THE ROLE OF THE NRF2/KEAP1 SIGNALING PATHWAY IN THE EARLY EVENTS FOLLOWING JURKAT T CELL ACTIVATION

By

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ABSTRACT

THE ROLE OF THE NRF2/KEAP1 SIGNALING PATHWAY IN THE EARLY EVENTS FOLLOWING JURKAT T CELL ACTIVATION

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Nuclear factor erythroid 2-related factor 2 (Nrf2) is a stress activated transcription factor, which is activated by reactive oxygen species, reactive electrophiles, and other xenobiotics. Under basal conditions, Nrf2 is tethered in the cytosol by its canonical repressor, Kelch-like ECH-associated protein 1 (Keap1). Keap1 acts as an adapter protein to an E3 ubiquitin ligase and facilitates the continual polyubiquitination and degradation of Nrf2 by the 26s proteasome, in unstressed conditions. In the presence of stressful stimulus, such as electrophilic compounds, key cysteines on Keap1 are modified, disrupting the ability of Keap1 to facilitate Nrf2 turnover, resulting in Nrf2 nuclear accumulation. In the nucleus, Nrf2 heterodimerizes with small Maf proteins and binds its consensus regulatory element, the antioxidant response element (ARE). To date, Nrf2 has been shown to drive the transcription of well over 100 genes, which are largely cytoprotective in function. Recently, Nrf2 has been shown to have a role beyond the antioxidant response. Studies have shown that Nrf2 plays a pivotal role in modulating immune response and inflammatory disease, ranging from autoimmunity to sepsis. Additionally, our lab demonstrated that activation of Nrf2 by the electrophilic food preservative tBHQ skews CD4 T cell fate toward a Th2 phenotype, in primary mouse CD4 T cells, which may increase susceptibility to allergy. In the present studies, we aimed to determine the role of the Nrf2/Keap1 system in human T cell activation, using the human Jurkat E6-1 cell line. In my first study, treatment of Jurkat T cells with the Nrf2 activator, tBHQ, resulted in a dramatic reduction in the early cytokine IL-2 at 24h. Although not as marked as the suppression of IL-2, tBHQ treatment also significantly decreased CD25 expression in these cells but had no effect on CD69 expression, indicating T cell activation was not suppressed as a whole. In correlation with

the decrease in IL-2 induction, tBHQ also inhibited the transcriptional activity of NF□B, an important transcription factor for IL-2 gene regulation. These were the first studies to demonstrate that the Nrf2 activator, tBHQ, suppresses the early events of T cell activation, using a human model; but it did not address whether the effects observed were due to the activation of Nrf2 or rather to off-target effects of tBHQ. Toward this end, we used CRISPR/Cas9 to generate Nrf2-null Jurkat cells. Treatment of wild-type and Nrf2-null Jurkat T cells with tBHQ demonstrated that the suppression of IL-2 and NFκB by tBHQ was largely independent of Nrf2, whereas tBHQ-mediated inhibition of CD25 induction was largely Nrf2 dependent. To further characterize the role of Nrf2 in this model, 1[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), a more potent and selective Nrf2 activator, was used. Interestingly, treatment of Jurkat cells with CDDO-Im resulted in a Nrf2-dependent suppression in IL-2 secretion. These were the first studies to demonstrate that activation of Nrf2 by tBHQ/CDDO-Im modulates immune function in a human model, but did not address basal genotype differences in the absence of xenobiotic. To address this, I utilized CRISPR/Cas9 to knock out the repressor of Nrf2, Keap1, resulting in Jurkat T cells with constitutive Nrf2 activity. Analysis of early endpoints of T cell activation in wild-type, Nrf2-null, and Keap1-null cells show a marked increase in IL-2 induction in Nrf2-null cells, which is consistent with the Nrf2-dependent suppression of IL-2 by CDDO-Im. Furthermore, Keap1-null cells, which have constitutive Nrf2 activity, show marked suppression of IL-2 secretion. Interestingly, Keap1-null cells also displayed a substantial increase in apoptosis, which may be the mechanism by which IL-2 is suppressed in these cells. In addition, modulation of nuclear translocation of c-jun and NFkB was observed in both Nrf2-null and Keap1-null genotypes. These are the first studies to demonstrate that the Nrf2/Keap1 system significantly modulates a number of early immune endpoints of T cell activation, including cytokine production, cell surface protein expression, and cell viability. These are the first studies, using a human model, to demonstrate a role for the Nrf2/Keap1 pathway in modulation of the early events of T cell activation.

This dissertation is dedicated to my loving wife, MacKenzie.
Thank you for taking part in my endeavors and supporting my dreams. I love you more than I could ever say in words.

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KEY TO ABBREVIATIONS

β-TrCP	Beta-transducin repeat containing
ɣc	Common gamma chain
15d-PGJ2	15-Deoxy-Delta-12,14-prostaglandin J2
AhR	Aryl-hydrocarbon receptor
ANOVA	Analysis of variance
AKT	Protein kinase B
ARE	Antioxidant Response Element
ATP	Adenosine triphosphate
BHA	Butylated hydroxyanisole
BLAST	Basic local alignment search tool
BLIMP1	B lymphocyte-induced maturation protein 1
BSA	Bovine serum albumin
bZip	Basic leuicine zipper
Cas9	CRISPR associated protein 9
CCL2	Chemokine ligand 2
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CD25	Cluster of differentiation 25
CD28	Cluster of differentiation 28
CD69	Cluster of differentiation 69
CD80	Cluster of differentiation 80
CD86	Cluster of differentiation 86

cDNA	Complementary DNA
CDDO-Im	1[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole
CNC	Cap n' Collar
CRAC	Calcium release activated calcium channel
CRISPR	Clustered regularly interspaced short palindromic repeats
CTLA4	Cytotoxic T-lymphocyte associated protein 4
Cul3	Cullin 3
DDCt	Delta-delta cycle threshold
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
EGTA	Egtazic acid
ERK	Extracellular signal–regulated kinase
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FRA-1	Fos-related antigen 1
Fyn	Proto-oncogene tyrosine-protein kinase Fyn
GFP	Green fluorescent protein
GSK3	Glycogen synthase kinase 3
gRNA	Guide RNA
H_2O_2	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMOX-1	Heme oxygenase (decycling) 1
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell

ICAM1	Intracellular adhesion molecule 1
IFN-ɣ	Interferon gamma
IκB	Inhibitor of kappa B
IKK	Inhibitor of kappa B kinase
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-2Rβ	Interleukin 2 receptor beta
INDEL	Insertions and deletions
JNK	c-jun N-terminal kindase
Keap1	Kelch ECH-associated protein 1
LCK	Lymphocyte-specific tyrosine protein kinase
MAF	Small musculo-aponeurotic fibro-sarcoma
MAPK	Mitogen-activated protein kinases
MHC	Major histocompatibility complex
MOG	Myelin Oligodendrocyte Glycoprotein
mRNA	Messenger RNA
Neh	Nrf2 ECH-homology
NFAT	Nuclear factor of activated T cells
NFDM	Nonfat dry milk
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NP40	4-Nonylphenyl-polyethylene glycol
Nrf1	Nuclear factor erythroid 2-related factor 1
Nrf2	Nuclear factor erythroid 2-related factor 2
Nrf3	Nuclear factor erythroid 2-related factor 3
NQO1	NAD(P)H quinone oxidoreductase 1

OCT1	Octamer-binding transcription factor 1
p62	Sequestosome 1
p65	RelA
PAM	Protospacer-adjacent motif
PBMC	Peripheral blood mononuclear cells
PBST	Phosphate buffered saline with tween 20
PCR	Polymerase chain reaction
PD-L1	Programmed death ligand 1
PERK	PKR-like endoplasmic reticulum kinase
PI3K	Phosphoinositide-3 kinase
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
Rac	Ras-related C3 botulinum toxin substrate
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
RBX1	RING-box protein 1
RNA	Ribonucleic acid
RPL13a	Ribosomal protein L13a
RPMI	Roswell Park Memorial Institute
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
shRNA	Small hairpin RNA
SLE	Systemic lupus erythematosus
siRNA	Small interfering RNA
SP	Sulforaphane
STAT5	Signal transducer and activator of transcription 5

Src	Proto-oncogene tyrosine-protein kinase Src
TCR	T cell receptor
TBET	T-box transcription factor 21
tBHQ	*tert*-butylhydroquinone
TBTO	Tri-n-butylin oxide
Th2	T-helper cell type 2
TNFα	Tumor necrosis factor alpha
Treg	Regulatory T cell
qPCR	Quantitative PCR
ZAP-70	Zeta-chain-associated protein kinase 70

CHAPTER 1

Literature review

Discovery of the ARE and Nrf2

Organisms experience an almost continual barrage of stressors from both endogenous and exogenous sources. As a result, cells have evolved mechanisms to both repair the damage caused by these insults as well as resolving the root cause. These responses include reducing agents, such as glutathione; induction of metabolic genes, such as cytochrome p450s; and efflux pumps. In the early 1990s a unique regulatory element was discovered in the rat phase II metabolism glutathione S-transferase Ya gene, which is responsive to the synthetic food preservative, and electrophilic xenobiotic, *tert*-butyl hydroquinone [1]. The newly discovered responsive element became known as the antioxidant response element (ARE). Similar AREs were, a short time later, discovered in the regulatory regions of cytoprotective genes in both humans and mice, implying an evolutionary conservation of this cis-regulatory sequence [2,3].

Nuclear factor erythroid 2-related factor 2 (Nrf2) was first cloned while determining proteins that bind to and drive the hypersensitivity site 2 within the beta globin locus [4,5]. Analysis of protein sequences of Nrf2 demonstrated that Nrf2 has a high degree of sequence homology to the basic leucine zipper (bZIP) family of transcription factors, as well as homology to structural Cap n' Collar proteins, shown to be necessary for drosophila mandibular development [4,6]. In mammals, this class of transcription factors is composed proteins such as Nrf1, Nrf2, and Nrf3 [4,5,7,8]. Furthermore, CNC transcription factors are known to heterodimerize with other bZIP proteins, including small Mafs [5,9]. In comparing Nrf2 homology between multiple species, including human and chicken, six highly conserved domains of Nrf2 were discovered and delineated as Neh1 through Neh6 (Fig. 1). The N-terminal Neh2 domain is necessary for Nrf2 turnover by its canonical repressor, Keap1. Beyond the Neh2 domain, the Neh4 and Neh5 domains are responsible for the transcriptional activator function of Nrf2, the Neh6 domain

interacts with a noncanonical repressor, β-TrCP, upon phosphorylation, and the C-terminal Neh1 and Neh3 domains function together to bind the ARE regulatory element [10].

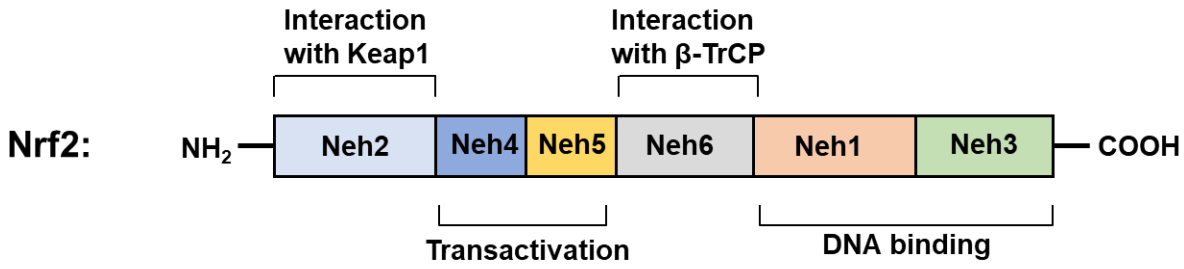

Figure 1: Diagram of Nrf2 functional domains

Keap1, the classical negative regulator of Nrf2

Although Nrf2 contains 6 highly conserved regions (Neh1-6), it is the most conserved region, Neh2, which was found to be necessary for Nrf2 turnover. Using the Neh2 domain of Nrf2 as bait, Keap1 was identified as the classical repressor of Nrf2 [10]. Keap1 is a cysteine-rich protein with at least 10 thiol groups adjacent to basic amino acids, serving to lower their pKa, and increase their reactivity to electrophilic compounds. These cysteine residues then act as electrophilic sensors, allowing electrophiles, such as *tert*-butylhydroquinone (tBHQ) to form adducts [11–13]. Under basal conditions Keap1 acts as a homodimer and is anchored to the actin cytoskeleton within the cytosol, where it serves as an adapter protein to an E3 ubiquitin ligase, RBX-1/Cul3 [14–17]. It is this interaction that facilitates the continual polyubiquitination and subsequent degradation of Nrf2. Two domains within the Neh2 region of Nrf2 are known to interact with Keap1. The DLG and ETGE have differing binding affinities to Keap1, with the ETGE domain binding with higher affinity, effectively tethering Nrf2 within proximity to the ubiquitin E3 ligase complex, RBX-1/Cul3, and resulting in the subsequent degradation of Nrf2 by the 26s proteasome [10,16,18–20]. Interestingly, Nrf2 has been shown to also facilitate its own turnover by driving the transcription of both RBX-1 and Keap1 [21]. Furthermore, though Keap1 is

classically thought to tether Nrf2 within the cytosol, evidence has shown that Keap1 itself can be shuttled into the nucleus, serving to further suppress Nrf2 activity and adding to the complexity of Nrf2 regulation [22].

Nrf2 activation and the ARE

The interaction of Keap1 with the ETGE and DLG motifs on the N-terminus portion of Nrf2, under physiologically unstressed conditions, results in a half-life of less than 20 minutes for Nrf2 [23–25]. The retention of Nrf2 within the cytosol is further facilitated by the tethering of Keap1 to the actin cytoskeleton [10,24]. Induction of Nrf2 can occur under a variety of stressful stimuli, including electrophilic xenobiotics and reactive oxygen species [26–28]. Endogenously, studies suggest that some proinflammatory stimuli, such as the prostaglandin 15d-PGJ2, can induce Nrf2 activity in macrophages [29]. The induction of Nrf2 largely occurs due to adduct formation between the toxicant and cysteines present on Keap1. Because of their lower Pka, these thiol residues act as electrophile sensors [11–13]. Additionally, studies have shown that induction of Nrf2 by modification of Keap1 cysteines is stimulus-specific. For instance, cysteine 151 is necessary for induction of Nrf2 by both tBHQ and another Nrf2 activator, sulfurophane (Sp) [26,27]. Furthermore, cysteines 273 and 288 are required for Keap1-mediated Nrf2 suppression [27]. Other stimuli, such as hydrogen peroxide, do not form adducts with Keap1, but instead cause conformational changes within Keap1, facilitating the liberation of Nrf2 [30]. Further adding to the complexity of the activation of Nrf2, some stimuli have been shown to fully liberate Nrf2 from Keap1; others act as a hinge and latch, only dissociating the low affinity DLG motif from Keap1, and others serve to cause the degradation of Keap1 itself [18,19,31,32].

Regardless of the mechanism of activation, activated Nrf2 accumulates within the nucleus, where it heterodimerizes with proteins, such as small Mafs, and acts as a master regulator of the cell stress response [33,34]. To date, Nrf2 has been shown to regulate well over 100 genes that contain the cis-acting regulatory element known as the antioxidant response element or ARE [35]. Binding of Nrf2 to the ARE results in the recruitment of transcriptional machinery and subsequent transcription of the gene target. Some examples of these genes include glutathione S-transferase Ya and NAD(P)H quinone oxidoreductase [33,34].

Post-translational regulation of Nrf2

Classical regulation of Nrf2 is often considered to be facilitated by Keap1, however a number of studies indicate that post-translational modification of Nrf2 plays a critical role, albeit complex. Whereas reactive oxygen species and electrophilic compounds control the entry of Nrf2 into the nucleus by modification of Keap1 directly, phosphorylation of Nrf2 has been shown to play a similar role [36,37]. For example, phosphorylation of Nrf2 within the Neh2 domain by PKC results in the liberation of Nrf2 from Keap1 and its subsequent accumulation in the nucleus [37–39]. Other kinases such as PI3K and PERK activate Nrf2 in a similar manner [40–42]. Although some studies have shown JNK and ERK to activate Nrf2, subsequent work mutating the sites of MAPK phosphorylation show similar activation, implying regulation of Nrf2 activation by MAPK may occur by indirect mechanisms, further adding to the complexity of Nrf2 regulation by multiple multiple pathways and interactions [37].

Phosphorylation of Nrf2 has, by contrast, also been shown to facilitate Nrf2 turnover [43]. Canonically, nuclear accumulation of Nrf2 is associated with activation of the transcription factor, but within the nucleus some kinases have been shown to phosphorylate Nrf2, resulting in Nrf2 nuclear export. Within the cytosol, Nrf2 is again polyubiquitinated and degraded,

facilitated by interaction with Keap1 [44]. Over the last several years, however, another major mode of Nrf2 turnover has been elucidated. Glycogen synthase kinase-3 (GSK3) has been shown to phosphorylate Nrf2 within the Neh6 domain, which is more C-terminal than the Neh2 domain, which interacts with Keap1 [45]. Phosphorylation of Neh6 results in recognition of Nrf2 by β-TrCP, which acts similarly to Keap1 to facilitate the interaction of Nrf2 with the Cul3/Rbx1 complex, resulting in Nrf2 degradation by the proteasome, completely independently of Keap1 [45,46]. Further strengthening the role of β-TrCP as a negative regulator of Nrf2, PI3K has been shown to inhibit the activity of GSK3, promoting the stabilization of Nrf2 [44–47]. Conversely, using inhibitors of the PI3K pathway results in lower levels of expression of Nrf2 target genes, such as NQO1.

Other mechanisms of Nrf2 regulation

As previously stated, in the presence of stressful stimuli, Nrf2 is no longer degraded by the proteasome, and accumulates within the nucleus of cells, where it serves as a key transcription factor, inducing cytoprotective genes. Protein turnover, however is only one mechanism of regulation. Typically, Nrf2 heterodimerizes within the nucleus with small Maf proteins, such as MafG and binds to the ARE, inducing gene transcription. Under certain circumstances, however, Nrf2 heterodimerizes with other binding partners, such as Fra-1, a member of the AP-1 family of transcription factors, among others [34,48–52]. This non-canonical partnering results in binding to the ARE and negative gene regulation. Interestingly, Nrf2 also autoregulates itself by upregulating key components of Nrf2 negative regulation, such as proteasomal subunits and BACH1, which can partner with Nrf2 to inhibit signaling through the ARE, similar to Fra-1. Conversely Nrf2 has been shown to drive its own transcription, adding to the complex regulation of the system [53–55]. Taken together, Nrf2 is regulated through a variety of mechanisms, involving several different pathways, and modifications.

Nrf2 and cancer

Shortly after the discovery of Nrf2 in the early 1990s, Nrf2-null mice were developed. Initial studies indicated that the mice developed normally and were fertile, implying that Nrf2 is not necessary for development [28,33]. Similarly, no changes in erythropoiesis were observed, which was interesting, given Nrf2 was discovered while cloning the beta globin control locus [28]. Although the Nrf2-null mice are both fertile and viable, they have been shown to be more sensitive to several disease states [56]. Interestingly, it has been shown that Nrf2 plays a critical role in the progression and chemoprevention of cancer [57,58]. This is somewhat counterintuitive, as Nrf2 is a master-regulator of the antioxidant response and is known to play a critical role in the amelioration of cell damage cause by oxidants, which would imply that Nrf2 functions as a tumor suppressor [11,56]. Consistent with this, studies done in human subjects from Qidong, China have shown that ingestion of two Nrf2 activators, Sp and oltipraz, resulted in an increase in the metabolism and excretion of aflatoxin, a fungal toxin and food contaminant known to cause hepatocellular carcinoma [59,60]. Furthermore, a number of cytoprotective genes are not induced in Nrf2-null mice after treatment with known oncogenic toxicants, resulting in a higher incidence of tumorigenesis [61,62]. Taken together, these studies demonstrate that Nrf2 has an endogenous role as a tumor suppressor, but like many biological systems, the role of Nrf2 in the development and progression of cancer is context-specific. Nrf2 has been shown to promote detoxification of cancer-causing toxicants, but the literature also shows a role for Nrf2 in the promotion of cancer. Specifically, Nrf2 is upregulated in endometrial, head and neck, lung, and gall bladder cancers, among others [61,63–68]. Additionally, upregulation of Nrf2 in cancer increases chemoresistance and radioresistance of tumors [57,65,69–71]. These studies contrast with the previously mentioned work demonstrating that Nrf2 functions endogenously as a tumor suppressor, implying Nrf2 also acts as a tumor promoter. The role of Nrf2 in the cytoprotection

of cancer cells has been termed the dark side of Nrf2 and demonstrates Nrf2 has a role in both prevention and progression of cancer [69].

Nrf2 and immune modulation

After the development of Nrf2-null mice, it was soon discovered that female Nrf2-null mice develop a disease which resembles lupus in humans, as evidenced by presence of auto-antibodies in serum targeting the host's double stranded DNA, as well as deposition of antibody complexes within the glomerulus, resulting in inflammation, glomerulonephritis, and eventual death [72,73]. Although the development of this autoimmune phenotype occurs in several mouse strains, the severity of disease is dependent on genetic background [72,74]. Further cementing the role of Nrf2 in the maintenance of proper immune response, Johnson *et. al.* further characterized the susceptibility of Nrf2-null mice to models of autoimmune neurodegeneration, such as experimental autoimmune encephalomyelitis, which is a model of multiple sclerosis [75]. In these studies mice were inoculated with the MOG peptide, which is a component of the myelin sheath, which resulted in significant increases in disease scores in Nrf2-null mice. By comparison, activation of Nrf2 by a variety of compounds protected wild-type mice from neurodegeneration in multiple models of autoimmune disease, functioning through modulation of T cell and macrophage populations [76–79]. Similarly, studies have found Nrf2-null mice to have worsened disease phenotypes in several autoimmune models, including hemolytic anemia and experimental autoimmune hepatitis, which can be ameliorated in wild-type mice by treatment with Nrf2-activating compounds, such as CDDO-ImSp [80–83]. Deletion of Nrf2 additionally increases the sensitivity of Nrf2-null mice to injury in inflammatory models other than autoimmunity, including traumatic brain injury, acute kidney injury, nephritis, sepsis, asthma, and injury due to hyperoxia, to name a few [84–91].

Numerous studies clearly demonstrate that the lack of Nrf2 results in an increased sensitivity to inflammatory disease, but the mechanism is unknown. Some early studies attempted to generate a constitutively-active Nrf2 model by knocking out Keap1, but the resulting mice died shortly after birth due to hyperkeratinization of the esophagus [92]. As a result, studies that focus on Nrf2 are largely restricted to the use of non-specific toxicants to activate the pathway, which frequently have both Nrf2-dependent and independent effects. In macrophages and dendritic cells, activation of Nrf2 has been shown to inhibit the expression of costimulatory markers, TNFα signaling, as well as dendritic cell maturation [85,93–95]. Studies from our lab have shown that activation of Nrf2 by tBHQ in isolated mouse CD4 T cells results in skewing of CD4 T cell polarization toward a Th2 phenotype, which may indicate an increase in susceptibility to the development of allergy and asthma [96]. Studies from our lab also show that activation of Nrf2 modulates early cytokines, such as Interleukin- 2 (IL-2) and Interferon-gamma (IFNɣ) [96].

The effect of Nrf2 activation upon human immune response has proven to be more difficult to elucidate, as no Nrf2-specific pharmacological agents are commercially available; and, until recently, no human Nrf2-null models were known. As a consequence, the results of such studies have been difficult to interpret, as Nrf2 activators, such as tBHQ, benzo[a]pyrene, and arsenic have Nrf2-independent effects [97–101]. Because of this, some investigators have turned to over-expression of Nrf2 or partial knock-down of the gene using RNA interference [102]. Recently, we have shown that the Nrf2 activator tBHQ suppresses IL-2 secretion by activated human T cells, in both a cell line and primary human models [97,98]. By knocking out Nrf2 in human Jurkat T cells, my research has shown the effect of tBHQ. However, a more potent Nrf2 activator, CDDO-Im, suppressed IL-2 secretion by activated Jurkat T cells in a Nrf2-dependent manner [101]. These studies were the first to demonstrate that Nrf2 activation modulates human CD4 T

cell response. Moreover, epidemiological studies have indicated that polymorphisms within the Nrf2 locus result in decreases in Nrf2 expression and an increase in TNFα in primary human PBMCs [103]. Similar studies have shown certain polymorphisms to cause an increase in the risk of certain diseases, including autoimmunity and lung injury [104,105]. In both mice and humans, numerous studies point to a role for Nrf2 in the modulation of innate and adaptive immune response, demonstrating a need for additional studies to fully elucidate the role of Nrf2 in the immune system, as well as the mechanism by which Nrf2 mediates its effects.

Nrf2 and NFκB cross-talk

As evidenced by the post-translational modification of Nrf2 by multiple kinase cascades, the Nrf2/Keap1 system interacts with many other pathways, which is, at least partially, stimulus- and context-dependent. These pathways range from the AhR and Notch to AP-1 and nuclear factor for κ chain in B cells (NFκB) [56]. One of the best established and most studied mechanisms of cross-talk between the Nrf2/Keap1 axis and another pathway is NFκB. NFκB is a ubiquitous transcription factor that controls varying cellular functions, from stress pathways such as glutathione to induction of several cytokines, including IL-2 [56,106]. The mechanism and result of these interactions is highly complex and context- and model-specific. For instance, Nrf2-null mice have shown an increase in NFκB activity in several models of disease, and activation of Nrf2 has been shown to inhibit NFκB activity by multiple mechanisms, including by indirect mechanism, such as the induction of Nrf2 target genes, such as HMOX-1 [84,85,107–111]. Conversely, NFκB has also been shown to antagonize the transcriptional activity of Nrf2 by competing for the Nrf2 binding partner, CREB [112]. Other stimuli, such as low-density lipoprotein and shear stress have been shown to activate both pathways simultaneously, demonstrating that, although there is a well-defined interplay between the NFκB pathway and the Nrf2/Keap1 pathway, they do not simply promote or antagonize one another [113,114].

T cell activation and signaling

T cells are a key player in both molding humoral responses, as well as playing an active role in direct cell killing [115]. Activation of T cells is a highly regulated process that requires two signals, and in the absence of both signals, T cells will become unresponsive and often apoptotic [116,117]. The first signal is generated by the interaction of the T cell receptor complex (TCR) with the major histocompatibility complex (MHC) on antigen presenting cells [106]. Mature MHC expressed on a cell's surface is loaded with peptides and therefore constitutively presents potential antigen to T cells, but these peptides are typically termed as self, as they are a part of the host's normal physiology [118]. Although these self-associated peptides are not typically capable of activating T cells, they generate the basal pro-survival signal needed to inhibit apoptosis [118,119]. When the proper MHC-antigen combination is recognized by a T cell, the interaction between MHC and TCR will increase from less than one second, to up to 10 seconds [120]. This increase in duration and strength of signal leads to the recruitment of two Src-family kinases LCK and FYN, which in turn phosphorylate the cytoplasmic tails of the ζ-chains of the TCR [121–125]. This phosphorylation event serves to change the conformation of the cytoplasmic tails, allowing their use as a scaffold for other proteins, ultimately resulting in the activation of other kinase cascades, such as MAPK, JNK, and PI3K, as well as a marked increase in intracellular calcium [106]. These events result in the activation of several transcription factors, such as AP-1, nuclear factor of activated T cells (NFAT), and NFκB, which are key in the upregulation of many cytokines, such as IL-2 [126].

The second signal necessary for T cell activation comes from one of several costimulatory markers. Costimulatory markers for activation of T cells are present on almost all cell types, but they are most abundantly expressed and studied on professional antigen presenting cells, such as dendritic cells and macrophages [127]. CD28 is a well-characterized costimulatory marker for T

cell priming and activation [128,129]. Ligation of the TCR by antibody or through interaction with loaded MHC results in the recruitment of CD28 to the immune synapse where it interacts with CD86 and/or CD80 on antigen presenting cells [123]. Interaction of CD28 with CD86/CD80 results in recruitment of PI3K and activation of several signal transduction cascades, including JNK, AKT, and ERK [130,131]. The amplification of signal through these branched pathways results in the further activation of NFκB, NFAT, and AP-1 [131]. The increased strength of signal through these transcription factors allows for proper induction of IL-2, which is a key early cytokine in many immune functions. Ligation of CD28 has been shown to be the integral costimulatory mechanism by which IL-2 is fully induced and IL-2 is induced to a much lesser extent when T cell stimulation occurs through other costimulatory molecules [131].

T cell stimulation is a highly controlled, inducible process and negative regulation of T cell response plays a key role in preventing potentially life-threatening damage to the host. There are several means for shutting down signaling through the T cell receptor, including inducing cell death, thereby promoting immune contraction after infection [132,133]. Another means of regulating T cell activation is the coinhibitory receptor CTLA-4. After stimulation, T cells upregulate CTLA-4, which competes with CD28 for space within the immune synapse [134–136]. Unlike CD28, however, CTLA-4 does not induce kinase cascades, but rather recruits phosphatases to the TCR. The phosphatases remove phosphates from the signaling ζ-chains of the TCR and Zap70, thereby inhibiting signaling most proximal to the engaged TCR [135,136].

IL-2 regulation

Several decades ago, scientists discovered that supernatants from activated T cells stimulate the proliferation of unstimulated T cells, when added in culture [137,138]. Shortly thereafter, IL-2

was discovered and was initially known as T cell growth factor. Since then, it has been determined that IL-2 plays a diverse role in many cell types, immune and non-immune alike. IL-2 is an early cytokine produced mainly by CD8 and CD4 T cells but is secreted in larger amounts in the latter [139,140]. After production, IL-2 acts in both an autocrine and paracrine fashion, being secreted directionally into the immune synapse [115,141]. Recognition of antigen loaded in the proper MHC by T cells results in a rapid upregulation of IL-2 mRNA which is stabilized by receipt of costimulatory signals propagated through CD28 [142–144]. The transient upregulation of IL-2 mRNA is mediated through the inducible transcription factors NFAT, AP-1, and NFκB, which accumulate within the nucleus and bind the promoter of IL-2 along with the constitutive transcription factor Oct1 [143,144].

Once transcribed and translated, the half-life of IL-2 is still short, about 10-20 minutes, depending on the model. The short half-life of IL-2 is largely due to its rapid uptake by target cells, as well as IL-2 secreting cells [145–147]. This process is mediated by the IL-2 receptor, which is a heterotrimeric receptor composed of CD25, IL-2Rβ, and the common ɣ chain ($ɣ_c$) [146]. CD25 is rapidly upregulated upon IL-2 production and has a higher affinity for IL-2 than either IL-2Rβ or $ɣ_c$, but signaling through the IL-2 receptor occurs through the cytoplasmic tails of IL-2Rβ and $ɣ_c$ [145,146]. Ligation of the IL-2 receptor by IL-2 protein results in rapid receptor-mediated endocytosis, where CD25 is recycled to the cell's surface and IL-2Rβ and $ɣ_c$ are targeted for lysosomal degradation, serving as a mechanism to control the availability of IL-2 as well as signaling through the IL-2 receptor [145].

The negative regulation of IL-2 is similarly as complex as its activation and is mediated by several mechanisms. Several mechanisms of negative IL-2 regulation exist, with one being

mediated by IL-2 itself. IL-2 has been shown to induce the transcription factor Stat5, which in turn induces transcription of Blimp1, a known negative regulator of the IL-2 promoter [148–150]. Furthermore, expression of TBET, the master regulator of Th1 polarization, serves to shut down the IL-2 promoter by out-competing the IL-2 promoter for p65, an integral subunit of the transcription factor NFκB, resulting in suppression of IL-2 as cells become terminally differentiated [151]. The negative regulation of IL-2 provides a means for metered regulation of immune cell proliferation, as well as peripheral tolerance, and T cell exhaustion [133,150].

Physiological role of IL-2

The physiological role of IL-2 is complex and cannot be easily categorized as either pro- or anti-inflammatory. In vivo and in vitro, IL-2 has been shown to play a key role in the clonal expansion of T and B cells, but in its absence, primary immune responses to pathogens and tissue transplants are mounted [152–156]. Although primary immune response is not abrogated, IL-2 knock-out mice have a decreased capacity for CD8 expansion to viral infection, as well as a substantial decrease in immune memory to infection [152,157]. Further analysis has shown that the timing and strength of IL-2 signal during T cell activation plays a critical role in terminal differentiation, where prolonged IL-2 signal results in an increase in effector T cell development in both CD4 and CD8 T cells, and acute signaling promotes the development of memory T cells [158–160]. Outside of response to pathogen, IL-2 is required for the development of regulatory T cells (Treg), which serve to control and suppress immune responses, as well as to promote peripheral tolerance [161–163]. Further strengthening the role of IL-2 in the maintenance and development of Treg cells is seen in mice lacking IL-2, which develop severe autoimmunity [164,165]. Aside from the T cell response, IL-2 plays a role in other cell types, as well. For instance, IL-2 has been shown to promote antibody secretion by B cells, and to promote the cytolytic activity of natural killer cells [153,166]. In non-immune cells, IL-2 induces the expression of the

chemokine CCL2 and the adhesion molecule ICAM1 in fibroblasts, which aids in the recruitment and extravasation of immune cells into tissue [167,168]. The body of scientific literature clearly demonstrates that IL-2 is a mediator of many cellular functions and is integral to proper immune function.

NFκB, NFAT, and AP-1 regulation of IL-2 transcription

The minimal IL-2 promoter is controlled by several transcription factors, which act in concert to maximally drive its expression. NFκB is a transcription factor that is comprised of several subunits in the Rel family of proteins, with the most prevalent being RelA (p65) [169]. When unstimulated, NFκB resides in the nucleus, where it is continually repressed by Inhibitor of kappa B (I□B). NFκB can be induced by several kinases, including PKC, but the final activating stimuli occurs through phosphorylation of of I□B by I□B kinases [170–172]. This phosphorylation event causes the nuclear accumulation of NFκB, where it binds the IL-2 promoter, which contains a single NFκB binding element [143,144].

AP-1 is a transcription factor that is most frequently comprised of a heterodimer of c-Fos and c-Jun, but AP-1 has been shown to be a complex of other proteins, such as Fra-1, JunD, and CREB proteins [173]. AP-1 is largely induced by phosphorylation events. Activation of the AP-1 pathway causes the accumulation of AP-1 in the nucleus, and subsequent binding to the IL-2 promoter [143,144]. Unlike NFκB, AP-1 has multiple binding sites within the promoter region of IL-2, both proximal and distal [174]. Although The proximal site is required for transcription of the gene, the distal binding site of AP-1 has been shown to serve to amplify transcription [174].

Unlike AP-1 and NFκB, NFAT responds in a Ca^{2+} dependent manner. Within the cytosol, NFAT is activated in a calmodulin/calcineurin-dependent manner, where activation of the phosphatase calcineurin, by the calcium-sensing protein calmodulin, results in dephosphorylation of NFAT [175,176]. This dephosphorylating event results in a conformational change in NFAT, which leads to the exposure of the NFAT nuclear localization sequence, resulting in NFAT nuclear accumulation [175]. Within the IL-2 promoter, there are at least two NFAT binding sties, although other sites have been proposed [143,144,177].

CRISPR/Cas9

Although genetic manipulation using molecular techniques has been reported and accomplished for several decades, the last five years have been a renaissance in genetic manipulation. This increase in interest and ease of targeted genetic alterations has largely occurred because of the advent of Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) as a viable and simple means to alter eukaryote genomes in a reliable manner [178–180]. CRISPR, endogenously, acts as a primitive bacterial immune system, providing a means for a bacterium to develop a memory of phages that have previously infected it [181–183]. In this system, an endonucleause, most frequently Cas9, is directed against a sequence of phage DNA which shares base complementarity with a short guide RNA (gRNA) that has been incorporated into the bacterial genome from previously survived infections [183]. Upon recognition of the gRNA and DNA complex, the CAS9 endonuclease will cleave the DNA three base pairs upstream of the Protospacer-Adjacent Motif (PAM), which is an NGG, where N depicts any DNA base, resulting in a double strand DNA break [184]. This process has, over the last several years, been successfully used to target mammalian DNA for genetic manipulation (Fig. 2). Double strand DNA breaks induced by Cas9 results in the endogenous repair machinery to undergo non-homologous end joining, which can be error prone. This process frequently introduced

insertions and deletions (INDEL) into the DNA at the cut site [178–180,185]. The presence of INDELs within a gene often results in a frameshift mutation and subsequent gene knock-out. Although shown to be highly efficient in the generation of gene knock-out models, CRISPR technology is capable of a more fine-tuned approach to genetic manipulation. With the development of catalytically dead Cas9 and Cas9 which only cuts one strand of DNA, CRISPR has the ability to, with high fidelity, induce genes, knock-in genes, as well as repress (but not knock-out) gene transcription [186,187]. Accordingly, this technology is a powerful tool to investigate molecular mchanism of biological processes.

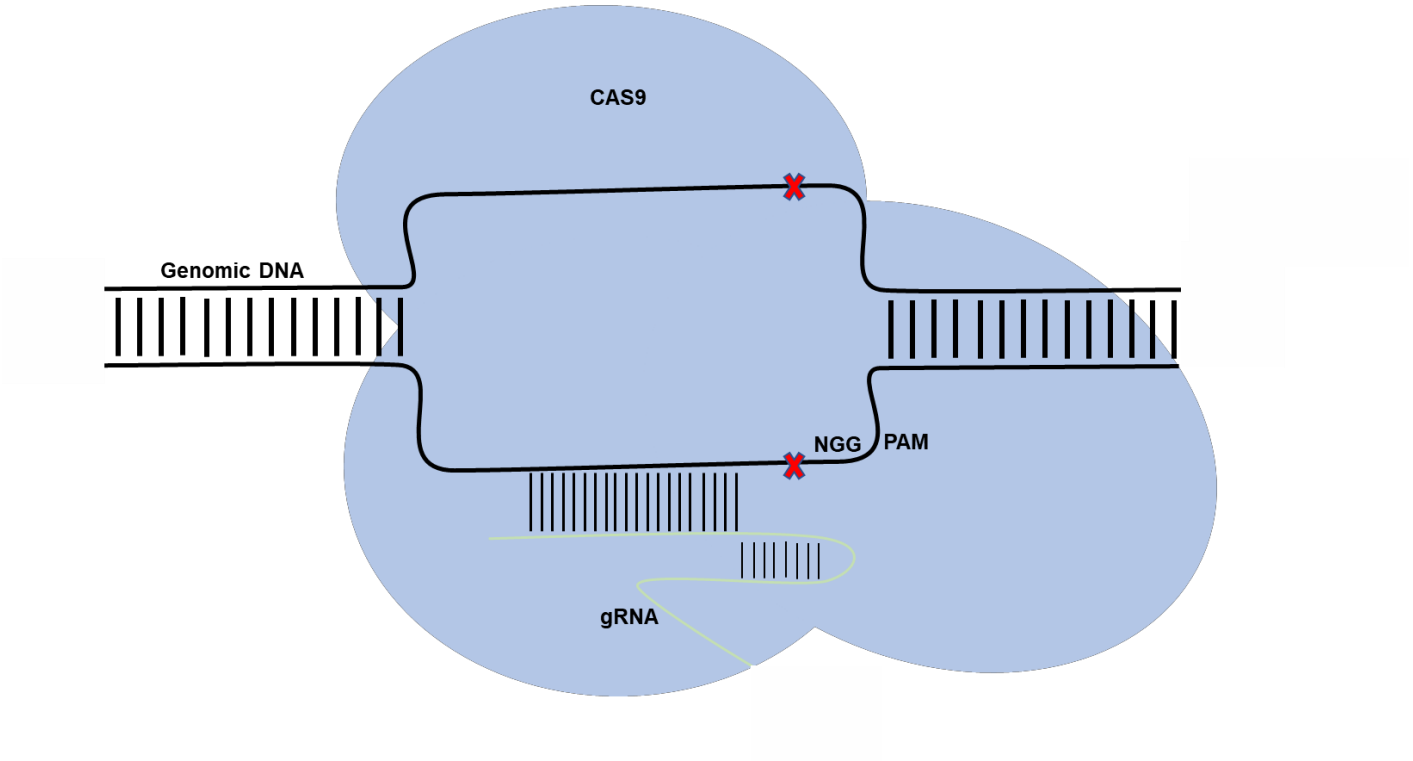

Figure 2: Depiction of CRISPR/Cas9 molecule interacting with genomic DNA

Rationale of the present studies

Work done by our group and others has shown that Nrf2 plays a role in the modulation of the immune response. Until recently, however, the role of the Nrf2/Keap1 system was largely studies in mouse models, with very few studies involving human immune models. The purpose of this dissertation was to address the role of the Nrf2/Keap1 system in the modulation of the early events of human Jurkat T cell activation. This was accomplished through use of multiple Nrf2 activating compounds, as well as development and implementation of both Nrf2-null and Keap1-null Jurkat cell lines. Together, these studies provide the models necessary to definitively determine the role of the Nrf2/Keap1 system in the early events of Jurkat T cell activation, as well as a model to study if the effects of other immunotoxicants, such as arsenic, are mediated through the Nrf2/Keap1 axis.

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CHAPTER 2

The Nrf2 activator, tBHQ, differentially affects early events following stimulation of Jurkat cells

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Abstract

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that is activated by cellular stresses, such as oxidative compounds. After activation, Nrf2 induces transcription of its target genes, many of which have cytoprotective functions. Previously, we have shown that activation of Nrf2 by tBHQ skews murine $CD4^+$ T cell differentiation. Although the role of Nrf2 in murine T cells is somewhat characterized, it is largely uncharacterized in human T cells. Therefore, the aim of the current studies was to characterize the effects of the Nrf2 activator, tBHQ, on the early events of human $CD4^+$ T cell activation. Pretreatment of Jurkat T cells with tBHQ, prior to activation with anti-CD3/anti-CD28, diminished the production of IL-2 at both the transcript and protein levels. Similarly, the expression of CD25 also diminished, albeit moderately, after pretreatment with tBHQ. The decrease in IL-2 production was not due to decreased nuclear translocation of c-fos or c-jun. Although tBHQ caused both a delay and a decrease in Ca^{2+} influx in activated Jurkat cells, no decrease in NFAT DNA binding or transcriptional activity was observed. In contrast to NFAT, tBHQ significantly decreased NFκB transcriptional activity. Collectively, our studies show that the Nrf2 activator, tBHQ, inhibits IL-2 and CD25 expression, which correlates with decreased NFκB transcriptional activity in activated Jurkat cells. Overall, our studies suggest that Nrf2 represents a novel mechanbism for the regulation of both human and mouse T cell function.

Introduction

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that is activated by cellular stresses, such as oxidative stimuli [1,2]. When inactive, Nrf2 is tethered to the actin cytoskeleton within the cytosol by its repressor protein, Kelch ECH associating protein 1 (Keap1). Interaction of Nrf2 with Keap1 causes its continual ubiquitination and degradation [3,4]. When activated by cellular stresses, Nrf2 translocates into the nucleus and binds to the promoters of genes containing the antioxidant response element (ARE), inducing their transcription[5-7]. Genes regulated by this pathway are involved in detoxification, antioxidant response, and glutathione homeostasis among other cytoprotective functions [8-10]. In mice, it has been shown that Nrf2 is inducible in leukocytes and up-regulates both Heme oxygenase-1 (Hmox-1) and NAD(P)H quinone oxidoreductase 1 (Nqo1) [11,12]. Accordingly, Nrf2 activity can be assessed by measuring induction of Hmox-1 and Nqo1 mRNA expression in addition to nuclear translocation of Nrf2.

Widely used as a preservative in food, *tert*-butylhydroquinone (tBHQ) is a well-characterized activator of Nrf2. It has been previously shown that tBHQ activates Nrf2 by interacting with thiol groups of cysteine molecules on the Keap1 protein, which interferes with the ability of Keap1 to repress Nrf2 [13]. In industry, tBHQ functions as a food preservative by preventing the rancification of lipids [14]. Previous studies have shown serum concentrations of tBHQ reaching the high µM range in healthy volunteers who ingested 100 – 150 mg of tBHQ [15].

Although a key player in xenobiotic detoxification and metabolism, a growing number of studies show Nrf2 also plays a role in immune cell function and in various inflammatory diseases, including autoimmune disease and allergy [16-18]. Our previous studies demonstrate that Nrf2

activation in primary mouse splenocytes modulates murine $CD4^+$ T cell differentiation [19]. Studies by other groups have demonstrated that female Nrf2 knockout mice are susceptible to an autoimmune disease resembling systemic lupus erythematosus, which is characterized by the presence of antibodies targeting host dsDNA [20,21]. Importantly, the majority of studies investigating the role of Nrf2 in immune cells have used murine models and thus the role of Nrf2 in human immune cells remains largely uncharacterized.

Activation of $CD4^+$ T cells is integral to initiating a healthy immune response to a pathogen. T cells are typically in a resting, quiescent state. In order for activation to occur, the cell requires two stimuli. First, the T cell receptor (CD3) must encounter an MHC class II molecule which is loaded with antigen. A costimulatory signal is also required and can be provided by numerous costimulatory receptors and ligands [22]. Experimentally, T cells can be activated with antibodies directed against CD3 and CD28, which is a costimulatory receptor. These interactions lead to a cascade of signaling events, bringing about T cell activation. One of the earliest events of T cell activation is the influx of Ca^{2+} through the calcium release activated channels (CRAC) [22]. This sudden change in the intracellular Ca^{2+} concentration activates the calcium-sensitive transcription factor, nuclear factor of activated T cells (NFAT), which translocates to the nucleus and binds to the promoters of target genes [22]. T cell activation also initiates several kinase cascades, including RAC-JNK, PKC-SEK1-JNK, and RAS-RAF-ERK [23]. The result of this signal transduction is the translocation of the transcription factors NFκB, c-fos, and c-jun which subsequently induce target genes, such as interleukin-2 (IL-2) [22]. In addition to induction of IL-2, T cell activation also upregulates expression of the canonical T cell activation markers, CD25 and CD69. CD25 is the high affinity chain of the IL-2 receptor, and CD69 is a lectin-type receptor, both become highly expressed after activation. These early events of T cell activation are vital in initiating an effective immune response.

Our previous studies demonstrated that activation of Nrf2 skews murine $CD4^+$ T cell differentiation; however the role of Nrf2 in T cell activation itself was not investigated. The purpose of the present studies was not only to determine the effect of the Nrf2 activator, tBHQ, on the early events of T cell activation, but to do so in a human T cell model.

Materials and Methods

Materials:

tBHQ, and all other reagents, were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

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% fetal bovine serum (FBS) (Biowest LLC, Kansas City, MO), 25 mM HEPES, 1 mM sodium pyruvate , 10 mM nonessential amino acids, 100 u/mL penicillin, and 100 u/mL streptomycin. All treatments and activation of Jurkat T cells are discussed in figure legends.

mRNA quantification by real-time PCR:

Total RNA was isolated from 2 x 10^6 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 ribosomal protein L13a, using Ct values

quantified by Life Technologies/Applied Biosystems Sequence Detection System 7500 (Grand Island, NY). All primer sequences were retrieved from qPrimerDepot (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'; Nrf2 forward primer, 5'-TCTTGCCTCCAAAGTATGTCAA-3' and Nrf2 reverse primer, 5'-CACGGTCCACAGCTCATC-3'; NQO1 forward primer, 5'-TCCTTTCTTCAAAGCCG-3' and NQO1 reverse primer, 5'-GGACTGCACCAGAGCCAT-3'; HMOX-1 forward primer, 5'-GGCTTCCCTCTGGGAGTCT-3' and HMOX-1 reverse primer, 5'-AGCTGCTGACCCATGACAC-3'; IL-2 forward primer, 5'-GCACTTCCTCCAGAGGTTTG-3' and IL-2 reverse primer 5'-TCACCAGGATGCTCACATTT-3'; CD69 forward primer, 5'-ACAGGAACTTGGAAGGACCC-3' and CD69 reverse primer, 5'-AGAACAGCTCTTTGCATCCG-3'; CD25 forward primer, 5'-TAGGCCATGGCTTTGAATGT-3' and CD25 reverse primer, 5'-ATACCTGCTGATGTGGGGAC-3'

IL-2 quantification by ELISA:

Jurkat T cells were cultured in 96-well plates (1 x 10^5 cells/well), treated as described and cell supernatants were harvested 24h after activation with anti-CD3/anti-CD28. IL-2 was quantified from cell supernatants by the use of a commercially available kit per the manufacturer's protocol (BioLegend, San Diego, CA). Relative light intensity was quantified at 450 nm by Bio-Tek µQuant microplate reader (Highland Park, VT). The IL-2 concentrations of each sample were then calculated using a linear standard curve on Microsoft Excel (Microsoft, Redmond, WA).

Measurement of extracellular markers by flow cytometry:

Jurkat T cells were labeled with anti-CD69/PECy7 (BioLegend, San Diego, CA) and anti-CD25/APC (eBioscience, San Diego, CA). The cells were then washed, resuspended in FACS buffer and subsequently 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).

Measurement of Ca^{2+} influx by flow cytometry:

Jurkat T cells were labeled, at a density of $1x10^6$ cells per mL, with Fluo-4, AM (Life Technologies) per the manufacturer's protocol. The cells were then washed and analyzed by flow cytometry using a BD Accuri C6 (BD Accuri, San Jose, CA). Fluorescence was quantified using CFlow software (BD Accuri, San Jose, CA).

Protein isolation:

Nuclei were extracted from $1x10^7$ Jurkat T cells, 3h after activation by anti-CD3/anti-CD28. Cells were lysed using a solution containing 10 mM HEPES, 100 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 0.5% NP-40 substitute, and 1x Halt protease inhibitor cocktail (Thermo Scientific, Waltham, MA). The cells were then spun down and resuspended in nuclear extraction buffer containing 10 mM HEPES, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol, and 1x Halt protease inhibitor cocktail. Samples were kept in nuclear extraction buffer for 1h, 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 blotting:

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 that were probed for histone H3 were blocked with 5% chicken albumin, from egg white (Sigma-Aldrich), in phosphate-buffered saline containing 0.05% Tween 20 (PBST). Blots targeting c-fos, c-jun, and Nrf2 were blocked with 5% nonfat dry milk (NFDM) in 0.05% PBST. Histone H3 primary antibody (FL-136) was diluted 1:100 in 0.05% PBST containing 2% chicken albumin. Primary antibodies for Nrf2 (H-300), c-Jun (D), and c-Fos (H-125) were diluted 1:1000 in 0.05% PBST containing 2% NFDM. All primary antibodies were 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% of the blocking compound. The anti-rabbit IgG was obtained from Cell Signaling (Danvers, MA). All blots were developed via ECL Western Blotting Substrate (Thermo Scientific) per the manufacturer's protocol. The bands were visualized by the LI-COR Odyssey FC infrared imaging system (Lincoln, NE).

ELISA based DNA binding assay:

One hour after activation by anti-CD3/anti-CD28, nuclear protein was extracted from $1x10^7$ Jurkat T cells, using a commercially available kit (Active Motif, Carlsbad, CA). After extraction, nuclear protein was quantified via the Bradford assay (BioRad). 1µg of nuclear protein was used to quantify NFAT DNA binding, using a commercially available ELISA-based DNA binding assay (Active Motif). Assays were done per the manufacturer's protocol.

Transient transfection and luciferase assay:

Human Jurkat T cells were reverse transfected with 1.5 µg of plasmid and 3 µl of Lipofectamine 2000 (Life Technologies) per $5x10^5$ cells for 12h in complete media. After 12h the cells were washed and resuspended at a concentration of $5x10^5$ 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 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).

Plasmids:

The NFAT luciferase reporter plasmid was obtained from Signosis, Inc. (Sunnyvale, CA). The AP-1 and NFκB luciferase reporters were purchased from Promega (Madison, WI).

Statistical analysis:

For each treatment group, the mean ± the standard error was calculated. One-way ANOVA followed by the Dunnett post hoc test was used to determine statistical significance at $p<0.05$, compared to the vehicle control. All statistical analyses were calculated by SigmaPlot 12.3 (Systat Software Inc., San Jose, CA)

Results

The Nrf2 activator, tBHQ, inhibits production of IL-2.

We have previously demonstrated that activation of Nrf2 by the food preservative, tBHQ, modulates T cell differentiation in primary murine $CD4^+$ T cells [19]. However, the role of Nrf2 and the effect of tBHQ in human $CD4^+$ T cells remain largely uncharacterized. Therefore, the purpose of our current studies was to investigate the effects of tBHQ on the early events of T cell activation in human Jurkat T cells. The Nrf2 activator, tBHQ, markedly decreased IL-2 production by Jurkat T cells activated with anti-CD3 and anti-CD28 (Fig. 1). The decrease in IL-2 by tBHQ was concentration-dependent and was observed at both the mRNA and protein levels. Decreased IL-2 protein production by tBHQ occurred at concentrations as low as 0.1 µM. No decrease in cellular viability were seen at the concentrations used in this study (data not shown). Consistent with our previous findings in murine T cells, these data suggest that tBHQ modulates cytokine production in human T cells as well.

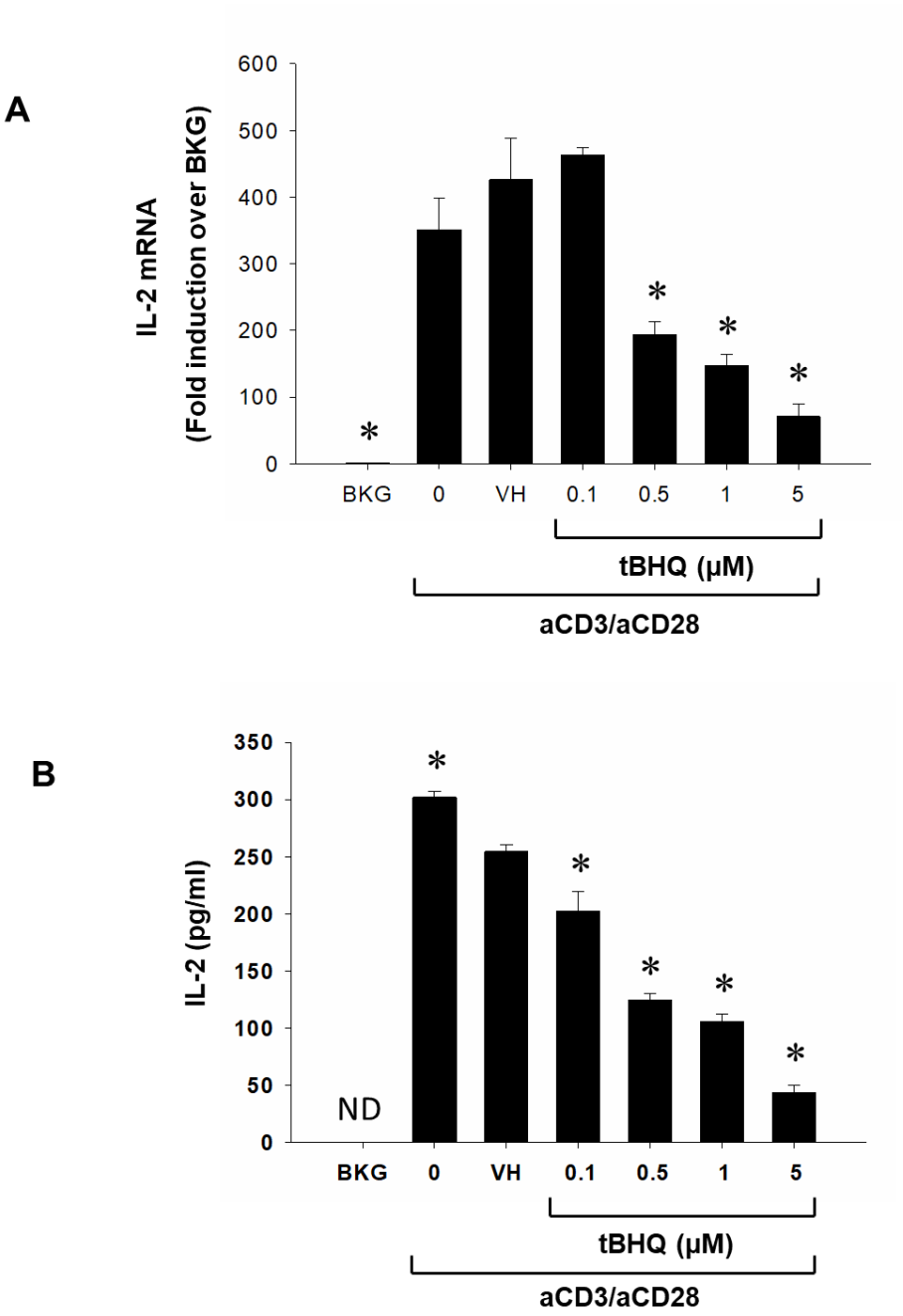

Figure 3: The Nrf2 activator, tBHQ, inhibits production of IL-2. The Nrf2 activator, tBHQ, inhibits both mRNA and protein production of IL-2 in human Jurkat T cells. Jurkat T cells received activator alone (0), were left untreated (BKG), or were pretreated with either tBHQ (0.1, 0.5, 1, or 5 µM) or the vehicle (VH) control (0.01% ethanol). Pretreatment of cells with both VH and tBHQ occurred 30 min prior to activation of the cells by anti-CD3/anti-CD28. A) 6h post activation, cells were harvested and mRNA collected. Quantification of IL2 mRNA was then performed by real-time PCR. B) 24h after activation with anti-CD3/anti-CD28, cell supernatants were collected and Il-2 protein quantified by ELISA. ***** represents $p<0.05$ versus VH.

Activation of Nrf2 by tBHQ in Jurkat T cells.

To determine whether activation of Nrf2 may play a role in inhibition of IL-2 by tBHQ, we investigated whether Nrf2 is activated by tBHQ in Jurkat cells. tBHQ caused an increase in Nrf2 in nuclear protein extracts as determined by western blot analysis, which is consistent with increased nuclear translocation (Fig. 2). Although tBHQ tended to increase Nrf2 mRNA levels, this effect was not statistically significant (Fig. 3a). In addition, mRNA levels of the Nrf2 target genes NQO1 and HMOX-1 were substantially increased by tBHQ, at concentrations as low as 0.1 µM (Fig. 3b and 3c). Overall, these data strongly suggest that tBHQ activates Nrf2 in Jurkat T cells, as has been observed in other cell types.

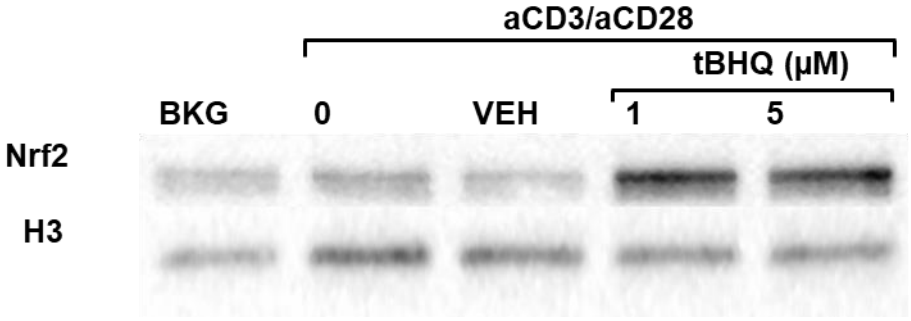

Figure 4: tBHQ, causes nuclear translocation of Nrf2 in Jurkat T cells. Jurkat T cells received activator alone (0), were left untreated (BKG), or were pretreated with either tBHQ (0.1, 0.5, 1, or 5 µM) or the vehicle (VH) control (0.01% ethanol). Pretreatment of cells with both VH and tBHQ occurred 30 min prior to activation of the cells by anti-CD3/anti-CD28. 3h after activation with anti-CD3/anti-CD28, cells were harvested and nuclear protein extracted. Western analysis was performed and the resulting blot was probed for Nrf2. Histone H3 was used as the loading control.

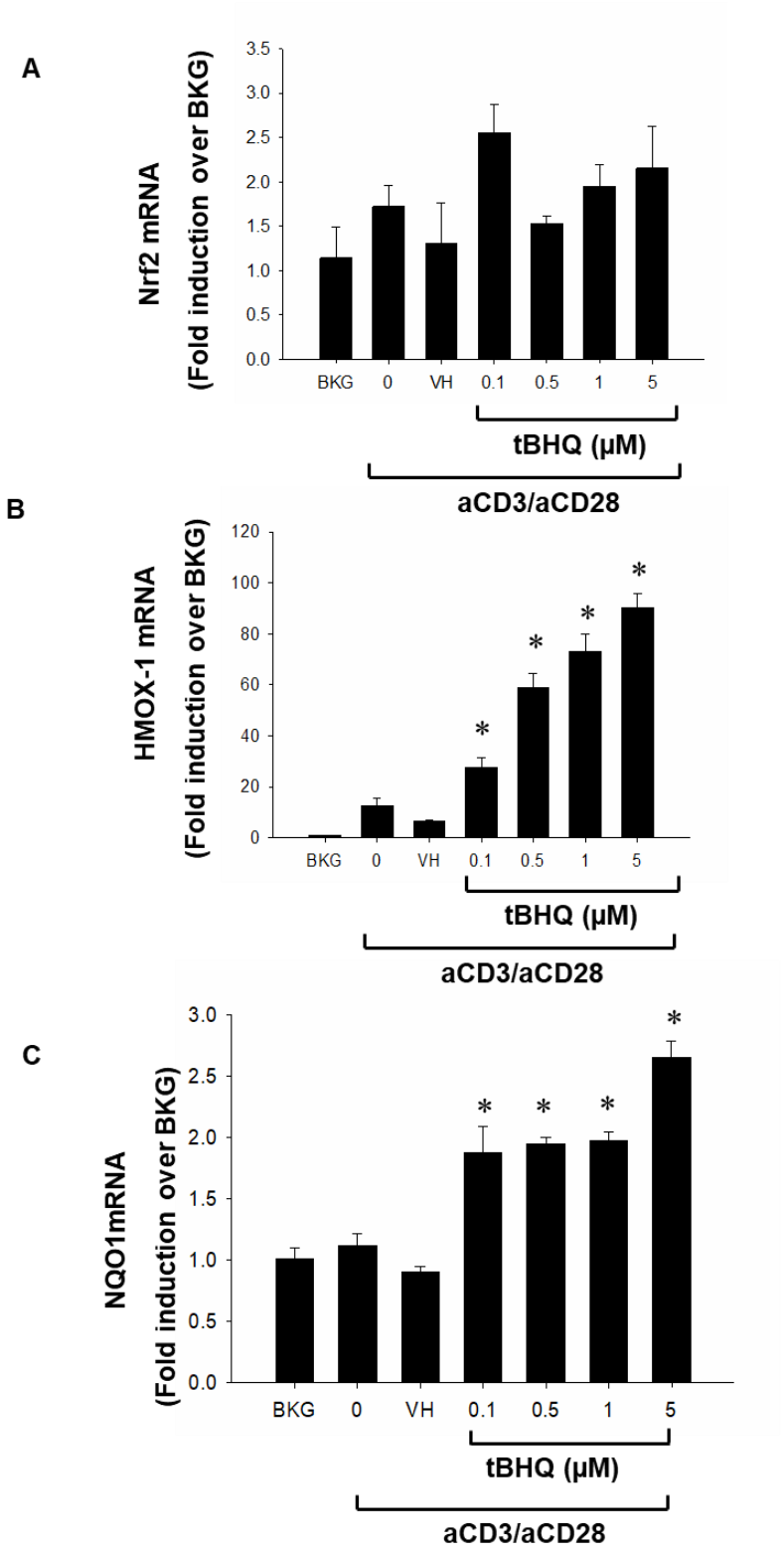

Figure 5: tBHQ causes induction of Nrf2 target genes in Jurkat T cells. Jurkat T cells received activator alone (0), were left untreated (BKG), or were pretreated with either tBHQ (0.1, 0.5, 1,

Figure 5: (cont'd) or 5 µM) or the vehicle (VH) control (0.01% ethanol). Pretreatment of cells with both VH and tBHQ occurred 30 min prior to activation of the cells by anti-CD3/anti-CD28. 6h post activation, cells were harvested and mRNA collected. Quantification of A) Nrf2, B.) HMOX-1 or C.) NQO1 mRNA was performed by real-time PCR. ***** represents $p<0.05$ versus VH.

Differential effects of tBHQ on CD25 and CD69 expression in Jurkat cells.

Because tBHQ markedly suppressed IL-2 production by activated Jurkat cells (Fig. 1), we next investigated the effects of tBHQ on other early events of T cell activation. The high affinity chain of the IL-2 receptor (CD25) is an early activation marker in $CD4^+$ T cells. Although treatment of Jurkat T cells with tBHQ caused a statistically-relevant decrease in CD25 expression at both the protein and mRNA levels, the effect of tBHQ on CD25 was more modest than that observed in IL-2 production (Fig. 4). In addition to CD25, CD69, a lectin-type receptor, is also a marker of early T cell activation. Although tBHQ caused a modest decrease in CD69 protein expression, this was not observed at the mRNA level. Taken together, the data suggest that Nrf2 activation may moderately decrease CD25 expression.

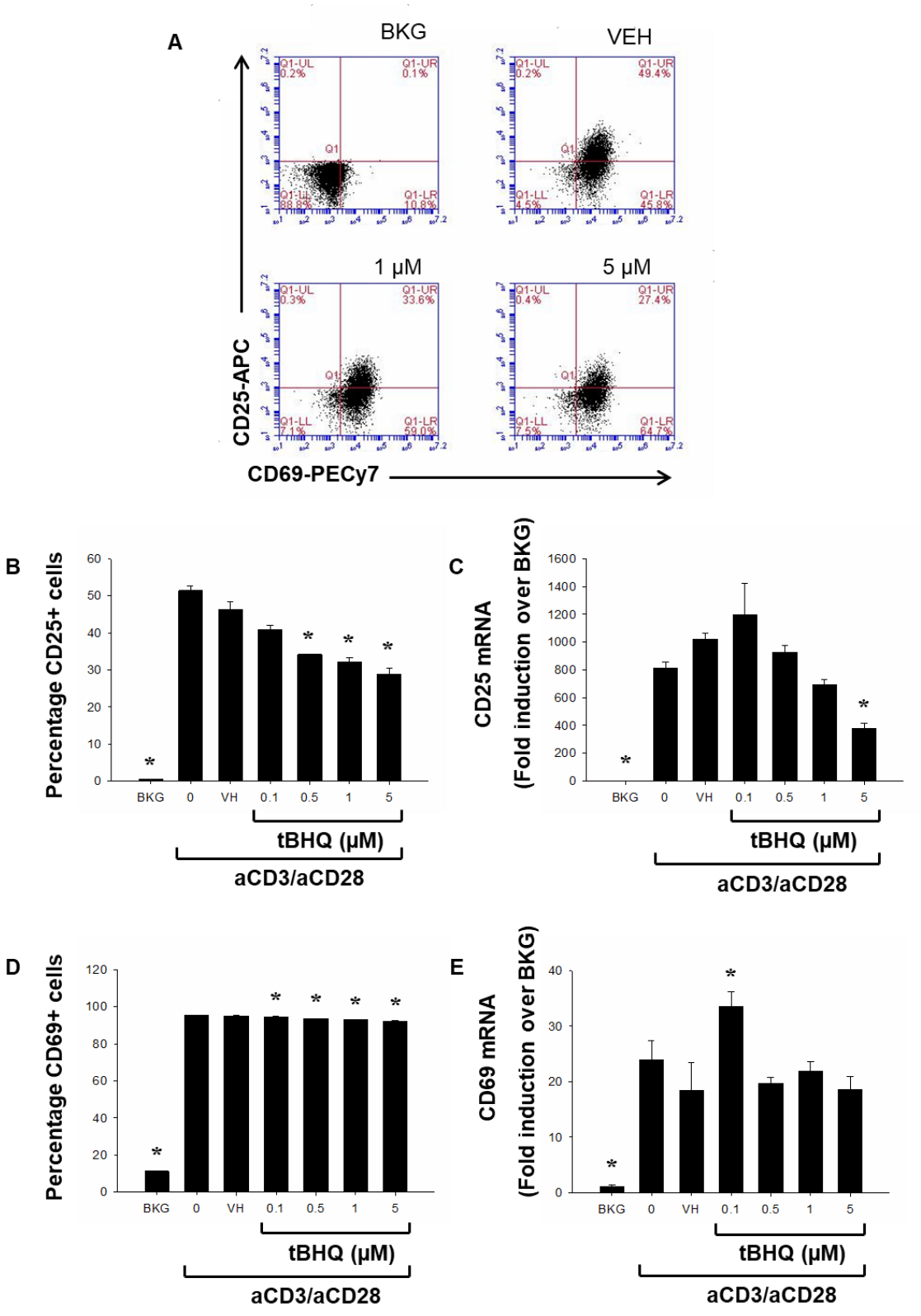

Figure 6: The Nrf2 activator, tBHQ, causes a modest reduction in CD25 expression in activated Jurkat cells. Jurkat T cells received activator alone (0), were left untreated (BKG), or were

Figure 6: (cont'd). pretreated with either tBHQ (0.1, 0.5, 1, or 5 µM) or the vehicle (VH) control (0.01% ethanol). Pretreatment of cells with both VH and tBHQ occurred 30 min prior to activation by anti-CD3/anti-CD28. 24h post activation with anti-CD3/anti-CD28, Jurkat T cells were isolated and labeled with anti-CD25/APC and anti-CD69/PECy7. CD25 and CD69 expression was analyzed by flow cytometry using the BD Accuri C6. The percentage of CD25- and CD69-positive cells was measured using CFlow software. For mRNA analysis, cells were harvested 6 h after activation with anti-CD3/anti-CD28 for RNA isolation. CD25 and CD69 mRNA were measured by real-time PCR. A) Representative dot plots from flow cytometry. B) Graphical representation of CD25-positive cells by flow cytometry. C) Quantification of CD25 mRNA. D) Graphical representation of CD69-positive cells by flow cytometry. E) Quantification of CD69 mRNA. ***** represents $p<0.05$ versus VH.

Effect of tBHQ on nuclear translocation of the transcription factors, c-fos and c-jun.

To determine the mechanism by which tBHQ suppresses IL-2 production in Jurkat cells, we next examined the effect of tBHQ on the nuclear translocation of c-fos and c-jun, known regulators of IL-2 transcription. As expected, activation of Jurkat T cells caused robust nuclear translocation of both c-fos and c-jun (Fig. 5). Pretreatment with tBHQ did not decrease nuclear translocation of c-fos or c-jun and in fact, appears to cause a modest increase at the highest concentration (5 µM). These data suggest that inhibition of IL-2 by tBHQ is not due to decreased nuclear translocation of c-fos/c-jun.

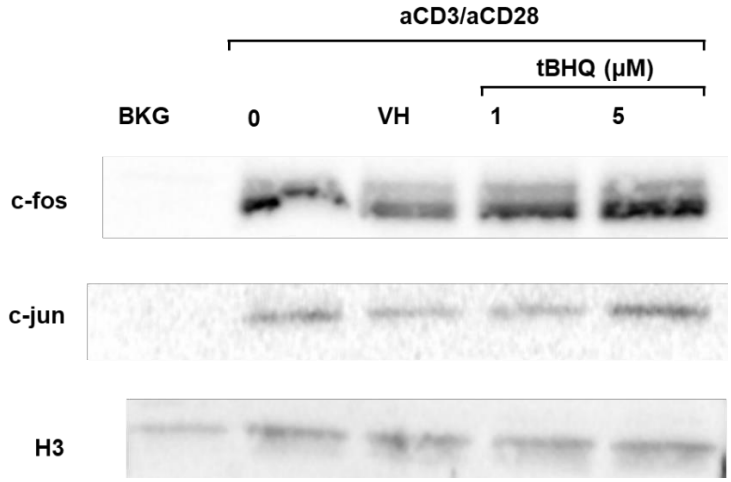

Figure 7: The Nrf2 activator, tBHQ, has a modest effect on the nuclear translocation of c-fos and c-jun. Jurkat T cells were left untreated (BKG), or were pretreated with nothing (0), tBHQ (1, or 5 µM) or the vehicle (VH) control (0.01% ethanol), 30 min prior to activation with anti-

Figure 7: (cont'd) CD3/anti-CD28. 3h after activation with anti-CD3/anti-CD28, cells were harvested and nuclear protein extracted. Nuclear translocation of c-fos and c-jun was determined by western analysis. Histone H3 was used as the loading control. Visualization of the resulting western blots was performed by the LI-COR Odyssey FC infrared imaging system.

tBHQ inhibits calcium influx in activated Jurkat T cells.

Calcium influx is a critical event in the activation of T cells. Calcium is integral in many cellular processes, including the activation of the transcription factor, NFAT [22]. NFAT is a calcium-sensitive transcription factor that has multiple binding sites on the promoter of the IL-2 gene. The lack of effect of tBHQ on either c-fos or c-jun suggests that Nrf2 is inhibiting the expression of this gene by another means, such as inhibition of calcium influx. Thus, calcium influx in Jurkat T cells was measured over time following cell activation. After measurement for 1.5 min to establish baseline, anti-CD3/anti-CD28 was added to the suspension, which caused a subsequent spike in calcium influx a couple of minutes later (Fig. 6a). Cells that were pretreated with tBHQ displayed significant reduction in both fluorescent intensity and number of cells that were positive for calcium influx at both 1 and 5 µM tBHQ (Fig. 6b-d). In addition, tBHQ caused a delay in calcium influx (Fig. 6a). Overall, these data demonstrate that tBHQ causes both a delay and a decrease in calcium influx in activated Jurkat T cells.

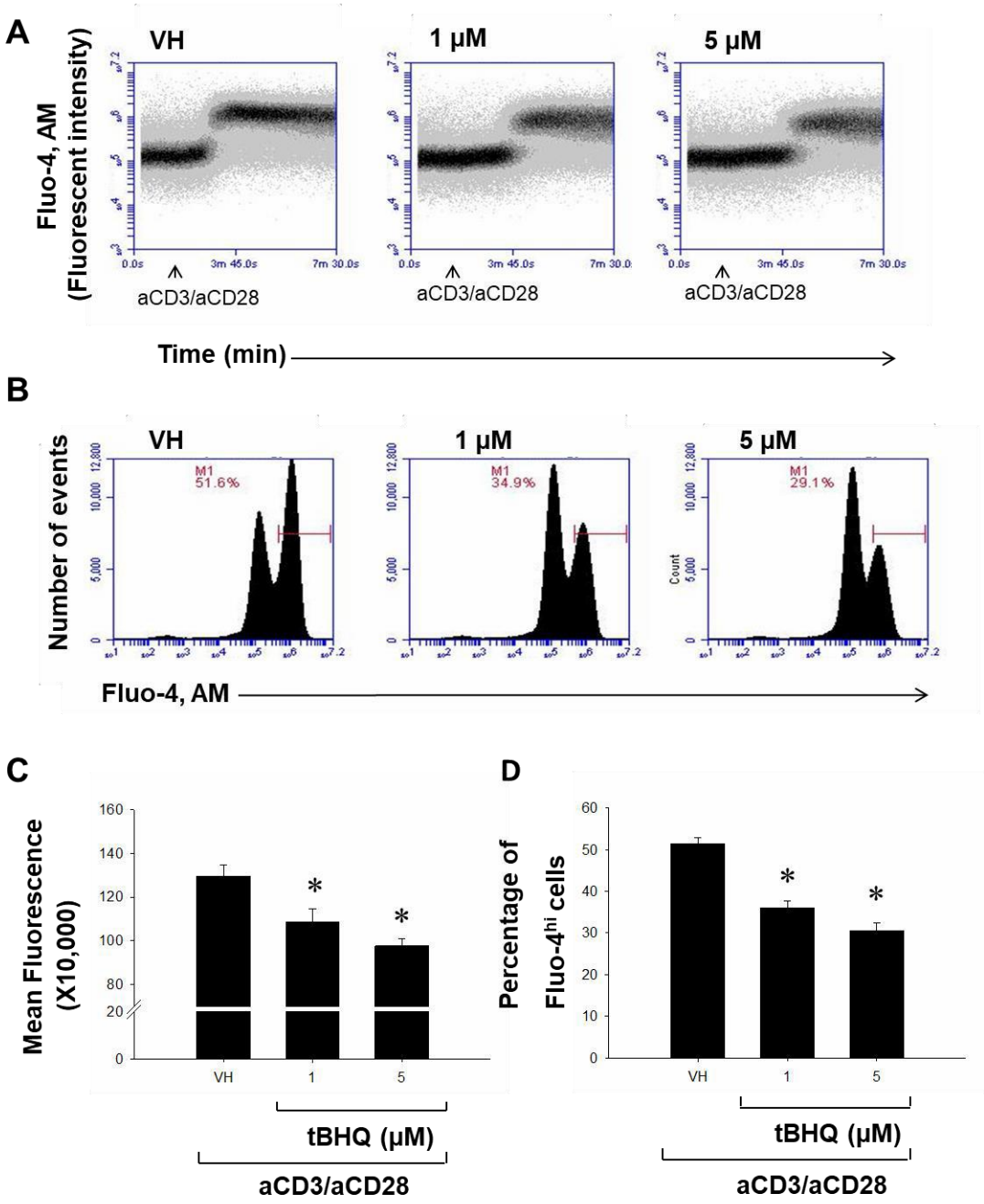

Figure 8: The Nrf2 activator, tBHQ, delays and decreases the magnitude of calcium influx in activated Jurkat T cells. Jurkat T cells were pretreated with either tBHQ (1 or 5 μM) or the vehicle (VH) control (0.01% ethanol) and loaded with Fluo-4/AM prior to activation with anti-CD3/anti-CD28. Calcium influx was determined by an increase in Fluo-4 fluorescence and measured over time by flow cytometry, using the BD Accuri C6 flow cytometer in conjunction with CFlow software. 1.5 minutes after beginning the analysis, anti-CD3/anti-CD28 was added to each sample and measurements continued for another 6 minutes. A) Fluo-4 fluorescence as measured over time by flow cytometry. B) Histograms of Fluo-4 fluorescence. Graphical representation of C) MFI or D) percentage Fluo-4^{hi} cells. ***** represents $p<0.05$ versus VH.

The activity of the transcription factor NFAT is unaffected by the Nrf2 activator, tBHQ.

The decrease in calcium influx by tBHQ prompted us to investigate DNA binding and transcriptional activity of the calcium-sensitive transcription factor, NFAT. Toward this end, we quantified NFAT DNA binding with an ELISA-based assay and transcriptional activity with an NFAT-luciferase reporter assay. Unexpectedly, NFAT DNA binding and transcriptional activity was largely unaffected by tBHQ (Fig. 7a-b). Collectively, these data indicate that tBHQ does not impair NFAT activity.

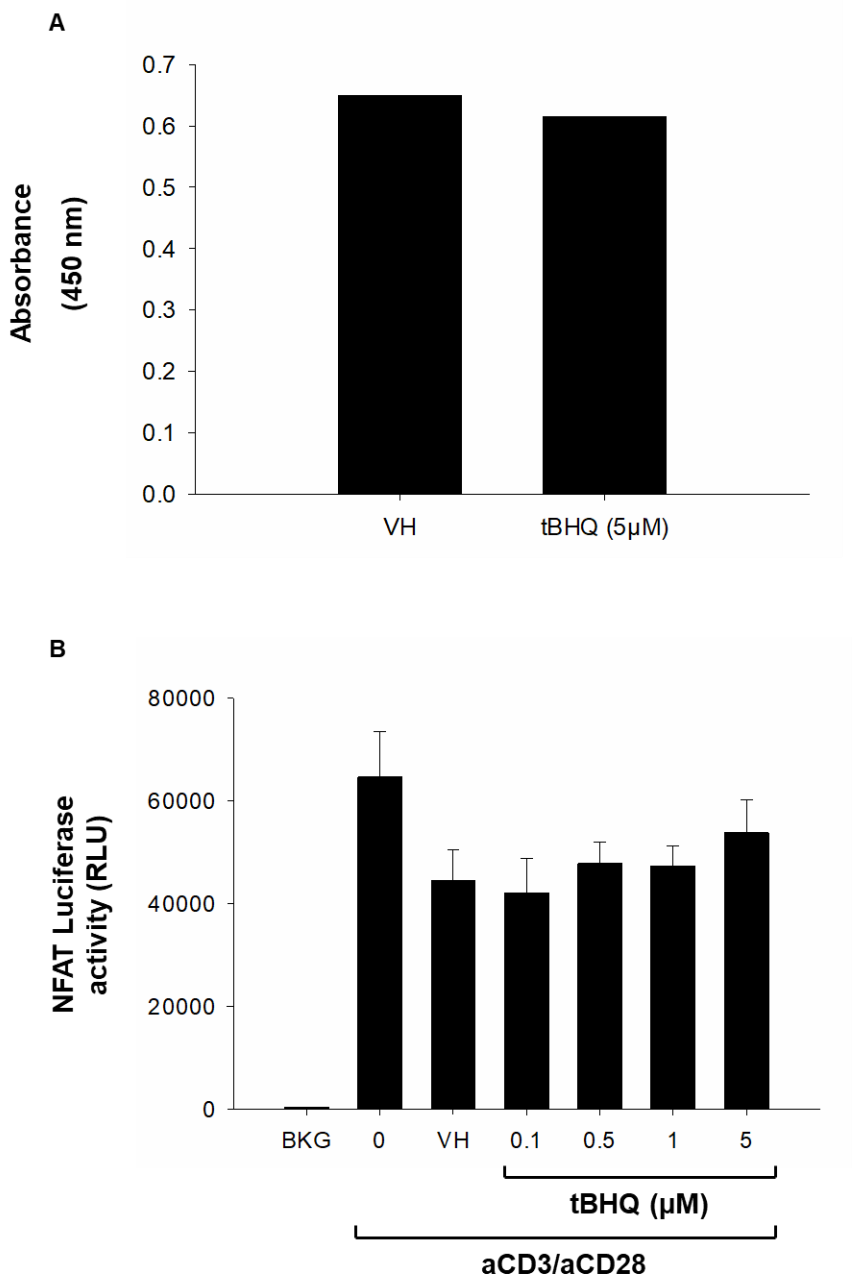

Figure 9: The Nrf2 activator, tBHQ, does not affect NFAT DNA binding or transcriptional activity in activated Jurkat cells. A) Jurkat T cells were pretreated with 5 µM tBHQ or vehicle (VH) control (0.01% ethanol), 30 min prior to activation with anti-CD3/anti-CD28. 1h post activation, nuclear proteins were extracted. NFAT DNA binding was quantified using an ELISA-based assay. B) Jurkat T cells were transiently transfected with an NFAT luciferase reporter plasmid. After transfection the cells received activator alone (0), were left untreated (BKG), or were pretreated with either tBHQ (0.1, 0.5, 1, or 5 µM) or vehicle (VH) control (0.01% ethanol),30 min prior to activation with anti-CD3/anti-CD28. 12h post activation, cells were harvested and luciferase luminescence was measured using a Tecan Infinite m1000 Pro Microplate Reader. None of the groups were statistically different from the VH control.

The Nrf2 activator, tBHQ, modulates AP-1 and NFκB activity

The lack of effect of tBHQ on NFAT activity was an unexpected finding and prompted us to investigate the activity of other transcription factors that regulate IL-2 transcription. Thus, the transcriptional activity of AP-1 (c-fos/c-jun) and NFκB, was investigated using luciferase reporter genes. At the highest concentration (5 □M), tBHQ markedly increased AP-1 luciferase activity (Fig. 8a). Conversely, tBHQ caused a concentration-dependent decrease in NFκB luciferase activity (Fig. 8b). Taken together, these data show that the Nrf2 activator, tBHQ, causes an increase in AP-1 transcriptional activity and a concurrent decrease in NFκB transcriptional activity in activated Jurkat T cells.

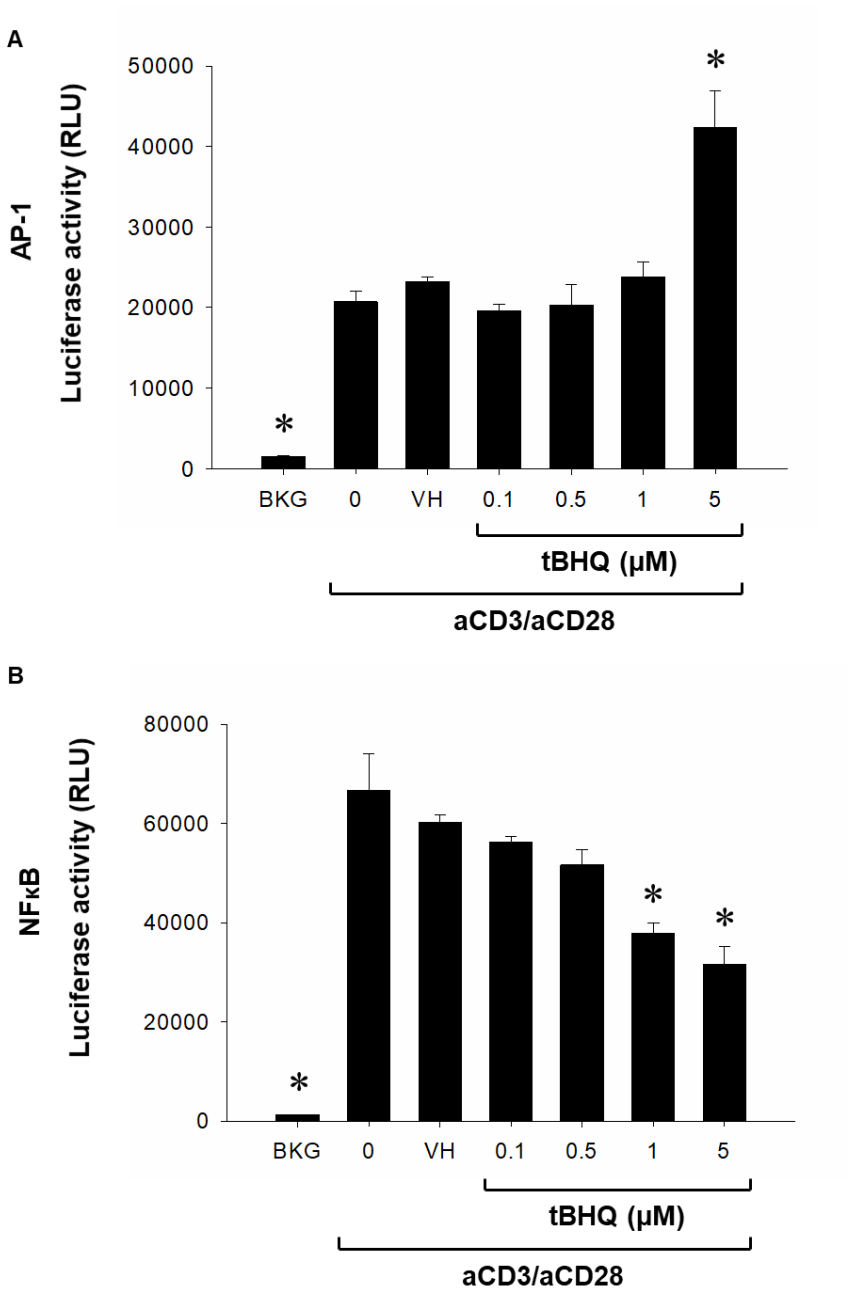

Figure 10: The Nrf2 activator, tBHQ, modulates AP-1 and NFκB transcriptional activity in activated Jurkat cells. Jurkat T cells were transiently transfected with either A.) AP-1 luciferase reporter or B) NFκB luciferase reporter plasmid. After transfection, the cells received activator alone (0), were left untreated (BKG), or were pretreated with either tBHQ (0.1, 0.5, 1, or 5 μM) or vehicle (VH) control (0.01% ethanol), 30 min prior to activation with anti-CD3/anti-CD28. 12h post activation, cells were harvested and luciferase luminescence was measured using a Tecan Infinite m1000 Pro Microplate Reader. * $p < 0.05$ as compared to the VH control.

Discussion

The purpose of the current studies was to characterize the effect of the Nrf2 activator, tBHQ, on the early events of T cell activation in a human T cell model. The data from these studies suggest that Nrf2 is activated by tBHQ in human Jurkat T cells, as evidenced by nuclear translocation of Nrf2 and up-regulation of the Nrf2 target genes, HMOX-1 and NQO1. The current studies also indicate that the Nrf2 activator, tBHQ, inhibits IL-2 secretion as well as the expression of CD25 on the cell surface, but only a modest reduction was observed in the expression of CD69. The decrease in IL-2 by tBHQ is consistent with what has recently been observed with benzo(a)pyrene, which is also a Nrf2 activator [24]. Although tBHQ decreased calcium influx in activated Jurkat cells, NFAT DNA binding and transcriptional activity were not diminished, which was an unexpected finding. In contrast, tBHQ increased AP-1 transcriptional activity at 5μM and dose-dependently decreased NFκB activity. Collectively, these data suggest that the inhibition of IL-2 secretion by tBHQ is due to decreased NFκB transcriptional activity.

Nrf2 is an important player in modulating the immune response in several models of inflammation and has been shown to be a potential therapeutic target in the treatment of inflammatory diseases, such as sepsis and brain inflammation [11,16,25]. Additionally, our lab has shown that activation of Nrf2 skews $CD4^+$ T cell differentiation in primary murine $CD4^+$ T cells [19]. Although the role of Nrf2 in the regulation of murine T cell function has been somewhat characterized, the role of Nrf2 in human T cells remains largely unexplored. Similar to our previous studies in murine T cells, the current study suggests Nrf2 also plays a role in the regulation of human T cell function.

The current study demonstrates that the Nrf2 activator tBHQ has an inhibitory effect on some of the early events of T cell activation, including calcium influx. In contrast to the current study, a previous study showed the Nrf2 activator, bis (tri-n-butyltin) oxide (TBTO), increased calcium influx in Jurkat cells [26]. There are many differences between the two studies, however, including the kinetics of calcium influx and the Nrf2 activators used. In addition to effects on T cell activation, treatment of Jurkat cells by tBHQ has also been shown to regulate chromatin remodeling in a mechanism that involves the tumor suppressor, phosphatase and tensin homolog (PTEN) [27].

Additives to food, such as tBHQ, are widely used today to increase shelf life and decrease spoilage [14]. The presence of the preservative, tBHQ, can be seen in products ranging from vegetable oil to crackers and cereal. Previously, the primary concern in using tBHQ, and similar compounds was carcinogenesis and thus early toxicological analyses focused on genotoxicity. In contrast, there are few published studies on the potential immunotoxic effects of tBHQ. The current study shows decreased IL-2 protein production at concentrations of tBHQ as low as 0.1 µM and a decrease in CD25 protein expression at concentrations as low as 0.5 µM. A previous study reported that treatment of human male subjects with 100-150 mg of tBHQ produces plasma concentrations of tBHQ in the high micromolar range suggesting that the treatment range in the current study (0.1 to 5 µM) is relevant to humans [15].

Within T cells, an increase in intracellular calcium is essential for full activation. Upon an increase in intracellular calcium, the transcription factor NFAT, translocates to the nucleus and binds to the promoters of its target genes, such as IL-2 [22]. Jurkat T cells treated with tBHQ prior to activation with anti-CD3/anti-CD28 demonstrated a decrease in number of cells positive for calcium influx, a delay in influx, and a decrease in the magnitude of calcium influx. The lack of effect of tBHQ on the binding and activity of the calcium-sensitive transcription factor, NFAT,

was unexpected and suggested that the decrease in calcium influx by tBHQ was not great enough to diminish NFAT activation. Alternatively, the decreased calcium influx by tBHQ may rebound at some later time point, ultimately allowing for full NFAT activation. These observations also suggested that the decrease in IL-2 production by tBHQ was not due to impaired NFAT activity.

Along with the influx of Ca^{2+} into the cell upon ligation of the TCR and coreceptor, one of the earliest events of T cell activation is the initiation and propagation of kinase cascades. These signal transduction cascades result in the activation of transcription factors that regulate IL-2 transcription, such as AP-1 (c-fos/c-jun) and NFκB. The current study shows that treatment of cells with 5 μM tBHQ causes a significant increase in AP-1 activity. It has been shown previously that Nrf2 can heterodimerize with AP-1 and increase the transcription of AP-1 target genes. In contrast, tBHQ caused a marked concentration-dependent decrease in NFκB activity. Previously published data have shown that Nrf2 has the ability to inhibit the activity of NFκB by inhibition of IKK, the kinase responsible for phosphorylation and activation of NFκB [28,29]. Activation of NFκB is necessary for IL-2 transcription, thus it seems likely that the decrease in IL-2 production by tBHQ in activated Jurkat T cells is due to the decrease in NFκB, transcriptional activity.

Although well known for its cytoprotective functions and detoxification activity, the role of Nrf2 in the immune system remains unclear. Numerous studies indicate an anti-inflammatory role for Nrf2 in models of inflammatory disease, but the specific cellular and molecular mechanisms are not known. Our previous study demonstrated that activation of Nrf2 moulates murine $CD4^+$ T cell differentiation and our current studies suggest suppression of several key events of early T

cell activation by tBHQ in human Jurkat T cells. Taken together, our studies in human and murine T cells suggest Nrf2 represents a novel mechanism for the regulation of T cell function and may provide insight into the mechanism of some disease states, such as SLE. These studies suggest that activation of Nrf2 by tBHQ may inhibit IL-2 production and CD25 expression, but further studies will be needed to clarify the role of Nrf2 more conclusively. In addition, further studies will be needed to determine the immunomodulatory effects of tBHQ in humans.

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CHAPTER 3

Nrf2-dependent and –independent effects of tBHQ, CDDO-Im, and H_2O_2 in human Jurkat T cells as determined by CRISPR/Cas9 gene editing

Zagorski, J. W., Maser, T. P., Liby, K. T. & Rockwell, C. E. Nrf2-Dependent and -Independent Effects of *tert* -Butylhydroquinone, CDDO-Im, and H $_2$O $_2$ in Human Jurkat T Cells as Determined by CRISPR/Cas9 Gene Editing. *J. Pharmacol. Exp. Ther.* **361,** 259–267 (2017).

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 NFκB 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 NFκB activation in a Nrf2-independent manner. This was an unexpected finding since Nrf2 inhibits NFκB activation in other models. These results lead us to investigate another more potent Nrf2 activator, the synthetic triterpenoid CDDO-Imidazolide (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 NFκB and Nrf2.

Introduction

As indicated previously in the first two chapters, Nrf2 is activated by cell stressors, such as electrophilic xenobiotics [1-3]. In the absence of stress, Nrf2 is quickly turned over, through interaction with Keap1, which is an E3 ubiquitin ligase adapter protein [4,5]. Activated Nrf2 accumulates in the nucleus, where it trans-activates well over 100 target genes, many of which are involved in the resolution of stress, including efflux pumps and glutathione metabolism. In the present study, and as was done previously, NQO1 mRNA levels and Nrf2 nuclear accumulation were used as markers of Nrf2 activation [3,7].

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 [8,9]. 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 [10]. It has been previously shown that human consumption of 100-150mg bolus doses of tBHQ can result in serum concentrations in the high micromolar range. Thus, in addition to serving as a useful tool for activating Nrf2, tBHQ is also relevant to human exposure at the concentrations used in the present studies [8,9,11].

The role of Nrf2 in antioxidant and metabolic responses to reactive toxicants is well described, but it is 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 [12-14]. 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 [13-18]. 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 [8,9].

In chapter 1, the necessity of two signals for proper activation of T cells was discussed, with the first signal occurring through interaction of the T cell receptor complex with antigen loaded and presented by MHC molecules. The second signal occurs through interaction of costimulatory markers, such as CD28 [19]. Experimentally, we activate T cells with antibodies targeting both CD3 and CD28 with antibodies, resulting in a signaling cascade and the activation of NFκB and other transcription factors, induction of the early cytokine IL-2, as well as the expression of early activation markers CD25 and CD69.

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 [20-23].

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 activated with tBHQ, CDDO-Im and H_2O_2.

Materials and Methods

Materials:

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

Plasmids

The NFκB 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 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 [24]. 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 $5x10^5$ cells for 12h in complete media. After 12h the cells were washed and resuspended at a concentration of $5x10^5$ 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' 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 x 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 [25], 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'-GGACTGCACCAGAGCCAT-3'.

IL-2 quantification by ELISA:

Wild-type and Nrf2-null Jurkat T cells were cultured in 96-well plates (1 x 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 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:

$2x10^5$ cells were stained with MitoProbe DilC1(5) per manufacturer's protocol (Invitrogen, Eugene, OR). Immediately prior to analysis by flow cytometry, 5µl 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 ($1x10^7$ 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 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 $MgCl_2$, 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

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 $5x10^5$ cells for 12h in complete media. After 12h, the cells were washed and resuspended at a concentration of $5x10^5$ 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 ± 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).

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 NF□B, in human Jurkat T cells [8]. 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 [22]. The selected sequences were cloned into empty Cas9 vectors coexpressing GFP, as described previously [24]. Sanger sequencing of the resulting Jurkat clones revealed that both guide RNAs were successful in generating INDELs at the Cas9 cut site (Fig. 9). Four clones with frame-shift mutations from each guide RNA were then selected to begin screening for Nrf2 disruption.

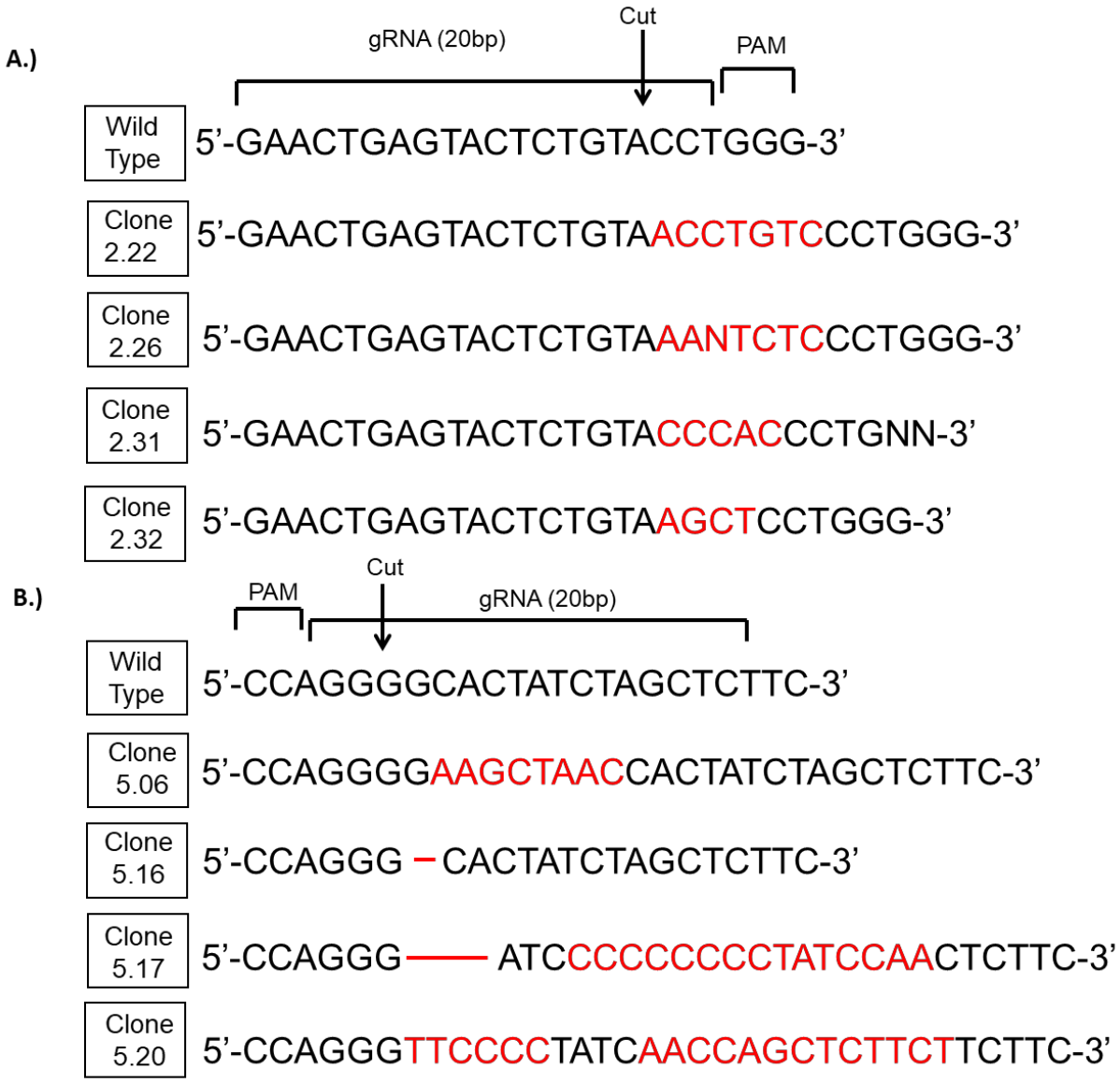

Figure 11: CRISPR-Cas9 induced INDEL formation in Jurkat T cells. Wild-type Jurkat E6-1 cells were transiently transfected with one of two CRISPR-Cas9 plasmids coexpressing GFP, targeting different sites within the genome. The two plasmids (panels A and B) contained different guide RNA sequences and 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.

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. 10a). Furthermore, tBHQ potently induced expression of the Nrf2 target gene Nqo1 in wild-type, but not pooled Nrf2-null clones (Fig. 10b). Likewise, basal expression of Nqo1 was decreased in the Nrf2-null Jurkat T cells as compared to wild-type cells.

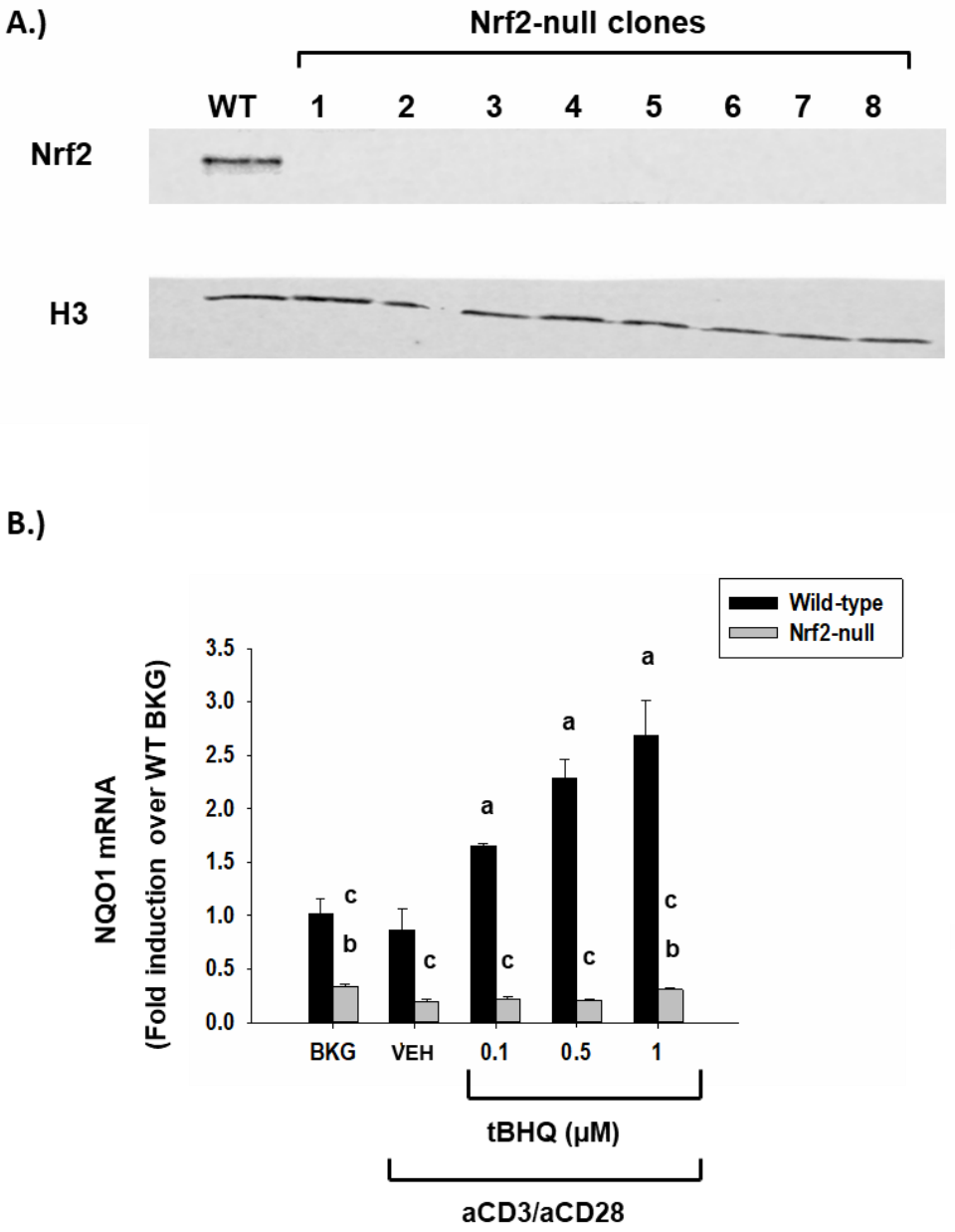

Figure 12: tBHQ increases Nrf2 nuclear accumulation and target gene expression in wild-type cells, but not Nrf2-null clones. (A) Wild-type Jurkat T cells or Nrf2-null Jurkat clones were treated with 10 μM tBHQ for 3 h. Nuclear protein was then isolated and expression of Nrf2 and the housekeeper histone 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 □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 ± 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.

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 [8]. To test whether this effect was dependent upon Nrf2, we pretreated wild-type and Nrf2-null Jurkat T cells with tBHQ (0.1-1μM) 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. 11). 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).

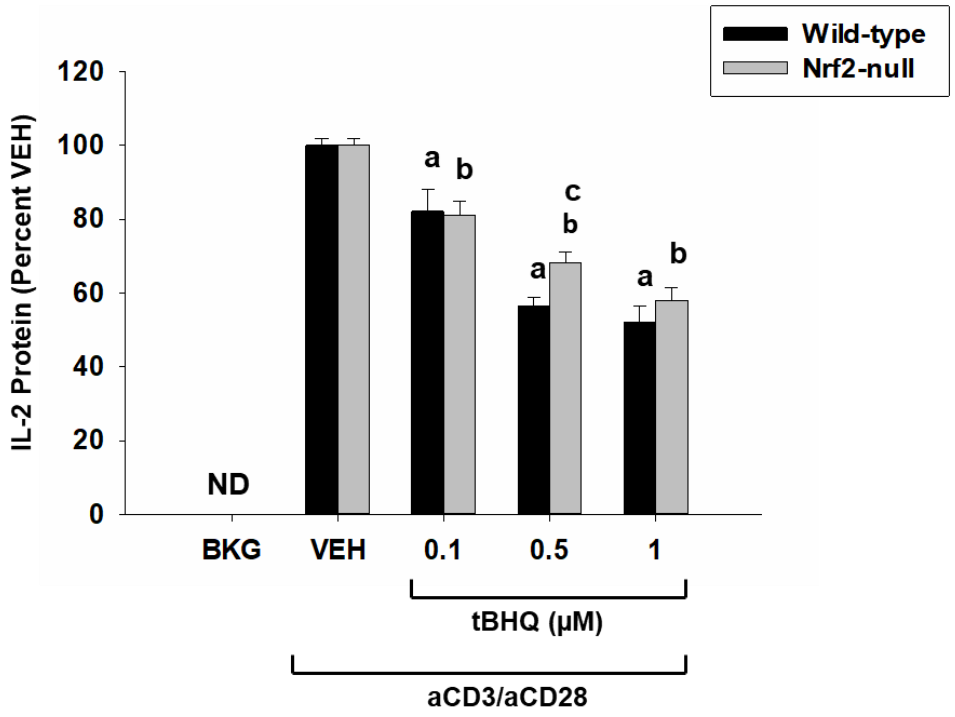

Figure 13: Suppression of IL-2 production by tBHQ is largely independent of Nrf2. Human Jurkat T cells were left untreated (BKG), pretreated with vehicle (VEH) control (0.001% ethanol), or tBHQ (0.1-1 μM) for 30 min prior to activation with anti-CD3/anti-CD28. After 24 h, the supernatants were collected and IL-2 was detected and quantified via ELISA. The data (n=3)

Figure 13: (cont'd) 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, ND = not detectable.

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 [8]. 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 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. 12).

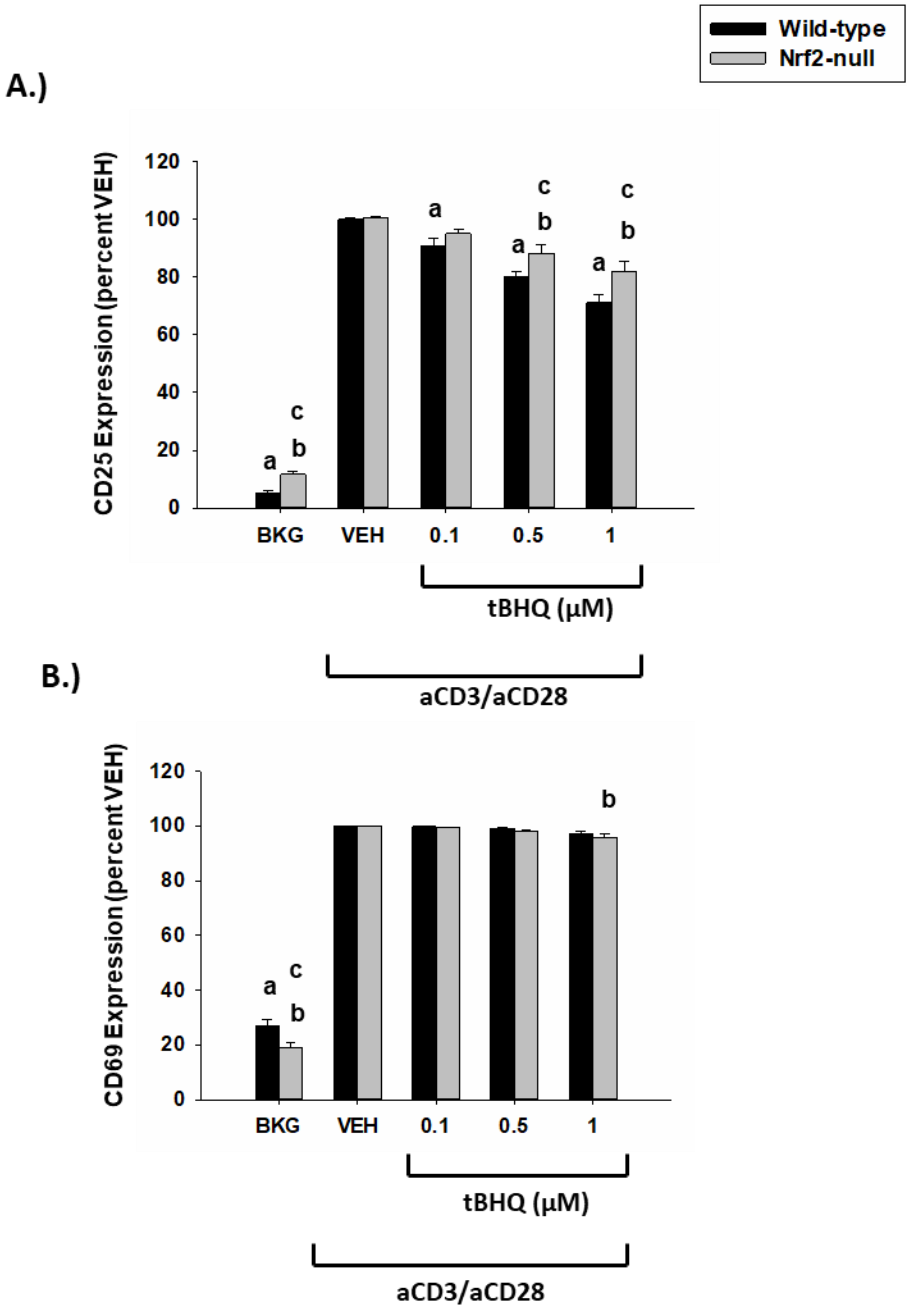

Figure 14: Treatment of Jurkat T cells with tBHQ suppresses CD25 in a partly Nrf2-dependent manner. Human Jurkat T cells were left untreated (BKG), pretreated with vehicle (VEH) control (0.001% ethanol), or tBHQ (0.1-1 µM) for 30 min prior to activation with anti-CD3/anti-CD28. After 24 h, the percentage of cells expressing (A) CD25 and (B) CD69 was quantified by flow cytometry. The data (n=3) are presented as mean ± 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.

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 [14,26]. We have previously shown that tBHQ suppresses NFκB transcriptional activity in Jurkat T cells [8]. 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. 13).

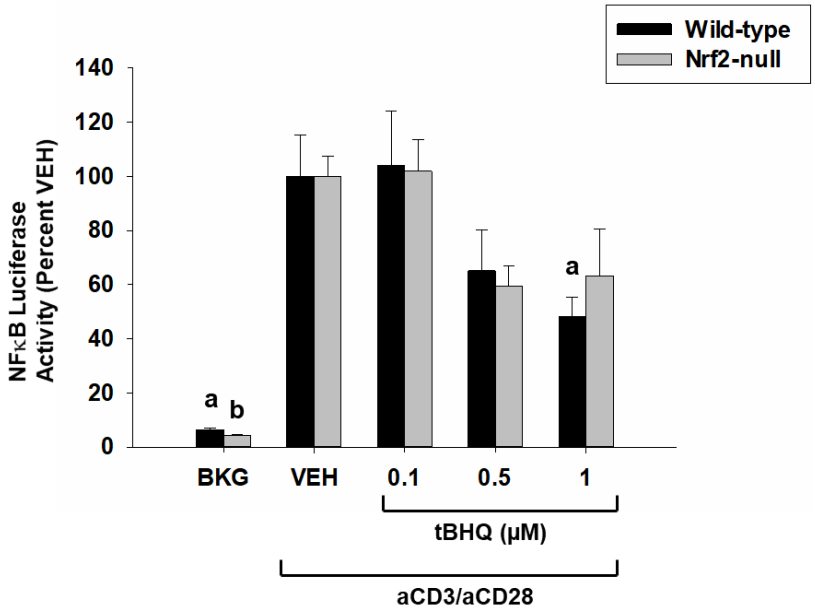

Figure 15: NFκB transcriptional activity is decreased by tBHQ treatment independently of Nrf2. Human Jurkat T cells were transfected with luciferase reporter plasmids driven by NFκB 12 h prior to tBHQ treatment and activation. Jurkat T cells were left untreated (BKG), pretreated with vehicle (VEH) control (0.001% ethanol), or tBHQ (0.1-1 μM) 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 ± SE. a = $p<0.05$

Figure 15: (cont'd) versus wild-type VEH, b = p<0.05 versus Nrf2-null VEH, c = p<0.05 between the wild-type and Nrf2-null genotypes.

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 [27]. 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. After 24h, analysis of cell supernatants demonstrated a Nrf2-dependent suppression of IL-2 secretion in wild-type Jurkat T cells (Fig. 14). Interestingly, treatment of Nrf2-null Jurkat T cells resulted in a Nrf2-independent increase in IL-2 secretion (Fig 14). 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.

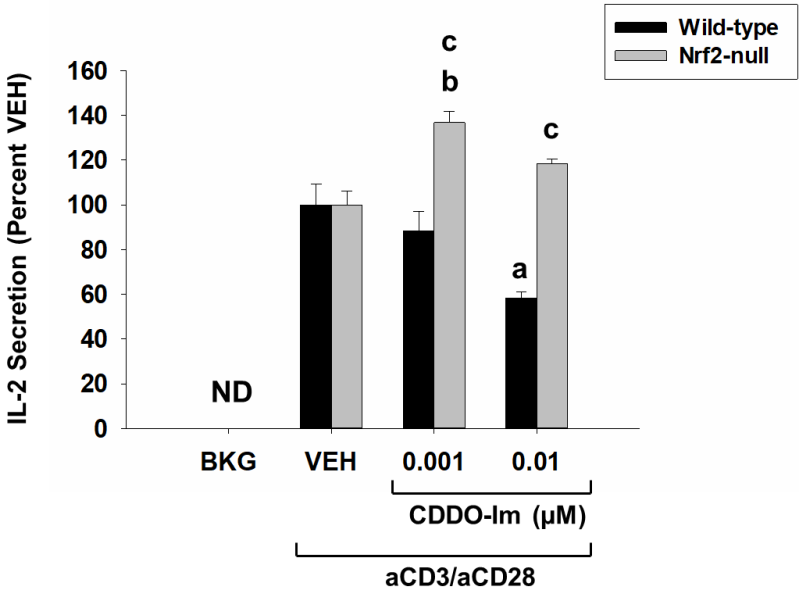

Figure 16: Suppression of IL-2 production by CDDO-Im is Nrf2-dependent. Human Jurkat T cells were left untreated (BKG), pretreated with vehicle (VEH) control (0.001% DMSO), or CDDO-Im (0.001-0.01 μM) for 30 min prior to activation with anti-CD3/anti-CD28. After 24 h, the

Figure 16: (cont'd). supernatants were collected and IL-2 was detected and quantified via ELISA. 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, ND = not detectable.

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 (0.001-0.01μM) 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 12a), CDDO-Im suppressed CD25 expression in a partially Nrf2-dependent manner (Fig. 15a), but had little effect on CD69 expression (Fig. 15b). Taken together, these results demonstrate that CDDO-Im has both Nrf2-dependent and -independent effects upon early events following activation of Jurkat T cells.

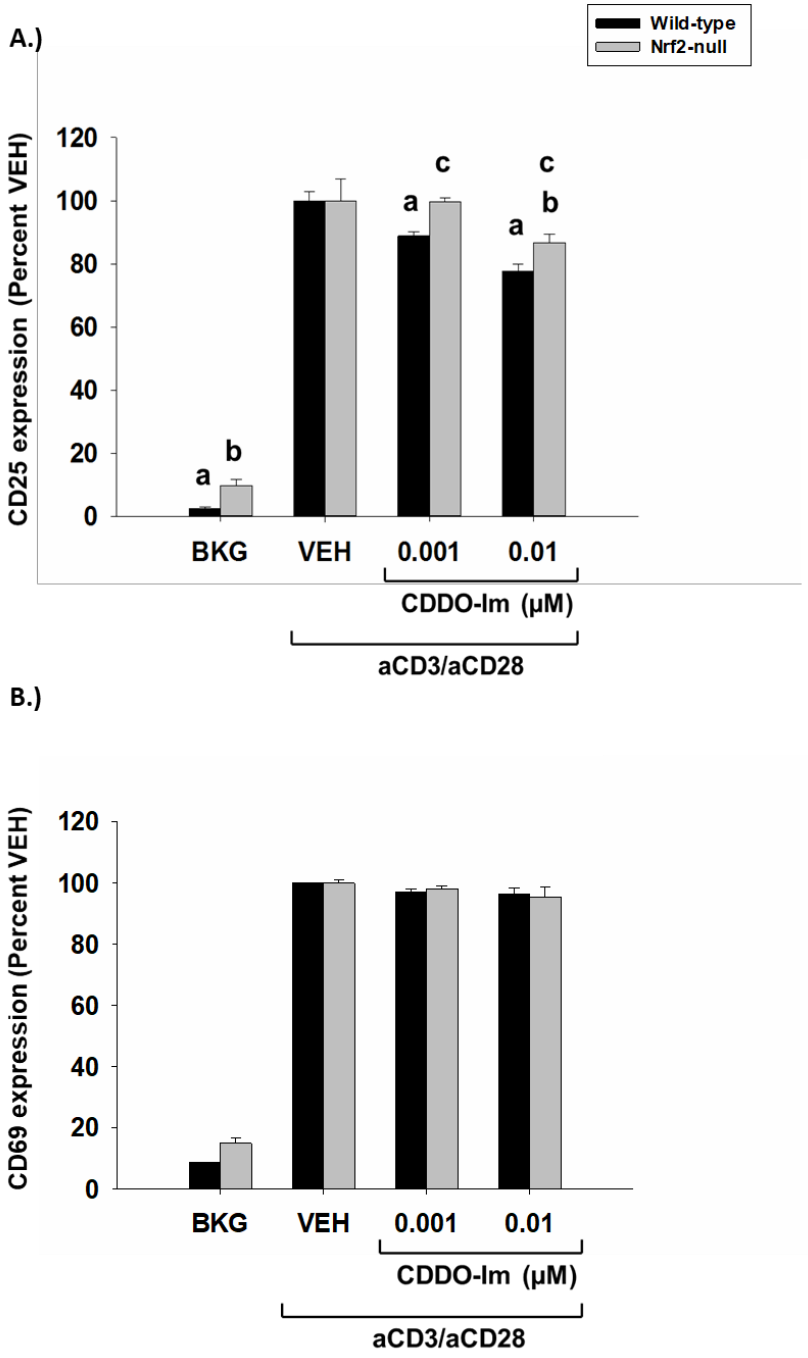

Figure 17: Treatment of Jurkat T cells with CDDO-Im suppresses CD25 in a partly Nrf2-dependent manner. Human Jurkat T cells were left untreated (BKG), pretreated with vehicle (VEH) control (0.001% DMSO), or CDDO-Im (0.001-0.01 μM) for 30 min prior to activation with anti-CD3/anti-CD28. After 24 h, the percentage of cells expressing (A) CD25 and (B) CD69 was quantified by flow cytometry. The data (n=3) are presented as mean ± 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.

Nrf2-null Jurkat T cells are more sensitive to H_2O_2

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 T cells were treated with cytotoxic levels of hydrogen peroxide (10 – 1000 □M). Mitochondrial membrane potential was assessed with the cell permeant dye, DilC, which accumulates in mitochondria with normal membrane potential. A reduction in DilC 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.

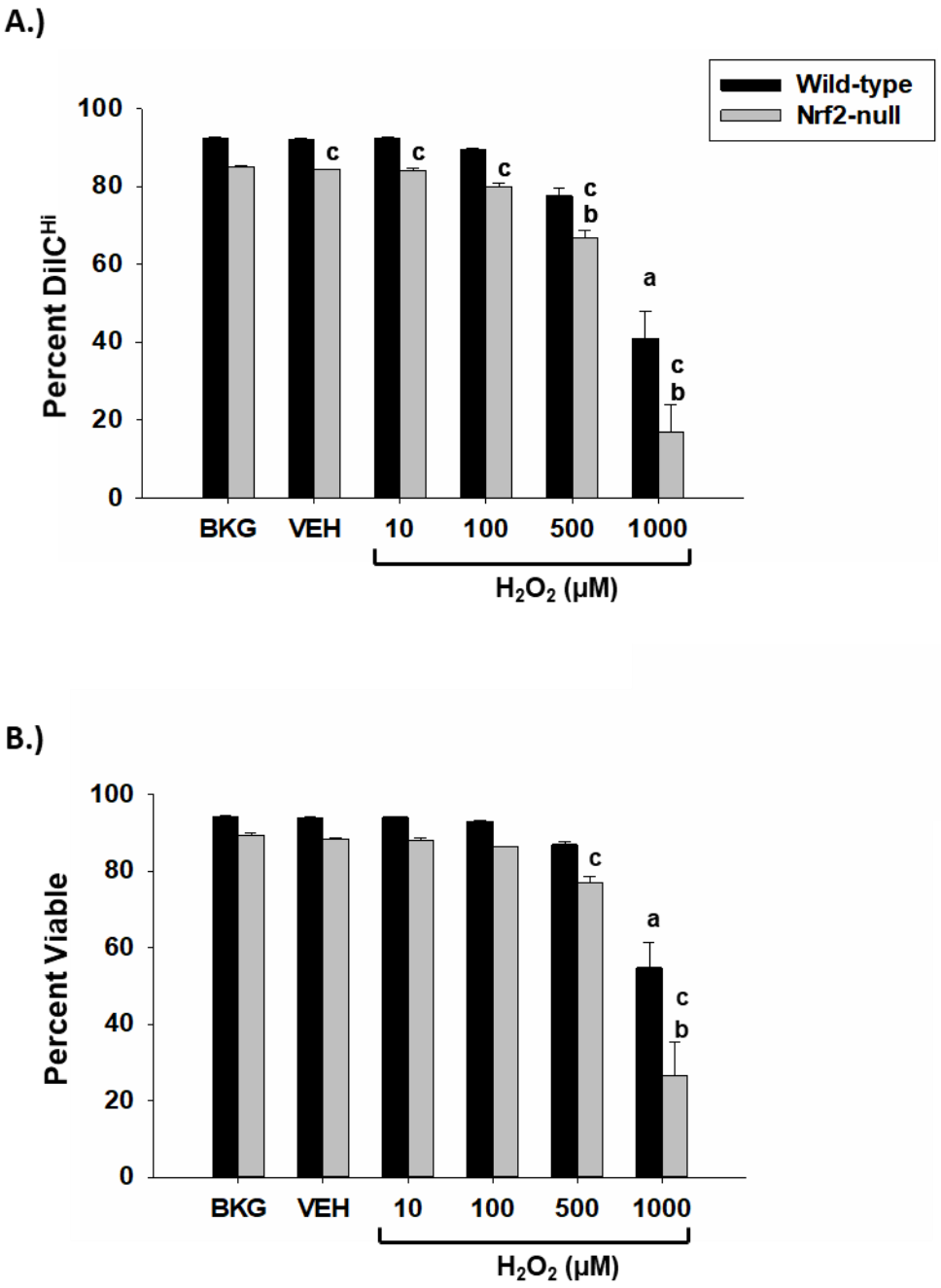

Figure 18: Nrf2-null Jurkat clones are more sensitive to exposure to hydrogen peroxide. Human Jurkat T cells were left untreated (BKG), treated with vehicle (VEH) control (0.01% water in RPMI), or H_2O_2 (1-1000μM) for 16 h prior to flow cytometry analysis for (A) mitochondrial membrane potential as measured using the cell permeable dye DilC1(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.

Discussion

Nrf2 has emerged as a critical signaling pathway in detoxification, cell survival, and immune regulation [3,28]. There is also a growing body of evidence to suggest Nrf2 may play an important role in the promotion of tumorigenesis in some models [29]. 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.

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□B[8,9]. Other groups have utilized other Nrf2 activators, including Benzo[a]pyrene and arsenic compounds, to both implicate and absolve Nrf2 in immune modulation [30-33]. 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 [34,35]. 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 NFκB 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 [27,36]. 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 NFκB 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 NFκB activation in immune cells [14]. The regulation of NFκB is quite complex. Classical activation of NFκB involves a series of phosphorylation events that culminates in the phosphorylation of inhibitor of □B□ (I□B□), which is subsequently degraded by the proteasome and thus liberates the p65/p50 homodimer, resulting in activation [37]. However, in addition to this component of NFκB 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 NFκB activation in immune cells, including butylated hydroxyanisole, an antioxidant that is structurally similar to tBHQ [38]. Thus, it seems plausible that the inhibition of NFκB 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 [39]. 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 [40]. 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 [41]. 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 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 NFκB 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.

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CHAPATER 4

The Nrf2/Keap1 system alters early human Jurkat T cell activation

Abstract

The stress activated transcription factor, Nrf2, has been previously shown to suppress IL-2 secretion in human Jurkat T cells, in response to the synthetic triterpenoid, CDDO-Im. The previous studies, did not address the question of basal genotype differences between wild-type and Nrf2-null cells; only changes in response to toxicant were reported. In the current study, we have generated Keap1-null Jurkat T cells by use of CRISPR-Cas9 technology. The purpose of the present study was to determine the effect of the removal of Nrf2 and Keap1 expression in Jurkat T cell activation. The data demonstrate that targeting of Keap1 by CRISPR-Cas9 is efficient in generating Keap1-null Jurkat clones, and the resulting transgenic clones have constitutive Nrf2 nuclear accumulation, as well as induction of the Nrf2 target gene NQO1. Additionally, Nrf2-null cells have increased IL-2 secretion. Conversely, Keap1-null cells have a significant decrease in IL-2 secretion, as compared to wild-type. Interestingly, Keap1-null cells also have a robust diminution in CD25 expression, post stimulation by the T cell receptor and co-receptor. Unexpectedly, little effect was observed on c-jun and p65 nuclear accumulation, indicating that the decrease in CD25 expression in the Keap1-null cells is not likely to be due to effects on AP-1 or NF□B. Furthermore, Keap1-null cells have a dramatic increase in caspase 3/7 activity, indicative of an increase in apoptosis. Additional studies are needed to determine if the suppression of IL-2 secretion is due to an increase in activation-induced cell death, or other mechanisms, such as an increase in uptake of IL-2 by the IL-2 receptor.

Introduction

Physiologically, Nrf2 is considered to be a master regulator of many detoxification pathways, as well as the anti-inflammatory response [1–6]. The study of Nrf2 has been, until recently, relegated to the use of compounds that activate Nrf2 through stress signals, such as reactive oxygen species and electrophilic compounds which are thiol-reactive [7,8]. As no known Nrf2-null human T cell models were previously known to exist, the investigation of Nrf2 in human systems was previously confined to imperfect systems, such as activators with off-target effects and/or partial knock down of Nrf2 by RNA interference [9]. Moreover, generation of Keap1-null mice was initially complicated by the death of Keap1-null mice shortly after birth due to hyperkeratinization of the esophagus, which resulted in starvation [10]. Conditional Keap1 knock-out mice have since demonstrated that after maturity, Keap1 removal does not result in a decrease in organismal viability, indicating that this may be a suitable target to generate a constitutively-active Nrf2 human cell line [11]. Although multiple mouse models exist, it has proven difficult to determine the role of Nrf2 and Keap1 in human models.

Over the past several years, our group and others have demonstrated that Nrf2 not only serves to induce antioxidant and anti-inflammatory pathways, but also plays a crucial role in proper immune homeostasis [5,11–14]. A number of Nrf2 activators have been shown to alter immune response in multiple species and models, including primary mouse, mouse cell lines, primary human, and in human cell line models [15–19]. Furthermore, Keap1 conditional knock-out mice, also display modulation of immune endpoints, including hematopoiesis [20,21]. Collectively, a compelling body of scientific literature shows the Nrf2/Keap1 pathway to be immune modulatory in multiple models, however the field needs additional methods, which do not rely on non-specific activators, to study the role of these proteins in the human immune response.

Discussed in more depth in chapter one, T cells requires signal transduction through both the T cell receptor complex, as well as a co-receptor [22,23]. Experimentally, in the present studies, we have used antibodies ligating CD3 and CD28, to mimic physiological activation. This process results in the activation of several signal transduction cascades and the eventual nuclear accumulation of key transcription factors AP-1, NFκB, and NFAT, which are responsible for the transcription of many immune related genes, including interleukin-2 (IL-2) [22]. IL-2 is a cytokine which acts in both an autocrine and paracrine fashion and has broad ranging effects on the immune response, including development of memory cells and clonal expansion of T cells in response to infection [24–26]. Alterations in IL-2 have been implicated in disease states, including autoimmune disease [27].

Our most recent study was the first to demonstrate that Nrf2 modulates human T cell response, in which we showed the potent Nrf2 activator, CDDO-Im, suppressed IL-secretion in a completely Nrf2-dependent manner and CD25 in a largely Nrf2-dependent manner [15]. The purpose of the present studies was to determine the basal genotype differences in Nrf2-null and Keap1-null Jurkat T cells, as compared to wild-type, by use of CRISPR-Cas9 gene editing. Genetic manipulation of Jurkat T cells allows for the analysis of the role of both Nrf2 and Keap1, without the confounding variable of potential off-target effects of Nrf2 activators.

Materials and Methods

Materials:

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

Plasmids

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).

Generation of CRISPR-Cas9 plasmids:

A CRISPR-Cas9 plasmid (PX458) lacking a gRNA sequence was purchased from Addgene (Cambridge, MA). The gRNA sequence targeting Kelch-like ECH-associated protein 1 (Keap1) was chosen from http://arep.med.harvard.edu/human_crispr/, and Nrf2 specificity was verified 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. 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 $5x10^5$ cells for 12h in complete media. After 12h the cells were washed and resuspended at a concentration of $5x10^5$ 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 for Keap1: forward primer: 5'-GGAAACAGCCTCAGGAAGAATA-3' reverse primer: 5'-GTCAAGTACGACTGCGAACA-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, 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'-GGACTGCACCAGAGCCAT-3'.

IL-2 quantification by ELISA:

Wild-type, Nrf2-null, and Keap1-null Jurkat T cells were cultured in 96-well plates (1×10^5 cells/well), 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 available ELISA kit (BioLegend, San Diego, CA), per the manufacturer's protocol. The absorbance was quantified at 450 nm using a Tecan m1000 Pro microplate reader (Männedorf, Switzerland).

Measurement of extracellular markers by flow cytometry:

Wild-type, Nrf2-null, and Keap1-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 caspase 3/7 activity by flow cytometry:

$2x10^5$ cells were stained with the fluorogenic substrate CellEvent and were treated per manufacturer's protocol (Thermo Scientific, Waltham, MA). All samples were analyzed by flow cytometry using the BD Accuri C6 to determine the functional activity of caspase 3/7 (BD Accuri, San Jose, CA).

Nuclear and whole cell lysate protein isolation:

Wild-type, Nrf2-null, and Keap1-null Jurkat T cells ($1x10^7$ 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 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 $MgCl_2$, 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).

Wild-type, Nrf2-null, and Keap1-null Jurkat T cells (4x10^6) were lysed using RIPA buffer in PBS containing 1% IGEPAL CA-630, 0.5% Sodium Deoxycholate, and 0.5% SDS. The resulting cell lysates were then spun at 10,000 x g for 10min, and the supernatants were collected.

Western Analysis:

After quantification, nuclear protein was diluted in Laemmli Sample Buffer (Bio-Rad) supplemented with 2-mercaptoethanol so that each well contained either 10µg of nuclear protein or 30µg of whole cell lysates. 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), Nrf2 (H-300), Keap1 (D1G10), c-jun (60A8), and p65 (7970) 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 Keap1 and c-jun antibodies were purchased from Cell signaling (Danvers, MA), and the primary p65 antibody was purchased from Abcam (Cambridge, UK) 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).

Statistical analysis:

The mean ± standard error was determined for each treatment group in the individual experiments. Homogeneous data were subsequently evaluated by two-way parametric analysis of variance. 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).

Results

CRISPR-Cas9 technology is efficient in generating Keap1-null Jurkat cells

The generation of CRISPR-Cas9 plasmids was accomplished as previously described, using a database containing gRNA sequences for 40% of the human exome. After selection of a gRNA sequence targeting Keap1, BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis was done to determine that the previously selected sequence was unique. The resulting unique sequence was cloned into an empty CRISPR-Cas9 vector, which co-expresses GFP, and this was done as has been previously reported. After transfection of the plasmid into wild-type Jurkat T cells, the positive transfectants were sorted by expression of GFP signal, and allowed to clonally expand. The resulting Jurkat clones were Sanger sequenced and those containing insertions and deletions at the gRNA cut-site (data not shown) were analyzed by Western blot for Keap1 protein expression. To determine Keap1 deficiency, whole cell lysates were analyzed by PAGE/Western blotting (Fig. 17). The resulting blot showed multiple Keap1-null clones were present, and eight were retained for analysis of immune endpoints, as was done previously with Nrf2-null Jurkat clones.

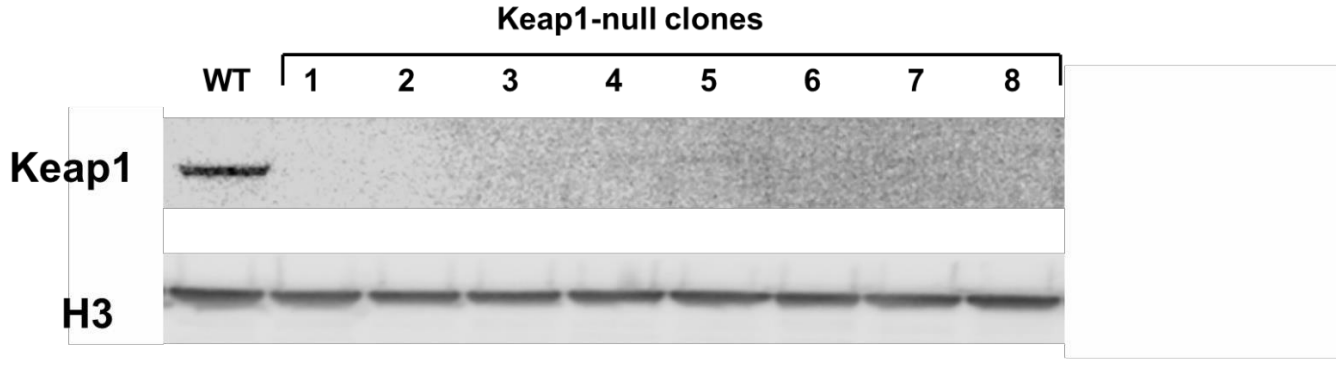

Figure 19: CRISPR-Cas9 targeting of Keap1 results in Keap1-null clones. Wild-type Jurkat T cells or Keap1-null Jurkat clones were lysed using RIPA buffer and whole cell lysates were analyzed for expression of Keap1 and the housekeeper GAPDH by western blot analysis.

Keap1 deficiency results in constitutive Nrf2 activity

The generation of Keap1 null clones led us to determine if Keap1 deficiency resulted in constitutive Nrf2 activity, as measured by nuclear accumulation and induction of the Nrf2 target gene NQO1. Wild-type, Nrf2-null, and Keap1-null Jurkat T cells were either left untreated or treated with 10μM tBHQ. tBHQ treatment resulted in a marked nuclear accumulation of Nrf2 in wild-type Jurkat cells, but no detectable Nrf2 protein was present in the Nrf2-null cell line, which has been reported previously. Additionally, Keap1-null Jurkat T cells had a robust nuclear accumulation of Nrf2 in the untreated background group, which was not observed in either the wild-type or Nrf2-null cells (Fig. 18a.). Furthermore, nuclear accumulation of Nrf2 was approximately the same in untreated and tBHQ-treated KEAP1-null cells, indicating that tBHQ was not able to boost nuclear accumulation any further in the absence of Keap1. We next measured NQO1, a known Nrf2-sensitive gene, in all three genotypes (Fig. 18b.). Similar to what was observed in the previous Western blot, wild-type cells had marked induction of NQO1 with treatment of tBHQ, and no induction was measured in Nrf2-null lines. Keap1-null clones, however, showed increased NQO1 expression in untreated cells, at levels well above the tBHQ-treated wild-type cells, which was maintained, but not increased, with tBHQ treatment. Taken together, these data demonstrate that Keap1-deficient cells have constitutive Nrf2 nuclear accumulation and transcriptional activity.

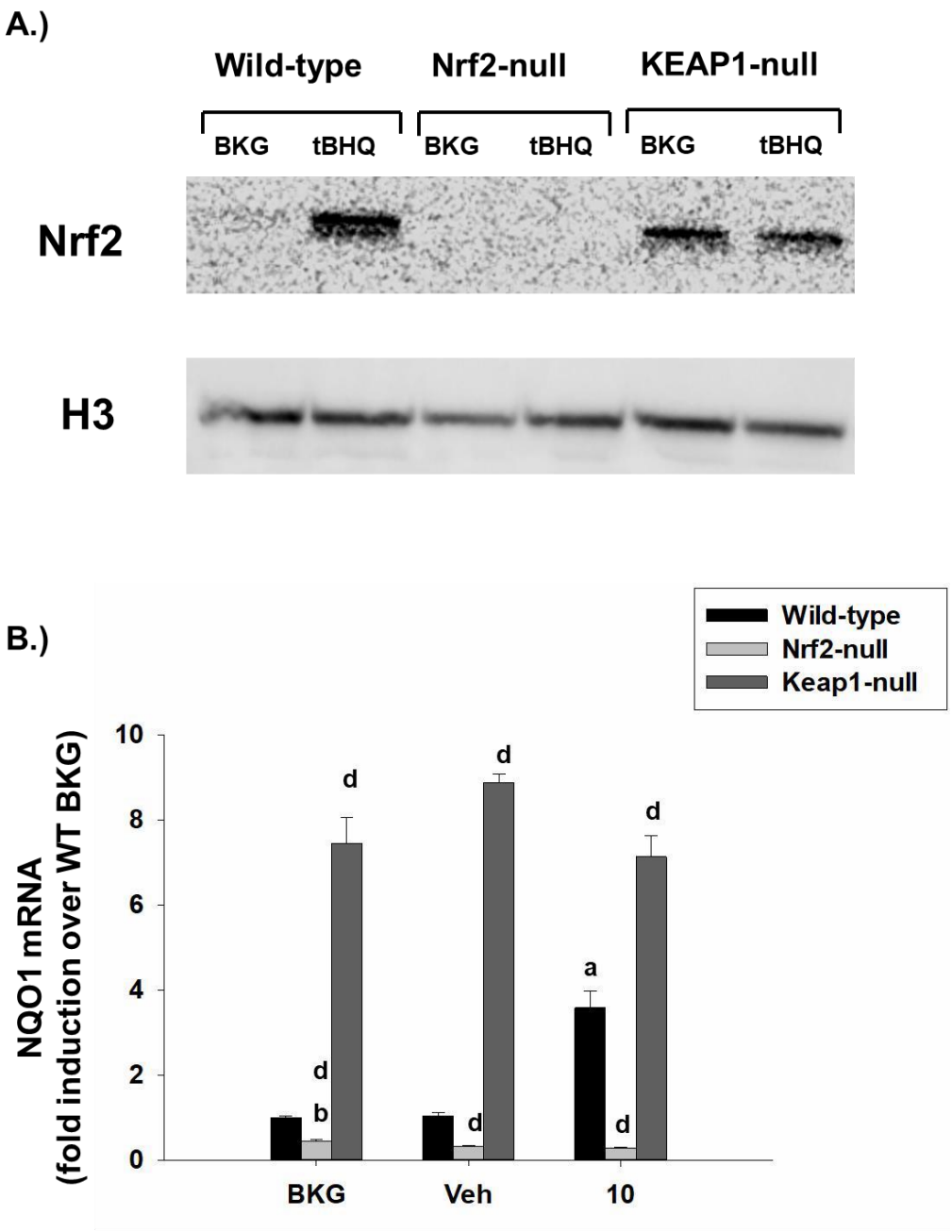

Figure 20: Keap1-null Jurkat T cells have constitutive Nrf2 activity. (A) Wild-type, Nrf2-null, or Keap1-null Jurkat T cells were left untreated (BKG) or treated with 10 µM tBHQ for 3 h. Nuclear protein was then isolated and expression of Nrf2 and the housekeeper histone H3 was probed by western analysis. (B) Wild-type, Nrf2-null, or Keap1-null Jurkat T cells were left untreated (BKG) or pretreated with vehicle (VEH) control (0.01% ethanol) or tBHQ (10 □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 are presented as mean ± SE. a = $p<0.05$ within WT, compared to aCD3/aCD28, b = $p<0.05$ within Nrf2-null, compared to aCD3/aCD28, c = $p<0.05$ within Keap1-null, compared to aCD3/aCD28, d= $p<0.05$ compared to WT.

Nrf2-deficiency increases IL-2 secretion, whereas Keap1-deficiency decreases IL-2 secretion in Jurkat T cells

Our previous studies have demonstrated that activation of Nrf2 suppresses secretion of IL-2 in the human Jurkat T cell line [17]. In the current studies, we measured basal genotype differences between wild-type, Nrf2-null, and Keap1-null cells with respect to the magnitude of IL-2 induction after T cell activation (Fig. 19). All three genotypes were either left untreated or activated with antibody targeting CD3 and CD28, the T cell receptor and a co-receptor. 24h later, cell supernatants were harvested and IL-2 was measured by commercially available sandwich ELISA. Analysis of IL-2 in supernatants demonstrated a more than two-fold increase in IL-2 secretion in Nrf2-null cells, as compared to wild type. Keap1-null cells, in contrast, secrete approximately half as much IL-2 as wild-type cells.

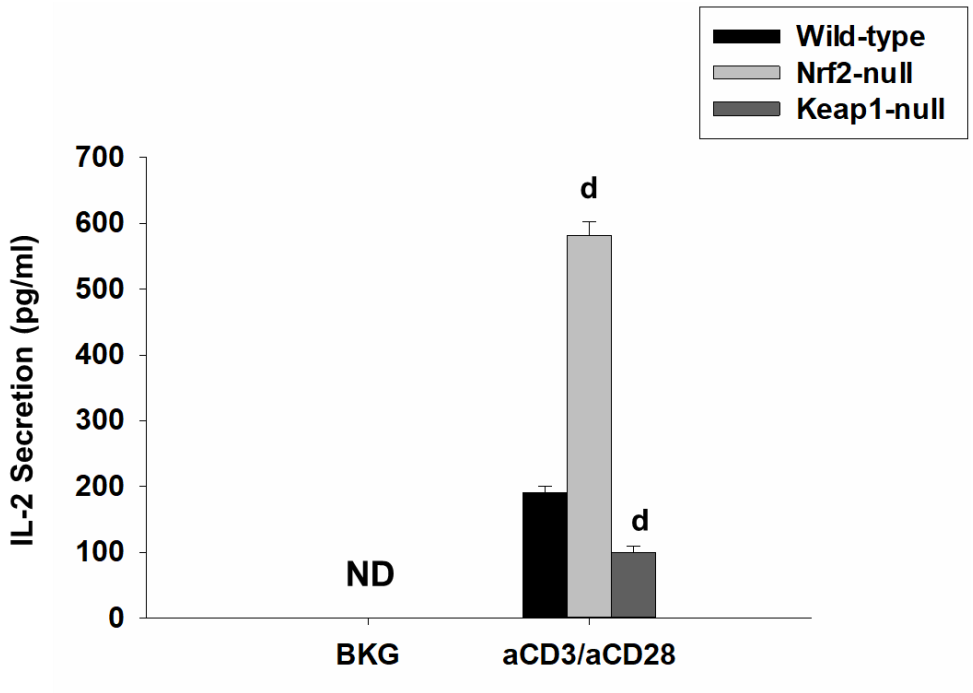

Figure 21: Expression of Nrf2 and Keap1 alters IL-2 secretion in Jurkat cells. Human Jurkat T cells were left untreated (BKG) or activated with anti-CD3/anti-CD28. After 24 h, the supernatants were collected and IL-2 was detected and quantified via ELISA. The data are

Figure 21: (cont'd) presented as mean ± SE. a = $p<0.05$ within WT, compared to aCD3/aCD28, b = $p<0.05$ within Nrf2-null, compared to aCD3/aCD28, c = $p<0.05$ within Keap1-null, compared to aCD3/aCD28, d= $p<0.05$ compared to WT, ND = not detectable.

Nrf2-null and Keap1-null Jurkat T cells express significantly less CD25 after stimulation

To further characterize Nrf2-null and Keap1-null cell lines, we analyzed the expression of two markers of T cell activation, CD25 and CD69, where CD25 is the high affinity chain of the IL-2 receptor, and CD69 is a C-type lectin. Both proteins are highly expressed within 24h after T cell activation. Cells were harvested 24h after activation and analyzed by flow cytometry, measuring cell surface expression of both CD25 (Fig. 20 a/b.) and CD69 (Fig. 20 b/c). Consistent with our previous studies, modulation of the Nrf2/Keap1 axis had little to no effect on the percentage of CD69 positive cells. In contrast, expression of CD25 was modestly decreased in the Nrf2-null cells and substantially decreased in the Keap1-null cells, which is consistent with our previous study, showing activation of Nrf2 inhibits CD25 expression.

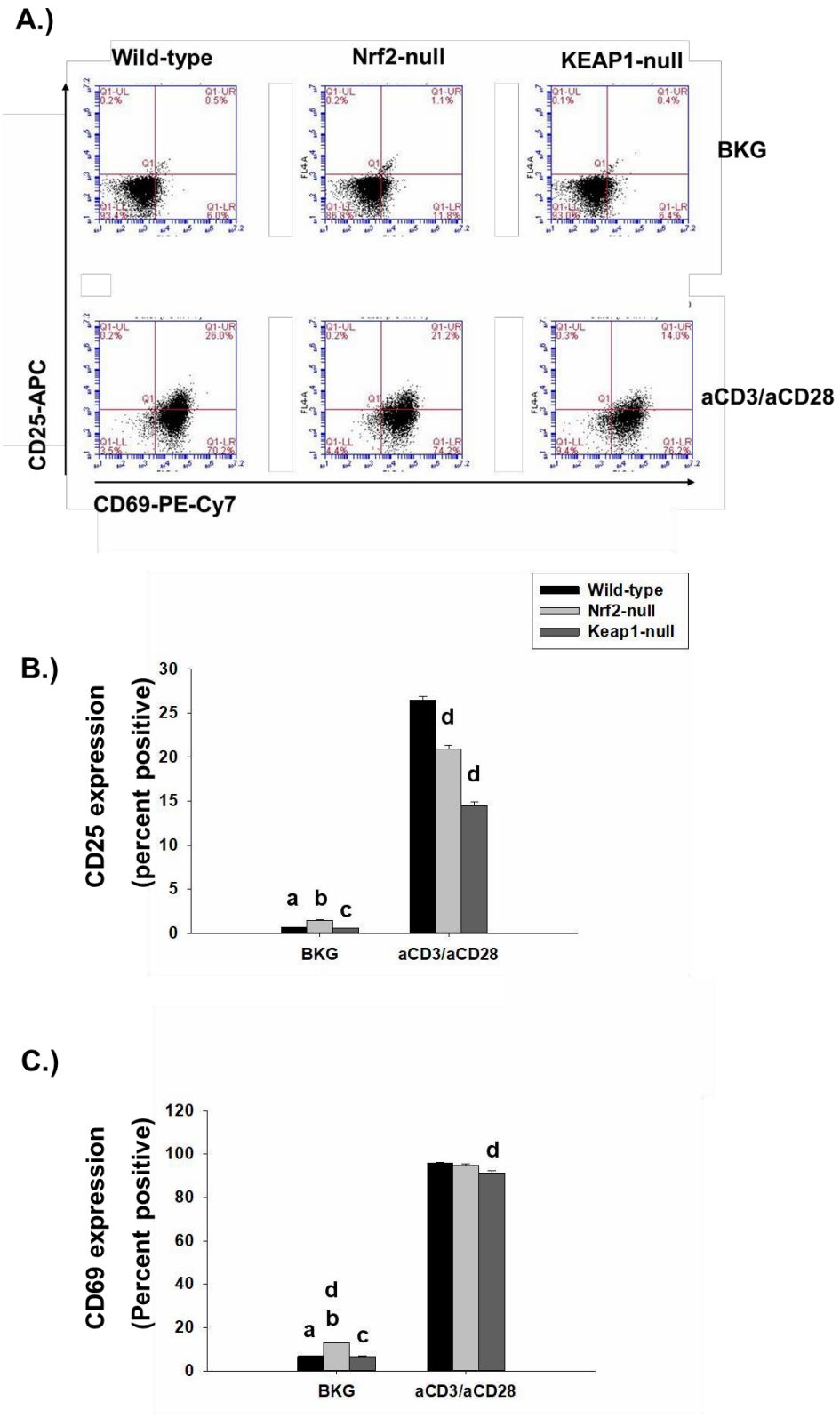

Figure 22: Keap1-null Jurkat clones express significantly less CD25 after stimulation, than either WT or Nrf2-null Jurkat cells. Wild-type, Nrf2-null, and Keap1-null human Jurkat T cells

Figure 22: (cont'd) were left untreated (BKG) or activated with with anti-CD3/anti-CD28 (aCD3aCD28). After 24 h, the percentage of cells expressing (A, B) CD25 and (A, C) CD69 was quantified by flow cytometry. The data are presented as mean ± SE. a = $p<0.05$ within WT, compared to aCD3/aCD28, b = $p<0.05$ within Nrf2-null, compared to aCD3/aCD28, c = $p<0.05$ within Keap1-null, compared to aCD3/aCD28, d= $p<0.05$ compared to WT.

Nrf2 inhibits c-jun nuclear accumulation

Our previous findings on the secretion of IL-2 in mutant cell lines led us to investigate the nuclear accumulation of c-jun, a member of the AP-1 family of transcription factors, which is known to be necessary for IL-2 transcription (Fig. 21). Wild-type, Nrf2-null, and Keap1-null cells were either left untreated or activated via antibody ligating CD3 and CD28. Three hours after activation, nuclear protein was isolated and analyzed for c-jun nuclear accumulation. Western blots of nuclear protein demonstrated a robust nuclear accumulation of c-jun, post activation of wild-type cells. Nrf2-null Jurkat cells showed a greater increase in c-jun nuclear accumulation, as compared to wild-type, as well as a marked increase in the untreated group. Conversely, Keap1-null cells had a modest decrease in the c-jun nuclear translocation.

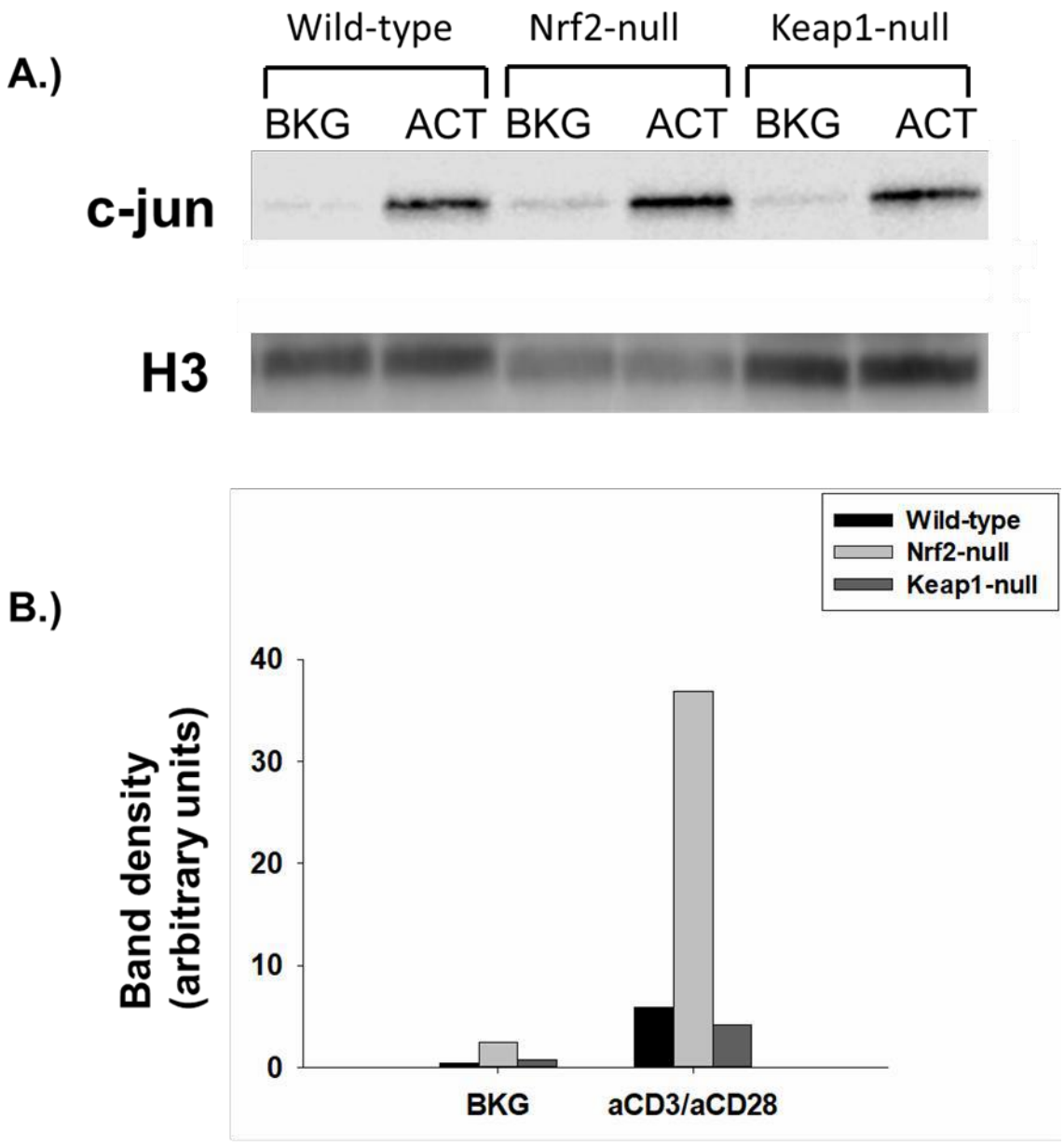

Figure 23: Nrf2 inhibits c-jun nuclear accumulation in Jurkat T cells. (A)Wild-type, Nrf2-null, or Keap1-null Jurkat T cells were left untreated (BKG) or activated with anti-CD3/anti-CD28 (ACT). After 3 hours, nuclear protein was then isolated and expression of c-jun and the housekeeper histone H3 was probed by western analysis, (B) and band density is depicted in arbitrary units, as compared to loading control.

Keap1 inhibits p65 nuclear accumulation

As Nrf2 and Keap1 were shown to have opposite effects on c-jun nuclear accumulation, we next investigated p65 nuclear accumulation. P65 (RelA) is a member of the NFκB family, which is known to be important in the control of cellular events, such as apoptosis, as well as many immune endpoints, such as transcription of IL-2. All three genotypes of Jurkat cells were either left untreated or activated via antibody ligating CD3 and CD28. Three hours after activation, nuclear protein was isolated and analyzed for p65 nuclear accumulation (Fig. 22). As was expected, T cell activation resulted in an increase in nuclear p65 in wild-type cells. Untreated Nrf2-null cells had comparable nuclear p65 as activated wild-type cells but was not inducible with T cell activation. The basal increase in p65 nuclear accumulation in the Nrf2-null cells is supported by published studies, which show Nrf2 can antagonize NFκB activity [(13)]. Interestingly, Keap1-null cells had an approximately two-fold increase in p65 nuclear accumulation after T cell stimulation as compared to wild-type, further demonstrating alteration of other signaling pathways by the Nrf2/Keap1 pathway.

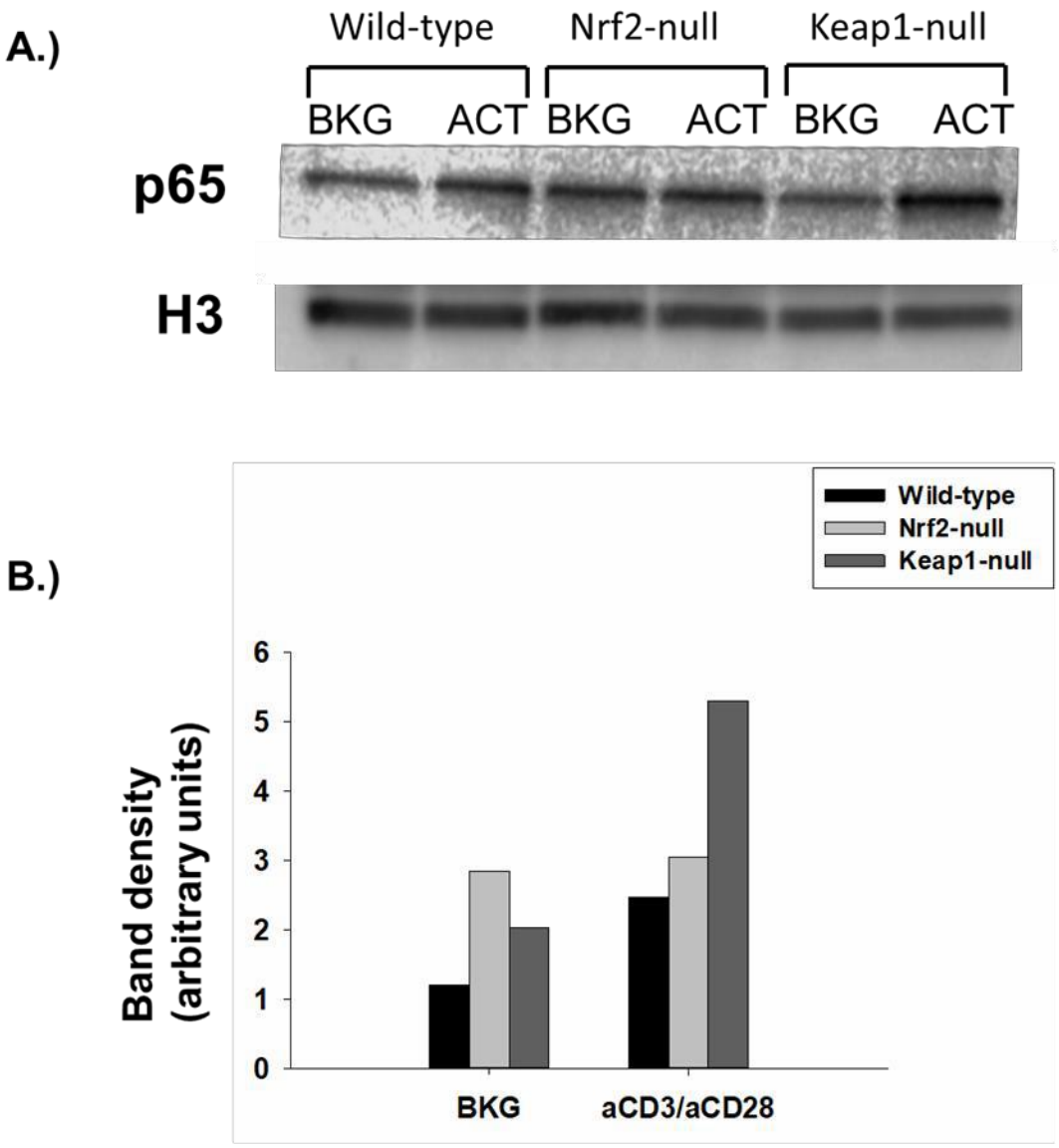

Figure 24: The Nrf2 Keap1 axis alters p65 nuclear accumulation. (A) Wild-type, Nrf2-null, or Keap1-null Jurkat T cells were left untreated (BKG) or activated with anti-CD3/anti-CD28 (ACT). After 3 hours, nuclear protein was then isolated and expression of c-jun and the housekeeper histone H3 was probed by western analysis, (B) and band density is depicted in arbitrary units, as compared to loading control.

Keap1-null T cells have increased apoptosis in response to CD3 and CD28 stimulation as compared to wild-type and Nrf2-null cells

Increases in p65 nuclear accumulation observed in the Keap1-null genotype in response to T cell stimulation, next led us to investigate cell death and apoptosis. Toward this end, Jurkat T cells were analyzed for caspase 3/7 catalytic activity and viability, by flow cytometry, using a commercially available kit (Fig. 23). Untreated or activated T cells were stained with fluorescent caspase substrate 24h after T cell activation and were subsequently analyzed by flow cytometry. Background samples of all three genotypes were over 90% viable, and had only minor caspase activity. As was expected, activation of both wild-type and Nrf2-null Jurkat T cells resulted in modest increases in early apoptosis and slight increases in cell death, consistent with activation-induced cell death. Unexpectedly and unlike both wild-type and Nrf2-null cells, Keap1-null cells demonstrated marked increases in both caspase activity, as well as cell death. These observations were surprising, given Keap1-null cells show constitutive Nrf2 activity, which has been associated with increased cell viability to toxicants and oxidative stress.

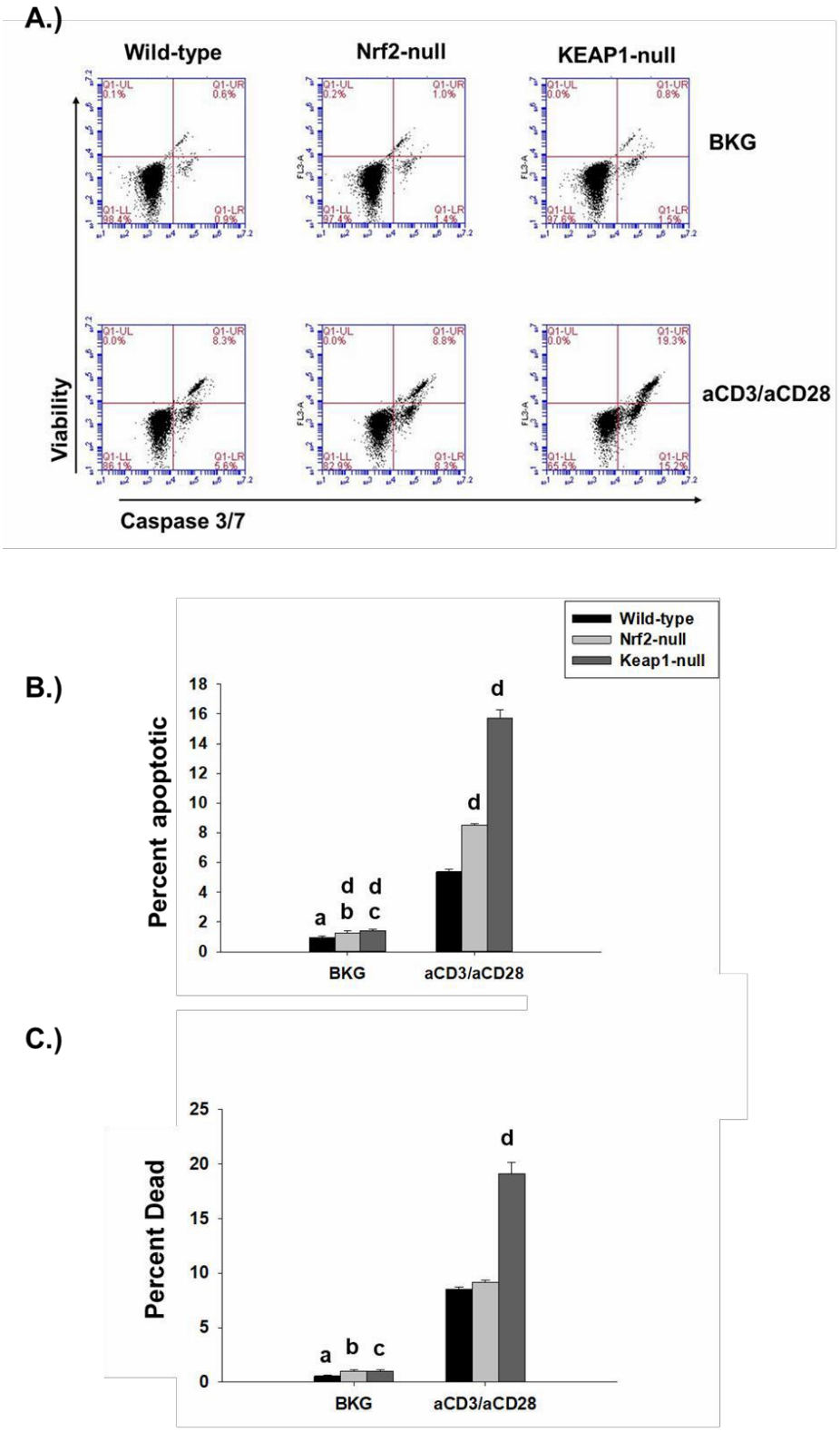

Figure 25: Keap1-null Jurkat clones have increased caspase activity and cell death after activation with anti-CD3/anti-CD28. Wild-type, Nrf2-null, and Keap1-null human Jurkat T cells were left untreated (BKG) or activated with with anti-CD3/anti-CD28 (aCD3aCD28). After 16 h, cells were analyzed, observing functional caspase 3/7 activity (A,B) and cell viability (A, C), by flow cytometry.

Discussion

Over the last several years, an increasing body of work has proven the role of endogenous role of Nrf2 and Keap1 to be quite complex. It is well established that the Nrf2/Keap1 pathway is integral in the amelioration of oxidative stress and inflammation [6,28,29]. In addition to these responses, Nrf2 and Keap1 have been shown to be intimately involved in formation of proper immune responses, as knock-out animals for both genes have alterations in immune response to pathogen, self-tolerance, hematopoiesis, and other immune anomalies [11–13,15,16,30]. In addition to the effects of genetic knockout of Nrf2/Keap1 on murine immune response, numerous studies also indicate that acute Nrf2 activation by pharmacological activators also modulates immune response [16,20,21].

Until recently, these studies had been done almost entirely in mouse models. This is largely due to the lack of commercially available compounds known to specifically target the pathway, as well as no known polymorphisms which confer knock-out of either protein in human populations. Therefore, we have been limited to indirect means, such as toxicants, or incomplete knock-down, to infer the role of both Nrf2 and Keap1 in human models [9,17,18]. In the present studies, we have aimed to answer questions left largely unanswered in our previous studies. Our previous studies determined Nrf2-dependent and independent effects of different Nrf2 activators on endpoints of early T cell activation, including cytokine secretion and expression of cell surface markers of activation [15]. These previous studies were the first to demonstrate Nrf2 activation to be immunomodulatory in human models, but it did not address

any basal genotype differences in human models of T cell activation. Therefore, we have again implemented CRISPR-Cas9 to knock-out Keap1. The resulting three genotypes were then used to observe the role of the Nrf2/Keap1-axis on a human model of T cell activation.

Using this approach, we demonstrated that, as expected, knocking out Keap1 in human Jurkat T cells resulted in constitutive Nrf2 activity, measured by constitutive, non-inducible, nuclear accumulation, as well as a marked increase in NQO1 transcription in untreated cells [11,20,31]. These data supported our hypothesis that lack of Keap1 expression is adequate to cause constitutive Nrf2 activation. Further analysis of wild-type, Nrf2-null, and Keap1-null Jurkat genotypes demonstrated that knocking out Nrf2 resulted in a marked increase in the amount of IL-2 secreted. This aligns with what we have previously observed with Nrf2 activation by CDDO-Im, which resulted in a suppression of IL-2 induction [15]. Furthermore, Keap1-null cells had a significant decrease in IL-2 secretion. These data are significant in that they demonstrate that alterations in Nrf2 expression alone, without the use of xenobiotic, markedly alter cytokine secretion. Likewise, Keap1-null cells demonstrated increased Nrf2 activity and suppressed IL-2 secretion, which further supports a role for Nrf2 as a regulator of IL-2 in this model.

Moreover, alterations in IL-2 secretion may have pleiotropic effects in the immune system. IL-2 is a key cytokine in the development and maintenance of regulatory T cells, which help to shut down immune responses and maintain peripheral tolerance. In addition, IL-2 is involved in the development of a proper memory response, facilitates clonal expansion, and can influence the polarization of T cell subsets [32–35]. Taken together, the current studies in conjunction with previously published studies, strongly suggest a critical role for the Nrf2/Keap1 pathway in regulating immune response.

The current studies suggest Nrf2 has differential effects on the induction of CD25 and CD69. In all three genotypes, CD69 was not dramatically affected. Although there was a very modest, albeit statistically significant, decrease in CD69 expression in the Keap1-null cells, this effect is not likely to be biologically relevant. In contrast, CD25 expression varied greatly between genotypes. There was a minor, albeit statistically significant, decrease in the number of CD25-positive cells in the Nrf2-null cells as compared to wild-type; whereas CD25 positive cells were diminished by approximately half in the Keap1-null genotype. These data indicate a potential deficiency in IL-2 signaling, as CD25 is the high affinity chain of the IL-2 receptor and necessary for optimal signal transduction [36,37]. Published studies have shown modulations in CD25 signaling can lead to dysregulation of immune cell polarization, leading to a Th2 phenotype by inducing STAT5 driven IL-4 expression [38,39].

Numerous studies have shown that Nrf2 and Keap1 directly interact with other signal transduction pathways, including NFκB and AP-1 [3]. In terms of IL-2, NFκB ad AP-1 are known to be necessary for optimal IL-2 transcription[40]. We first analyzed the nuclear accumulation of c-jun, a member of the AP-1 family, which has been shown in previous studies to be suppressed by Nrf2. As was expected, Nrf2-null cells had a robust increase in c-jun nuclear accumulation as compared to wild-type cells. Similar to what was observed with IL-2 secretion, Keap1-null Jurkat cells had slightly less nuclear c-jun protein than wild-type cells. The robust increase in nuclear c-jun in Nrf2-null cells may be the mechanism by which IL-2 is increased in the Nrf2-null genotype, as there are multiple known AP-1 binding sites in the IL-2 promoter, with at least one being necessary for transcription [40,41].

Investigation of p65 nuclear accumulation proved to be somewhat more complex. It is known that activation of T cells results in nuclear accumulation of NFκB within the nucleus, which we observed in wild-type cells [22,40]. In Nrf2-null cells, both unactivated and activated groups exhibited nuclear accumulation of p65 at approximately the same level as activated wild-type cells. These data indicate that Nrf2 inhibits NFκB activity, which has been shown by other groups [13,42]. Surprisingly, Keap1-null cells had more nuclear p65 than either genotype after T cell activation. Previous studies have shown that Keap1 can interact directly with IKKβ, which is responsible for NFκB activation. Nonetheless, these data were unexpected because the Keap1-null cells have constitutive activation of Nrf2, which is also known to be inhibitory to NFκB [13,43]. The potential effects of increased NFκB signaling are wide ranging, from inducing transcription of genes such as IL-2, but under some circumstances NFκB also induces apoptosis [44].

The results from the p65 Western blots lead us to investigate potential changes in apoptosis after T cell activation. We observed the catalytic activity of executioner caspases 3 and 7 by flow cytometry. After activation, it is physiologically normal for a fraction of T cells to undergo apoptosis, a process known as activation-induced cell death [45]. This aids in the protection of the host from an over-response to pathogen. In the cell lines used in these studies, a minor increase in functional caspase was observed in both wild-type and Nrf2-null genotypes after T cell activation, as predicted. In contrast, Keap1-null cells had a profound increase in caspase activity, indicating a large apoptotic population, which was also accompanied by a distinct increase in cell death in these cells, which may be responsible for the decrease in IL-2 secretion observed in Keap1-null cells.

Taken together, these studies are both novel and high impact for several reasons. First, this is the first time, to our knowledge, that a human model has shown that changes in expression of Nrf2 and Keap1 result in marked changes in immune cell function. Our present studies show that the overall expression of Nrf2 and Keap1, independent of the activation of Nrf2 by xenobiotics and cell stress, alter early events of human T cell activation. Second, these results have implications beyond immunology. Nrf2 and Keap1 have been shown to be involved in the progression of many types of cancer. This is profound because the Jurkat E6-1 cell line is a T cell leukemia, and previously, we showed ablation of Nrf2 to cause an increase in sensitivity to oxidative stress induced by hydrogen peroxide, which further implicates Nrf2 to be a pseudo-oncogene. These studies go a step further. Although Keap1-null Jurkat cells have constitutively active Nrf2, they also show a marked decrease in viability by apoptosis after stimulation through the T cell receptor and co-receptor. Finally, these studies demonstrate that targeting Keap1 to investigate the effects of constitutive Nrf2 activation comes with its own complications, as Keap1 knock-out appears to have effects on T cell activation that may be separate from Nrf2. To determine Nrf2-specific from Keap1-specific effects, additional studies using Nrf2/Keap1 double-knock-out cells will be needed.

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CHAPTER 5

Summary, significance, and future directions

Summary of findings

Previously, the work done implicating the stress activated transcription factor Nrf2 in the modulation of immune response had primarily been accomplished in mouse models. Although mouse models are extremely useful for in vivo and mechanistic studies, species differences between mice and humans exist. Therefore, our initial studies aimed at determining the role of the synthetic food preservative and Nrf2 activator, tBHQ, in the early events of human CD4 T cell activation, implementing a human Jurkat T cell model [1]. Our hypothesis was that pretreatment of Jurkat T cells with tBHQ, prior to T cell stimulation through the T cell receptor and co-receptor would result in suppression of cytokine production (chapter 2). These studies demonstrated that tBHQ has differential effects on the early events following Jurkat T cell activation. As hypothesized, tBHQ suppressed induction of IL-2, a key early cytokine. Furthermore, pretreatment with tBHQ also inhibited the expression of CD25 mRNA and protein expression, but there was no effect on the level of CD69 mRNA or protein expression. Continued analysis demonstrated a similar differential effect on transcription factors known to drive the IL-2 promoter, with NFκB being suppressed with increasing concentration of tBHQ, but there was no observed suppression of either NFAT or AP-1. These data indicate that overall T cell activation is not inhibited as a whole, but rather, only specific endpoints are affected by tBHQ. These studies indicated that Nrf2 may have a role in the regulation of human T cell activation, but the question remained whether the effects of tBHQ were due to activation of Nrf2 or were occurring through off-target effects of tBHQ.

One difficulty of many human studies within the Nrf2 field is the off-target effects of many of the Nrf2 activators. Because no commercially available Nrf2 specific activators or inhibitors are currently available, studies rely on overexpression of Nrf2, as well as its incomplete knock down using siRNA and shRNA techniques. The resulting data are often difficult to interpret, leaving

the role of Nrf2 in the human immune response largely unanswered. These same issues left the role of Nrf2 in our previous studies ambiguous. To address this, wenext developed a Nrf2-null human model, using CRISPR-Cas9 gene editing. These studies aimed at determining if the previously described immunotoxicological effects of tBHQ were Nrf2-dependent (chapter 3). After development of a Nrf2-null model, we hypothesized that the suppression of IL-2 by tBHQ is dependent upon Nrf2 expression, leading us to expect to observe suppression of IL-2 in only wild-type Jurkat T cells treated with tBHQ. However, in opposition to our hypothesis, a similar suppression of IL-2 secretion was observed in both wild-type and Nrf2-null genotypes, showing the effects of tBHQ on this endpoint to be independent of Nrf2 [2]. Although counter to the hypothesis, these data are not entirely surprising, as tBHQ activates Nrf2 through modifications of key cysteines on Keap1, and similar modifications are likely occurring on other molecules within the cell. Additionally, tBHQ also inhibited p65, a member of the NFκB family, in a Nrf2-independent manner, which may be the means by which tBHQ suppresses IL-2 secretion in Jurkat T cells. We next went on to investigate the role of other Nrf2 activators on Jurkat cell function. Treatment of Jurkat T cells with the synthetic triterpenoid CDDO-Im resulted in a completely Nrf2-dependent suppression of IL-2 induction and a partially Nrf2-dependent suppression of CD25 expression, while again having no effect on CD69. These data again demonstrate that activation of Nrf2 does not completely block T cell activation which would result in a decrease in all parameters, but rather modulates only specific endpoints.

Nrf2-null mice have been shown to be deficient in the resolution of oxidative stress caused by reactive oxygen species. The immune system is uniquely sensitive to reactive oxygen species because cells within the immune system utilize reactive oxygen species as a both a means of host-defense and as a second messenger [3,4]. In light of this, we treated wild-type and Nrf2-null Jurkat T cells with hydrogen peroxide for 16 hours, prior to measurement of cell viability and

mitochondrial membrane potential. These data show that Nrf2-null cells have a lower basal mitochondrial membrane potential, as well as being more sensitive to H_2O_2 than wild-type cells. These studies are interesting because T cells are exposed to oxidative stress through normal immune responses. In addition, these studies also have implications in cancer biology, as Nrf2 has been shown to promote chemo- and radio-resistance of tumors, a role which these studies support, as Jurkat T cells are a T cell leukemia cell line.

Although the studies of tBHQ and CDDO-Im in Nrf2-null Jurkat cells were critical for defining the role of Nrf2 in the effects of these two activators, these studies did not approach the question of basal genotype differences between wild-type and Nrf2-null cells. Since activation of Nrf2 causes a suppression of IL-2 secretion, we hypothesized that Nrf2-null cells would basally produce more IL-2 upon T cell stimulation. Conversely, we hypothesized that ablation of the Nrf2-repressor Keap1 would result in subsequent suppression of IL-2 and constitutive Nrf2 activity (chapter 4). The initial studies using all three genotypes demonstrated that, indeed, knocking-out Keap1 results in constitutive Nrf2 nuclear accumulation and NQO-1 mRNA induction, strongly suggesting that Nrf2 is constitutively active. Furthermore, increased IL-2 induction was observed in Nrf2-null cells and decreased IL-2 induction in Keap1-null cells as compared to wild-type, which was consistent with our hypothesis. In addition, Keap1 null cells expressed about half as much CD25 after T cell stimulation compared to wild-type, which was consistent with our previous observations with the Nrf2 activator CDDO-Im.

Investigations into the mechanism by which these effects are mediated resulted in interesting and somewhat unexpected findings. Nrf2-null Jurkat T cells had increased c-jun nuclear accumulation in the background group, and a substantial increase upon T cell activation.

Conversely, Keap1-null cells had a slight decrease in c-jun nuclear accumulation. Taken together, it is plausible that the increase in IL-2 secretion within the Nrf2-null genotype is due to the robust increase in c-jun activity, as AP-1 has been shown to be necessary for IL-2 transcription and has several binding sites in the IL-2 promoter. The role of the Nrf2/Keap1 system on regulation of p65 nuclear accumulation was more complex. Within the Nrf2-null genotype, untreated cells had increased p65 nuclear accumulation as compared to wild-type. After T cell stimulation, there was no notable further increase in p65 nuclear accumulation within this genotype. Interestingly, Keap1-null cells had a slight increase in basal p65 nuclear accumulation, but a more robust induction of p65 than either wild-type or Nrf2-null stimulated cells. These findings were unexpected, given Nrf2 has been shown to suppress p65 activity, and both, AP-1 and NFκB are integral in driving the IL-2 promoter. Additionally, NFκB signaling has been shown to drive apoptosis in some models [5]. This is significant as another key event in T cell activation is activation-induced cell death [6]. In this process, some activated T cells undergo apoptosis, which is important for maintaining peripheral tolerance and immune contraction after resolution of infection. Our studies show that T cell activation results in a marked increase in caspase 3/7 activity in Keap1-null cells, which is not observed in either wild-type or Nrf2-null genotypes. Further studies must be done to determine if the changes in Keap1-null cytokine secretion are due to cell death, or some other mechanism, such as IL-2 uptake, autophagy, or mRNA stability. These studies are of importance because they are the first to show that manipulation of Nrf2 and Keap1 expression changes human T cell function. Beyond that, manipulation of Keap1 is used as a means to overexpress Nrf2 [7–9]. Our current data suggest that this approach may have effects aside from an increase in Nrf2 signaling, which is not surprising as Keap1 has been shown to interact with several different signal transduction pathways.

Significance of the findings

The role of Nrf2 in the resolution of cell stress, including oxidative stress and electrophilic compounds, has been widely studied over the last several decades [10–12]. Recently, the role of Nrf2 in the murine innate and adaptive immune responses has been an accelerating area of research, as Nrf2-null mice have aberrant immune reactions, including autoimmunity [13–17]. Also, activation of Nrf2 in mouse models has proven to skew T cell polarization and may play a role in the development of allergy and asthma [17]. With that in mind, little has been elucidated in regard to the role of Nrf2 in the human immune response, partially due to inadequate models to determine these effects. The work presented in this dissertation shows a key role for Nrf2 in the modulation of the early effects of T cell activation [2]. This was done by implementing several different Nrf2 activators, as well as knocking out Nrf2 and Keap1 expression in the human Jurkat T cell line. These are the first studies to our knowledge in which these genetic models have been used to study Nrf2 and Keap1 expression on human T cells, showing modulation of immune endpoints from cytokine production to activation-induced cell death. Moreover, the cell lines that have been generated from these studies can be used to determine the Nrf2 specific effects of other immunotoxicants that activate Nrf2, such as arsenic and cadmium. Prior to these studies, it was difficult to determine if the effects these compounds have on the immune response are due to Nrf2 activation, as these studies frequently relied on incomplete knock-down of Nrf2. Aside from different toxicants, Nrf2-null cells provide a blank slate of sorts. A system that lacks Nrf2 will allow us to re-introduce different Nrf2-mutants into the cells, providing means by which to investigate different polymorphisms within the human population and to study the effects of those within the model. Moreover, the CRISPR-Cas9 plasmids that were utilized to generate the gene knock-outs can, in turn, be used to produce Nrf2 and Keap1 knock-outs in human hematopoietic stem cells, a model in which differentiation and polarization of T cells can be studied. Taken together, the data generated in this dissertation moves the

field forward on multiple fronts and is the first to show definitively that Nrf2 modulates human immune responses.

Future directions

Our most recent studies examining the differences between wild-type, Nrf2-null, and Keap1-null Jurkat T cells demonstrate that changes in gene expression for both Nrf2 and Keap1 result in marked changes in cytokine production, expression of cell surface markers of activation, as well as cell viability after activation. Additional studies need to be done to determine other potential pathways and mechanisms by which disruption of Nrf2 and Keap1 modulates early events of T cell activation. Recently, recent studies indicate an increasingly important role for Nrf2 and Keap1 in the maintenance of mitochondrial homeostasis, where increases in Nrf2 signaling result in increases in mitochondrial membrane potential and ATP production, and depletion of Nrf2 had the inverse effect [18,19]. Additionally, it has been shown that Nrf2 can be tethered to the outer membrane of the mitochondria by Keap1, where it serves to regulate mitophagy, a process in which damaged mitochondria are removed from the cell [20–22].

At the site of interaction between Nrf2 and Keap1 is p62, an autophagic adapter protein and Nrf2 target gene, which in turn has been shown to induce Nrf2 in a positive regulatory loop [20,21]. Activation of Nrf2 can induce the transcription of p62 and target the mitochondrion for turnover. In our system, the interplay between Nrf2, Keap1, and the mitochondria may be playing multiple roles. Increased Nrf2 signaling in Keap1-null cells may be resulting in a concurrent increase in p62. Induction of p62 may increase the rate of Keap1 turnover and thereby Nrf2 signaling, which would ultimately be beneficial in mitigating stress. Overexpression of Nrf2 may, however, be detrimental to the cell, which has been shown in other systems, as increased Nrf2 signaling

has been shown to promote chemo resistance of tumors, as well as dysregulating immune endpoints, such as hematapoesis. Moreover, maintenance of mitochondrial membrane potential and apoptosis can be conjoined phenomena, where a decrease in mitochondrial membrane potential can lead to cytochrome c leakage and subsequent caspase activity, as well as decreases in ATP production [23]. Stimulation of T cells cause a rapid increase in the metabolic state of the cell, as immune responses, such as cytokine production and clonal expansion are induced [24]. It is reasonable to hypothesize, therefore, changes in the metabolic state of T cells may cause both increases and decreases in endpoints of activation, including cytokine production and activation-induced cell death.

Analysis of mitochondrial function, in addition to the completed studies in immune endpoints and cell death, in all three Jurkat genotypes takes strides in determining the immune modulatory effects of the Nrf2/Keap1 system within a human model. These studies do not definitively determine if the phenotype observed in the Keap1-null cells is due to overexpression of Nrf2 or ablation of Keap1, as both proteins have been shown to interact with and modulate several different pathways, including AP-1, NFκB, as well as other P45 family members [10]. Furthermore, numerous studies demonstrate that modulation of both Nrf2 and Keap1 individually can modulate the immune response in murine models [8,25]. Accordingly, it is plausible that, although knocking out Keap1 results in constitutive Nrf2 activity, it also has effects on T cell activation that are Nrf2-independent. One approach to determine Nrf2-specific vs. Nrf2-independent effects on the early events of human T cell activation, is the development of Nrf2/Keap1 double knock out clones, by CRISPR-Cas9. Analysis of Nrf2 and Keap1 double-knock outs will provide the tools necessary to delineate the role of each individual protein on T cell activation. Given the heterogeneity of the Jurkat cell line, as well as the possible off-target effects of techniques, such as CRISPR-Cas9, double knock-outs must be analyzed from both

Nrf2-null and Keap1-null parent populations. Analysis of single knock-out clones, as well as double knock-out clones will additional insight on the role of Nrf2 upon the early events of T cell activation. Currently, the data suggest that modulation of Keap1 expression to generate constitutively-active Nrf2 clones has its shortcomings, which suggests the need for alternative approaches.

One significant contribution of this dissertation is the development of a Nrf2-null human T cell model, which was previously unavailable. This model provides a system in which one can re-express varying polymorphisms of Nrf2 and study their function on T cell activation. This is important because there are a number of Nrf2 polymorphisms that have been identified within the human population; many of these have been shown to be either associated with, or causative, in human disease [26]. These diseases include systemic lupus erythematosus, several cancers, and acute lung injury [26]. Because of the limitations of the field, however, the mechanisms by which these diseases arise have gone understudied in human models. As many of the diseases which are associated with Nrf2 polymorphisms are mediated by aberrant immune responses, including asthma and lupus, re-expression of these polymorphisms into the pre-existing Nrf2-null T cell line will begin to allow the study of the mechanism(s) by which altered Nrf2 function may modulate T cell activation and, in turn, result in dysregulation of immune function and the development of disease.

Understanding the role of known Nrf2 polymorphisms within the human population is paramount, but much can still be gleaned from a model in which Nrf2 is not able to be adequately repressed and has constitutive activity. Modulation of Keap1 has been previously shown to affect immune endpoints in mouse models, including hematopoiesis. Discussed in

chapter one, canonically, negative regulation of Nrf2 occurs by interaction with Keap1 and subsequent degradation by the proteasome [12]. The interaction of Nrf2 with Keap1 is mediated through the N-terminal Neh2 domain, specifically the ETGE and DLG motifs, with the first having greater affinity for Keap1 [10]. Studies have shown that mutations within the ETGE and DLG motifs results in an increase in Nrf2 signaling [27]. One approach to generating a constitutively-active Nrf2 model, with minimal off targets, is the lentiviral transduction of Nrf2 mutants, which lack the DLG, ETGE, or both DLG and ETGE motifs within the Nrf2-null Jurkat cell line. Expression of these Nrf2 mutant constructs within Nrf2-null cells will provide a model in which the only functional Nrf2 present can no longer be repressed by Keap1. Although other means of Nrf2 degradation exist, such as β-TrCP, our previous data, as well as studies by other groups, suggest that the major pathway of Nrf2 negative regulation is through Keap1, suggesting that disruption of the interaction between Nrf2 and Keap1 may be sufficient for constitutive Nrf2 activation [28,29]. Ultimately, this could produce a model which no longer requires non-specific stimuli to activate Nrf2, such as tBHQ and CDDO-Im, which are known to interact with other pathways, including NFκB.

Generation of constitutive Nrf2-mutants, by removal of either the DLG or ETGE is applicable in models beyond the Jurkat T cell. Currently, as the field lacks pharmacologic Nrf2-specific activators or suppressors, Nrf2 activation is studied by use of compounds, such as arsenic, which activate pathways other than Nrf2. There is a need for models in which the function of Nrf2 can be studied, without the off-target effects of many of the compounds currently in use. One approach to meeting this need would be the generation of transgenic mice, which confer the same mutations described above, lacking the DLG, ETGE, or both motifs of Nrf2. This approach will allow for the in-vivo analysis of immune endpoints, using wild-type, Nrf2-null, and constitutively-active Nrf2 models. With respect to previous studies in our lab, this model could

be used to further characterize the mechanism by which Nrf2 skews CD4 T cells toward a Th2 phenotype. Beyond this, Th2 polarization has effects on other immune endpoints, such as the development of Th1 cells, which is suppressed by Th2 polarization. In terms of immune response, Th1 cells are integral in the response to intracellular pathogens, such as viruses. It is reasonable to hypothesize that, if Nrf2 skews CD4 T cells toward a Th2 phenotype, DLG/ETGE mutant mice would be more sensitive to viral infection. Conversely, Nrf2-null mice would have increased viral clearance, as we have previously shown that Nrf2-null mice have an increase in Th1 cytokine secretion, and Th1 master regulators, such as T-BET [17].

Although these studies push the field forward in the determination of the role of Nrf2 in human T cell responses, Jurkat T cells are a leukemic cell line, and as such, are inherently different than primary cells and are not used to study T cell polarization or differentiation. Additionally, mice provide a model to determine molecular mechanism and T cell differentiation in vivo and ex vivo, but species differences can arise. Therefore, researchers must be diligent to ensure the endpoints measured are indicative of normal human physiology. The development of human CRISPR constructs to remove Nrf2 expression in Jurkat cells can also be used in other human models, including commercially available hematopoietic stem cells (HSC). As we have already shown these constructs to be efficient in targeting Nrf2, it is feasible that the same plasmids may be used in the HSCs [1]. Comparison of wild-type and Nrf2-null HSCs will allow for a beginning-to-end approach to T cell differentiation and polarization. Additionally, we have shown that mouse CD4 T cells treated with the food additive tBHQ are skewed toward the Th2 phenotype, but these studies need to be repeated in a human model. Generation of Nrf2-null human HSCs would provide the opportunity to determine the Nrf2-specificity of not just tBHQ, but also other Nrf2 activators, such as cadmium, on primary human immune cell function.

Taken together, the work that has been already accomplished in this dissertation pushes the field forward in the understanding of the role of the stress-activated transcription factor Nrf2 in modulating human T cell function. Furthermore, it provides the foundation and framework necessary to generate additional models, in which we can determine the mechanism by which human polymorphisms of Nrf2 promote the pathogenesis of various diseases. Additionally, the insight gained from the previous studies may allow for the development of constitutively-active Nrf2 models, which do not alter the expression of Keap1, decreasing the likelihood of unwanted off-target effects. Such a model could be potentially applicable to human cell lines, primary human cells and various mouse models. Finally, the CRISPR-Cas9 vectors which have been generated can be used to generate transgenic human HSCs, which will allow for the analysis of the development of the T cell niche, as well as polarization of T cells toward a terminally differentiated state, such as the Th2 subset. Furthermore, immunomodulatory molecules such as tBHQ, can be used in this model to determine the role of Nrf2 in their effects on the development and polarization of T cells. These data, as well as the other proposed future directions, would make major advances in the understanding of the role Nrf2 plays in human T cell biology.

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