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Nrf2-dependent and –independent effects of tBHQ, CDDO-Im, and H₂O₂ in human Jurkat T cells as determined by CRISPR/Cas9 gene editing

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JPET #238899

Running title: CRISPR/Cas9 to assess Nrf2 and tBHQ in T cell function

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JPET #238899

Abbreviations: tBHQ, *tert*-butylhydroquinone; CDDO-Im, 1[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole; CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9; IL-2, interleukin-2; Keap1, Kelch ECH associating protein 1; Nqo1, NADPH quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2

JPET #238899

Abstract

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a stress activated transcription factor, activated by stimuli such as electrophilic compounds and other reactive xenobiotics. Previously, we have shown that the commonly-used food additive and Nrf2 activator, *tert*-butylhydroquinone (tBHQ), suppresses IL-2 production, CD25 expression, and NF κ B activity in human Jurkat T cells. The purpose of the current studies was to determine whether these effects were dependent upon Nrf2 by developing a human Nrf2-null T cell model using CRISPR/Cas9 technology. The current studies show that suppression of CD25 expression by tBHQ is partially dependent on Nrf2, whereas inhibition of IL-2 secretion is largely Nrf2-independent. Interestingly, tBHQ inhibited NF κ B activation in a Nrf2-independent manner. This was an unexpected finding since Nrf2 inhibits NF κ B activation in other models. These results lead us to investigate another more potent Nrf2 activator, the synthetic triterpenoid CDDO-Imidazolidine (CDDO-Im). Treatment of wild-type and Nrf2-null Jurkat T cells with CDDO-Im resulted in a Nrf2-dependent suppression of IL-2. Furthermore, susceptibility to reactive oxygen species was significantly enhanced in the Nrf2-null clones as determined by decreased mitochondrial membrane potential and cell viability. Importantly, this study is the first to describe the generation of a human Nrf2-null model, which is likely to have multiple applications in immunology and cancer biology. Collectively, this study demonstrates a role for Nrf2 in the effects of CDDO-Im on CD25 and IL-2 expression, whereas the effect of tBHQ on these parameters is complex and likely involves modulation of multiple stress-activated transcription factors, including NF κ B and Nrf2.

Introduction

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that is activated by cell stress, including the presence of reactive oxygen species, electrophilic compounds, and other reactive xenobiotics (Chan et al., 1996; Dinkova-Kostova et al., 2002; Kensler et al., 2007). In the absence of cell stress, Nrf2 is tethered within the cytosol by its repressor protein Kelch ECH associated protein 1 (Keap1), which facilitates the continual degradation of Nrf2 by the 26S proteasome (Itoh et al., 1999; McMahon et al., 2003). Upon activation, Nrf2 accumulates in the nucleus and binds to its consensus sequence in the promoters of target genes, inducing transcription (Venugopal and Jaiswal, 1996). Genes regulated by Nrf2 are often cytoprotective in function and include genes involved in glutathione homeostasis, detoxification, and metabolism. Within leukocytes, Nrf2 has been shown to regulate transcription of NADPH quinone oxidoreductase 1 (Nqo1), making it a good target to assess Nrf2 activity (Itoh et al., 1997; Kensler et al., 2007).

Nrf2 is activated by numerous chemicals, including environmental contaminants, such as heavy metals; synthetic compounds, such as CDDO-Imidazolide (CDDO-Im); and food preservatives, such as *tert*-butylhydroquinone (tBHQ) and butylated hydroxyanisole (BHA). The present studies focused on tBHQ, as it activates Nrf2, is a widely used food preservative, and has robust immunomodulatory effects in T cells at low concentrations (Turley et al., 2015; Zagorski et al., 2013). In the food industry, tBHQ is often added to oils (vegetable, soybean, etc.) to increase shelf life, but it has numerous applications and is found in a variety of foods (Shahidi, 2000). Previous studies have shown that human volunteers who ingested between 100 and 150mg of tBHQ achieved a range of serum concentrations in some cases in excess of 200µM. Thus, in addition to serving as a useful tool for activating Nrf2, tBHQ is also relevant to human

JPET #238899

exposure at the concentrations used in the present studies (Turley et al., 2015; WHO, 1975; Zagorski et al., 2013).

The role of Nrf2 in antioxidant and metabolic responses to reactive toxicants is well described, but it also has a strong anti-inflammatory role. For instance, Nrf2-null mice are more sensitive to insults in a host of inflammatory models, including traumatic brain injury, models of multiple sclerosis, and models of sepsis, among others (Johnson et al., 2010; Saykally et al., 2012; Thimmulappa et al., 2006). Nrf2 has also been implicated in the development of autoimmunity, as female Nrf2-null mice develop a disease similar to systemic lupus erythematosus in humans (Johnson et al., 2010; Li et al., 2004; Ma et al., 2006; Rangasamy et al., 2005; Thimmulappa et al., 2006; Yoh et al., 2001). In humans, the role of Nrf2 is less clear, but our previous studies demonstrate that a known Nrf2 activator, tBHQ, inhibits several events of T cell activation in both primary human T cells as well as in human Jurkat T cells, which suggests Nrf2 may play a role in regulating human T cell activation (Turley et al., 2015; Zagorski et al., 2013).

T cell activation requires two signals. The first is stimulation and signaling through the T cell receptor complex, one member of which is CD3. The second signal is propagated through a co-stimulatory receptor, such as CD28 (Cantrell, 1996). Experimentally, we activate T cells with antibodies targeting both CD3 and CD28, resulting in a signaling cascade and the activation of NF κ B and other transcription factors, induction of the early cytokine IL-2, as well as the expression of early activation markers CD25 and CD69.

JPET #238899

Use of clustered regularly interspaced short palindromic repeats (CRISPR) in conjunction with the endonuclease CRISPR associated protein 9 (Cas9), isolated from *Streptococcus pyogenes*, to mediate targeted gene disruption, has greatly increased over the last several years due to the high efficiency of CRISPR/Cas9 over other methods of gene editing. This technology relies on Watson-Crick base pairing and complementarity of a guide RNA to a specific site within the genome to target the endonuclease Cas9 to the gene of interest and facilitate a double strand DNA break. The cell then initiates non-homologous end joining, which can be error prone. Subsequent insertions or deletions (INDEL) at the site of DNA repair can result in a frame shift mutation, early stop codon, and/or a gene knock out (Cong et al., 2013; Mali et al., 2013a; Mali et al., 2013b; Wang et al., 2013).

The characterization of the physiological and pathophysiological effects of Nrf2 activators on human cells is vital given the ubiquitous nature of such chemicals in the human environment. However, these studies are hampered by lack of adequate tools for mechanistic research, including availability of Nrf2-null human cells. The purpose of the present studies was to address this problem by generating Nrf2-null Jurkat T cell clones as well as to use these clones to determine the role of Nrf2 on T cell function when stimulated with tBHQ, CDDO-Im and H₂O₂.

JPET #238899

Materials and Methods

Materials:

Unless specified, reagents, including tBHQ, were purchased from Sigma-Aldrich (St. Louis, MO).

Plasmids

The NF κ B luciferase reporter was purchased from Promega (Madison, WI). The CRISPR-Cas9 plasmid (PX458) was purchased from Addgene (Cambridge, MA).

Cell culture:

Human Jurkat E6-1 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were then cultured in RPMI 1640 media, with the addition of 10% FBS, 25 mM HEPES, 1 mM sodium pyruvate, 1x nonessential amino acids, 100 U/mL penicillin, and 100 U/mL streptomycin. FBS was purchased from BioWest (Riverside, MO). The protocols for tBHQ treatment of Jurkat T cells are described in the corresponding figure legends.

Generation of CRISPR-Cas9 plasmids:

A CRISPR-Cas9 plasmid (PX458) lacking a gRNA sequence was purchased from Addgene (Cambridge, MA). gRNA sequences targeting different locations within *Nfe2l2* (the Nrf2 gene) were chosen from http://arep.med.harvard.edu/human_crispr/, and Nrf2 specificity was verified

JPET #238899

using <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. Oligos for the identified gRNA sequences were generated by Integrated DNA Technologies (Coralville, IA), and restriction digest cloning was performed as described by the Zhang lab (Ran et al., 2013). After CRISPR-Cas9-transformed bacterial clones were identified, the plasmids were isolated by QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Plasmids were sent to Genewiz (South Plainfield, NJ), using the following primer sequence: 5'-GGGCCTATTTCCCATGATTC-3', to ensure proper cloning of the gRNA sequence into the PX458 vector.

Transient transfection of CRISPR-Cas9 plasmids:

Human Jurkat T cells were reverse transfected with 1.5 µg of plasmid and 3 µl of Lipofectamine 2000 (Life Technologies) per 5×10^5 cells for 12h in complete media. After 12h the cells were washed and resuspended at a concentration of 5×10^5 cells/mL, in complete media. After resuspension, the cells were seeded in a 48-well plate at a volume of 1 mL per well. The cells were then incubated at 37° C for 36 h. After incubation, the cells were sorted by GFP expression and a single GFP-positive cell was deposited per well of a 96-well tissue culture plate, using a BD Influx flow cytometer (San Jose, CA) and allowed to clonally expand.

Identification of Nrf2 mutants by Sanger sequencing:

Genomic DNA was extracted from Jurkat clones, using DNeasy Blood and Tissue kit from Qiagen (Valencia, CA). The region of Nrf2 containing the targeted cut site was then amplified using the high fidelity Herculase II fusion DNA polymerase kit by Agilent Genomics (Santa Clara, CA). The primers used are as follows: exon 2 of Nrf2 forward primer: 5'-GAGGCTGAGGTTGGAAAGTAG-3' reverse primer: 5'-CAGCGACGGAAAGAGTATGAG-3'

JPET #238899

exon 5 of Nrf2 forward primer 5'-TCTACAGGGAATGGGATATGGA-3' reverse primer 5'-CTACTTGGCCTCAGTGATTCTG-3'. DNA extracts from individual clones were then sent to Genewiz (South Plainfield, NJ) for Sanger sequencing, using the forward primer from the PCR reaction for sequencing.

mRNA quantification by real-time PCR:

Total RNA was isolated from 2×10^6 wild-type and Nrf2-null Jurkat T cells using TRIzol Reagent, per the manufacturer's protocol (Life Technologies, Grand Island, NY). After isolation, reverse transcription was performed prior to Sybr green real-time PCR analysis. Relative mRNA expression was calculated by DDCT, normalized to the housekeeping gene, ribosomal protein RPL13a, which has previously been shown to be a stable housekeeping gene in T cell analysis (Mane et al., 2008), using Ct values quantified by Life Technologies/Applied Biosystems Sequence Detection System 7500 (Grand Island, NY). All primer sequences were retrieved from Primer Depot (<http://primerdepot.nci.nih.gov/>) and synthesized by Integrated DNA Technologies (Coralville, IA). The primer sequences are as follows: RPL13A forward primer, 5'-GTTGATGCCTTCACAGCGTA-3' and RPL13A reverse primer, 5'-AGATGGCGGAGGTGCAG-3'; NQO1 forward primer, 5'-TCCTTTCTTCAAAGCCG-3' and NQO1 reverse primer, 5'-GGAAGTGCACCAAGAGCCAT-3'.

IL-2 quantification by ELISA:

Wild-type and Nrf2-null Jurkat T cells were cultured in 96-well plates (1×10^5 cells/well), treated as described and cell supernatants were harvested 24 h after activation with anti-CD3/anti-CD28. IL-2 was quantified from cell supernatants by the use of a commercially

JPET #238899

available ELISA kit (BioLegend, San Diego, CA), per the manufacturer's protocol. The relative light intensity was quantified at 450 nm using a Tecan m1000 Pro microplate reader (Männedorf, Switzerland).

Measurement of extracellular markers by flow cytometry:

Wild-type and Nrf2-null Jurkat T cells were labeled with anti-CD69/PECy7 (BioLegend, San Diego, CA) and anti-CD25/APC (eBioscience, San Diego, CA). After labeling, cells were washed and resuspended in FACS buffer. After resuspension, cells were analyzed by flow cytometry using the BD Accuri C6 (BD Accuri, San Jose, CA). Fluorescence was quantified using CFlow software (BD Accuri, San Jose, CA)

Quantification of mitochondrial membrane potential and viability by flow cytometry:

2×10^5 cells were stained with MitoProbe DiIC1(5) per manufacturer's protocol (Invitrogen, Eugene, OR). Immediately prior to analysis by flow cytometry, 5 μ l of propidium iodide (Biolegend, San Diego, CA) was added to each sample. All samples were analyzed by flow cytometry using the BD Accuri C6 (BD Accuri, San Jose, CA). Fluorescence was quantified using CFlow software (BD Accuri, San Jose, CA)

Nuclear protein isolation:

Wild-type and Nrf2-null Jurkat T cells (1×10^7 cells) were collected 3 h after activation by anti-CD3/anti-CD28. Cells were lysed using a solution containing 10mM HEPES, 100mM KCl, 1.5mM $MgCl_2$, 0.1 mM EGTA, 0.5mM DTT, 0.5% NP-40 substitute, and 1x Halt protease

JPET #238899

inhibitor cocktail (Thermo Scientific, Waltham, MA). After lysis, lysates were spun down and resuspended in nuclear extraction buffer containing 10mM HEPES, 420mM NaCl, 1.5 mM MgCl₂, 0.1mM EGTA, 0.5mM DTT, 5% glycerol, and 1x Halt protease inhibitor cocktail. Samples were kept in nuclear extraction buffer for 1 h, vortexing intermittently. After incubation, samples were spun down and supernatants containing the nuclear fraction of the protein extract were collected. Nuclear protein was quantified via Bradford assay (Bio-Rad, Hercules, CA).

Western Analysis:

After quantification, nuclear protein was diluted in Laemmli Sample Buffer (Bio-Rad) supplemented with 2-mercaptoethanol so that each well contained 10µg of nuclear protein. Samples were subjected to SDS-PAGE and subsequently transferred to a PVDF membrane. Membranes were blocked with 5% NFDM (non-fat dehydrated milk) in PBS containing 0.05% Tween 20 (PBST). Histone H3 (7074S) and Nrf2 (H-300) primary antibodies were diluted 1:1000 in PBST containing 5% NFDM. The H3 primary antibody was purchased from Cell Signaling (Danvers, MA). The Nrf2 primary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibody was HRP-linked and was diluted 1:2000 in 0.05% PBST containing 2% NFDM. The anti-rabbit IgG secondary was obtained from Cell Signaling (Danvers, MA). All blots were developed via Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL) per the manufacturer's protocol. The bands were visualized by the LI-COR Odyssey FC infrared imaging system (Lincoln, NE).

Transient transfection and luciferase assay

JPET #238899

Wild-type and Nrf2-null human Jurkat T cells were reverse transfected with 1.5 µg of plasmid and 3 µl of Lipofectamine 2000 (Life Technologies) per 5×10^5 cells for 12h in complete media. After 12h, the cells were washed and resuspended at a concentration of 5×10^5 cells/mL in complete media. After resuspension, the cells were seeded in a 96-well plate at a volume of 200µl per well. The cells were then treated as described in the figure legends and incubated for 12h. After incubation, the luciferase activity was measured via a commercially-available luciferase assay system kit, using the manufacturer's protocol (Promega, Madison, WI). Luciferase luminescence was quantified by the Tecan Infinite M1000 Pro Microplate Reader (Tecan, San Jose, CA).

Statistical analysis:

The mean \pm standard error was determined for each treatment group in the individual experiments. Data were determined to be homogenous upon passing both the Shapiro-Wilk normality test and equal variance test. Homogeneous data were subsequently evaluated by two-way parametric analysis of variance (in which genotype was one factor and cell treatment was a second factor). When significant differences were observed, the Holm-Sidak post-hoc test was used to compare treatment groups to the vehicle (VEH) control using SigmaPlot 12.3 software (Systat Software, Inc., Chicago, IL).

JPET #238899

Results

CRISPR-Cas9 gene editing causes INDEL formation in Nrf2

Previously, we have shown that the Nrf2 activator, tBHQ, causes a significant suppression in the secretion of IL-2, cell surface expression of CD25, and transactivation activity of NFκB, in human Jurkat T cells (Zagorski et al., 2013). The purpose of the present studies was to determine if these effects are dependent upon Nrf2. Toward that end, CRISPR-Cas9 mediated gene editing was implemented. Guide RNA sequences were selected from a database listing the optimal guide RNA sequences for 40% of the human exome (Mali et al., 2013b). The selected sequences were cloned into empty Cas9 vectors coexpressing GFP, as described previously (Ran et al., 2013). Sanger sequencing of the resulting Jurkat clones revealed that both guide RNAs were successful in generating INDELs at the Cas9 cut site (Fig. 1). Four clones with frame-shift mutations from each guide RNA were then selected to begin screening for Nrf2 disruption.

CRISPR/Cas9-mediated gene editing results in Nrf2 deficiency

Jurkat clones identified as having frameshift mutations were then screened for Nrf2 activity, as ascertained by Nrf2 translocation and induction of Nrf2 target genes when treated with tBHQ. Wild-type Jurkat T cells displayed robust nuclear accumulation 3 h after treatment with 10μM tBHQ. In contrast, none of the CRISPR-Cas9 clones showed nuclear accumulation under the same conditions (Fig. 2a). Furthermore, tBHQ potently induced expression of the Nrf2 target gene Nqo1 in wild-type, but not pooled Nrf2-null clones (Fig. 2b). Likewise, basal expression of Nqo1 was decreased in the Nrf2-null Jurkat T cells as compared to wild-type cells. Taken

JPET #238899

together, the data strongly suggest that targeting Nrf2 by CRISPR-Cas9 successfully generated Nrf2-null clones.

The effect of tBHQ on IL-2 is largely independent of Nrf2

We have previously shown that the secretion of IL-2 by activated Jurkat T cells is suppressed by the Nrf2 activator tBHQ (Zagorski et al., 2013). To test whether this effect was dependent upon Nrf2, we pretreated wild-type and Nrf2-null Jurkat T cells with tBHQ (0.1-1 μ M) 30 min prior to T cell stimulation. IL-2 secretion was evaluated 24 h later. Our data show that suppression of IL-2 by tBHQ is largely independent of Nrf2, as tBHQ inhibits IL-2 production in both genotypes, although the Nrf2-null Jurkat cells were slightly less sensitive (Fig. 3). In the present study, only non-cytotoxic concentrations of tBHQ were used. In order to determine whether the effect of tBHQ on T cells is similar at physiological oxygen concentrations, we treated Jurkat cells with tBHQ and cultured the cells at 5% oxygen to better mimic physiological oxygen concentrations in blood. However, the effect of tBHQ on IL-2 production was similar at 5% oxygen concentration (data not shown).

Suppression of CD25 by tBHQ is partially Nrf2-dependent

In addition to the suppression of IL-2, we have previously shown that tBHQ suppresses the cell surface expression of CD25, the high affinity chain of the IL-2 receptor (Zagorski et al., 2013). To determine the role of Nrf2 in the suppression of CD25 by tBHQ, wild-type and Nrf2-null Jurkat T cells were pretreated with tBHQ prior to T cell stimulation with anti-CD3/anti-CD28. 24h post stimulation, the expression of CD25 and CD69 was quantified by flow cytometry. CD25 and CD69 are cell surface molecules that are rapidly upregulated after T cell activation and thus

JPET #238899

serve as early markers of T cell activation. The data indicate that the inhibition of CD25 expression by tBHQ is partially dependent on the expression of Nrf2, as Nrf2-null Jurkat T cells are more sensitive than wild-type cells to tBHQ treatment. Additionally, tBHQ had little effect on CD69 expression, which is consistent with previous studies (Fig. 4).

NFκB luciferase activity is inhibited by tBHQ in a Nrf2-independent manner

Previous studies have established there is cross-talk between the Nrf2 and NFκB signaling pathways, where activation of Nrf2 inhibits the phosphorylation of inhibitor of kappa B kinase, resulting in a loss of NFκB activity (Liby et al., 2008; Thimmulappa et al., 2006). We have previously shown that tBHQ suppresses NFκB transcriptional activity in Jurkat T cells (Zagorski et al., 2013). To assess the role of Nrf2 in tBHQ-induced suppression of NFκB, we transiently transfected a NFκB luciferase reporter into wild-type and Nrf2-null Jurkat T cells 12 h prior to treatment with tBHQ and T cell activation. Genotypes were normalized to their own vehicle control to control for potential differences in transfection efficiency. These data demonstrate that the inhibition of NFκB activity by tBHQ occurs independently of Nrf2, as both genotypes display similar inhibition of NFκB (Fig. 5).

CDDO-Im inhibits IL-2 secretion in a Nrf2-dependent manner

The current data show that the inhibition of IL-2 secretion and NFκB activity by tBHQ is largely independent of the transcription factor Nrf2. To further investigate the role of Nrf2 in the early events following T cell activation, we investigated the effects of another, more potent, Nrf2 activator, CDDO-Im (Liby and Sporn, 2012). Wild-type and Nrf2-null T cells were treated with CDDO-Im (0.001-0.01μM) for 30 min prior to stimulation of T cells with anti-CD3/anti-CD28.

JPET #238899

After 24h, analysis of cell supernatants demonstrated a Nrf2-dependent suppression of IL-2 secretion in wild-type Jurkat T cells (Fig. 6). Interestingly, treatment of Nrf2-null Jurkat T cells resulted in a Nrf2-independent increase in IL-2 secretion (Fig 6). In the present study, only non-cytotoxic concentrations of CDDO-Im were used. Overall, these data demonstrate that the inhibition of IL-2 secretion by CDDO-Im in activated Jurkat T cells occurs through Nrf2.

Suppression of CD25 by CDDO-Im is partially Nrf2-dependent

To further characterize CDDO-Im in activated Jurkat T cells, we quantified expression of CD25 and CD69. Wild-type and Nrf2-null T cells were treated with CDDO-Im (0.001-0.01 μ M) for 30 min prior to T cell stimulation. After a 24h incubation, the expression of CD25 and CD69 was quantified by flow cytometry. Similar to tBHQ (Fig 4a), CDDO-Im suppressed CD25 expression in a partially Nrf2-dependent manner (Fig. 7a), but had little effect on CD69 expression (Fig. 7b). Taken together, these results demonstrate that CDDO-Im has both Nrf2-dependent and -independent effects upon early events following activation of Jurkat T cells.

Nrf2-null Jurkat T cells are more sensitive to H₂O₂

Reactive oxygen species play an important role in the immune response, both as cytotoxic agents against pathogens as well as signaling molecules. Accordingly, such reactive moieties contribute to and are frequently present during the course of an immune response and thus represent potential endogenous activators of Nrf2 in immune cells. Although important contributors to host defense, reactive oxygen species produced by classically-activated macrophages and other leukocytes can also damage immune cells in the vicinity of the pathogens. To determine the role of Nrf2 in providing protection against oxidative insult, Jurkat

JPET #238899

T cells were treated with cytotoxic levels of hydrogen peroxide (10 – 1000 μ M). Mitochondrial membrane potential was assessed with the cell permeant dye, DiIC, which accumulates in mitochondria with normal membrane potential. A reduction in DiIC fluorescence indicates a disruption of mitochondrial membrane potential and mitochondrial stress. Hydrogen peroxide caused a significant decrease in mitochondrial membrane potential in Jurkat T cells that was more pronounced in the Nrf2-null clones (Fig. 8a). The mitochondrial stress correlated with a decrease in cell viability as assessed by propidium iodide exclusion. The loss of cell viability by hydrogen peroxide was significantly greater in the Nrf2-null clones (Fig. 8b). These data demonstrate that Nrf2-null Jurkat T cells are more sensitive to oxidative stress induced by hydrogen peroxide than wild-type cells, which suggests that Nrf2 may provide protection against endogenous reactive oxygen species produced during the course of an immune response.

Discussion

Nrf2 has emerged as a critical signaling pathway in detoxification, cell survival, and immune regulation (Kensler et al., 2007; Ma, 2013). There is also a growing body of evidence to suggest Nrf2 may play an important role in the promotion of tumorigenesis in some models (De Nicola et al., 2011). Accordingly, investigation into the multiple and complex roles of Nrf2 has become a very active area of research. We have developed the first human Nrf2-null model using CRISPR/Cas9 technology. As such, we believe this model will be a useful tool for a variety of different studies. In addition, the model may help to distinguish between therapeutically beneficial and adverse effects of Nrf2 activation in human immune response and in human cancer, which has been difficult to parse apart thus far.

JPET #238899

In the literature, the role of Nrf2 in human T cell function has been unclear. We have previously shown in both cell lines and primary human T cells that the Nrf2 activator, tBHQ, suppresses cytokine production, expression of surface markers of activation, and activity of NF κ B (Turley et al., 2015; Zagorski et al., 2013). Other groups have utilized other Nrf2 activators, including Benzo[a]pyrene and arsenic compounds, to both implicate and absolve Nrf2 in immune modulation (Katika et al., 2011; Kaufmann et al., 2016; Morzadec et al., 2014; Murugaiyan et al., 2013). In addition, the Jurkat cell line has been used to observe the role of Nrf2 in cellular function apart from T cell activation, including measurement of glutathione induction by Nrf2, as well as antioxidant response (Kataoka et al., 2001; Ogasawara et al., 2014). As pharmacological tools specifically modulating Nrf2 are not readily available, the field as a whole has been reliant upon techniques such as siRNA and shRNA, which result in less than total knockout and thus make the data difficult to interpret. Ultimately, these technical problems have left the question of Nrf2-specific effects in human T cells largely unanswered.

For the current studies, we used this model to address a long-standing question in our laboratory, and the Nrf2 community, concerning the role of Nrf2 in the early events following T cell activation. Our results suggest that the activation of Nrf2 by tBHQ in this setting is complex; while inhibition of IL-2 production by tBHQ appears to be largely Nrf2-independent, the suppression of CD25 expression by tBHQ appears to be partially Nrf2-dependent. The decrease in NF κ B activity by tBHQ appears to be Nrf2-independent. With respect to cell stress caused by a Nrf2 activator that is endogenously produced, hydrogen peroxide, we found that absence of Nrf2 increased the susceptibility of Jurkat T cells to mitochondrial stress and loss of viability.

Although the effects of tBHQ upon the early events of human T cell activation were determined to be independent of Nrf2, the overarching goal of these studies was to determine the role of Nrf2 in human T cell activation. Toward this end, we utilized the synthetic triterpenoid, CDDO-Im, which is widely used as a potent Nrf2 activator. Treatment of wild-type and Nrf2-null human Jurkat T cells with CDDO-Im resulted in a Nrf2-dependent suppression of IL-2 secretion. Importantly, this is the first time to our knowledge, in which activation of Nrf2 has been shown to modulate a human immune response, which is consistent with a role for Nrf2 as an important regulator of human immune function. The concentrations of CDDO-Im used in the present studies were markedly lower than those of tBHQ because CDDO-Im is a more potent activator of Nrf2 (Dinkova-Kostova et al., 2005; Liby and Sporn, 2012). Furthermore, higher concentrations of CDDO-Im were cytotoxic to both wild-type and Nrf2- null cells (data not shown).

One of the unexpected findings of the current studies was that the inhibition of NF κ B activation by tBHQ seems to be independent of Nrf2. This was surprising since tBHQ is a strong Nrf2 activator and activation of Nrf2 has been previously shown to inhibit NF κ B activation in immune cells (Thimmulappa et al., 2006). The regulation of NF κ B is quite complex. Classical activation of NF κ B involves a series of phosphorylation events that culminates in the phosphorylation of inhibitor of κ B α (I κ B α), which is subsequently degraded by the proteasome and thus liberates the p65/p50 homodimer, resulting in activation (Flohe et al., 1997). However, in addition to this component of NF κ B activation, it is also well established that reactive oxygen intermediates also play an essential role. Accordingly, a number of structurally disparate antioxidants have been shown to inhibit NF κ B activation in immune cells, including butylated hydroxyanisole, an

JPET #238899

antioxidant that is structurally similar to tBHQ (Schulze-Osthoff et al., 1993). Thus, it seems plausible that the inhibition of NF κ B transactivation activity by tBHQ may instead be due to direct antioxidant effects.

Recent evidence has led some researchers to suggest that Nrf2 may be a proto-oncogene because it functions endogenously to resolve cell stress to xenobiotics and reactive oxygen species, but the system appears to be hijacked in certain types of cancer (Shelton and Jaiswal, 2013). One of the hallmarks of cancer is immune cell evasion, and this occurs by several mechanisms, such as the upregulation of the T cell inhibitory marker PD-L1. Notably, we have shown previously that tBHQ inhibits Th1 differentiation in a Nrf2-dependent manner (Rockwell et al., 2012). This potentially adds another mechanism by which Nrf2 may promote the survival of tumor cells, as the Th1 response is known to be crucial for tumor cell surveillance. Our current model is unique in that it has applicability beyond immune endpoints. The Jurkat E6-1 cell line is a T cell leukemia. Until recently, determining the role of Nrf2 in human cancer biology has been difficult in that it has relied upon either overexpression or knock down of Nrf2 mRNA, both of which may result in the generation of artifacts (Sanchez-Rivera and Jacks, 2015). The implementation of CRISPR-Cas9 methodology has, for the first time, provided a model in which the role of Nrf2 can be studied, without the addition of such confounding factors. Furthermore, our data demonstrate that in the absence of Nrf2, Jurkat T cells are significantly more sensitive to oxidative stress induced by hydrogen peroxide, further supporting the hypothesis that Nrf2 promotes tumor cell survival.

Overall, this study is novel because, to our knowledge, it is the first to generate a human Nrf2-null model using a genome editing approach, which is a significant improvement over RNA

JPET #238899

interference previously utilized by our lab and others. In most model systems and particularly in immune cells, targeting Nrf2 using shRNA or siRNA has fallen far short of full gene knockout. Our data demonstrate that targeting Nrf2 by CRISPR/Cas9 genome editing essentially abrogated Nrf2 expression and function. Using this model, we determined that the inhibition of IL-2 and its receptor, CD25, by the widely used food additive tBHQ is complex, with Nrf2-dependent and Nrf2-independent components. The data also suggest a role for NF κ B in this process, which likely occurs through direct antioxidant effects of tBHQ. Furthermore, utilization of a second, more potent, Nrf2 activator, CDDO-Im, resulted in a Nrf2-dependent suppression of IL-2 secretion, as well as a partially Nrf2-dependent suppression of the early activation marker, CD25. Collectively, these studies demonstrate that low concentrations of tBHQ that are relevant to human exposure modulate multiple stress-activated transcription factors, which ultimately impairs early T cell response following activation. Furthermore, these studies also showed that Nrf2 activation by CDDO-Im suppresses IL-2 secretion after T cell stimulation, demonstrating that Nrf2 is a regulator of early events following activation of human Jurkat T cells.

JPET #238899

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Authorship contributions

Participated in research design: Zagorski and Rockwell

Conducted experiments: Zagorski and Maser

Contributed new reagents or analytic tools: Zagorski and Liby

Performed data analysis: Zagorski and Rockwell

Wrote or contributed to the writing of the manuscript: Zagorski, Maser, Liby, and Rockwell

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JPET #238899

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Footnotes

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JPET #238899

Figure 1. Wild-type Jurkat E6-1 cells were transiently transfected with one of two CRISPR-Cas9 plasmids coexpressing GFP, targeting different sites within the genome. The two plasmids (panels A and B) contained different guide RNA sequences and targeted different exons on the plus and minus strands of Nrf2, respectively. After sorting positive transfectants, cells were clonally expanded and analyzed by Sanger sequencing. INDEL mutations are listed in red font.

Figure 2. tBHQ increases Nrf2 nuclear accumulation and target gene expression in wild-type cells, but not Nrf2-null clones. (A) Wild-type Jurkat T cells or Nrf2-null Jurkat clones were treated with 10 μ M tBHQ for 3 h. Nuclear protein was then isolated and expression of Nrf2 and the housekeeper histone H3 was probed by western analysis. (B) Wild-type and Nrf2-null Jurkat T cells were left untreated (BKG) or pretreated with vehicle (VEH) control (0.001% ethanol) or tBHQ (0.1 – 1 μ M) prior to activation by anti-CD3/anti-CD28. 6 h post activation, cells were harvested and total RNA was collected. Quantification of NQO1 was performed by SYBR Green real-time PCR. The data (n=3) are presented as mean \pm SE. a = p<0.05 versus wild-type VEH, b = p<0.05 versus Nrf2-null VEH, c = p<0.05 between the wild-type and Nrf2-null genotypes.

JPET #238899

Figure 3. Suppression of IL-2 production by tBHQ is largely independent of Nrf2. Human Jurkat T cells were left untreated (BKG), pretreated with vehicle (VEH) control (0.001% ethanol), or tBHQ (0.1-1 μ M) for 30 min prior to activation with anti-CD3/anti-CD28. After 24 h, the supernatants were collected and IL-2 was detected and quantified via ELISA. The data (n=3) are presented as mean \pm SE. a = p<0.05 versus wild-type VEH, b = p<0.05 versus Nrf2-null VEH, c = p<0.05 between the wild-type and Nrf2-null genotypes, ND = not detectable.

Figure 4. Treatment of Jurkat T cells with tBHQ suppresses CD25 in a partly Nrf2-dependent manner. Human Jurkat T cells were left untreated (BKG), pretreated with vehicle (VEH) control (0.001% ethanol), or tBHQ (0.1-1 μ M) for 30 min prior to activation with anti-CD3/anti-CD28. After 24 h, the percentage of cells expressing (A) CD25 and (B) CD69 was quantified by flow cytometry. The data (n=3) are presented as mean \pm SE. a = p<0.05 versus wild-type VEH, b = p<0.05 versus Nrf2-null VEH, c = p<0.05 between the wild-type and Nrf2-null genotypes.

Figure 5. NF κ B transcriptional activity is decreased by tBHQ treatment independently of Nrf2. Human Jurkat T cells were transfected with luciferase reporter plasmids driven by NF κ B 12 h prior to tBHQ treatment and activation. Jurkat T cells were left untreated (BKG), pretreated with vehicle (VEH) control (0.001% ethanol), or tBHQ (0.1-1 μ M) for 30 min prior to activation with anti-CD3/anti-CD28. 12 h post stimulation, luciferase activity was measured by luminescence, using a Tecan microplate reader. The data (n=3) are presented as mean \pm SE. a = p<0.05

JPET #238899

versus wild-type VEH, b = $p < 0.05$ versus Nrf2-null VEH, c = $p < 0.05$ between the wild-type and Nrf2-null genotypes.

Figure 6. Suppression of IL-2 production by CDDO-Im is Nrf2-dependent. Human Jurkat T cells were left untreated (BKG), pretreated with vehicle (VEH) control (0.001% DMSO), or CDDO-Im (0.001-0.01 μ M) for 30 min prior to activation with anti-CD3/anti-CD28. After 24 h, the supernatants were collected and IL-2 was detected and quantified via ELISA. The data (n=3) are presented as mean \pm SE. a = $p < 0.05$ versus wild-type VEH, b = $p < 0.05$ versus Nrf2-null VEH, c = $p < 0.05$ between the wild-type and Nrf2-null genotypes, ND = not detectable.

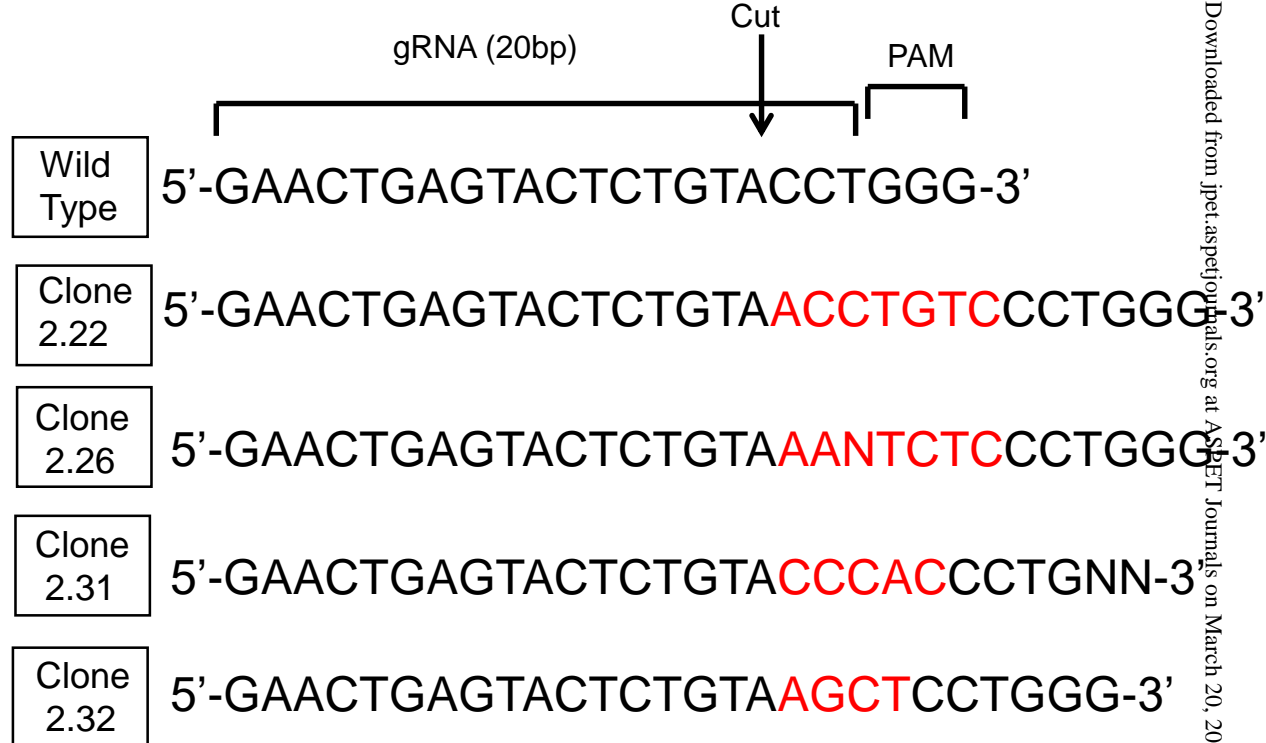
Figure 7. Treatment of Jurkat T cells with CDDO-Im suppresses CD25 in a partly Nrf2-dependent manner. Human Jurkat T cells were left untreated (BKG), pretreated with vehicle (VEH) control (0.001% DMSO), or CDDO-Im (0.001-0.01 μ M) for 30 min prior to activation with anti-CD3/anti-CD28. After 24 h, the percentage of cells expressing (A) CD25 and (B) CD69 was quantified by flow cytometry. The data (n=3) are presented as mean \pm SE. a = $p < 0.05$ versus wild-type VEH, b = $p < 0.05$ versus Nrf2-null VEH, c = $p < 0.05$ between the wild-type and Nrf2-null genotypes.

JPET #238899

Figure 8. Nrf2-null Jurkat clones are more sensitive to exposure to hydrogen peroxide. Human Jurkat T cells were left untreated (BKG), treated with vehicle (VEH) control (0.01% water in RPMI), or H₂O₂ (1-1000μM) for 16 h prior to flow cytometry analysis for (A) mitochondrial membrane potential as measured using the cell permeable dye DiIC1(5) and (B) cell viability as quantified by propidium iodide exclusion. The data (n=3) are presented as mean ± SE. a = p<0.05 versus wild-type VEH, b = p<0.05 versus Nrf2-null VEH, c = p<0.05 between the wild-type and Nrf2-null genotypes.

Figure 1

A.)



B.)

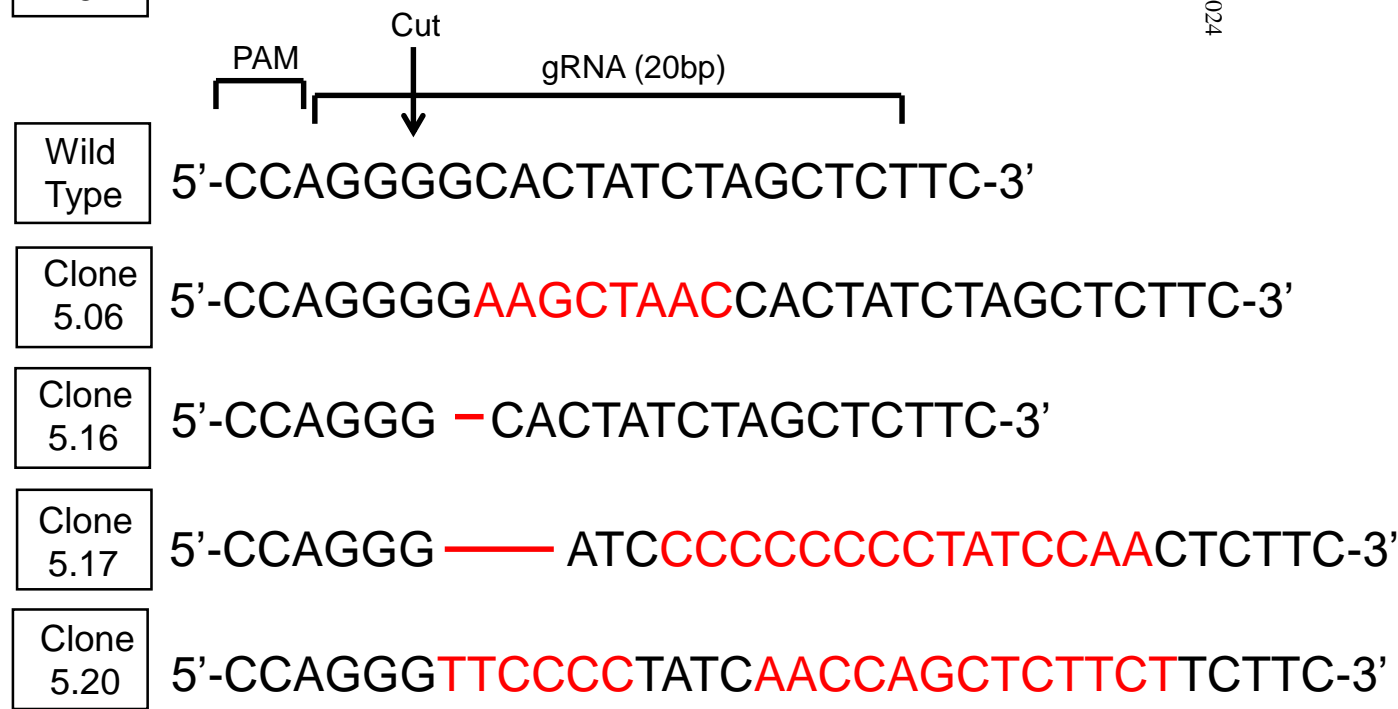


Figure 2

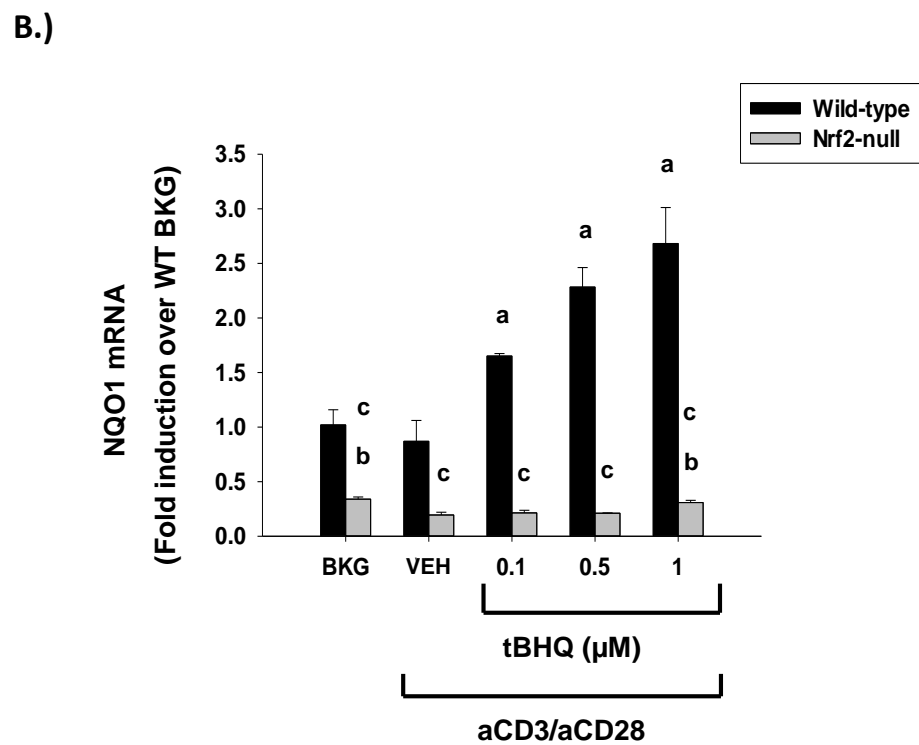
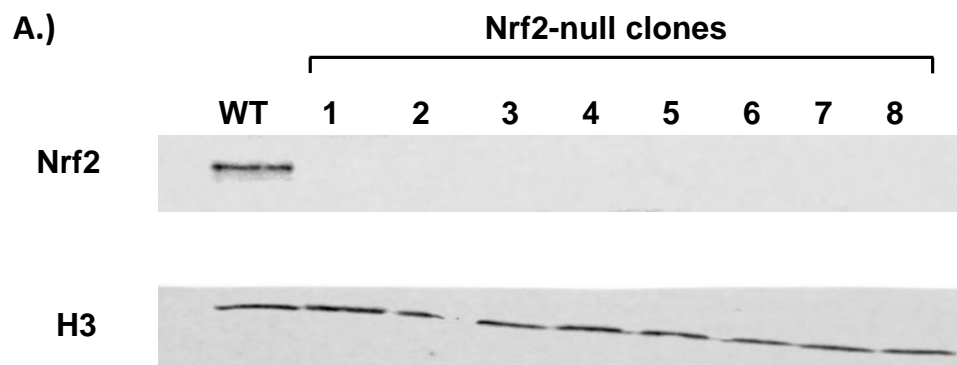


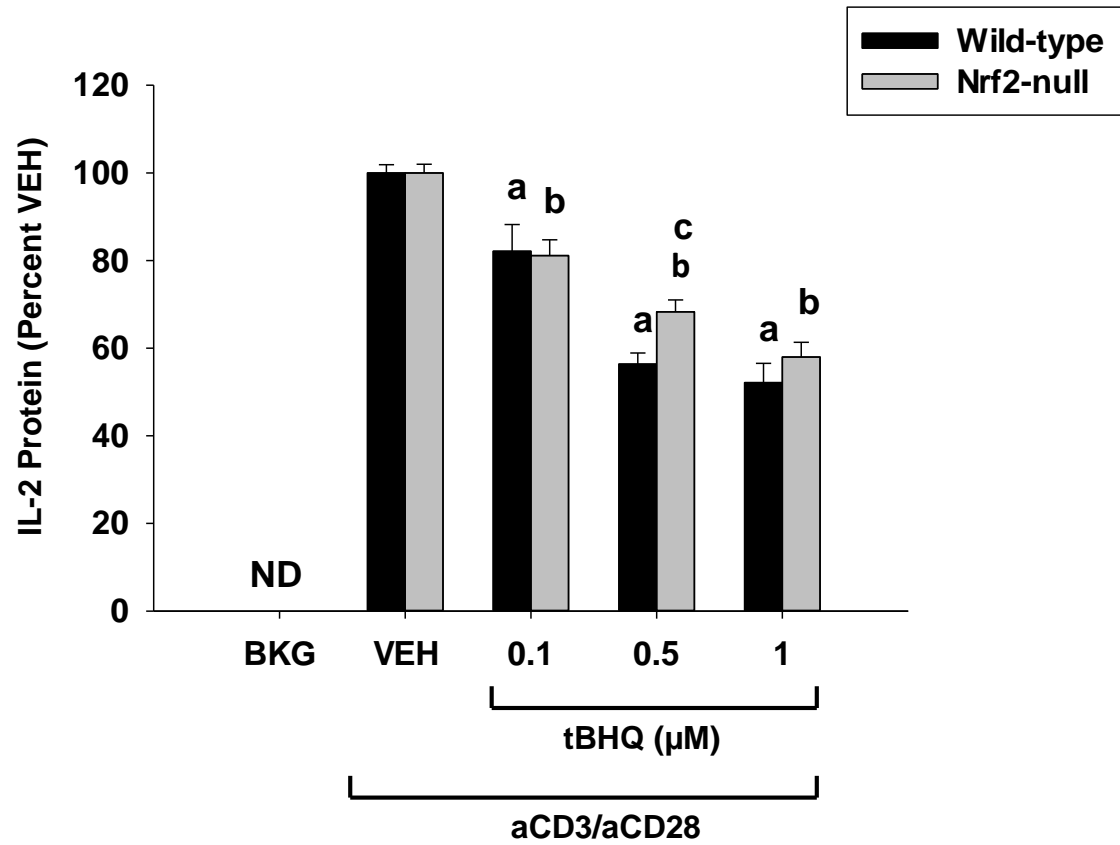
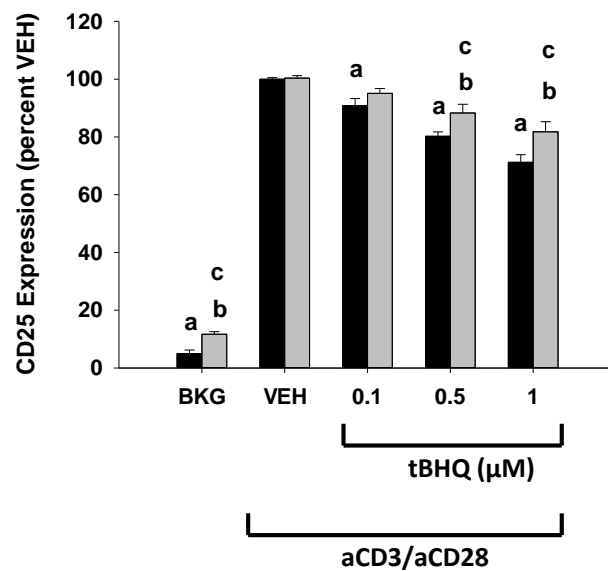
Figure 3

Figure 4

A.)



B.)

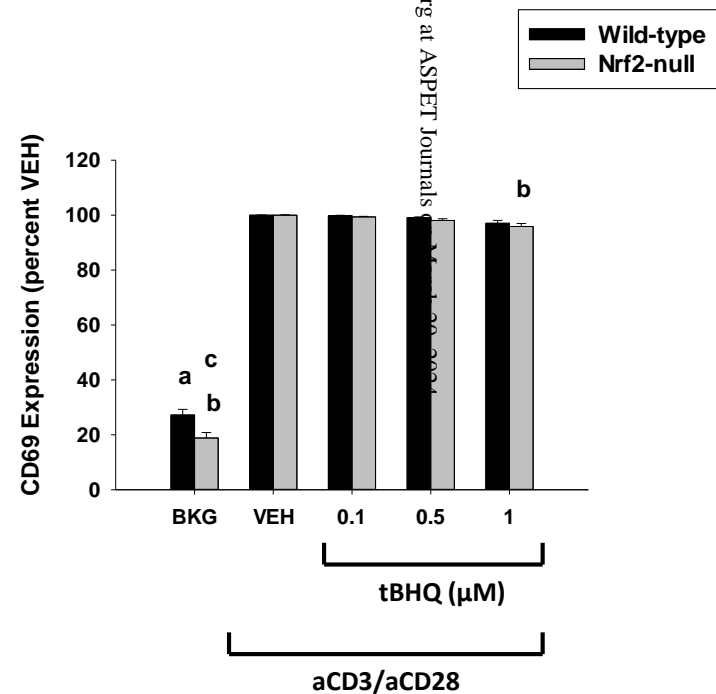


Figure 5

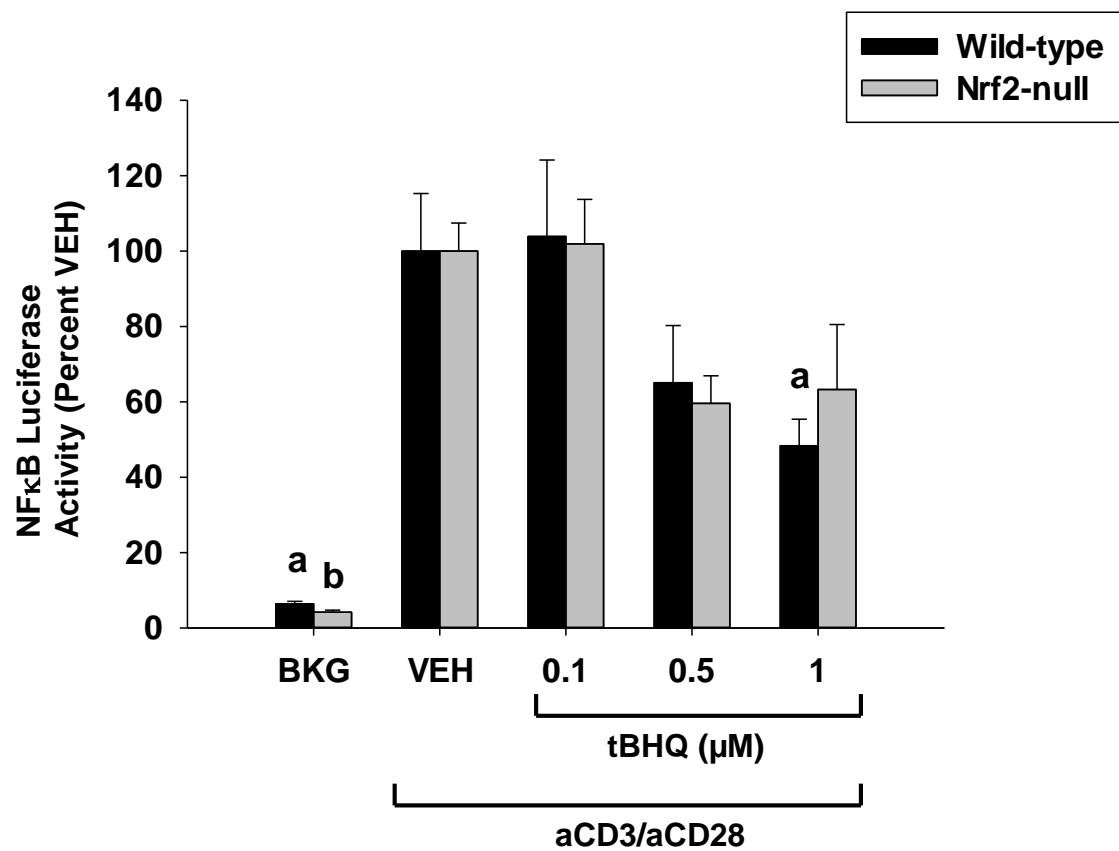


Figure 6

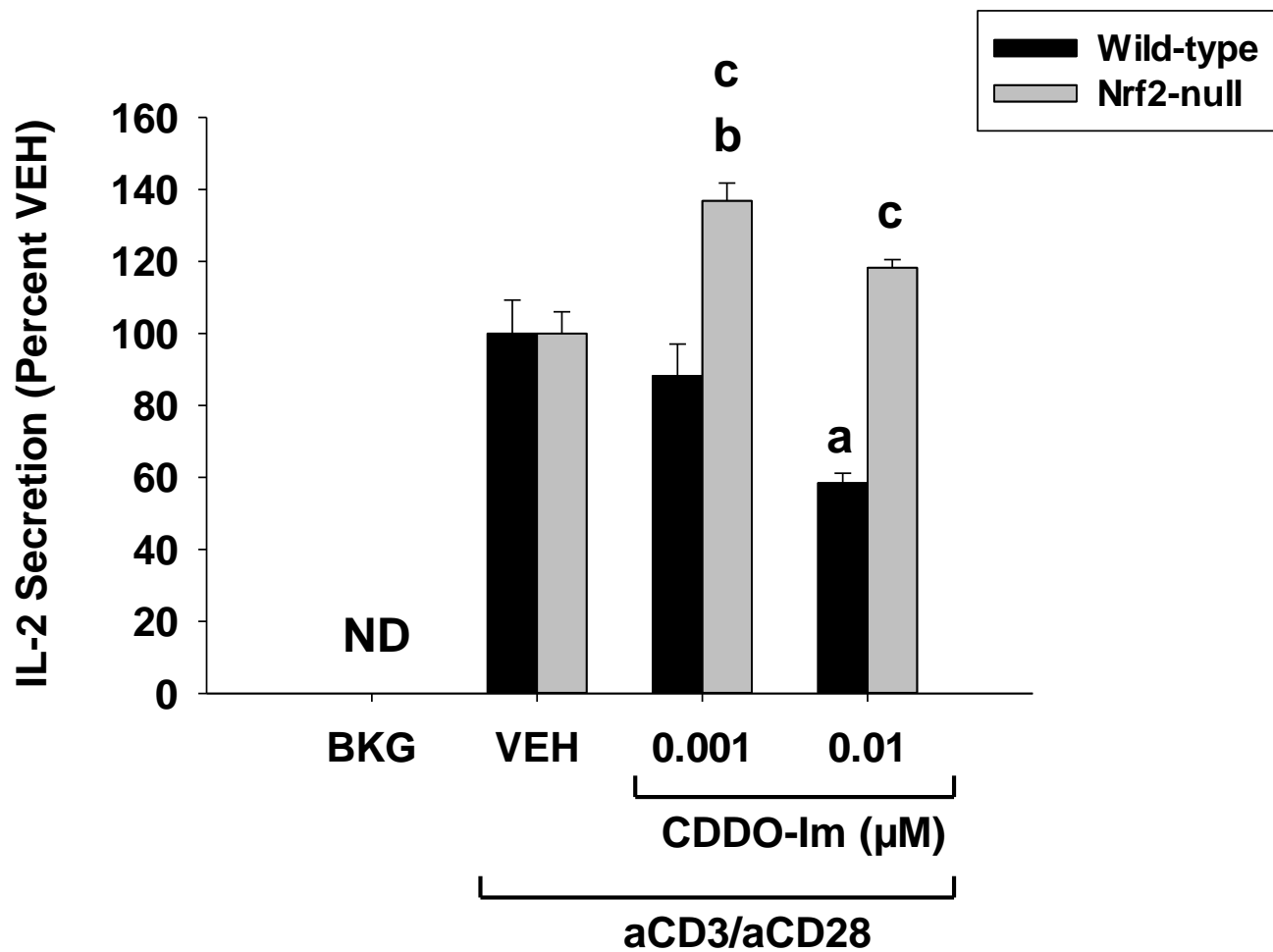


Figure 7

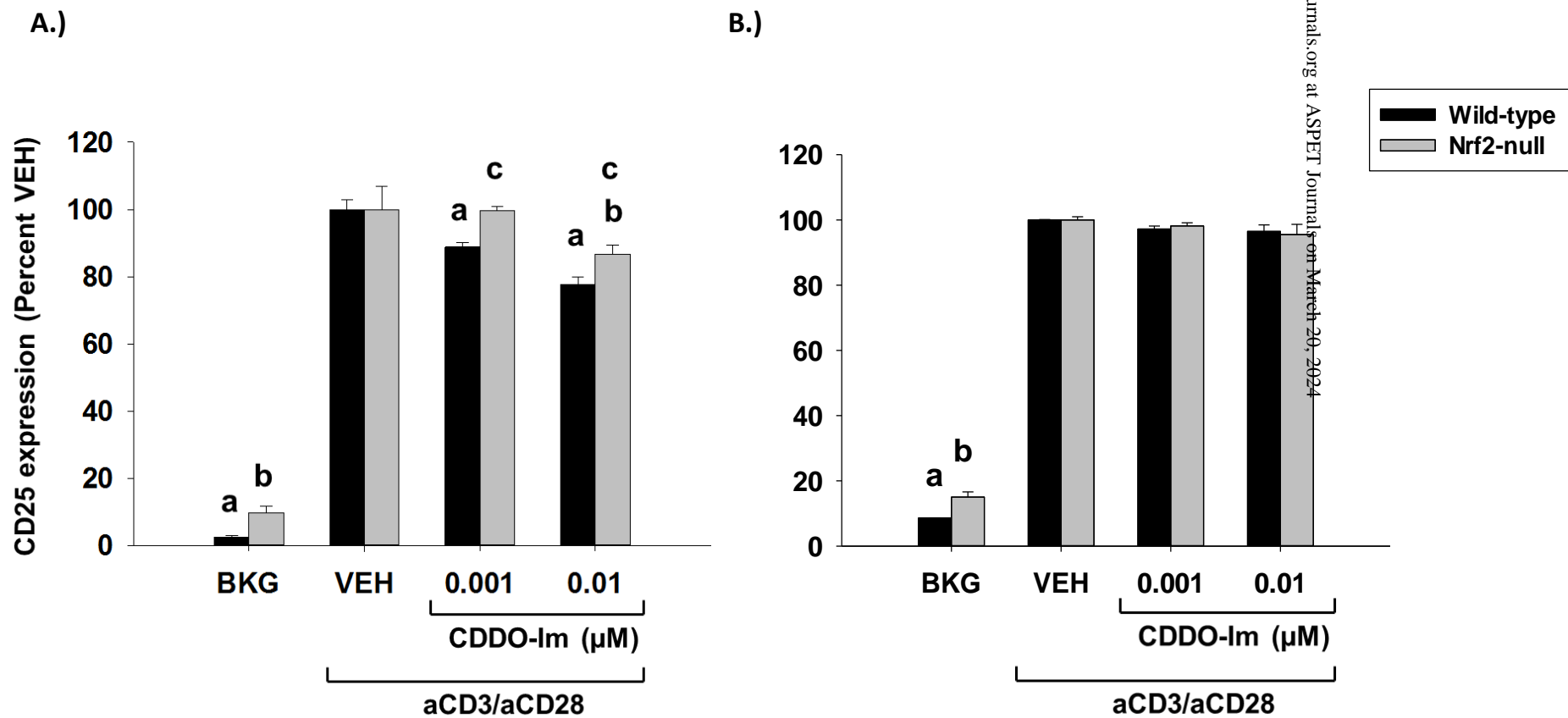


Figure 8

