQ. Importantly, PMA/ionomycin activation of NK cells in the presence of tBHQ significantly decreased production of IFNγ, granzyme B, and perforin in a concentration dependent manner. Additionally, induction of activation markers, CD69 and CD25, was significantly reduced in response to tBHQ. Maturation was also altered with tBHQ, which was demonstrated by an increase in terminally mature NK cells with 5 μM tBHQ.

As we hypothesized tBHQ would impair NK cell activation with tBHQ treatment via Nrf2-dependent mechanisms, we used a Nrf2-null mouse model to assess the role of Nrf2 in the tBHQ-mediated effects on NK cell activation. We utilized two NK cell activators in this study, including PMA/ionomycin and IL-12/IL-18. We used PMA/ionomycin to be consistent with our previous published study and IL-12/IL-18 because, in contrast to PMA/ionomycin, it is a relatively selective NK cell activator. Notably, the presence of Nrf2 promoted NK cell maturation towards the terminally mature phenotype, CD27-CD11b+, whereas the absence of Nrf2 resulted in a more immature NK cell phenotype. Furthermore, NK cell expression of the

activating markers, CD25 and CD69, was decreased via a partially Nrf2-dependent mechanism when the NK cells were activated with PMA/ionomycin, but not IL-12/IL-18. FasL expression in NK cells activated with IL-12/IL-18 was inhibited in a Nrf2-dependent manner. Similarly, IFNγ expression was also inhibited by tBHQ in NK cells through a partially Nrf2-dependent mechanism at 1 μM, but not 5 μM, tBHQ. Whereas IFNγ protein accumulation in the supernatant was diminished by tBHQ in a partially Nrf2-dependent manner in the 5 μM tBHQ group. This study demonstrates a differential role of Nrf2 activation by tBHQ on NK cell responses with two different NK cell activators.

Based on these findings, we wanted to assess whether these tBHQ-mediated effects on NK cells *in vitro* could translate into an *in vivo* model. We hypothesized that tBHQ would impair NK cell responses against influenza A infection. In our influenza model, mice were fed diets containing either no tBHQ or tBHQ, at a concentration relevant to human exposure, prior to influenza infection. NK cell responses were analyzed at days 2 and 3 after infection. At day two of influenza infection, dietary tBHQ decreased expression of IFNγ and FasL in NK cells. Additionally, induction of genes important in NK cell effector function, such as IFNγ and granzyme B, were diminished in mice fed tBHQ at day two. At day three of infection, tBHQ suppressed the expression of CD107a expression in NK cells. Furthermore, tBHQ decreased expression of a number of genes important in antiviral immunity, including IFNα, IFNγ, T-bet, and perforin. Taken together, these data suggest that exposure to a low dose of tBHQ in the diet reduces NK cell response to influenza A virus.

Significance of Findings

In the United States, influenza vaccinations have been estimated to prevent millions of illnesses and medical visits and thousands of hospitalizations and deaths each season.[8] Despite the advancements in vaccination effectiveness and coverage, the burden of disease remains high, affecting millions in the United States alone.[9] Individual factors, including demographic, genetic, and environmental, are known to influence immune responses, which provides an explanation for the wide variations in susceptibility and severity of infection.[10] Impaired immune responses often result in increased morbidity and mortality from influenza, and therefore, the identification of factors and mechanisms involved in the impairment of host immunity warrants further investigation.

tBHQ is a commonly used preservative found in foods to prevent rancidification and oxidation of fats.[185] tBHQ was first approved in the 1970's with an ADI of 0.7 mg/kg/day, which was based on toxicity findings in dogs. However, model diets estimating tBHQ consumption have found high consumers of fats, oils, and frozen fish products to exceed recommended ADI for tBHQ with levels up to 7.7 mg/kg/day.[186] Additionally, studies in healthy volunteers have shown serum levels of tBHQ reaching the high micromolar range following ingestion of 150 mg tBHQ.[191] To our knowledge, the following studies are the first to demonstrate the negative impact of dietary tBHQ on NK cell responses to influenza A virus infection. These studies agree with other experiments suggesting that NK cells are highly influenced by a variety of environmental factors, and impairment of NK cells during influenza infection may lead to decreased viral clearance and survival. For instance, in calorie-restricted mice, influenza infection resulted in higher mortality rates and decreased NK cell cytotoxicity. In addition, aged

mice put on a calorie-restricted diet had more severe mortality and morbidity rates compared to young, calorie-restricted mice and aged mice fed ad libitum.[71–73,258,259] Diet-induced obese mice also display hampered NK cell responses against influenza infection, which resulted in decreased NK cell cytotoxicity and anti-viral cytokines.[74] Accordingly, it is clear that environmental factors influence NK cell antiviral response and it is important to identify such factors that may impair NK cell function and compromise host defense to influenza. The current studies suggest tBHQ impairs NK cell responses at early timepoints in infection.

In addition to the effects of tBHQ on NK cells *in vivo*, we also gained insight on the role of Nrf2 in NK cells *in vitro.* Our lab has previously shown T cell modulation through the activation of the transcription factor, Nrf2, by tBHQ in numerous models, including primary mouse CD4+ T cells, Jurkat T cells, and primary human CD4+ T cells.[142,143,145–147] Studies from our lab and others have demonstrated the immunomodulatory effects of tBHQ through Nrf2 activation in other immune cell types.[134,139,236,237] However, the current findings are the first to elucidate the role of Nrf2 in NK cell activation in primary murine splenocytes.

Future Directions

The current studies identify the effects of tBHQ on NK cell activation and a potential role for Nrf2. Additionally, these findings suggest dietary tBHQ dampens early NK cell responses to influenza infection. However, further studies are necessary to understand the role of Nrf2 activation in NK cells and how these effects occur. The following *in vitro* studies help identify the molecular effects of tBHQ. However, the studies do not directly show Nrf2 activation in NK cells. Previous studies in T cells show increased Nrf2 DNA binding and upregulation of Nrf2 target genes.[142] The current studies could be bolstered by quantifying Nrf2 target gene

induction following tBHQ treatment in isolated NK cells derived from wild-type and Nrf2-null mice.

Additionally, NK cells are considered a rare immune population. Therefore, the *in vitro* experiments assessed NK cell population within the lymphocyte populations of the spleen, which is rich with immune cells. Future studies on the effects of tBHQ on isolated NK cells without outside stimuli from surrounding immune cells are needed. It is reported that three average sized spleens from C57Bl/6 mice are estimated to return an average of approximately 2.5×10^6 purified NK cells.[260] Thus, although large studies identifying the effects of tBHQ at various concentrations would require a large number of mice, a limited number of experiments could be performed with one or two concentrations of tBHQ to determine effects in isolated NK cells.

For the influenza experiments, tBHQ was fed to mice for the duration of the studies. It is unclear whether tBHQ has lasting effects on NK cell responses to influenza A virus infection. For example, previous study on calorie-restricted mice showed short-term refeeding to restore body weight and attenuate the decline in NK cell function following primary influenza infection.[261] Although diet consumption decreases during infection, it is unclear from these studies if tBHQ exposure is sufficient to decrease response to infection. Future studies should address whether tBHQ has long term effects or if stopping tBHQ consumption rescues these effects.

It is well established that NK cells are recruited to the lung following influenza infection.[262] However, the current studies examine NK cell responses at day two and three of infection where induction of cytokines and effector molecules is high, but NK cell lung

infiltration is minimal. Therefore, experiments examining the effects of tBHQ at later timepoints in infection are necessary to determine if tBHQ impacts NK cell recruitment to the lung.

Lastly, experiments assessing human relevance of the tBHQ-mediated effects in NK cells is needed. As previously discussed, tBHQ exposure data is limited in humans. Therefore, experts have estimated tBHQ consumption using model diets. This limitation makes identifying an epidemiological link between influenza infections and tBHQ consumption unlikely. Furthermore, tBHQ is not found on food labels. Where tBHQ is listed on labels, the amount of tBHQ is often not available. Humanized immune system mice could be an option in assessing the effects of tBHQ on human NK cell responses against influenza. Additionally, using an *in vitro* model to assess NK cell responses from PBMCS following tBHQ treatment can be performed. However, depending on the individual diet and baseline consumption of tBHQ, tBHQ-treatment may have an additive effect.

Together, the studies presented provide an outline to assess Nrf2 activation in NK cells with tBHQ-treatment and to further expand the effects of tBHQ on NK cell immune responses in the context of influenza A virus infection.

WORKS CITED

1. Samji T. Influenza A: Understanding the Viral Life Cycle. *Yale J Biol Med*. 2009;82(4):153. Accessed January 1, 2023. /pmc/articles/PMC2794490/

2. Shim JM, Kim J, Tenson T, Min JY, Kainov DE. Influenza Virus Infection, Interferon Response, Viral Counter-Response, and Apoptosis. *Viruses 2017, Vol 9, Page 223*. 2017;9(8):223. doi:10.3390/V9080223

3. Influenza (seasonal). Accessed January 3, 2023. https://www.who.int/health-topics/influenza-seasonal#tab=tab_1

4. Tokars JI, Olsen SJ, Reed C. Seasonal Incidence of Symptomatic Influenza in the United States. *Clinical Infectious Diseases*. 2018;66(10):1511-1518. doi:10.1093/cid/cix1060

5. Sellers SA, Hagan RS, Hayden FG, Fischer WA. The hidden burden of influenza: A review of the extra-pulmonary complications of influenza infection. *Influenza Other Respir Viruses*. 2017;11(5):372-393. doi:10.1111/IRV.12470

6. Lee VJ, Ho ZJM, Goh EH, et al. Advances in measuring influenza burden of disease. *Influenza Other Respir Viruses*. 2018;12(1):3-9. doi:10.1111/IRV.12533

7. Paget J, Spreeuwenberg P, Charu V, et al. Global mortality associated with seasonal influenza epidemics: New burden estimates and predictors from the GLaMOR Project. *J Glob Health*. 2019;9(2). doi:10.7189/JOGH.09.020421

8. Rolfes MA, Foppa IM, Garg S, et al. Annual estimates of the burden of seasonal influenza in the United States: A tool for strengthening influenza surveillance and preparedness. *Influenza Other Respir Viruses*. 2018;12(1):132-137. doi:10.1111/irv.12486

9. Services USD of H& H. Estimated Influenza Illnesses, Medical visits, Hospitalizations, and Deaths in the United States- 2018-19 influenza season. Centers for Diease Control and Prevention. Published 2020. Accessed May 26, 2020. https://www.cdc.gov/flu/about/burden/2018-2019.html

10. Clohisey S, Baillie JK. Host susceptibility to severe influenza A virus infection. *Crit Care*. 2019;23(1). doi:10.1186/s13054-019-2566-7

11. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat Immunol*. 2008;9(5):503-510. doi:10.1038/ni1582

12. Vivier E, Nunès JA, Vély F. Natural Killer Cell Signaling Pathways. *Science (1979)*. 2004;306(5701):1517-1519. doi:10.1126/SCIENCE.1103478

13. Lanier LL. NK CELL RECOGNITION. *Annu Rev Immunol*. 2005;23:225-274. doi:10.1146/annurev.immunol.23.021704.115526

14. Sivori S, Falco M, della Chiesa M, et al. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: Induction of cytokine release and cytotoxicity against tumors and dendritic cells. *Proceedings of the National Academy of Sciences*. 2004;101(27):10116-10121. doi:10.1073/PNAS.0403744101

15. Johansson MH, Bieberich C, Jay G, Kärre K, Höglund P. Natural Killer Cell Tolerance in Mice with Mosaic Expression of Major Histocompatibility Complex Class I Transgene. *Journal of Experimental Medicine*. 1997;186(3):353-364. doi:10.1084/JEM.186.3.353

16. Kumar S. Natural killer cell cytotoxicity and its regulation by inhibitory receptors. *Immunology*. http://doi.wiley.com/10.1111/imm.12921. Published July 2018.

17. Long EO. Ready for Prime Time: NK Cell Priming by Dendritic Cells. *Immunity*. 2007;26(4):385-387. doi:10.1016/J.IMMUNI.2007.04.001

18. Laouar Y, Sutterwala FS, Gorelik L, Flavell RA. Transforming growth factor-β controls T helper type 1 cell development through regulation of natural killer cell interferon-γ. *Nature Immunology 2005 6:6*. 2005;6(6):600-607. doi:10.1038/ni1197

19. Townsend MJ, Weinmann AS, Matsuda JL, et al. T-bet Regulates the Terminal Maturation and Homeostasis of NK and Vα14i NKT Cells. *Immunity*. 2004;20(4):477-494. doi:10.1016/S1074-7613(04)00076-7

20. van Helden MJ, Goossens S, Daussy C, et al. Terminal NK cell maturation is controlled by concerted actions of T-bet and Zeb2 and is essential for melanoma rejection. *Journal of Experimental Medicine*. 2015;212(12):2015-2025. doi:10.1084/JEM.20150809

21. Chiossone L, Chaix J, Fuseri N, Roth C, Vivier E, Walzer T. Maturation of mouse NK cells is a 4-stage developmental program. *Blood*. 2009;113(22):5488-5496. doi:10.1182/blood-2008-10-187179

22. Pesce S, Squillario M, Greppi M, et al. New miRNA signature heralds human NK cell subsets at different maturation steps: Involvement of miR-146a-5p in the regulation of KIR expression. *Front Immunol*. 2018;9(OCT):2360. doi:10.3389/FIMMU.2018.02360/BIBTEX

23. Romagnani C, Juelke K, Falco M, et al. CD56brightCD16− Killer Ig-Like Receptor− NK Cells Display Longer Telomeres and Acquire Features of CD56dim NK Cells upon Activation. *The Journal of Immunology*. 2007;178(8):4947-4955. doi:10.4049/JIMMUNOL.178.8.4947

24. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol*. 2001;22(11):633-640. doi:10.1016/S1471-4906(01)02060-9

25. di Vito C, Mikulak J, Mavilio D. On the way to become a natural killer cell. *Front Immunol*. 2019;10:1812. doi:10.3389/FIMMU.2019.01812/BIBTEX

26. Boos MD, Yokota Y, Eberl G, Kee BL. Mature natural killer cell and lymphoid tissue–inducing cell development requires Id2-mediated suppression of E protein activity. *Journal of Experimental Medicine*. 2007;204(5):1119-1130. doi:10.1084/JEM.20061959

27. Vosshenrich CAJ, Ranson T, Samson SI, et al. Roles for Common Cytokine Receptor γ-Chain-Dependent Cytokines in the Generation, Differentiation, and Maturation of NK Cell Precursors and Peripheral NK Cells in Vivo. *The Journal of Immunology*. 2005;174(3):1213-1221. doi:10.4049/JIMMUNOL.174.3.1213

28. Kennedy MK, Glaccum M, Brown SN, et al. Reversible Defects in Natural Killer and Memory Cd8 T Cell Lineages in Interleukin 15–Deficient Mice. *Journal of Experimental Medicine*. 2000;191(5):771-780. doi:10.1084/JEM.191.5.771

29. Lodolce JP, Boone DL, Chai S, et al. IL-15 Receptor Maintains Lymphoid Homeostasis by Supporting Lymphocyte Homing and Proliferation. *Immunity*. 1998;9(5):669-676. doi:10.1016/S1074-7613(00)80664-0

30. Gordon SM, Chaix J, Rupp LJ, et al. The Transcription Factors T-bet and Eomes Control Key Checkpoints of Natural Killer Cell Maturation. *Immunity*. 2012;36(1):55-67. doi:10.1016/J.IMMUNI.2011.11.016

31. Brillard E, Pallandre JR, Chalmers D, et al. Natural killer cells prevent CD28-mediated Foxp3 transcription in CD4+CD25- T lymphocytes. *Exp Hematol*. 2007;35(3):416-425. doi:10.1016/j.exphem.2006.12.004

32. Jinushi M, Takehara T, Tatsumi T, et al. Natural killer cell and hepatic cell interaction via NKG2A leads to dendritic cell-mediated induction of CD4+ CD25+ T cells with PD-1-dependent regulatory activities. *Immunology*. 2007;120(1):73-82. doi:10.1111/J.1365-2567.2006.02479.X

33. Ferlazzo G, Morandi B, D'Agostino A, et al. The interaction between NK cells and dendritic cells in bacterial infections results in rapid induction of NK cell activation and in the lysis of uninfected dendritic cells. *Eur J Immunol*. 2003;33(2):306-313. doi:10.1002/IMMU.200310004

34. Paust S, Senman B, von Andrian UH. Adaptive immune responses mediated by natural killer cells. *Immunol Rev*. 2010;235(1):286-296. doi:10.1111/j.0105-2896.2010.00906.x

35. della Chiesa M, Vitale M, Carlomagno S, Ferlazzo G, Moretta L, Moretta A. The natural killer cell-mediated killing of autologous dendritic cells is confined to a cell subset expressing CD94/NKG2A, but lacking inhibitory killer Ig-like receptors. *Eur J Immunol*. 2003;33(6):1657-1666. doi:10.1002/EJI.200323986

36. Piccioli D, Sbrana S, Melandri E, Valiante NM. Contact-dependent Stimulation and Inhibition of Dendritic Cells by Natural Killer Cells. *Journal of Experimental Medicine*. 2002;195(3):335-341. doi:10.1084/JEM.20010934

37. Carbone E, Terrazzano G, Ruggiero G, et al. Recognition of autologous dendritic cells by human NK cells. doi:10.1002/(SICI)1521-4141(199912)29:12

38. Lünemann A, Lünemann JD, Roberts S, et al. Human NK Cells Kill Resting but Not Activated Microglia via NKG2D- and NKp46-Mediated Recognition. *The Journal of Immunology*. 2008;181(9):6170-6177. doi:10.4049/JIMMUNOL.181.9.6170

39. Gao N, Dang T, Yuan D. IFN-γ-Dependent and -Independent Initiation of Switch Recombination by NK Cells. *The Journal of Immunology*. 2001;167(4):2011-2018. doi:10.4049/JIMMUNOL.167.4.2011

40. Martín-Fontecha A, Thomsen LL, Brett S, et al. Induced recruitment of NK cells to lymph nodes provides IFN-γ for TH1 priming. *Nature Immunology 2004 5:12*. 2004;5(12):1260-1265. doi:10.1038/ni1138

41. Morandi B, Bougras G, Muller WA, Ferlazzo G, Münz C. NK cells of human secondary lymphoid tissues enhance T cell polarization via IFN-γ secretion. *Eur J Immunol*. 2006;36(9):2394-2400. doi:10.1002/EJI.200636290

42. Lu L, Ikizawa K, Hu D, Werneck MBF, Wucherpfennig KW, Cantor H. Regulation of Activated CD4+ T Cells by NK Cells via the Qa-1–NKG2A Inhibitory Pathway. *Immunity*. 2007;26(5):593-604. doi:10.1016/J.IMMUNI.2007.03.017

43. Maroof A, Beattie L, Zubairi S, Svensson M, Stager S, Kaye PM. Posttranscriptional Regulation of Il10 Gene Expression Allows Natural Killer Cells to Express Immunoregulatory Function. *Immunity*. 2008;29(2):295-305. doi:10.1016/J.IMMUNI.2008.06.012

44. Takahashi K, Aranami T, Endoh M, Miyake S, Yamamura T. The regulatory role of natural killer cells in multiple sclerosis. *Brain*. 2004;127(9):1917-1927. doi:10.1093/BRAIN/AWH219

45. Loza MJ, Zamai L, Azzoni L, Rosati E, Perussia B. Expression of type 1 (interferon gamma) and type 2 (interleukin-13, interleukin-5) cytokines at distinct stages of natural killer cell differentiation from progenitor cells. *Blood*. 2002;99(4):1273-1281. doi:10.1182/BLOOD.V99.4.1273

46. Takahashi K, Miyake S, Kondo T, et al. Natural killer type 2 bias in remission of multiple sclerosis. *J Clin Invest*. 2001;107(5):R23-R29. doi:10.1172/JCI11819

47. Johansson S, Berg L, Hall H, Höglund P. NK cells: elusive players in autoimmunity. *Trends Immunol*. 2005;26(11):613-618. doi:10.1016/J.IT.2005.08.008

48. Vivier E, Spits H, Cupedo T. Interleukin-22-producing innate immune cells: new players in mucosal immunity and tissue repair? *Nature Reviews Immunology 2009 9:4*. 2009;9(4):229-234. doi:10.1038/nri2522

49. Colonna M. Interleukin-22-Producing Natural Killer Cells and Lymphoid Tissue Inducer-like Cells in Mucosal Immunity. *Immunity*. 2009;31(1):15-23. doi:10.1016/J.IMMUNI.2009.06.008

50. Stein-Streilein J, Bennett M, Mann D, Kumar V. Natural killer cells in mouse lung: surface phenotype, target preference, and response to local influenza virus infection. *J Immunol*. 1983;131(6):2699-2704. Accessed February 10, 2021. http://www.ncbi.nlm.nih.gov/pubmed/6644021

51. Biron CA, Byron KS, Sullivan JL. Severe Herpesvirus Infections in an Adolescent without Natural Killer Cells. *New England Journal of Medicine*. 1989;320(26):1731-1735. doi:10.1056/NEJM198906293202605

52. Culley FJ. Natural killer cells in infection and inflammation of the lung. *Immunology*. 2009;128(2):151-163. doi:10.1111/J.1365-2567.2009.03167.X

53. Carlin LE, Hemann EA, Zacharias ZR, Heusel JW, Legge KL. Natural killer cell recruitment to the lung during influenza A virus infection is dependent on CXCR3, CCR5, and virus exposure dose. *Front Immunol*. 2018;9(APR):781. doi:10.3389/FIMMU.2018.00781/BIBTEX

54. Brandstadter JD, Yang Y. Natural killer cell responses to viral infection. *J Innate Immun*. Published online 2011. doi:10.1159/000324176

55. Bartel Y, Bauer B, Steinle A. Modulation of NK cell function by genetically coupled C-type lectin-like receptor/ligand pairs encoded in the human natural killer gene complex. *Front Immunol*. 2013;4(NOV):362. doi:10.3389/FIMMU.2013.00362/BIBTEX

56. Biassoni R, Cantoni C, Pende D, et al. Human natural killer cell receptors. Published online 2001. doi:10.1034/j.1600-065X.2001.1810117.x

57. Walzer T, Bléry M, Chaix J, et al. Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. *Proc Natl Acad Sci U S A*. 2007;104(9):3384-3389. doi:10.1073/PNAS.0609692104/SUPPL_FILE/09692FIG12.PDF

58. Jost S, Altfeld M. Control of Human Viral Infections by Natural Killer Cells. doi:10.1146/annurev-immunol-032712-100001

59. Ochoa MC, Minute L, Rodriguez I, et al. Antibody-dependent cell cytotoxicity: immunotherapy strategies enhancing effector NK cells. *Immunol Cell Biol*. 2017;95(4):347-355. doi:10.1038/ICB.2017.6

60. Sirén J, Sareneva T, Pirhonen J, et al. Cytokine and contact-dependent activation of natural killer cells by influenza A or Sendai virus-infected macrophages. *Journal of General Virology*. 2004;85(8):2357-2364. doi:10.1099/VIR.0.80105-0/CITE/REFWORKS

61. Fauriat C, Long EO, Ljunggren HG, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood*. 2010;115(11):2167-2176. doi:10.1182/BLOOD-2009-08-238469

62. Wu Y, Tian Z, Wei H. Developmental and functional control of natural killer cells by cytokines. *Front Immunol*. 2017;8(AUG):930. doi:10.3389/FIMMU.2017.00930/BIBTEX

63. Nogusa S, Ritz BW, Kassim SH, Jennings SR, Gardner EM. Characterization of age-related changes in natural killer cells during primary influenza infection in mice. *Mech Ageing Dev*. 2008;129(4):223-230. doi:10.1016/j.mad.2008.01.003

64. Stein-Streilein J, Guffee J, Fan W. Locally and systemically derived natural killer cells participate in defense against intranasally inoculated influenza virus. *Reg Immunol*. 1988;1(2):100-105. Accessed January 10, 2023. https://europepmc.org/article/med/3275212

65. O'Brien KB, Schultz-Cherry S, Knoll LJ. Parasite-Mediated Upregulation of NK Cell-Derived Gamma Interferon Protects against Severe Highly Pathogenic H5N1 Influenza Virus Infection. *J Virol*. 2011;85(17):8680-8688. doi:10.1128/JVI.05142-11/ASSET/3F0DB25D-00F6-4972-BE0E-076156CD9031/ASSETS/GRAPHIC/ZJV9990949750007.JPEG

66. Stein-Streilein2*' And J, Guffeei J. In vivo treatment of mice and hamsters with antibodies to asialo GM1 increases morbidity and mortality to pulmonary influenza infection. *The Journal of Immunology*. 1986;136(4):1435-1441. doi:10.4049/JIMMUNOL.136.4.1435

67. Liu B, Mori I, Hossain MJ, Dong L, Takeda K, Kimura Y. Interleukin-18 improves the early defence system against influenza virus infection by augmenting natural killer cell-mediated cytotoxicity. *Journal of General Virology*. 2004;85(2):423-428. doi:10.1099/VIR.0.19596-0/CITE/REFWORKS

68. Dong L, Mori I, Hossain MJ, Kimura Y. The Senescence-Accelerated Mouse Shows Aging-Related Defects in Cellular but Not Humoral Immunity against Influenza Virus Infection. *J Infect Dis*. 2000;182(2):391-396. doi:10.1086/315727

69. Zhou G, Juang SWW, Kane KP, Juang WW, Kane KP. NK cells exacerbate the pathology of influenza virus infection in mice. *Eur J Immunol*. 2013;43(4):929-938. doi:10.1002/eji.201242620

70. Abdul-Careem MF, Mian MF, Yue G, et al. Critical Role of Natural Killer Cells in Lung Immunopathology During Influenza Infection in Mice. *J Infect Dis*. 2012;206(2):167-177. doi:10.1093/infdis/jis340

71. Beli E, Clinthorne JF, Duriancik DM, Hwang Ii, Kim S, Gardner EM. Natural killer cell function is altered during the primary response of aged mice to influenza infection. *Mech Ageing Dev*. 2011;132(10):503-510. doi:10.1016/j.mad.2011.08.005

72. Gardner EM. Caloric restriction decreases survival of aged mice in response to primary influenza infection. *J Gerontol A Biol Sci Med Sci*. 2005;60(6):688-694. doi:10.1093/gerona/60.6.688

73. Ritz BW, Aktan I, Nogusa S, Gardner EM. Energy Restriction Impairs Natural Killer Cell Function and Increases the Severity of Influenza Infection in Young Adult Male C57BL/6 Mice. *J Nutr*. 2008;138(11):2269-2275. doi:10.3945/jn.108.093633

74. Smith AG, Sheridan PA, Harp JB, Beck MA. Diet-induced obese mice have increased mortality and altered immune responses when infected with influenza virus. *J Nutr*. 2007;137(5):1236-1243. doi:10.1093/jn/137.5.1236

75. Ritz BW, Nogusa S, Ackerman EA, Gardner EM. Supplementation with Active Hexose Correlated Compound Increases the Innate Immune Response of Young Mice to Primary Influenza Infection. *J Nutr*. 2006;136(11):2868-2873. doi:10.1093/jn/136.11.2868

76. Heuser G, Vojdani A. Enhancement of Natural Killer Cell Activity and T and B Cell Function by Buffered Vitamin C In Patients Exposed to Toxic Chemicals: The Role of Protein Kinase-C. *Immunopharmacol Immunotoxicol*. 1997;19(3):291-312. doi:10.3109/08923979709046977

77. Zhang H, Meadows GG. Chronic alcohol consumption perturbs the balance between thymus-derived and bone marrow-derived natural killer cells in the spleen. *J Leukoc Biol*. 2008;83(1):41-47. doi:10.1189/jlb.0707472

78. Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proceedings of the National Academy of Sciences*. 1994;91(21):9926-9930. doi:10.1073/PNAS.91.21.9926

79. Chan K, Lu R, Chang JC, Kan YW. NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proc Natl Acad Sci U S A*. 1996;93(24):13943-13948. doi:10.1073/PNAS.93.24.13943/ASSET/72DF3EAC-94D6-49F7-B696-4D9B3994B202/ASSETS/GRAPHIC/PQ2262554006.JPEG

80. Lacher SE, Lee JS, Wang X, Campbell MR, Bell DA, Slattery M. Beyond antioxidant genes in the ancient Nrf2 regulatory network. *Free Radic Biol Med*. 2015;88(Part B):452-465. doi:10.1016/J.FREERADBIOMED.2015.06.044

81. Venugopal R, Jaiswal AK. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. *Proceedings of the National Academy of Sciences*. 1996;93(25):14960-14965. doi:10.1073/pnas.93.25.14960

82. Itoh K, Chiba T, Takahashi S, et al. An Nrf2/Small Maf Heterodimer Mediates the Induction of Phase II Detoxifying Enzyme Genes through Antioxidant Response Elements. *Biochem Biophys Res Commun*. 1997;236(2):313-322. doi:10.1006/BBRC.1997.6943

83. Itoh K, Igarashi K, Hayashi N, Nishizawa M, Yamamoto M. Cloning and characterization of a novel erythroid cell-derived CNC family transcription factor heterodimerizing with the small Maf family proteins. *Mol Cell Biol*. 1995;15(8):4184-4193. doi:10.1128/MCB.15.8.4184

84. Katsuoka F, Otsuki A, Takahashi M, Ito S, Yamamoto M. Direct and Specific Functional Evaluation of the Nrf2 and MafG Heterodimer by Introducing a Tethered Dimer into Small Maf-Deficient Cells. *Mol Cell Biol*. 2019;39(20). doi:10.1128/MCB.00273-19/ASSET/8111348D-6F06-4B49-B7E0-0375C64989EA/ASSETS/GRAPHIC/MCB.00273-19-F0008.JPEG

85. Wasserman WW, Fahl WE. Functional antioxidant responsive elements. *Proc Natl Acad Sci U S A*. 1997;94(10):5361. doi:10.1073/PNAS.94.10.5361

86. Venugopal R, Jaiswal AK. Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. *Oncogene*. 1998;17(24):3145-3156. doi:10.1038/sj.onc.1202237

87. Iso T, Suzuki T, Baird L, Yamamoto M. Absolute Amounts and Status of the Nrf2-Keap1-Cul3 Complex within Cells. *Mol Cell Biol*. 2016;36(24):3100-3112. doi:10.1128/MCB.00389-16/ASSET/CBC66FFE-8ECF-49F5-B921-47E634AF43CA/ASSETS/GRAPHIC/ZMB9991013680011.JPEG

88. Ramos-Gomez M, Kwak MK, Dolan PM, et al. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proceedings of the National Academy of Sciences*. 2001;98(6):3410-3415. doi:10.1073/PNAS.051618798

89. Aoki Y, Sato H, Nishimura N, Takahashi S, Itoh K, Yamamoto M. Accelerated DNA Adduct Formation in the Lung of the Nrf2 Knockout Mouse Exposed to Diesel Exhaust. *Toxicol Appl Pharmacol*. 2001;173(3):154-160. doi:10.1006/TAAP.2001.9176

90. Enomoto A, Itoh K, Nagayoshi E, et al. High Sensitivity of Nrf2 Knockout Mice to Acetaminophen Hepatotoxicity Associated with Decreased Expression of ARE-Regulated Drug Metabolizing Enzymes and Antioxidant Genes. *Toxicological Sciences*. 2001;59(1):169-177. doi:10.1093/TOXSCI/59.1.169

91. Chan K, Kan YW. Nrf2 is essential for protection against acute pulmonary injury in mice. *Proceedings of the National Academy of Sciences*. 1999;96(22):12731-12736. doi:10.1073/PNAS.96.22.12731

92. Yamamoto T, Yoh K, Kobayashi A, et al. Identification of polymorphisms in the promoter region of the human NRF2 gene. *Biochem Biophys Res Commun*. 2004;321(1):72-79. doi:10.1016/J.BBRC.2004.06.112

93. McMahon M, Itoh K, Yamamoto M, Hayes JD. Keap1-dependent Proteasomal Degradation of Transcription Factor Nrf2 Contributes to the Negative Regulation of Antioxidant Response Element-driven Gene Expression *. *Journal of Biological Chemistry*. 2003;278(24):21592-21600. doi:10.1074/JBC.M300931200

94. Kobayashi A, Kang MI, Okawa H, et al. Oxidative Stress Sensor Keap1 Functions as an Adaptor for Cul3-Based E3 Ligase To Regulate Proteasomal Degradation of Nrf2. *Mol Cell Biol*. 2004;24(16):7130-7139. doi:10.1128/MCB.24.16.7130-7139.2004/ASSET/8F5A37B3-36B6-4F80-B503-123613A07590/ASSETS/GRAPHIC/ZMB0160443020008.JPEG

95. McMahon M, Thomas N, Itoh K, Yamamoto M, Hayes JD. Dimerization of substrate adaptors can facilitate Cullin-mediated ubiquitylation of proteins by a “tethering” mechanism: A two-site interaction model for the Nrf2-Keap1 complex. *Journal of Biological Chemistry*. 2006;281(34):24756-24768. doi:10.1074/JBC.M601119200/ATTACHMENT/AE22B8B3-C033-402D-917C-DC8068556086/MMC1.ZIP

96. Furukawa M, Xiong Y. BTB Protein Keap1 Targets Antioxidant Transcription Factor Nrf2 for Ubiquitination by the Cullin 3-Roc1 Ligase. *Mol Cell Biol*. 2005;25(1):162-171. doi:10.1128/MCB.25.1.162-171.2005/ASSET/847416E3-DA3A-4447-B240-769CDADA338B/ASSETS/GRAPHIC/ZMB0010546710007.JPEG

97. McMahon M, Thomas N, Itoh K, Yamamoto M, Hayes JD. Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron. *Journal of Biological Chemistry*. 2004;279(30):31556-31567. doi:10.1074/jbc.M403061200

98. Stewart D, Killeen E, Naquin R, Alam S, Alam J. Degradation of Transcription Factor Nrf2 via the Ubiquitin-Proteasome Pathway and Stabilization by Cadmium *. *Journal of Biological Chemistry*. 2003;278(4):2396-2402. Accessed January 11, 2023. http://www.jbc.org/article/S0021925819309986/fulltext

99. Itoh K, Wakabayashi N, Katoh Y, et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev*. 1999;13(1):76-86. doi:10.1101/GAD.13.1.76

100. Dinkova-Kostova AT, Holtzclaw WD, Cole RN, et al. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc Natl Acad Sci U S A*. 2002;99(18):11908-11913. doi:10.1073/PNAS.172398899/ASSET/A31A9E07-FB8C-458E-A989-E1680946A68A/ASSETS/GRAPHIC/PQ1723988008.JPEG

101. Tong KI, Kobayashi A, Katsuoka F, Yamamoto M. Two-site substrate recognition model for the Keap1-Nrf2 system: A hinge and latch mechanism. *Biol Chem*. 2006;387(10-11):1311-1320. doi:10.1515/BC.2006.164/MACHINEREADABLECITATION/RIS

102. Tong KI, Padmanabhan B, Kobayashi A, et al. Different Electrostatic Potentials Define ETGE and DLG Motifs as Hinge and Latch in Oxidative Stress Response. *Mol Cell Biol*. 2007;27(21):7511-7521. doi:10.1128/MCB.00753-07/ASSET/21139396-E48F-4379-8033-6FA62EA4525C/ASSETS/GRAPHIC/ZMB0210770740005.JPEG

103. Baird L, Llères D, Swift S, Dinkova-Kostova AT. Regulatory flexibility in the Nrf2-mediated stress response is conferred by conformational cycling of the Keap1-Nrf2 protein complex. *Proc Natl Acad Sci U S A*. 2013;110(38):15259-15264. doi:10.1073/PNAS.1305687110/-/DCSUPPLEMENTAL

104. Liu Y, Kern JT, Walker JR, Johnson JA, Schultz PG, Luesch H. A genomic screen for activators of the antioxidant response element. *Proc Natl Acad Sci U S A*. 2007;104(12):5205-5210. doi:10.1073/PNAS.0700898104/SUPPL_FILE/00898FIG7.JPG

105. Fan W, Tang Z, Chen D, et al. Keap1 facilitates p62-mediated ubiquitin aggregate clearance via autophagy. *http://dx.doi.org/104161/auto6512189*. 2010;6(5):614-621. doi:10.4161/AUTO.6.5.12189

106. Lau A, Wang XJ, Zhao F, et al. A Noncanonical Mechanism of Nrf2 Activation by Autophagy Deficiency: Direct Interaction between Keap1 and p62. *Mol Cell Biol*. 2010;30(13):3275-3285. doi:10.1128/MCB.00248-10/ASSET/F8BD7F3D-F1D1-4B45-B0CD-C8ED45012A1E/ASSETS/GRAPHIC/ZMB9991086610005.JPEG

107. Huang HC, Nguyen T, Pickett CB. Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *Journal of Biological Chemistry*. 2002;277(45):42769-42774. doi:10.1074/jbc.M206911200

108. Huang HC, Nguyen T, Pickett CB. Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2. *Proc Natl Acad Sci U S A*. 2000;97(23):12475-12480. doi:10.1073/PNAS.220418997/ASSET/39AE8301-391D-421F-9E6E-5CB4D9FC38E0/ASSETS/GRAPHIC/PQ2204189006.JPEG

109. Apopa PL, He X, Ma Q. Phosphorylation of Nrf2 in the transcription activation domain by casein kinase 2 (CK2) is critical for the nuclear translocation and transcription activation function of Nrf2 in IMR-32 neuroblastoma cells. *J Biochem Mol Toxicol*. 2008;22(1):63-76. doi:10.1002/JBT.20212

110. Pi J, Bai Y, Reece JM, et al. Molecular mechanism of human Nrf2 activation and degradation: Role of sequential phosphorylation by protein kinase CK2. *Free Radic Biol Med*. 2007;42(12):1797-1806. doi:10.1016/J.FREERADBIOMED.2007.03.001

111. Kawai Y, Garduño LK, Theodore M, Yang J, Arinze IJ. Acetylation-deacetylation of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) regulates its transcriptional activity and nucleocytoplasmic localization. *Journal of Biological Chemistry*. 2011;286(9):7629-7640. doi:10.1074/jbc.M110.208173

112. Zimmermann K, Baldinger J, Mayerhofer B, Atanasov AG, Dirsch VM, Heiss EH. Activated AMPK boosts the Nrf2/HO-1 signaling axis—A role for the unfolded protein response. *Free Radic Biol Med*. 2015;88(Part B):417-426. doi:10.1016/J.FREERADBIOMED.2015.03.030

113. Holtzclaw WD, Dinkova-Kostova AT, Talalay P. Protection against electrophile and oxidative stress by induction of phase 2 genes: the quest for the elusive sensor that responds to inducers. *Adv Enzyme Regul*. 2004;44(1):335-367. doi:10.1016/J.ADVENZREG.2003.11.013

114. Kobayashi M, Li L, Iwamoto N, et al. The Antioxidant Defense System Keap1-Nrf2 Comprises a Multiple Sensing Mechanism for Responding to a Wide Range of Chemical Compounds. *Mol Cell Biol*. 2009;29(2):493-502. doi:10.1128/MCB.01080-08/SUPPL_FILE/SF4.PDF

115. Takaya K, Suzuki T, Motohashi H, et al. Validation of the multiple sensor mechanism of the Keap1-Nrf2 system. *Free Radic Biol Med*. 2012;53(4):817-827. doi:10.1016/J.FREERADBIOMED.2012.06.023

116. Dinkova-Kostova AT, Holtzclaw WD, Wakabayashi N. Keap1, the sensor for electrophiles and oxidants that regulates the phase 2 response, is a zinc metalloprotein. *Biochemistry*. 2005;44(18):6889-6899. doi:10.1021/BI047434H/ASSET/IMAGES/LARGE/BI047434HH00002.JPEG

117. Wang XJ, Sun Z, Chen W, Li Y, Villeneuve NF, Zhang DD. Activation of Nrf2 by arsenite and monomethylarsonous acid is independent of Keap1-C151: enhanced Keap1–Cul3 interaction. *Toxicol Appl Pharmacol*. 2008;230(3):383-389. doi:10.1016/J.TAAP.2008.03.003

118. Pi J, Qu W, Reece JM, Kumagai Y, Waalkes MP. Transcription factor Nrf2 activation by inorganic arsenic in cultured keratinocytes: involvement of hydrogen peroxide. *Exp Cell Res*. 2003;290(2):234-245. doi:10.1016/S0014-4827(03)00341-0

119. Saito R, Suzuki T, Hiramoto K, et al. Characterizations of Three Major Cysteine Sensors of Keap1 in Stress Response. *Mol Cell Biol*. 2016;36(2):271-284. doi:10.1128/MCB.00868-15/ASSET/F2FEEF79-205C-4D68-B1E6-5BB09D32CED7/ASSETS/GRAPHIC/ZMB9991010850010.JPEG

120. Wang XJ, Hayes JD, Higgins LG, Wolf CR, Dinkova-Kostova AT. Activation of the NRF2 Signaling Pathway by Copper-Mediated Redox Cycling of Para- and Ortho-Hydroquinones. *Chem Biol*. 2010;17(1):75-85. doi:10.1016/J.CHEMBIOL.2009.12.013

121. Imhoff BR, Hansen JM. Tert-butylhydroquinone induces mitochondrial oxidative stress causing Nrf2 activation. *Cell Biol Toxicol*. 2010;26(6):541-551. doi:10.1007/S10565-010-9162-6/FIGURES/7

122. Kobayashi EH, Suzuki T, Funayama R, et al. Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nat Commun*. 2016;7. doi:10.1038/NCOMMS11624

123. Feng R, Morine Y, Ikemoto T, et al. Nrf2 activation drive macrophages polarization and cancer cell epithelial-mesenchymal transition during interaction. *Cell Communication and Signaling*. 2018;16(1):1-12. doi:10.1186/S12964-018-0262-X/FIGURES/6

124. Luo D, Guo Y, Cheng Y, Zhao J, Wang Y, Rong J. Natural product celastrol suppressed macrophage M1 polarization against inflammation in diet-induced obese mice via regulating Nrf2/HO-1, MAP kinase and NF-κB pathways. *Aging (Albany NY)*. 2017;9(10):2069. doi:10.18632/AGING.101302

125. Li J, Wang H, Zheng Z, et al. Mkp-1 cross-talks with Nrf2/Ho-1 pathway protecting against intestinal inflammation. *Free Radic Biol Med*. 2018;124:541-549. doi:10.1016/J.FREERADBIOMED.2018.07.002

126. Wenzel P, Rossmann H, Müller C, et al. Heme oxygenase-1 suppresses a pro-inflammatory phenotype in monocytes and determines endothelial function and arterial hypertension in mice and humans. *Eur Heart J*. 2015;36(48):3437-3446. doi:10.1093/EURHEARTJ/EHV544

127. Harvey CJ, Thimmulappa RK, Sethi S, et al. Targeting Nrf2 Signaling Improves Bacterial Clearance by Alveolar Macrophages in Patients with COPD and in a Mouse Model. Accessed January 11, 2023. https://www.science.org

128. Reddy NM, Suryanarayana V, Kalvakolanu D v., et al. Innate Immunity against Bacterial Infection following Hyperoxia Exposure Is Impaired in NRF2-Deficient Mice. *The Journal of Immunology*. 2009;183(7):4601-4608. doi:10.4049/JIMMUNOL.0901754

129. Wang Z, Sun D, Chen G, et al. Tim-3 inhibits macrophage control of Listeria monocytogenes by inhibiting Nrf2. *Scientific Reports 2017 7:1*. 2017;7(1):1-14. doi:10.1038/srep42095

130. Williams MA, Rangasamy T, Bauer SM, et al. Disruption of the Transcription Factor Nrf2 Promotes Pro-Oxidative Dendritic Cells That Stimulate Th2-Like Immunoresponsiveness upon Activation by Ambient Particulate Matter. *The Journal of Immunology*. 2008;181(7):4545-4559. doi:10.4049/JIMMUNOL.181.7.4545

131. Yeang HXA, Hamdam JM, Al-Huseini LMA, et al. Loss of transcription factor nuclear factor-erythroid 2 (NF-E2) p45-related factor-2 (Nrf2) leads to dysregulation of immune functions, redox homeostasis,and intracellular signaling in dendritic cells. *Journal of Biological Chemistry*. 2012;287(13):10556-10564. doi:10.1074/jbc.M111.322420

132. Li N, Wang M, Barajas B, Sioutas C, Williams MA, Nel AE. Nrf2 Deficiency in Dendritic Cells Enhances the Adjuvant Effect of Ambient Ultrafine Particles on Allergic Sensitization. *J Innate Immun*. 2013;5(6):543-554. doi:10.1159/000347060

133. Rangasamy T, Williams MA, Bauer S, et al. Nuclear Erythroid 2 p45-Related Factor 2 Inhibits the Maturation of Murine Dendritic Cells by Ragweed Extract. *https://doi.org/101165/rcmb2008-0438OC*. 2012;43(3):276-285. doi:10.1165/RCMB.2008-0438OC

134. Macoch M, Morzadec C, Génard R, et al. Nrf2-dependent repression of interleukin-12 expression in human dendritic cells exposed to inorganic arsenic. *Free Radic Biol Med*. 2015;88(Part B):381-390. doi:10.1016/J.FREERADBIOMED.2015.02.003

135. Saddawi-Konefka R, Seelige R, Gross ETE, et al. Nrf2 Induces IL-17D to Mediate Tumor and Virus Surveillance. *Cell Rep*. 2016;16(9):2348-2358. doi:10.1016/j.celrep.2016.07.075

136. Kumar P, Rajasekaran K, Nanbakhsh A, Gorski J, Thakar MS, Malarkannan S. IL-27 promotes NK cell effector functions via Maf-Nrf2 pathway during influenza infection. *Sci Rep*. 2019;9(1):4984. doi:10.1038/s41598-019-41478-6

137. Boss AP, Freeborn RA, Duriancik DM, Kennedy RC, Gardner EM, Rockwell CE. The Nrf2 activator tBHQ inhibits the activation of primary murine natural killer cells. *Food and Chemical Toxicology*. 2018;121:231-236. doi:https://doi.org/10.1016/j.fct.2018.08.067

138. Pyaram K, Kumar A, Kim YH, et al. Keap1-Nrf2 System Plays an Important Role in Invariant Natural Killer T Cell Development and Homeostasis. *Cell Rep*. 2019;27(3):699-707.e4. doi:10.1016/j.celrep.2019.03.052

139. Bursley JK, Rockwell CE. Nrf2-dependent and -independent effects of tBHQ in activated murine B cells. *Food and Chemical Toxicology*. Published online July 2020:111595. doi:10.1016/j.fct.2020.111595

140. Lugade AA, Vethanayagam RR, Nasirikenari M, Bogner PN, Segal BH, Thanavala Y. Nrf2 Regulates Chronic Lung Inflammation and B-Cell Responses to Nontypeable Haemophilus influenzae. *https://doi.org/101165/rcmb2010-0321OC*. 2012;45(3):557-565. doi:10.1165/RCMB.2010-0321OC

141. Kikuchi N, Ishii Y, Morishima Y, et al. Nrf2 protects against pulmonary fibrosis by regulating the lung oxidant level and Th1/Th2 balance. *Respir Res*. 2010;11(1):1-12. doi:10.1186/1465-9921-11-31/FIGURES/6_874

142. Rockwell CE, Zhang M, Fields PE, Klaassen CD. Th2 Skewing by Activation of Nrf2 in CD4+ T Cells. *The Journal of Immunology*. 2012;188(4):1630 LP - 1637. doi:10.4049/jimmunol.1101712

143. Turley AE, Zagorski JW, Rockwell CE. The Nrf2 activator tBHQ inhibits T cell activation of primary human CD4 T cells. *Cytokine*. 2015;71(2):289-295. doi:10.1016/j.cyto.2014.11.006

144. Noel S, Martina MN, Bandapalle S, et al. T lymphocyte-specific activation of Nrf2 protects from AKI. *Journal of the American Society of Nephrology*. 2015;26(12):2989-3000. doi:10.1681/ASN.2014100978/-/DCSUPPLEMENTAL

145. Zagorski JW, Turley AE, Freeborn RA, et al. Differential effects of the Nrf2 activators tBHQ and CDDO-Im on the early events of T cell activation. *Biochem Pharmacol*. 2018;147:67-76. doi:10.1016/j.bcp.2017.11.005

146. Zagorski JW, Turley AE, Dover HE, VanDenBerg KR, Compton JR, Rockwell CE. The Nrf2 activator, tBHQ, differentially affects early events following stimulation of Jurkat cells. *Toxicol Sci*. 2013;136(1):63-71. doi:10.1093/toxsci/kft172

147. Zagorski JW, Maser TP, Liby KT, Rockwell CE. Nrf2-Dependent and -Independent Effects of tert-Butylhydroquinone, CDDO-Im, and H2O2 in Human Jurkat T Cells as Determined by CRISPR/Cas9 Gene Editing. *Journal of Pharmacology and Experimental Therapeutics*. 2017;361(2):259-267. doi:10.1124/jpet.116.238899

148. Noel S, Lee SA, Sadasivam M, Hamad ARA, Rabb H. KEAP1 Editing Using CRISPR/Cas9 for Therapeutic NRF2 Activation in Primary Human T Lymphocytes. *The Journal of Immunology*. 2018;200(5):1929-1936. doi:10.4049/JIMMUNOL.1700812

149. Klemm P, Rajendiran A, Fragoulis A, et al. Nrf2 expression driven by Foxp3 specific deletion of Keap1 results in loss of immune tolerance in mice. *Eur J Immunol*. 2020;50(4):515-524. doi:10.1002/EJI.201948285

150. Li J, Stein TD, Johnson JA. Genetic dissection of systemic autoimmune disease in Nrf2-deficient mice. *Physiol Genomics*. 2004;18(3):261-272. doi:10.1152/physiolgenomics.00209.2003

151. Ma Q, Battelli L, Hubbs AF. Multiorgan autoimmune inflammation, enhanced lymphoproliferation, and impaired homeostasis of reactive oxygen species in mice lacking the antioxidant-activated transcription factor Nrf2. *American Journal of Pathology*. 2006;168(6):1960-1974. doi:10.2353/ajpath.2006.051113

152. Jiang T, Tian F, Zheng H, et al. Nrf2 suppresses lupus nephritis through inhibition of oxidative injury and the NF-κB-mediated inflammatory response. *Kidney Int*. 2014;85(2):333. doi:10.1038/KI.2013.343

153. McDougald DS, Dine KE, Zezulin AU, Bennett J, Shindler KS. SIRT1 and NRF2 Gene Transfer Mediate Distinct Neuroprotective Effects Upon Retinal Ganglion Cell Survival and Function in Experimental Optic Neuritis. *Invest Ophthalmol Vis Sci*. 2018;59(3):1212. doi:10.1167/IOVS.17-22972

154. Linker RA, Lee DH, Ryan S, et al. Fumaric acid esters exert neuroprotective effects in neuroinflammation via activation of the Nrf2 antioxidant pathway. *Brain*. 2011;134(3):678-692. doi:10.1093/BRAIN/AWQ386

155. Hayashi M, Takai J, Yu L, Motohashi H, Moriguchi T, Yamamoto M. Whole-Body In Vivo Monitoring of Inflammatory Diseases Exploiting Human Interleukin 6-Luciferase Transgenic Mice . *Mol Cell Biol*. 2015;35(20):3590-3601. doi:10.1128/MCB.00506-15/ASSET/C067EE1F-3553-4B97-846C-63AF613BAEF4/ASSETS/GRAPHIC/ZMB9991009860008.JPEG

156. Liu Y, Gao Y, Yang J, Shi C, Wang Y, Xu Y. Nrf2/ARE pathway inhibits inflammatory infiltration by macrophage in rats with autoimmune myositis. *Mol Immunol*. 2019;105:165-172. doi:10.1016/J.MOLIMM.2018.11.014

157. Johnson DA, Amirahmadi S, Ward C, Fabry Z, Johnson JA. The Absence of the Pro-antioxidant Transcription Factor Nrf2 Exacerbates Experimental Autoimmune Encephalomyelitis. *Toxicological Sciences*. 2010;114(2):237-246. doi:10.1093/TOXSCI/KFP274

158. Dong W, Jia Y, Liu X, et al. Sodium butyrate activates NRF2 to ameliorate diabetic nephropathy possibly via inhibition of HDAC. *Journal of Endocrinology*. 2017;232(1):71-83. doi:10.1530/JOE-16-0322

159. Wu WJ, Jia WW, Liu XH, et al. S-propargyl-cysteine attenuates inflammatory response in rheumatoid arthritis by modulating the Nrf2-ARE signaling pathway. *Redox Biol*. 2016;10:157-167. doi:10.1016/J.REDOX.2016.08.011

160. Hu X, Li L, Yan S, Li Z. Arsenic trioxide suppresses acute graft-versus-host disease by activating the Nrf2/HO-1 pathway in mice. *Br J Haematol*. 2019;186(5):e145-e148. doi:10.1111/BJH.15982

161. Scannevin RH, Chollate S, Jung MY, et al. Fumarates Promote Cytoprotection of Central Nervous System Cells against Oxidative Stress via the Nuclear Factor (Erythroid-Derived 2)-Like 2 Pathway. *Journal of Pharmacology and Experimental Therapeutics*. 2012;341(1):274-284. doi:10.1124/JPET.111.190132

162. Yang L, Fan X, Cui T, Dang E, Wang G. Nrf2 Promotes Keratinocyte Proliferation in Psoriasis through Up-Regulation of Keratin 6, Keratin 16, and Keratin 17. *Journal of Investigative Dermatology*. 2017;137(10):2168-2176. doi:10.1016/j.jid.2017.05.015

163. Ivanciuc T, Sbrana E, Casola A, Garofalo RP. Protective role of nuclear factor erythroid 2-related factor 2 against respiratory syncytial virus and human metapneumovirus infections. *Front Immunol*. 2018;9(APR):854. doi:10.3389/FIMMU.2018.00854/BIBTEX

164. Cho HY, Imani F, Miller-DeGraff L, et al. Antiviral Activity of Nrf2 in a Murine Model of Respiratory Syncytial Virus Disease. *https://doi.org/101164/rccm200804-535OC*. 2012;179(2):138-150. doi:10.1164/RCCM.200804-535OC

165. Komaravelli N, Tian B, Ivanciuc T, et al. Respiratory syncytial virus infection down-regulates antioxidant enzyme expression by triggering deacetylation-proteasomal degradation of Nrf2. *Free Radic Biol Med*. 2015;88(Part B):391-403. doi:10.1016/J.FREERADBIOMED.2015.05.043

166. Komaravelli N, Ansar M, Garofalo RP, Casola A. Respiratory syncytial virus induces NRF2 degradation through a promyelocytic leukemia protein - ring finger protein 4 dependent pathway. *Free Radic Biol Med*. 2017;113:494-504. doi:10.1016/J.FREERADBIOMED.2017.10.380

167. Kesic MJ, Simmons SO, Bauer R, Jaspers I. Nrf2 expression modifies influenza A entry and replication in nasal epithelial cells. *Free Radic Biol Med*. 2011;51(2):444-453. doi:10.1016/j.freeradbiomed.2011.04.027

168. Noah TL, Zhang H, Zhou H, et al. Effect of Broccoli Sprouts on Nasal Response to Live Attenuated Influenza Virus in Smokers: A Randomized, Double-Blind Study. *PLoS One*. 2014;9(6):e98671. doi:10.1371/JOURNAL.PONE.0098671

169. Yageta Y, Ishii Y, Morishima Y, et al. Role of Nrf2 in Host Defense against Influenza Virus in Cigarette Smoke-Exposed Mice. *J Virol*. 2011;85(10):4679-4690. doi:10.1128/JVI.02456-10

170. Yageta Y, Ishii Y, Morishima Y, et al. Carbocisteine reduces virus-induced pulmonary inflammation in mice exposed to cigarette smoke. *Am J Respir Cell Mol Biol*. 2014;50(5):963-973. doi:10.1165/RCMB.2012-0292OC/SUPPL_FILE/DISCLOSURES.PDF

171. Huang H, Falgout B, Takeda K, Yamada KM, Dhawan S. Nrf2-dependent induction of innate host defense via heme oxygenase-1 inhibits Zika virus replication. *Virology*. 2017;503:1-5. doi:10.1016/J.VIROL.2016.12.019

172. Olagnier D, Peri S, Steel C, et al. Cellular Oxidative Stress Response Controls the Antiviral and Apoptotic Programs in Dengue Virus-Infected Dendritic Cells. *PLoS Pathog*. 2014;10(12):e1004566. doi:10.1371/JOURNAL.PPAT.1004566

173. Cheng YL, Lin YS, Chen CL, et al. Activation of Nrf2 by the dengue virus causes an increase in CLEC5A, which enhances TNF-α production by mononuclear phagocytes. *Scientific Reports 2016 6:1*. 2016;6(1):1-15. doi:10.1038/srep32000

174. Cherupanakkal C, Ramachadrappa V, Kadhiravan T, et al. Differential expression of NADPH oxidase-2 (Nox-2) and nuclear factor-erythroid 2-related factor 2 (Nrf2) transcripts in peripheral blood mononuclear cells isolated from dengue patients. *Virusdisease*. 2017;28(1):54-60. doi:10.1007/S13337-017-0365-9/FIGURES/2

175. Cross SA, Cook DR, Chi AWS, et al. Dimethyl Fumarate, an Immune Modulator and Inducer of the Antioxidant Response, Suppresses HIV Replication and Macrophage-Mediated Neurotoxicity: A Novel Candidate for HIV Neuroprotection. *The Journal of Immunology*. 2011;187(10):5015-5025. doi:10.4049/JIMMUNOL.1101868

176. Furuya AKM, Sharifi HJ, Jellinger RM, Cristofano P, Shi B, de Noronha CMC. Sulforaphane Inhibits HIV Infection of Macrophages through Nrf2. *PLoS Pathog*. 2016;12(4):e1005581. doi:10.1371/JOURNAL.PPAT.1005581

177. Zhang HS, Li HY, Zhou Y, Wu MR, Zhou H sen. Nrf2 is involved in inhibiting Tat-induced HIV-1 long terminal repeat transactivation. *Free Radic Biol Med*. 2009;47(3):261-268. doi:10.1016/J.FREERADBIOMED.2009.04.028

178. Schaedler S, Krause J, Himmelsbach K, et al. Hepatitis B virus induces expression of antioxidant response element-regulated genes by activation of Nrf2. *Journal of Biological Chemistry*. 2010;285(52):41074-41086. doi:10.1074/jbc.M110.145862

179. Sugiyama K, Ebinuma H, Nakamoto N, et al. Prominent Steatosis with Hypermetabolism of the Cell Line Permissive for Years of Infection with Hepatitis C Virus. *PLoS One*. 2014;9(4):e94460. doi:10.1371/JOURNAL.PONE.0094460

180. Nio Y, Sasai M, Akahori Y, et al. Bardoxolone methyl as a novel potent antiviral agent against hepatitis B and C viruses in human hepatocyte cell culture systems. *Antiviral Res*. 2019;169:104537. doi:10.1016/J.ANTIVIRAL.2019.104537

181. Wyler E, Franke V, Menegatti J, et al. Single-cell RNA-sequencing of herpes simplex virus 1-infected cells connects NRF2 activation to an antiviral program. *Nature Communications 2019 10:1*. 2019;10(1):1-14. doi:10.1038/s41467-019-12894-z

182. Gunderstofte C, Iversen MB, Peri S, et al. Nrf2 Negatively Regulates Type I Interferon Responses and Increases Susceptibility to Herpes Genital Infection in Mice. *Front Immunol*. 2019;10:2101. doi:10.3389/FIMMU.2019.02101/BIBTEX

183. Lee J, Koh K, Kim YE, Ahn JH, Kim S. Upregulation of Nrf2 expression by human cytomegalovirus infection protects host cells from oxidative stress. *Journal of General Virology*. 2013;94(PART7):1658-1668. doi:10.1099/VIR.0.052142-0/CITE/REFWORKS

184. Seelige R, Saddawi-Konefka R, Adams NM, et al. Interleukin-17D and Nrf2 mediate initial innate immune cell recruitment and restrict MCMV infection. *Scientific Reports 2018 8:1*. 2018;8(1):1-14. doi:10.1038/s41598-018-32011-2

185. Shahidi F. Antioxidants in food and food antioxidants. *Nahrung - Food*. 2000;44(3):158-163. doi:10.1002/1521-3803(20000501)44:3<158::AID-FOOD158>3.0.CO;2-L

186. WHO. Safety evaluation of certain food additives. *WHO Food Additive Series no 42*. Published online 1999.

187. Li W, Kong AN. Molecular mechanisms of Nrf2-mediated antioxidant response. *Mol Carcinog*. 2009;48(2):91-104. doi:10.1002/mc.20465

188. Asti BL, Terhaar C, Krasavage W, et al. Safety Evaluation and Biochemical Behavior of Monotertiarybutylhydroqu inone I. doi:10.1007/BF02901824

189. Conning DM, Phillips JC. Comparative metabolism of BHA, BHT and other phenolic antioxidants and its toxicological relevance. *Food and Chemical Toxicology*. 1986;24(10-11):1145-1148. doi:10.1016/0278-6915(86)90300-5

190. van Esch GJ. Toxicology of tert-butylhydroquinone (TBHQ). *Food and Chemical Toxicology*. 1986;24(10-11):1063-1065. doi:10.1016/0278-6915(86)90289-9

191. WHO. Toxicological evaluation of some food colours, thickening agents, and certain other substances. *WHO Food Additive Series no 8*. Published online 1975.

192. Khezerlou A, Akhlaghi A pouya, Alizadeh AM, Dehghan P, Maleki P. Alarming impact of the excessive use of tert-butylhydroquinone in food products: A narrative review. *Toxicol Rep*. 2022;9:1066-1075. doi:10.1016/J.TOXREP.2022.04.027

193. Peters MMCG, Lau SS, Dulik D, et al. Metabolism of tert-butylhydroquinone to S-substituted conjugates in the male fischer 344 rat. *Chem Res Toxicol*. 1996;9(1):133-139. doi:10.1021/TX950122I/ASSET/IMAGES/MEDIUM/TX950122IU00001A.GIF

194. Huang W, Niu H, Gu Y. Metabolic kinetics of tert-butylhydroquinone and its metabolites in rat serum after oral administration by LC/ITMS. *Lipids*. 2008;43(8):757-763. doi:10.1007/S11745-008-3196-Z/FIGURES/1

195. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol*. 2008;9(5):495-502. doi:10.1038/ni1581

196. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol*. 1999;17:189-220. doi:10.1146/annurev.immunol.17.1.189

197. Henkart PA. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity*. 1994;1(5):343-346.

198. Kim S, Iizuka K, Kang HS, et al. In vivo developmental stages in murine natural killer cell maturation. *Nat Immunol*. 2002;3(6):523-528. doi:10.1038/ni796

199. Chiossone L, Chaix J, Fuseri N, Roth C, Vivier E, Walzer T. Maturation of mouse NK cells is a 4-stage developmental program. *Blood*. 2009;113(22):5488-5496. doi:10.1182/blood-2008-10-187179

200. Hayakawa Y, Smyth MJ. CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. *J Immunol*. 2006;176(3):1517-1524.

201. Yokoyama WM. Natural killer cell receptors. *Curr Opin Immunol*. 1998;10(3):298-305.

202. Clinthorne JF, Beli E, Duriancik DM, Gardner EM. NK cell maturation and function in C57BL/6 mice are altered by caloric restriction. *J Immunol*. 2013;190(2):712-722. doi:10.4049/jimmunol.1201837

203. Gardner EM, Beli E, Clinthorne JF, Duriancik DM. Energy intake and response to infection with influenza. *Annu Rev Nutr*. 2011;31:353-367. doi:10.1146/annurev-nutr-081810-160812

204. Ritz BW, Aktan I, Nogusa S, Gardner EM. Energy restriction impairs natural killer cell function and increases the severity of influenza infection in young adult male C57BL/6 mice. *J Nutr*. 2008;138(11):2269-2275. doi:10.3945/jn.108.093633

205. Heuser G, Vojdani A. Enhancement of natural killer cell activity and T and B cell function by buffered vitamin C in patients exposed to toxic chemicals: the role of protein kinase-C. *Immunopharmacol Immunotoxicol*. 1997;19(3):291-312. doi:10.3109/08923979709046977

206. Ritz BW, Nogusa S, Ackerman EA, Gardner EM. Supplementation with active hexose correlated compound increases the innate immune response of young mice to primary influenza infection. *J Nutr*. 2006;136(11):2868-2873. doi:10.1093/jn/136.11.2868

207. Smith AG, Sheridan PA, Harp JB, Beck MA. Diet-induced obese mice have increased mortality and altered immune responses when infected with influenza virus. *J Nutr*. 2007;137(5):1236-1243. doi:10.1093/jn/137.5.1236

208. Zhang H, Meadows GG. Chronic alcohol consumption perturbs the balance between thymus-derived and bone marrow-derived natural killer cells in the spleen. *J Leukoc Biol*. 2008;83(1):41-47. doi:10.1189/jlb.0707472

209. Shahidi F. Antioxidants in food and food antioxidants. *Nahrung/Food*. 2000;44(3):158-163. doi:10.1002/1521-3803(20000501)44:3<158::AID-FOOD158>3.0.CO;2-L

210. USFDA. FDA food and drug regulations 21CFR172.185. International programme on chemical safety. *Title 21- Food and Drugs*. Published online 2018.

211. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol*. 2007;47:89-116. doi:10.1146/annurev.pharmtox.46.120604.141046

212. Li W, Kong AN. Molecular mechanisms of Nrf2-mediated antioxidant response. *Mol Carcinog*. 2009;48(2):91-104. doi:10.1002/mc.20465

213. Rockwell CE, Zhang M, Fields PE, Klaassen CD. Th2 skewing by activation of Nrf2 in CD4(+) T cells. *Journal of immunology*. 2012;188(4):1630.

214. Zagorski JW, Turley AE, Dover HE, VanDenBerg KR, Compton JR, Rockwell CE. The Nrf2 activator, tBHQ, differentially affects early events following stimulation of Jurkat cells. *Toxicol Sci*. 2013;136(1):63-71. doi:10.1093/toxsci/kft172

215. Zagorski JW, Turley AE, Freeborn RA, et al. Differential effects of the Nrf2 activators tBHQ and CDDO-Im on the early events of T cell activation. *Biochem Pharmacol*. 2018;147:67-76. doi:10.1016/j.bcp.2017.11.005

216. Turley AE, Zagorski JW, Rockwell CE. The Nrf2 activator tBHQ inhibits T cell activation of primary human CD4 T cells. *Cytokine*. 2015;71(2):289-295. doi:10.1016/j.cyto.2014.11.006

217. Sun JC. Transcriptional Control of NK Cells. *Curr Top Microbiol Immunol*. 2016;395:1-36. doi:10.1007/82_2015_452

218. Babcock GF. Intracellular Cytokines. In: *Current Protocols in Cytometry*. John Wiley & Sons, Inc.; 2004:9.9.1-9.9.11. doi:10.1002/0471142956.cy0909s28

219. Gregoire C, Chasson L, Luci C, et al. The trafficking of natural killer cells. *Immunol Rev*. 2007;220:169-182. doi:10.1111/j.1600-065X.2007.00563.x

220. Macoch M, Morzadec C, Genard R, et al. Nrf2-dependent repression of interleukin-12 expression in human dendritic cells exposed to inorganic arsenic. *Free Radic Biol Med*. 2015;88(Pt B):381-390. doi:10.1016/j.freeradbiomed.2015.02.003

221. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat Immunol*. 2008;9(5):503-510. doi:10.1038/ni1582

222. Ma Q, Kinneer K. Chemoprotection by Phenolic Antioxidants. *Journal of Biological Chemistry*. Published online 2002. doi:10.1074/jbc.M106685200

223. Wei J, Chen G, Shi X, et al. Nrf2 activation protects against intratracheal LPS induced mouse/murine acute respiratory distress syndrome by regulating macrophage polarization. *Biochem Biophys Res Commun*. Published online 2018. doi:10.1016/j.bbrc.2018.04.161

224. Itoh K, Wakabayashi N, Katoh Y, et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev*. 1999;13(1):76-86. doi:10.1101/GAD.13.1.76

225. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol*. 2007;47(1):89-116. doi:10.1146/annurev.pharmtox.46.120604.141046

226. Itoh K, Chiba T, Takahashi S, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun*. 1997;236(2):313-322. doi:10.1006/BBRC.1997.6943

227. Kobayashi A, Kang MI, Watai Y, et al. Oxidative and Electrophilic Stresses Activate Nrf2 through Inhibition of Ubiquitination Activity of Keap1. *Mol Cell Biol*. 2006;26(1):221-229. doi:10.1128/MCB.26.1.221-229.2006/FORMAT/EPUB

228. Rangasamy T, Guo J, Mitzner WA, et al. Disruption of Nrf2 enhances susceptibility to severe airway inflammation and asthma in mice. *J Exp Med*. 2005;202(1):47-59. doi:10.1084/jem.20050538

229. Li YJ, Takizawa H, Azuma A, et al. Nrf2 is closely related to allergic airway inflammatory responses induced by low-dose diesel exhaust particles in mice. *Clinical Immunology*. 2010;137(2):234-241. doi:10.1016/J.CLIM.2010.07.014

230. Thimmulappa RK, Lee H, Rangasamy T, et al. Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. *Journal of Clinical Investigation*. 2006;116(4):984-995. doi:10.1172/JCI25790

231. Kim JH, Choi YK, Lee KS, et al. Functional dissection of Nrf2-dependent phase II genes in vascular inflammation and endotoxic injury using Keap1 siRNA. *Free Radic Biol Med*. 2012;53(3):629-640. doi:10.1016/J.FREERADBIOMED.2012.04.019

232. Osburn WO, Yates MS, Dolan PD, et al. Genetic or Pharmacologic Amplification of Nrf2 Signaling Inhibits Acute Inflammatory Liver Injury in Mice. *Toxicological Sciences*. 2008;104(1):218-227. doi:10.1093/TOXSCI/KFN079

233. Cho HY, Reddy SP, Kleeberger SR. Nrf2 Defends the Lung from Oxidative Stress. *https://home.liebertpub.com/ars*. 2006;8(1-2):76-87. doi:10.1089/ARS.2006.8.76

234. Yoh K, Itoh K, Enomoto A, et al. Nrf2-deficient female mice develop lupus-like autoimmune nephritis. *Kidney Int*. 2001;60(4):1343-1353. doi:10.1046/j.1523-1755.2001.00939.x

235. Macoch M, Morzadec C, Genard R, et al. Nrf2-dependent repression of interleukin-12 expression in human dendritic cells exposed to inorganic arsenic. *Free Radic Biol Med*. 2015;88(Pt B):381-390. doi:10.1016/j.freeradbiomed.2015.02.003

236. Ma Q, Kinneer K. Chemoprotection by Phenolic Antioxidants. *Journal of Biological Chemistry*. 2002;277(4):2477-2484. doi:10.1074/jbc.M106685200

237. Wei J, Chen G, Shi X, et al. Nrf2 activation protects against intratracheal LPS induced mouse/murine acute respiratory distress syndrome by regulating macrophage polarization. *Biochem Biophys Res Commun*. 2018;500(3):790-796. doi:10.1016/j.bbrc.2018.04.161

238. Kobayashi EH, Suzuki T, Funayama R, et al. Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nat Commun*. 2016;7(1):11624. doi:10.1038/ncomms11624

239. Chan K, Lu R, Chang JC, Kan YW. NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proc Natl Acad Sci U S A*. 1996;93(24):13943-13948. doi:10.1073/PNAS.93.24.13943

240. Seelige R, Saddawi-Konefka R, Adams NM, et al. Interleukin-17D and Nrf2 mediate initial innate immune cell recruitment and restrict MCMV infection OPEN. *Sci Rep*. 2011;8:13670. doi:10.1038/s41598-018-32011-2

241. Prager I, Watzl C. Mechanisms of natural killer cell-mediated cellular cytotoxicity. *J Leukoc Biol*. 2019;105(6):1319-1329. doi:10.1002/JLB.MR0718-269R

242. Nogusa S, Murasko DM, Gardner EM. Differential effects of stimulatory factors on natural killer cell activities of young and aged mice. *J Gerontol A Biol Sci Med Sci*. 2012;67(9):947-954. doi:10.1093/gerona/gls079

243. Ai W, Li H, Song N, Li L, Chen H. Optimal Method to Stimulate Cytokine Production and Its Use in Immunotoxicity Assessment. *Int J Environ Res Public Health*. 2013;10(9):3834. doi:10.3390/IJERPH10093834

244. Kim S, Iizuka K, Kang HSPS, et al. In vivo developmental stages in murine natural killer cell maturation. *Nat Immunol*. 2002;3(6):523-528. doi:10.1038/ni796

245. Schulze-Osthoff K, Beyaert R, Vandevoorde V, Haegeman G, Fiers W. Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. *EMBO J*. 1993;12(8):3095-3104. doi:10.1002/J.1460-2075.1993.TB05978.X

246. Gharavi N, El-Kadi AOS. tert-BUTYLHYDROQUINONE IS A NOVEL ARYL HYDROCARBON RECEPTOR LIGAND. *Drug Metabolism and Disposition*. 2005;33(3):365-372. doi:10.1124/DMD.104.002253

247. Centers for Disease Control and Prevention NC for I and RD. *Estimated Influenza Illnesses, Medical Visits, Hospitalizations, and Deaths in the United States — 2017–2018 Influenza Season*.; 2019. Accessed May 31, 2019. https://www.cdc.gov/flu/about/burden/2017-2018.htm

248. Healthcare Workers: Infectious Agents | NIOSH | CDC. Accessed January 12, 2023. https://www.cdc.gov/niosh/topics/healthcare/infectious.html

249. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. NATURAL KILLER CELLS IN ANTIVIRAL DEFENSE: Function and Regulation by Innate Cytokines. *Annu Rev Immunol*. 1999;17(1):189-220. doi:10.1146/annurev.immunol.17.1.189

250. Lanier LL. Evolutionary struggles between NK cells and viruses. *Nat Rev Immunol*. 2008;8(4):259-268. doi:10.1038/nri2276

251. Kim DH, Chung WC, Chun S hyun, Han JH, Song MJ, Lee KW. Enhancing the natural killer cell activity and anti-influenza effect of heat-treated Lactobacillus plantarum nF1-fortified yogurt in mice. *J Dairy Sci*. 2018;101(12):10675-10684. doi:10.3168/jds.2018-15137

252. Kamei M, Nishimura H, Takahashi T, et al. Anti-influenza virus effects of cocoa. *J Sci Food Agric*. 2016;96(4):1150-1158. doi:10.1002/jsfa.7197

253. Balish AL, Katz JM, Klimov AI. Influenza: propagation, quantification, and storage. *Curr Protoc Microbiol*. 2013;Chapter 15(SUPPL.29):15G.1.1-15G.1.24. doi:10.1002/9780471729259.mc15g01s29

254. Matsuoka Y, Lamirande EW, Subbarao K. The Mouse Model for Influenza. *Curr Protoc Microbiol*. 2009;13(1):15G.3.1-15G.3.30. doi:10.1002/9780471729259.MC15G03S13

255. Frank K, Paust S. Dynamic Natural Killer Cell and T Cell Responses to Influenza Infection. *Front Cell Infect Microbiol*. 2020;10:425. doi:10.3389/FCIMB.2020.00425/BIBTEX

256. Hayakawa Y, Smyth MJ. CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. *J Immunol*. 2006;176(3):1517-1524.

257. Abel AM, Yang C, Thakar MS, Malarkannan S. Natural killer cells: Development, maturation, and clinical utilization. *Front Immunol*. 2018;9(AUG):1. doi:10.3389/fimmu.2018.01869

258. Clinthorne JF, Beli E, Duriancik DM, Gardner EM. NK cell maturation and function in C57BL/6 mice are altered by caloric restriction. *J Immunol*. 2013;190(2):712-722. doi:10.4049/jimmunol.1201837

259. Gardner EM, Beli E, Clinthorne JF, Duriancik DM. Energy Intake and Response to Infection with Influenza. *Annu Rev Nutr*. 2011;31(1):353-367. doi:10.1146/annurev-nutr-081810-160812

260. Pak-Wittel MA, Piersma SJ, Plougastel BF, Poursine-Laurent J, Yokoyama WM. Isolation of murine natural killer cells. *Curr Protoc Immunol*. 2014;(SUPPL.105). doi:10.1002/0471142735.im0322s105

261. Clinthorne JF, Adams DJ, Fenton JI, Ritz BW, Gardner EM. Short-term re-feeding of previously energy-restricted C57BL/6 male mice restores body weight and body fat and attenuates the decline in natural killer cell function after primary influenza infection. *J Nutr*. 2010;140(8):1495-1501. doi:10.3945/jn.110.122408

262. Carlin LE, Hemann EA, Zacharias ZR, Heusel JW, Legge KL. Natural Killer Cell Recruitment to the Lung During Influenza A Virus Infection Is Dependent on CXCR3, CCR5, and Virus Exposure Dose. *Front Immunol*. 2018;9(APR):1. doi:10.3389/fimmu.2018.00781

263. UN. *United Nations Synthesis Rport on Arsenic in Drinking-Water*.; 2001. Accessed September 22, 2022. http://www.who.int/water_sanitation_health/dwq/arsenic3/en/

264. Ayotte JD, Medalie L, Qi SL, Backer LC, Nolan BT. Estimating the High-Arsenic Domestic-Well Population in the Conterminous United States. *Environ Sci Technol*. 2017;51(21):12443-12454. doi:10.1021/acs.est.7b02881

265. Kenny JF, Barber NL, Hutson SS, Linsey KS, Lovelace JK, Maupin MA. Estimated use of water in the United States in 2005. *US Geological Survey Circular*. 2009;(1344):1-50. doi:10.3133/CIR1405

266. Ratnaike RN. Acute and chronic arsenic toxicity. *Postgrad Med J*. 2003;79(933):391-396. doi:10.1136/pmj.79.933.391

267. Ramsey K. Arsenic and Respiratory Disease. In: *Handbook of Arsenic Toxicology*. Elsevier; 2015:335-347. doi:10.1016/B978-0-12-418688-0.00013-7

268. Sanchez TR, Powers M, Perzanowski M, George CM, Graziano JH, Navas-Acien A. A Meta-analysis of Arsenic Exposure and Lung Function: Is There Evidence of Restrictive or Obstructive Lung Disease? *Curr Environ Health Rep*. 2018;5(2):244-254. doi:10.1007/s40572-018-0192-1

269. Liao CM, Chio CP, Cheng YH, Hsieh NH, Chen WY, Chen SC. Quantitative Links Between Arsenic Exposure and Influenza A (H1N1) Infection-Associated Lung Function Exacerbations Risk. *Risk Analysis*. 2011;31(8):1281-1294. doi:10.1111/J.1539-6924.2010.01575.X

270. Farzan SF, Li Z, Korrick SA, et al. Infant Infections and Respiratory Symptoms in Relation to in Utero Arsenic Exposure in a U.S. Cohort. *Environ Health Perspect*. 2016;124(6):840-847. doi:10.1289/ehp.1409282

271. George CM, Brooks WA, Graziano JH, et al. Arsenic exposure is associated with pediatric pneumonia in rural Bangladesh: A case control study. *Environ Health*. 2015;14(1):83. doi:10.1186/s12940-015-0069-9

272. Amouzougan EA, Lira R, Klimecki WT. Chronic exposure to arsenite enhances influenza virus infection in cultured cells. *Journal of Applied Toxicology*. 2020;40(4):458-469. doi:10.1002/JAT.3918

273. Kozul CD, Ely KH, Enelow RI, Hamilton JW. Low-Dose Arsenic Compromises the Immune Response to Influenza A Infection in Vivo. *Environ Health Perspect*. 2009;117(9):1441-1447. doi:10.1289/EHP.0900911

274. Ramsey KA, Foong RE, Sly PD, Larcombe AN, Zosky GR. Early life arsenic exposure and acute and long-term responses to influenza A infection in mice. *Environ Health Perspect*. 2013;121(10):1187-1193. doi:10.1289/ehp.1306748

275. Hsu KS, Goodale BC, Ely KH, Hampton TH, Stanton BA, Enelow RI. Single-cell RNA-seq Analysis Reveals That Prenatal Arsenic Exposure Results in Long-term, Adverse Effects on Immune Gene Expression in Response to Influenza A Infection. *Toxicological Sciences*. 2020;176(2):312-328. doi:10.1093/toxsci/kfaa080

276. Freeborn RA, Boss AP, Kaiser LM, Gardner EM, Rockwell CE. Trivalent arsenic impairs the effector response of human CD4+ and CD8+ T cells to influenza A virus ex vivo. *Food and Chemical Toxicology*. 2022;165:113122. doi:10.1016/j.fct.2022.113122

277. Kim JY, Bae JH, Lee SH, et al. Induction of NKG2D ligands and subsequent enhancement of NK cell-mediated lysis of cancer cells by arsenic trioxide. *Journal of Immunotherapy*. 2008;31(5):475-486. doi:10.1097/CJI.0b013e3181755deb

278. Alex AA, Ganesan S, Palani HK, et al. Arsenic Trioxide Enhances the NK Cell Cytotoxicity Against Acute Promyelocytic Leukemia While Simultaneously Inhibiting Its Bio-Genesis. *Front Immunol*. 2018;9(JUN). doi:10.3389/FIMMU.2018.01357

279. Sumi D, Tsuyama H, Ogawa T, Ogawa M, Himeno S. Arsenite suppresses IL-2-dependent tumoricidal activities of natural killer cells. *Toxicol Appl Pharmacol*. 2021;412:115353. doi:10.1016/J.TAAP.2020.115353

280. Prasad P, Sarkar N, Sinha D. Effect of low- and high-level groundwater arsenic on peripheral blood and lung function of exposed rural women. *Regulatory Toxicology and Pharmacology*. 2020;115:104684. doi:10.1016/J.YRTPH.2020.104684

281. Naujokas MF, Anderson B, Ahsan H, et al. The broad scope of health effects from chronic arsenic exposure: Update on a worldwide public health problem. *Environ Health Perspect*. 2013;121(3):295-302. doi:10.1289/EHP.1205875

282. Hughes MF, Beck BD, Chen Y, Lewis AS, Thomas DJ. Arsenic Exposure and Toxicology: A Historical Perspective. *Toxicological Sciences*. 2011;123(2):305-332. doi:10.1093/TOXSCI/KFR184

283. Hall M, Chen Y, Ahsan H, et al. Blood arsenic as a biomarker of arsenic exposure: Results from a prospective study. *Toxicology*. 2006;225(2-3):225-233. doi:10.1016/J.TOX.2006.06.010

284. Wagar LE, Gentleman B, Pircher H, McElhaney JE, Watts TH. Influenza-Specific T Cells from Older People Are Enriched in the Late Effector Subset and Their Presence Inversely Correlates with Vaccine Response. Tripp R, ed. *PLoS One*. 2011;6(8):e23698. doi:10.1371/journal.pone.0023698

285. de La Fuente H, Portales-Pérez D, Baranda L, et al. Effect of arsenic, cadmium and lead on the induction of apoptosis of normal human mononuclear cells. *Clin Exp Immunol*. 2002;129(1):69-77. doi:10.1046/J.1365-2249.2002.01885.X

286. Xu H, Wang X, Wang W. Monomethylarsonous acid: Induction of DNA damage and oxidative stress in mouse natural killer cells at environmentally-relevant concentrations. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 2018;832-833:1-6. doi:10.1016/J.MRGENTOX.2018.05.017

287. Ma H, Song X, Huang P, et al. Myricetin protects natural killer cells from arsenite induced DNA damage by attenuating oxidative stress and retaining poly(ADP-Ribose) polymerase 1 activity. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 2021;865:503337. doi:10.1016/J.MRGENTOX.2021.503337

288. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol*. 2001;22(11):633-640. doi:10.1016/S1471-4906(01)02060-9

289. Luczo JM, Ronzulli SL, Tompkins SM. Influenza A Virus Hemagglutinin and Other Pathogen Glycoprotein Interactions with NK Cell Natural Cytotoxicity Receptors NKp46, NKp44, and NKp30. *Viruses*. 2021;13(2):156. doi:10.3390/v13020156

290. Sivori S, Vitale M, Morelli L, et al. p46, a Novel Natural Killer Cell–specific Surface Molecule That Mediates Cell Activation. *Journal of Experimental Medicine*. 1997;186(7):1129-1136. doi:10.1084/JEM.186.7.1129

291. de Maria A, Fogli M, Costa P, et al. The impaired NK cell cytolytic function in viremic HIV-1 infection is associated with a reduced surface expression of natural cytotoxicity receptors (NKp46, NKp30 and NKp44). *Eur J Immunol*. 2003;33(9):2410-2418. doi:10.1002/EJI.200324141

292. Costello RT, Sivori S, Marcenaro E, et al. Defective expression and function of natural killer cell–triggering receptors in patients with acute myeloid leukemia. *Blood*. 2002;99(10):3661-3667. doi:10.1182/BLOOD.V99.10.3661

293. Sivori S, Pende D, Bottino C, et al. NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface

density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. doi:10.1002/(SICI)1521-4141(199905)29:05

294. DeWitt J, Buck B, Goossens D, et al. Health effects following subacute exposure to geogenic dusts from arsenic-rich sediment at the Nellis Dunes Recreation Area, Las Vegas, NV. *Toxicol Appl Pharmacol*. 2016;304:79-89. doi:10.1016/J.TAAP.2016.05.017

295. Keil DE, Buck B, Goossens D, et al. Health effects from exposure to atmospheric mineral dust near Las Vegas, NV, USA. *Toxicol Rep*. 2016;3:785-795. doi:10.1016/J.TOXREP.2016.09.009

296. Liao CMM, Chio CPP, Cheng YHH, Hsieh NHH, Chen WYY, Chen SCC. Quantitative links between arsenic exposure and influenza A (H1N1) infection-associated lung function exacerbations risk. *Risk Analysis*. 2011;31(8):1281-1294. doi:10.1111/j.1539-6924.2010.01575.x

297. Alex AA, Ganesan S, Palani HK, et al. Arsenic trioxide enhances the NK cell cytotoxicity against acute promyelocytic leukemia while simultaneously inhibiting its bio-genesis. *Front Immunol*. 2018;9(JUN). doi:10.3389/fimmu.2018.01357

APPENDIX

Arsenic trioxide impairs primary human NK cell responses against influenza A virus

Abstract

Arsenic is a naturally occurring contaminant that is commonly found in water supplies. Chronic exposure to arsenic has been associated with multisystem disease, including respiratory diseases such as viral infections. Arsenic is known to regulate immune cell activity and has been shown to alter NK cell frequency and function. However, our knowledge on effects of arsenic on NK cell response to influenza remains limited. The following study aims to identify the effects of arsenic trioxide on NK cell activation and function against influenza A virus in primary human immune cells. Primary human peripheral blood mononuclear cells were treated with environmentally relevant concentrations of arsenic trioxide prior to an influenza A virus challenge. Flow cytometric analysis of NK cells showed arsenic trioxide significantly decreased NK cell viability. Additionally, NK cell activation markers, CD69 and NKp46, were reduced with increasing concentrations of arsenic. Consistently, the production of NK cell effector mediators, including IFNγ, granzyme B and perforin, were significantly impaired with treatment of arsenic trioxide. Overall, these data suggest that environmentally relevant concentrations of arsenic trioxide impaired NK cell responses to influenza A virus, which could negatively impact NK cell-mediated host defense.

Introduction

Arsenic is a naturally occurring chemical that is commonly found in drinking water, and long-term exposure has been link to numerous diseases including cancer.[263] Although the maximum contaminant levels for arsenic in public-water supplies has been lowered from 50 μg/L to 10 μg/L, domestic wells in the U.S. have been found to contain arsenic at levels greater than 10 μg/L.[264] It is estimated that 14% of the total population in the U.S. relies on domestic wells for water use.[265]

Chronic exposure to arsenic can lead to multisystem disease, including skin changes, diarrhea, cardiovascular disease, neuropathy, prostate cancer, bladder cancer, lung cancer, kidney cancer, skin cancer, and diabetes.[266] In addition, arsenic has been associated with a wide spectrum of respiratory diseases.[267] A meta-analysis found arsenic to impair pulmonary function [268], which agrees with several epidemiological studies that have shown a negative relation between arsenic exposure and respiratory infection.[269–271]

In addition to the epidemiological studies, arsenic has been reported to impact several immune cells in response to influenza infection. A study found sodium arsenite to enhance influenza infection in epithelial cells and decrease effectiveness of anti-influenza drug, oseltamivir.[272] In mice chronically exposed to arsenic, influenza infection resulted in higher influenza virus titers and decreased the number of dendritic cells early in influenza infection.[273] Additionally, mice exposed to sodium arsenite in utero and postnatal resulted in enhanced inflammatory responses which were attributed to the dysregulation of innate immune functions, including natural killer (NK) cells.[274,275] More recently, our group showed arsenic trioxide to impair human T cell responses against influenza A virus *ex vivo*.[276]

NK cells are innate lymphocytes that play a vital role in limiting viral spread through the rapid release of cytokines and cytotoxic granules.[249] Currently, several studies have assessed the effects of arsenic in human and mouse NK cells. Numerous cancer disease models have shown arsenic trioxide to enhance NK cell cytolytic activity in both human cell lines and mouse models.[277,278] Conversely, pretreatment of NK-92 cells and primary murine NK cells with sodium arsenite significantly reduced NK cell cytotoxicity in response to target cells.[279] In mice, prenatal exposure to sodium arsenite was shown to increase NK cell activity and enhance inflammation in response to IAV infection.[275] Additionally, women chronically exposed to arsenic via ground water had significantly less circulating CD56+ NK cells compared to women with acute exposure.[280] Despite these findings, the effects of arsenic on NK cells in response to influenza in humans remain unclear. Therefore, the objective of this study is to determine how arsenic, in the form of arsenic trioxide impacts human NK cell responses against IAV infection *ex vivo.*

Materials and Methods

Materials

All reagents, including arsenic (III) oxide (arsenic trioxide, ATO, catalog number A1010) were purchased from Sigma-Alrdich (Saint Louis, MO) except where otherwise stated. Complete PBMC medium used in this study consisted of RPMI 1640 (Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Biowest LLC, Kansas City, MO), 25 mM HEPES, 1 mM sodium pyruvate, 1x nonessential amino acids, 100 U/mL penicillin (Life Technologies, Carlsbad, CA), and 100 U/mL streptomycin (Life Technologies, Carlsbad, CA).

Peripheral Blood Mononuclear Cell (PBMC) Isolation and Treatment

Source leukocytes were purchased from the Gulf Coast Regional Blood Center (Houston, TX). Isolation of PBMCs was done by Lymphoprep (Stem Cell Technologies, Vancouver, BC) density gradient centrifugation following the manufacturer's protocol. Cells were resuspended in complete medium to $1x10^6$ cells/mL and plated in triplicates at 0.2 mL/well in a 96-well U-bottom plate. Cells were either left untreated (background, BKG) or treated with vehicle (VEH, 0.004% PBS), 0.5 μM, 1 μM, or 2 μM ATO. Concentrations of arsenic were based on high environmental levels of arsenic in drinking water (10 ppb to 1000 ppb) where pathogenic effects have been observed with concentrations greater than 5 μM [375-750 ppb AS(III)].[281,282] Additionally, blood arsenic in humans exposed via drinking water containing levels >50 ppb resulted in blood levels of 2-23 ppb.[283] After 30 min, VEH- and ATO-exposed cells were infected with 0.5 hemagglutination units (HAU) of influenza A/PR/8/34 (H1N1) while the BKG cells had no virus added. The amount of virus added to cells was based on a previous study.[284] Cells were maintained at 37°C, 5% CO_2 for 96 h. Monensin (1x) (Biolegend, San Diego, CA) was added to all cells for the remaining 6 h of stimulation for intracellular cytokine accumulation.

Flow Cytometry

The following anti-human mAbs used in this study are listed in **Table 6**. After 96 h of stimulation, supernatants were collected for later cytokine analysis and cells were processed for FACs analysis. To assess viability, cells were initially washed in PBS and stained with Zombie Aqua Fixable Viability Dye (Biolegend, San Diego, CA). After, cells were washed in FACS buffer (1% FBS in PBS) and labeled for surface markers for 30 min at 4°C. For intracellular cytokine detection, cells previously stained for surface markers were permeabilized using FoxP3/Transcription Buffer Staining Kit (Invitrogen, Waltham, MA) following the manufacturer's

protocol, then labeled with antibodies against interferon gamma (IFNγ), granzyme B (GZMB), and perforin for 30 min at room temperature. Cells were then washed and immediately analyzed on an Attune NxT Flow Cytometer (ThermoFisher, Waltham, MA). Compensation for this study was done with Ultra-Comp eBeads following manufacturer's protocol (Invitrogen, Waltham, MA).

Table 6: List of Antibodies

Antibody	Fluorochrome	Clone	Supplier
Live/Dead	Zombie Aqua Dye		Biolegend
CD3	PerCP/Cyanine5.5	OKT3	Biolegend
CD56 (NCAM)	FITC	HCD56	Biolegend
CD335 (NKp46)	Brilliant Violet 711	9E2	Biolegend
CD69	Brilliant Violet 605	FN50	Biolegend
CD25	APC	BC96	Invitrogen
CD107a (LAMP-1)	APC/Cyanine7	H4A3	Biolegend
CD16	PE/Cy5	3G8	Biolegend
Granzyme B	Pacific Blue	QA16A02	Biolegend
IFN-γ	PE	4S.B3	Biolegend
Perforin	PE/Cyanine7	B-D48	Biolegend

IFNγ ELISA

Supernatants were collected from samples 96 hours after treatment. Secreted IFNγ was measured using the human IFNγ ELISA MAX Standard Kit (Biolegend, San Diego, CA) following

manufacturer's protocol. Tecan Infinite M1000 Pro Microplate Reader (Tecan, San Jose, CA) was used to quantify absorbance at 450 nm.

Statistical Analysis

Individual data points were converted to a percentage of the vehicle mean for each independent experiment. The mean ± standard error (SE) was determined for each treatment group and analyzed by one-way ANOVA followed by a Dunnett's post hoc test to compare treatments to the VEH group. Analyses were done using GraphPad PRISM software version 8.4.2 (GraphPad Software, La Jolla, CA).

Results

Arsenic Trioxide Reduces Viability of PBMCs.

The percentage of viable PBMCs was determined since arsenic has been shown to induce apoptosis in PBMCs.[285] We assessed the viability of cells by flow cytometry using Zombie Aqua fixable viability dye. The percentage of viable cells was significantly attenuated at concentrations greater than 1 μM ATO (**Figure 24A**), which occurred in a concentration-dependent manner.

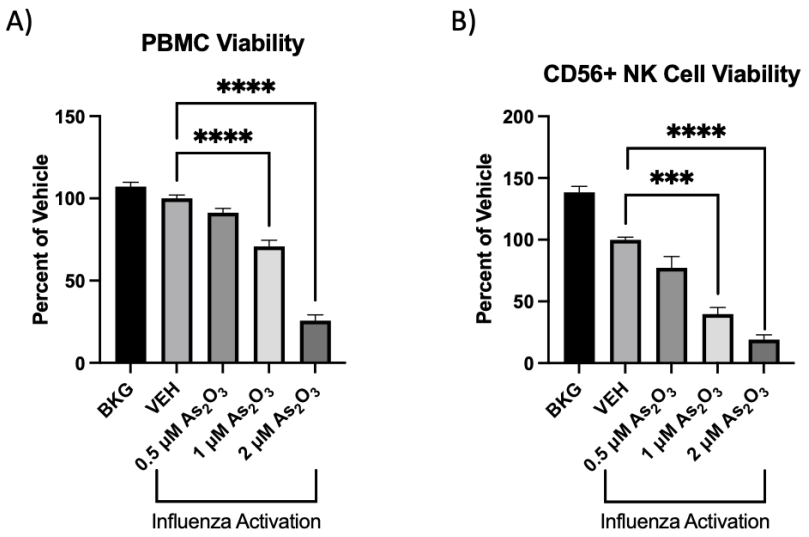

Figure 24: Arsenic trioxide reduced overall PBMC and NK cell viability. Human PBMCs were isolated and treated with arsenic trioxide (0.5- 2 μM As_2O_3), dPBS (VEH), or left untreated (BKG). Samples were either unactivated (BKG) or activated with influenza A virus at 5 HAU/sample (VEH and 0.5- 2 μM As_2O_3) for 96 h. Cells were stained with viability dye and fluorescently conjugated antibodies against CD3 and CD56 and were analyzed by flow cytometry. (A) PBMC viability was assessed using Live/Dead stain. (B) Viability of NK cells was determined by identifying $CD56^+CD3^-$ cells followed by Live/Dead stain. Percent vehicle was calculated by dividing individual cell count by the average cell count of the vehicle group. Three experiments were performed in triplicates.
* Denotes P ≤ 0.5, ** denotes P ≤ 0.01, *** denotes P ≤ 0.001, **** denotes P ≤ 0.0001 compared to VEH.

Arsenic Decreased NK Cell Population.

In addition to overall cell viability, we examined the effects of ATO on CD56+CD3- NK cells, as previous studies have shown arsenic to induce DNA damage in murine NK cells.[286,287] NK cells were identified as CD56+CD3- prior to gating of NK cell subsets, $CD56^{bright}$CD16- and $CD56^{dim}$CD16+. ATO caused a concentration-dependent decrease in viable NK cells (**Figure 24B**). When further divided into NK cell subsets, most NK cells displayed $CD56^{dim}$CD16+ phenotype (data not shown). This was expected since $CD56^{dim}$CD16+ NK cells are the main population of NK cells found in peripheral blood.[288] In $CD56^{dim}$CD16+ NK cells, a reduction was observed with 1 μM and 2 μM ATO, whereas $CD56^{bright}$CD16- NK cells were decreased with all

ATO-treatments (**Figure 25**). These data suggest that the NK cell subset, CD56^{bright}CD16-, are more sensitive to ATO at lower concentrations.

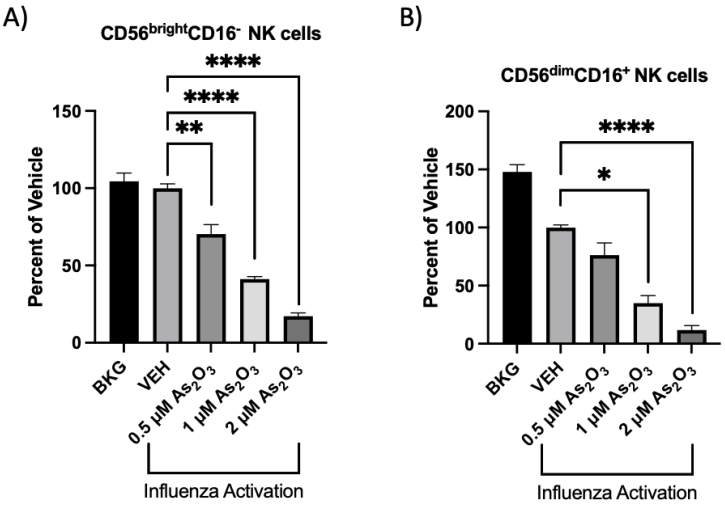

Figure 25: NK cell subsets decreased with arsenic trioxide. Human PBMCs were isolated and treated with arsenic trioxide (0.5- 2 µM As_2O_3), dPBS (VEH), or left untreated (BKG). Samples were either unactivated (BKG) or activated with influenza A virus at 5 HAU/sample (VEH and 0.5- 2 µM As_2O_3) for 96 h. Cells were stained with viability dye and fluorescently conjugated antibodies against CD3, CD56, and CD16 were analyzed by flow cytometry. NK cells were identified as viable CD56$^+$CD3$^-$ prior to analysis of subsets, (A) CD56^{bright}CD16$^-$ and (B) CD56^{dim}CD16$^+$. Percent vehicle was calculated by dividing individual cell count by the average cell count of the vehicle group. Three experiments were performed in triplicates.
* Denotes P ≤ 0.5, ** denotes P ≤ 0.01, *** denotes P ≤ 0.001, **** denotes P ≤ 0.0001 compared to VEH.

Arsenic Trioxide Suppressed Expression of the Activating Receptor, NKp46, and Early Activation Marker, CD69, in NK Cells.

NKp46 is an important NK cell activating receptor which is involved in the recognition of hemagglutinin which is expressed on virally infected cells. Although NKp46 is constitutively expressed on NK cells, the level of expression has been shown to correlate with NK cell cytolytic activity.[289] In the present study, we found IAV exposure to modestly increase NKp46 expression in NK cells (**Figure 26**). However, ATO-treatment diminished NKp46 expression to levels

comparable to the BKG group, which suggests impaired NK cell activation in response to influenza (**Figure 26B**). Likewise, ATO-treatment significantly reduced expression of the activation marker, CD69, in NK cells compared to the vehicle group (**Figure 27A**). Overall, the data suggest that ATO inhibits NK cell activation.

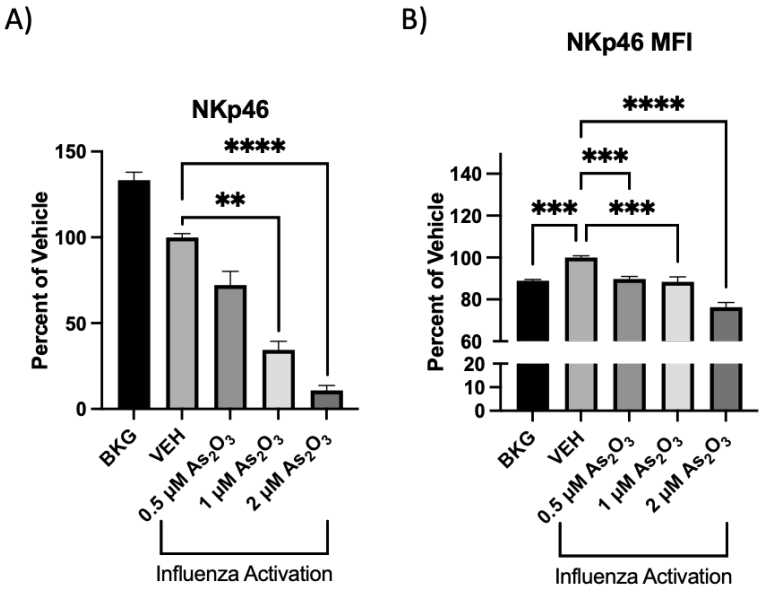

Figure 26: Arsenic trioxide suppressed expression of activating receptor NKp46. Human PBMCs were isolated and treated with arsenic trioxide (0.5- 2 µM As_2O_3), dPBS (VEH), or left untreated (BKG). Samples were either unactivated (BKG) or activated with influenza A virus5 HAU/sample (VEH and 0.5- 2 µM As_2O_3) for 96 h. Cells were stained with viability dye and fluorescently conjugated antibodies against CD3, CD56, and NKp46 and were analyzed by flow cytometry. NK cells were identified as viable $CD56^+CD3^-$ cells prior to analysis of NKp46. Percent vehicle was calculated by dividing individual (A) cell count or (B) median fluorescence intensity (MFI) by the average of the vehicle group. Three experiments were performed in triplicates.
* Denotes P ≤ 0.5, ** denotes P ≤ 0.01, *** denotes P ≤ 0.001, **** denotes P ≤ 0.0001 compared to VEH.

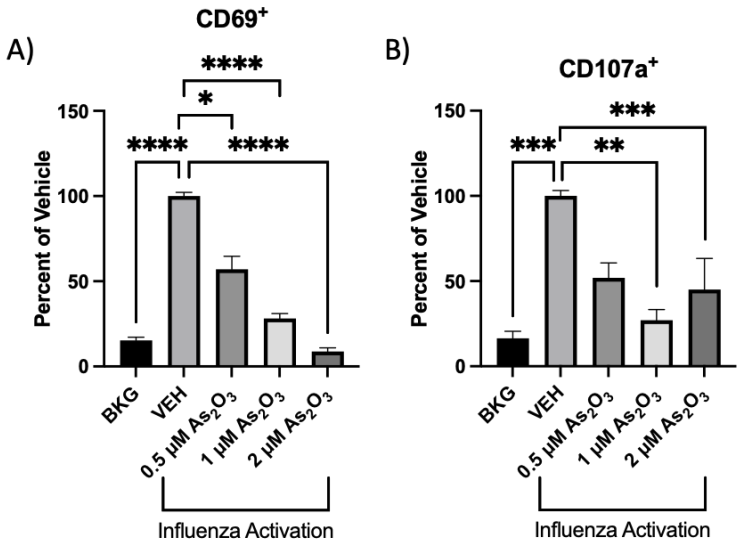

Figure 27: Arsenic trioxide reduced activation and active degranulation in NK cells. Human PBMCs were isolated and treated with arsenic trioxide (0.5- 2 µM As_2O_3), dPBS (VEH), or left untreated (BKG). Samples were either unactivated (BKG) or activated with influenza A virus at 5 HAU/sample (VEH and 0.5- 2 µM As_2O_3) for 96 h. Cells were stained with viability dye and fluorescently conjugated antibodies against CD3, CD56, CD69, and CD107a and were analyzed by flow cytometry. NK cells were identified as viable $CD56^+CD3^-$ cells prior to analysis of surface markers (A) CD69 and (B) CD107a. Percent vehicle was calculated by dividing individual cell count by the average cell count of the vehicle group. Three experiments were performed in triplicates.
* Denotes P ≤ 0.5, ** denotes P ≤ 0.01, *** denotes P ≤ 0.001, **** denotes P ≤ 0.0001 compared to VEH.

NK Cell Cytotoxicity is Impaired with Arsenic Trioxide.

NK cell activation is a necessary precursor to NK cell effector function. Thus, we investigated the effect of ATO on NK cell effector function. NK cells play an essential role in limiting viral replication by rapidly killing virally infected cells through the release of perforin and granzyme.[249] A previous study found arsenic to enhanced NK cell activation and effector potential in response to influenza A infection in mice. More specifically, cell surface expression of CD107a, a marker of active degranulation, was greater in mice exposed to arsenic.[275] In comparison, our study demonstrated a significant decrease in NK cell expression of CD107a at 1

μM and 2 μM ATO compared to the vehicle group (**Figure 27B**). Similarly, intracellular granzyme and perforin, were also reduced in NK cells at 1 μM and 2 μM ATO (**Figure 28**).

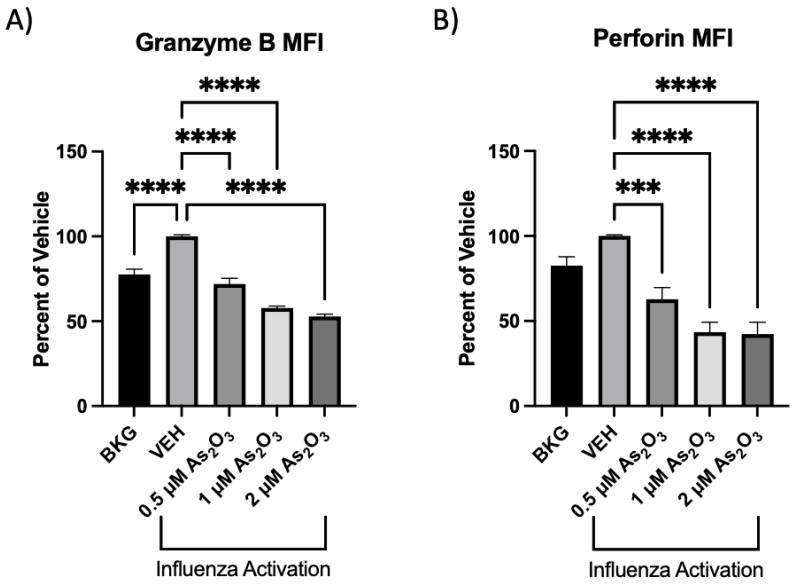

Figure 28: NK cell cytotoxicity was decreased with arsenic trioxide. Human PBMCs were isolated and treated with arsenic trioxide (0.5- 2 μM As_2O_3), dPBS (VEH), or left untreated (BKG). Samples were either unactivated (BKG) or activated with influenza A virus at 5 HAU/sample (VEH and 0.5- 2 μM As_2O_3) for 96 h. Monensin was added 6 h prior to flow cytometry analysis (90 h post treatment). Cells were stained with viability dye and fluorescently conjugated antibodies against CD3, CD56, granzyme B, and perforin and were analyzed by flow cytometry. NK cells were identified as viable $CD56^+CD3^-$ cells prior to analysis of intracellular markers: (A) granzyme B and (B) perforin. Percent vehicle was calculated by dividing individual median fluorescence intensity (MFI) by the average MFI of the vehicle group. Three experiments were performed in triplicates.
* Denotes P ≤ 0.5, ** denotes P ≤ 0.01, *** denotes P ≤ 0.001, **** denotes P ≤ 0.0001 compared to VEH.

Arsenic Trioxide Diminishes IFNγ Expression in NK Cells.

IFNγ is an important antiviral cytokine secreted by NK cells during influenza infection to limit viral replication.[249] Therefore, we also wanted to assess the effects of ATO on NK cell production of IFNγ when exposed to IAV. With influenza, IFNγ expression in NK cells sharply increased in the vehicle group, which confirms NK cell activation. However, cells treated with

ATO exhibited a concentration-dependent decrease in IFNγ to levels similar to the BKG group (**Figure 29A**). Consistent with our FACS analysis, IFNγ secretion detected in the supernatant was also markedly reduced in ATO-treated cells (**Figure 29B**).

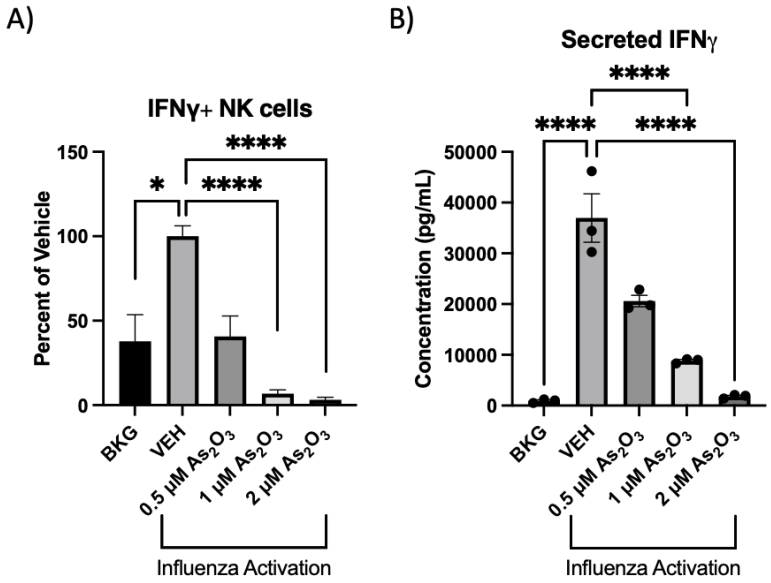

Figure 29: Arsenic diminishes NK cell expression of IFNγ. Human PBMCs were isolated and treated with arsenic trioxide (0.5- 2 μM As_2O_3), dPBS (VEH), or left untreated (BKG). Samples were either unactivated (BKG) or activated with influenza A virus at 5 HAU/sample (VEH and 0.5- 2 μM As_2O_3) for 96 h. Monensin was added 6 h prior to flow cytometry analysis (90 h post treatment). Cells were stained with viability dye and fluorescently conjugated antibodies against CD3, CD56, and IFNγ and were analyzed by flow cytometry. NK cells were identified as viable $CD56^+CD3^-$ cells prior to analysis of intracellular marker, (A) IFNγ. Percent vehicle was calculated by dividing individual cell count by the average cell count of the vehicle group. Three experiments were performed in triplicates. Supernatants collected prior to FACS analysis were used to quantify (B) secreted IFNγ protein by ELISA. Data are representative of an individual experiment done in triplicate.
* Denotes P ≤ 0.5, ** denotes P ≤ 0.01, *** denotes P ≤ 0.001, **** denotes P ≤ 0.0001 compared to VEH.

Discussion

Overall, this study demonstrated ATO decreased overall PBMC and NK cell viability. Additionally, ATO significantly reduced NK cell activation, as demonstrated by decreased

CD107a and CD69 expression and reduced NKp46 MFI in NK cells exposed to influenza virus. Consistent with NK cell activation, ATO significantly reduced important antiviral effector functions, including the production of IFNγ and induction of the cytotoxic molecules, granzyme B and perforin, in NK cells. In addition to the decrease in intracellular expression of IFNγ in NK cells, we also showed an overall decrease in secreted IFNγ protein from supernatants treated with ATO. However, the reduction in IFNγ may also be attributed to other immune cell populations, such as T cells since it was previously reported that ATO attenuated IFNγ production in T cells from IAV-exposed PBMCs.[276]

NK cells are divided into subsets based on the density of surface markers CD56 and CD16. $CD56^{dim}CD16+$ subset compromises about 90% of circulating NK cells, whereas $CD56^{bright}CD16-$ subset make up the remaining 10%, which aligned with what we observed (data not shown).[288] $CD56^{dim}CD16+$ NK cells are known to express higher levels of natural cytotoxicity receptors (NCRs), have increased granularity, and are more cytotoxic compared to $CD56^{bright}CD16-$ NK cells.[289] Human NCRs include the activating receptor, NKp46, which is constitutively expressed on resting and activated NK cells.[290] However, studies have shown a positive correlation between the levels of NK cell surface expression of NKp46 and NK cell cytolytic activity.[291–293] In agreement with our NK viability data (**Figure 24B**), we found NK cells identified as NKp46+ to significantly decrease with ATO-treatment (**Figure 26A**). However, when examining the MFI of NKp46, ATO-treatment decreased induction of NKp46 expression in NK cells compared to the vehicle group (**Figure 26B**), which may indicate diminished NK cell activity in response to IAV infection.

In addition, we found arsenic to decrease NK cell activation, CD69 (**Figure 27A**) and CD107a (**Figure 27B**), and effector function, as demonstrated by reduced NK cell expression of IFNγ, granzyme B, and perforin (**Figures 28** and **29**). In contrast, a previous study found arsenic to enhance expression of genes relating to NK cell effector function and increase cytolytic activity in mice infected with influenza A virus.[275] Specifically, single-cell RNA sequencing showed enhanced inflammatory response which was mediated by dysregulation of innate immune cells.[275] However, arsenic exposure via dust particles impaired NK cell lysis of target cells in mice.[294,295] Disparities in outcomes across studies are likely due to differences in the form of arsenic exposure, species, and experimental model used.

This study is novel because it is the first to investigate the effects of arsenic, at concentrations achievable in humans, on the response of primary human NK cells to influenza. Although there are conflicting studies showing increased NK cell activity with arsenic exposure in mice and NK cell lines, it should be noted that our study is specific to primary human NK cells and aligns with epidemiological studies evaluating arsenic exposure and respiratory disease.[268,270,275,277,296,297] Specifically, in utero arsenic exposure was associated with increased risk of infection during the first year of life.[270] Additionally, children with higher urinary arsenic concentrations were more likely to have pneumonia.[271] Lastly, chronic arsenic exposure was found to exacerbate risk of lung infection.[296] Together, this study furthers our knowledge of the impacts of arsenic trioxide exposure on primary human NK cells in response to influenza A virus, which could negatively impact NK cell-mediated host defense. This study is also unique because we developed an *in vitro* model that can be used to screen the effects of chemicals on the antiviral response of primary human NK cell *in vitro.*