THE ROLE OF NRF2 IN THE ACTIVATION OF PRIMARY CD4 T CELLS FROM MICE AND HUMANS

By

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ABSTRACT

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Nuclear factor erythroid 2-related factor 2 (Nrf2) is a cytoprotective transcription factor activated by cellular stressors such as reactive oxygen species, electrophilic xenobiotics, and other forms of oxidative stress that regulates the transcription of a number of antioxidant, detoxification, and cytoprotective genes. Commonly used Nrf2 activators include 1[2-Cyano-3,12-di oxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) and the food additive *tert*-butylhydroquinone (tBHQ). Nrf2 was initially characterized as a cytoprotective transcription factor, and has since been shown to have additional effects, including a role in the immune system. Nrf2-null mice are more sensitive to a number of inflammatory stimuli, and cell-type specific effects of Nrf2 have been observed, mainly in macrophages and dendritic cells. However, the role of Nrf2 in T cell function has not been well studied. Prior studies from our lab have shown that activation of Nrf2 skews murine CD4 T cell differentiation towards a Th2 (allergy-like) phenotype. However, the role of Nrf2 in T cell activation, which sets up T cells for differentiation, and in human T cells is unknown. The purpose of the present studies is to determine the role of Nrf2 in primary CD4 T cell activation to fill this gap. To do this, the Nrf2 activators tBHQ and CDDO-Im were used to determine effects on T cell activation in murine or human primary CD4 T cells. In mice, tBHQ inhibited production of the cytokines interleukin-2 (IL-2), interferon gamma (IFNγ), and granulocyte-macrophage

colony stimulating factor (GM-CSF) in both wild-type and Nrf2-null splenocytes, and inhibited tumor necrosis factor alpha (TNFα) only in wild-type splenocytes. CDDO-Im inhibited IFNγ and TNFα production, and increased IL-2 production, in a largely Nrf2-dependent manner, and increased GM-CSF independently of Nrf2. Both tBHQ and CDDO-Im had little effect on expression of the cell surface proteins CD25 and CD69. Both tBHQ and CDDO-Im increased nuclear translocation of the transcription factor c-Jun in wild-type but not Nrf2-null splenocytes, and inhibited p65 NF-κB DNA binding in wild-type splenocytes. Overall, the data suggest that Nrf2 activation has differential effects on murine T cell activation. Nrf2 activation inhibits IFNγ and TNFα secretion and p65 NF-κB DNA binding, and increases production of IL-2 and translocation of c-Jun, with little effect on CD25 and CD69 expression. In primary human CD4 T cells, tBHQ inhibits events of T cell activation, including production of IL-2 and IFNγ, expression of CD25 and CD69, and p65 NF-κB DNA binding. CDDO-Im also inhibits IL-2 and IFNγ production, and CD25 and CD69 expression in primary human CD4 T cells. However, the role of Nrf2 in mediating these effects was undetermined, so a primary human CD4 T cell Nrf2 knockdown model was developed. Nucleofection with siRNA directed against Nrf2 knocked down Nrf2 protein expression by ~90% 12 h after transfection. Suppression of IL-2, IFNγ, TNFα, and GM-CSF production by tBHQ occurred in both the scrambled control and Nrf2-deficient CD4 T cells, as did inhibition of CD25 and CD69 expression. RNA-seq analysis was performed and identified a number of differentially expressed genes between the scrambled control and Nrf2-deficient CD4 T cells, as well as genes differentially expressed with tBHQ treatment, indicating potential areas in which Nrf2 may play a role in primary human CD4 T cell activation.

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KEY TO ABBREVIATIONS

15d-PGJ2	15-deoxy-delta-12,14-prostaglandin J2
ADI	Acceptable daily intake
AhR	Aryl hydrocarbon receptor
AKT	Protein kinase B
ANOVA	Analysis of variance
AP-1	Activator protein 1
APC	Antigen presenting cell
ARE	Antioxidant response elements
ATF4	Activating transcription factor 4
ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BLIMP1	B-lymphocyte-induced maturation protein 1
BMDC	Bone marrow-derived dendritic cell
BTB	Broad complex, tram track, brick-a-brac
bZIP	Basic leucine zipper
CaMK	Ca^{2+}-calmodulin-dependent kinase
Cas9	CRISPR associated protein 9
CD122	Interleukin 2 receptor beta, IL-2Rβ
CD132	Common cytokine receptor γ chain

CD25	Cluster of differentiation 25, IL-2Rα
CD28	Cluster of differentiation 28, co-stimulatory receptor
CD3	Cluster of differentiation 3, component of the T cell receptor
CD4	Cluster of differentiation 4, marker of helper T cells
CD69	Cluster of differentiation 69
CD8	Cluster of differentiation 8, marker of cytotoxic T cells
CD80	Cluster of differentiation 80
CD86	Cluster of differentiation 86
CDDO-Im	1[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole
cDNA	Complementary DNA
c-Fos	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit
c-Jun	Jun Proto-Oncogene, AP-1 Transcription Factor Subunit
CK2	Casein kinase 2
CLP	Cecal-ligation and puncture
CNC	Cap-n-collar
ConA	Concanavalin A
CREB	cAMP response element binding protein
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Cul3	Cullin-3
DAG	Diacylglycerol
DDCt	Delta-delta cycle threshold (ΔΔCT)
DEC1	Deleted in esophageal cancer 1 (Bhlhe40)
DEG	Differentially expressed gene

DJ-1	Parkinson disease protein 7
DMEM	Dulbecco Modified Eagle Medium
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EGR	Early Growth Response
ELISA	Enzyme-linked immunosorbent assay
EpRE	electrophile response element
ERK	Extracellular signal-regulated kinase
Ets1	ETS Proto-Oncogene 1, Transcription Factor
FACS	Fluorescence-activated cell sorting
Fas	Fas cell surface death receptor
FBS	Fetal bovine serum
FDA	U.S. Food and Drug Administration
FoxP3	Forkhead box P3
Fra-1	Fos-related antigen 1
Fyn	Proto-oncogene, Src Family Tyrosine Kinase Fyn
GADS	GRB2-related adaptor protein 2
GATA-3	GATA-Binding Factor 3
GCLC	Glutamine-cysteine ligase catalytic subunit
GCLM	Glutamine-cysteine ligase regulatory subunit
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRAS	Generally recognized as safe

GRB2	Growth factor receptor bound protein 2
GSH	Glutathione
GSK-3β	Glycogen synthase kinase 3β
GSTYa	Glutathione-s-transferase Ya subunit
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMOX	Heme oxygenase-1
HSC	Hematopoietic stem cell
IFNγ	Interferon gamma
IKKγ	IκB kinase gamma
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-12p70	Interleukin 12 p70
IL-13	Interleukin 13
IL-17A	Interleukin 17A
IL-17F	Interleukin 17F
IL-18	Interleukin 18
IL-1β	Interleukin 1 beta
IL-23	Interleukin 23
IL-2Rα	Interleukin 2 receptor alpha, CD25
IL-2Rβ	Interleukin 2 receptor beta, CD122
IL-32	Interleukin 32
IL-4	Interleukin 4
IL-5	Interleukin 5

IL-6	Interleukin 6
IL-7	Interleukin 7
iNOS	Inducible nitric oxide synthase
IO	Ionomycin
IP3	Inositol trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
Itk	Interleukin-2-inducible T cell kinase
IκB	Inhibitor of kappa B
JAK	Janus kinase
JNK	JUN N-terminal kinase
KC	Keratinocyte chemoattractant
Keap1	Kelch-like ECH-associated protein 1
LAT	Linker for activation of T cells
Lck	Lymphocyte Cell Specific Protein Tyrosine Kinase
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCP1	Monocyte chemoattractant protein 1 (CCL2)
MEFs	Mouse embryonic fibroblasts
MEK1	Mitogen-activated protein kinase kinase 1
MHC	Major histocompatibility complex
MIP-2	Macrophage inflammatory protein 2 (CXCL2)
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin

NAC	N-acetyl cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NCK1	Non-catalytic region of tyrosine kinase adaptor protein 1
Neh	Nrf2-ECH homology
NFAT	Nuclear factor of activated T cells
NFDM	Nonfat dry milk
NF-E2	Nuclear factor erythroid 2
NFE2L2	Nuclear factor erythroid-derived 2 like-2, Nrf2 official gene name
NF-κB	Nuclear Factor Kappa B
NK Cell	Natural Killer Cell
NKT Cell	Natural Killer T Cell
NQO1	NAD(P)H:Quinone oxidoreductase
Nrf1	Nuclear factor erythroid 2-related factor 1
Nrf2	Nuclear factor erythroid 2-related factor 2, also called Nuclear factor erythroid-derived 2 like-2
p300/CBP	CREB binding protein
p38	P38 Mitogen activated protein kinase
p62/SQSTM1	Sequestome 1
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with tween 20
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase 1
PERK	Protein kinase R (PKR)-like endoplasmic reticulum kinase

PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PKCθ	Protein kinase C theta
PLCγ	Phospholipase C gamma
PMA	Phorbol myristate acetate
PVDF	Polyvinylidene difluoride
qPCR	Quantitative PCR
RARα	Retinoic acid receptor alpha
RNA	Ribonucleic acid
RORγt	Retinoid orphan receptor gamma t
ROS	Reactive oxygen species
RPL13A	Ribosomal protein L13a
RPMI	Roswell Park Memorial Institute (culture medium)
RUNX3	Runt-related transcription factor 3
RXRα	Retinoic X receptor alpha
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SFN	Sulforaphane
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa, Lymphocyte cytosolic protein 2
SOS	Son of sevenless
SPLC	Splenocyte
Src	Proto-Oncogene Tyrosine Protein Kinase Src

STAT4	Signal transducer and activator of transcription 4
STAT5	Signal transducer and activator of transcription 5
STAT6	Signal transducer and activator of transcription 6
T-bet	T-Box expressed in T cells
tBHQ	tert-butylhydroquinone
TCR	T cell receptor
TGF-β	Transforming growth factor beta
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
TNFα	Tumor necrosis factor alpha
Treg	Regulatory T cell
VAV1	Vav guanine nucleotide exchange factor 1
XRE	Xenobiotic response elements
β-TrCP	Beta-transducin repeat containing E3 ubiquitin protein ligase

Chapter 1

Introduction

The immune system and T cells

The immune system is the organ system charged with protecting the body against foreign invaders. It must distinguish between self and non-self or altered-self material in the body, determine if the detected material is dangerous, and then, if necessary, mount an appropriate response to rid the body of the threat. This process is complex and must be tightly regulated to ensure that the detected invader is eliminated with minimal damage to the surrounding tissue. Because immune responses can be highly cytotoxic, responses that are too strong or are directed against inappropriate stimuli, such as self-tissue, can lead to permanent damage and illness. Furthermore, a great variety of organisms can infect the body, from viruses and intracellular bacteria to parasitic worms, each demanding a different response. The appropriate response for each type of threat must be determined and mounted, as an inappropriate immune response will not resolve the infection.

T cells are a crucial part of the immune response. They integrate signals from a variety of innate first responder immune cells to identify breaches in barrier defense as well as to determine the nature of the threat. The T cell response is tailored to the pathogen or tumor and results in the production of a variety of proteins to coordinate and direct a number of other immune cell types[1]. T cells are a part of the adaptive immune response, meaning that upon re-exposure with the same pathogen, T cells that participated in the initial response can respond much more quickly and effectively upon secondary exposure to eliminate the threat, a phenomenon known as immunological memory[2,3]. Cytotoxic (CD8+) T cells specialize in killing virally-infected cells and tumor

cells, whereas helper (CD4+) T cells specialize in coordinating immune responses and providing help to other immune cell subsets[4].

Overview of T cell responses

T cells develop from hematopoietic stem cells in the bone marrow, and mature in the thymus to become mature naïve T cells. Naïve T cells are fully functional T cells that have never encountered antigen (a molecule that stimulates T and B cell responses) and thus have never undergone T cell activation[5]. Naïve T cells circulate through the body in a quiescent state and become activated upon encountering antigen that is specific to the T cell receptor uniquely expressed by the T cell. T cell activation refers to the initial events that occur after the T cell receptor binds its specific antigen, which initiates a signaling cascade[6,7]. T cell activation results in profound cytoskeletal and metabolic changes that prepare the T cell to proliferate and coordinate a specific immune response. This includes early production of cytokines and chemokines, induction of numerous cell surface molecules, and upregulation of factors that promote cell survival and proliferation[8–11]. After the initial events following T cell activation, the T cell undergoes clonal expansion, and then differentiation into one of a number of functionally distinct helper T cell subsets.

Differentiation allows the T cell to tailor an immune response to a specific infection or disease state and is critical for the development of an effective adaptive immune response (Fig. 1)[12]. The Th1 subset of CD4 T cells secrete IFNγ and support responses to intracellular pathogens, including viruses and intracellular bacteria. Th2 cells secrete IL-4, IL-5, and IL-13, support mast cell and eosinophil responses, plasma cell differentiation, and class switching to IgE antibody production, all of which are

targeted against large extracellular pathogens such as helminths[13]. Th17 cells secrete IL-17A and IL-17F, and support immunity directed against extracellular pathogens, including antibacterial and antifungal responses[14]. Regulatory T cells (Tregs) produce IL-10 and TGF-β, and function to suppress the immune response[14]. Lineage decisions are determined by the signals the T cell receives during activation and differentiation. Th2 differentiation is supported by the cytokine IL-4 and the transcription factors GATA-3 and STAT6[15,16]. Th1 differentiation is supported by the cytokine IL-12 and the transcription factor T-bet[17]. Th17 differentiation is supported by the cytokines TGFβ, IL-23, and IL-6 and the transcription factor RORγt[18]. Treg differentiation is supported by TGFβ and IL-2, and the transcription factor FoxP3[19,20]. Each subtype also has supporting transcription factors in addition to the ones detailed above, and other signals that integrate with the cytokines signals to support differentiation.

Different Th cell subsets are also associated with different disease states. Th2 differentiation has a causative role in the development of allergies and asthma, with excessive Th2 responses to common antigens initiating the pathogenesis associated with these diseases[21]. In contrast, Th1 and Th17 responses are associated with autoimmune diseases[22]. In healthy individuals, the immune system contracts after successful resolution of the infection, with many of the differentiated effector CD4 T cells undergoing apoptosis[4]. Some of the CD4 T cells stimulated in the immune response become long-lived memory T cells. At the end of an immune response, memory T cells return to a quiescent state to function as a reservoir of antigen-experienced cells that can respond more quickly upon re-exposure to the same antigen[23]. This process of T cell activation, differentiation, and contraction is crucial to

generate a successful immune response, and to generate memory T cells to incur long lasting defense against the same stimuli. When functioning properly, the system provides defense against infection and cancer, but when dysregulated, results in immune-mediated diseases such as allergy, asthma, and autoimmunity. As such, each part of the response is tightly regulated and interdependent. The initial events of T cell activation provide an initial response against infection while the immune response is ramping up, and set the stage for T cell proliferation and differentiation.

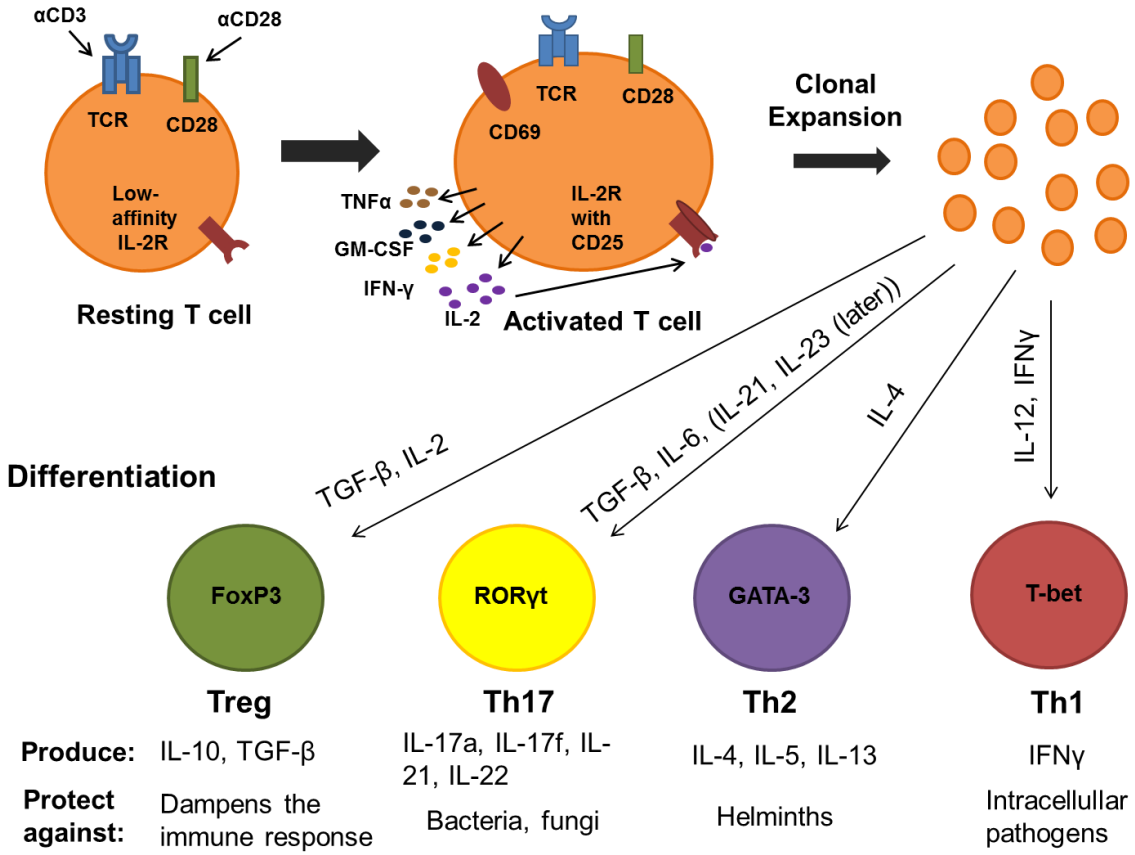

Figure 1. Helper (CD4) T cell activation and differentiation.
Upon ligation of the T cell receptor and co-stimulatory receptors such as CD28, the T cell turns on a program of gene transcription that results in the early production of a number of cytokines and cell surface proteins including IL-2, TNFα, IFNγ, GM-CSF, CD25, and CD69. Subsequently, the CD4 T cell undergoes clonal expansion and differentiation into one of a number of different effector T cell subsets.

T cell activation

T cell activation is initiated when a T cell receptor encounters antigen that is specific for that particular receptor. T cells only recognize antigen in the context of MHC molecules. CD4 T cells recognize antigen bound by the MHC II receptor, which is expressed by specialized cells known as antigen presenting cells (APCs)[24]. APCs include cells, such as dendritic cells, that have the capability to phagocytose particles and pathogens in their surrounding environment, digest the phagocytosed materials, and display peptides derived from the digested materials on MHC II molecules on the cell surface. The presence of co-stimulatory signals from the APC cues the T cells that the displayed molecules came from something that has features of a pathogen. T cell specificity lies in the T cell receptor (TCR), which is a complex of proteins that resides on the cell surface of the T cell[25]. The TCR consists of the TCR dimer, two CD3 dimers, and a zeta chain (ζζ) dimer[6,26,27]. The TCR dimer contains both constant and variable regions, which are generated by gene rearrangement during T cell development and provide each T cell with a different TCR capable of recognizing a specific antigen[28]. The TCR dimer provides recognition of antigen, but does not have intrinsic signaling ability. The CD3 and ζζ dimers confer the signaling ability of the TCR complex through intracellular tails containing immunoreceptor tyrosine-based activation motifs (ITAMs), which are phosphorylated upon binding of antigen and recruit downstream adaptor molecules to facilitate signaling[27,29,30]. This can be mimicked *in vitro* using antibodies directed against CD3.

Signaling through the TCR complex alone is not enough to induce T cell activation. T cells also require a second signal through co-stimulatory receptors to

become activated[7,31]. Antigen presenting cells phagocytose potential antigens, and display the antigens bound to the MHC II molecules on the cell surface[32]. If the APC also detects signs of damage or infection, co-stimulatory molecules are upregulated and displayed along with the antigen[31]. This indicates to the T cell that the current antigen presented by the APC is a threat and an immune response should be mounted. CD28 is one such co-stimulatory receptor expressed on T cells, which binds CD80 and CD86 expressed on APCs[31,33]. This can be mimicked *in vitro* using antibodies directed against CD28.

Ligation of the TCR with MHC II-bound antigen leads to phosphorylation of ITAMs on CD3 and the zeta chain by Src-family kinases Lck and Fyn (Fig. 2)[34,35]. This allows for the recruitment of Zap70, another tyrosine kinase, to the TCR[36,37]. These events initiate a phosphorylation cascade that results in the formation of a signaling complex, the backbone of which is formed by the adaptor proteins LAT and SLP-76[38,39]. Recruited to this complex are additional signaling molecules, including kinases such as interleukin-2-inducible T cell kinase (Itk), adaptor proteins such as GRB2 and NCK1, the RHO-family GTPase exchange factor VAV1, phospholipase C gamma 1 (PLCγ1), and others[6,40–43]. This complex coordinates the induction of several signaling pathways that together initiate T cell activation. PLCγ activation results in the production of inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 causes the release of intracellular calcium, which leads to activation of downstream factors including the phosphatase calcineurin and Ca2+-calmodulin-dependent kinase (CaMK), and ultimately leads to nuclear factor of activated T cells (NFAT) activation[44]. DAG induces Ras, which activates the MAPK cascades and ultimately leads to activation of activator protein

1(AP-1), a dimeric transcription factor composed of Fos and Jun family members[6,45]. DAG also induces protein kinase C theta (PKCθ), which facilitates the phosphorylation of the Iκb kinase (IKK) complex that induces NF-κB activation through degradation of IκB[46].

Each of the transcription factors that drive T cell activation are regulated in multiple ways following T cell receptor stimulation, and defects in any component leads to suboptimal activation. For example, the Ras and MAPK cascades, as well as PKCθ, are also induced by guanine nucleotide exchange factors such as VAV1 and SOS that are recruited to the signaling complex[47,48]. TCR stimulation also results in activation of phosphoinositide 3-kinase (PI3K), which induces phosphoinositide-dependent kinase 1 (PDK1) and PKCθ, as well as Akt, and enhances NF-κB and NFAT activation[49]. Akt also induces mammalian target of rapamycin (mTOR), which, along with other factors, helps regulate the shift in metabolism towards increased glycolysis and anabolic programs that occurs upon T cell activation[11,50]. These three transcription factors – NFAT, AP-1, and NF-κB – are the main transcription factors that mediate changes in gene transcription upon T cell activation. The TCR signaling complex also induces remodeling of the actin cytoskeleton, and initiates shifts in the metabolic program of the cells necessary for T cell activation[6]. Co-stimulatory receptor signaling supports and enhances this signaling program. Ligation of CD28 induces binding of PI3K, which in turn activates PDK1, as well as binding of and activation of Lck, GADS, and GRB2[51].

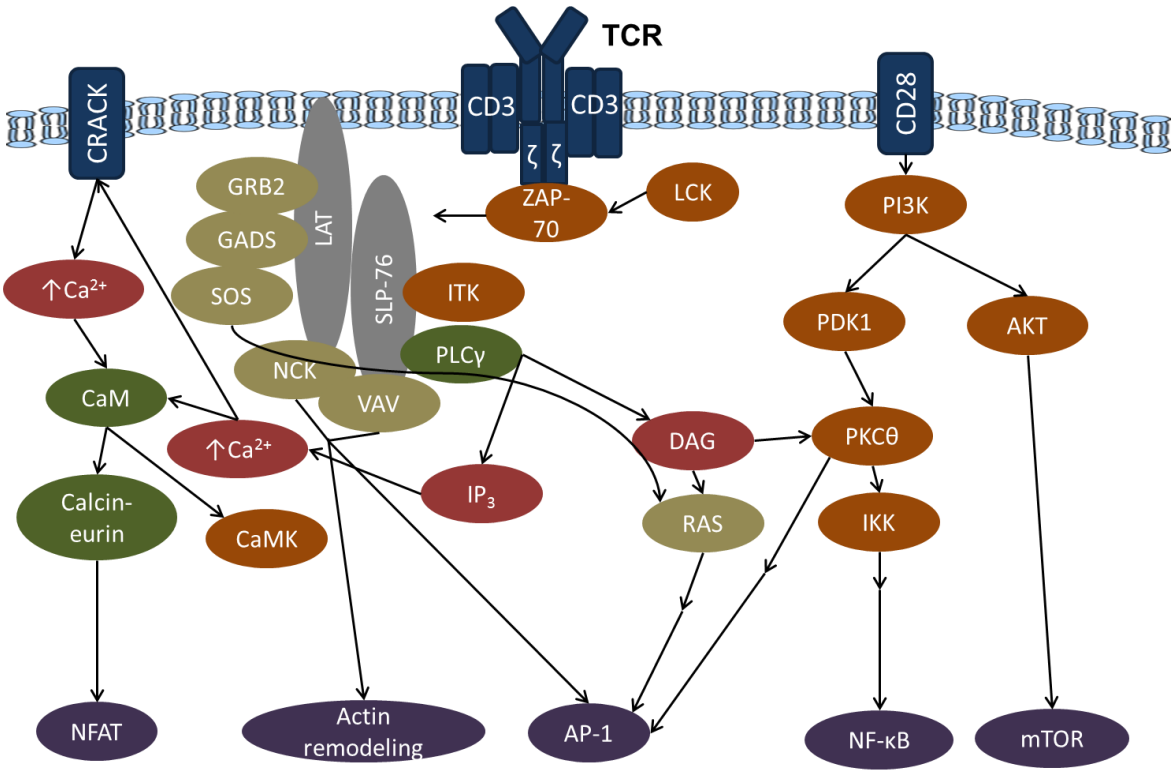

Figure 2. Signaling downstream of the T cell receptor and co-stimulatory receptors.

Early cytokines produced upon T cell activation

IL-2 and CD25

Several cytokines are produced rapidly after CD4 T cell activation, including interleukin-2 (IL-2), interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and granulocyte-macrophage colony-stimulating factor (GM-CSF). IL-2 is a cytokine that supports T cell proliferation and survival, and is necessary for optimal T cell responses. IL-2 is produced primarily by activated CD4 T cells, and to a lesser degree by activated CD8 T cells, natural killer (NK) cells, and natural killer T (NKT) cells. IL-2 production is rapidly induced upon TCR and CD28 co-stimulation, and remains increased for a few days before decreasing[52]. This induction is controlled by the transcription factors NFAT,

NF-κB, AP-1,which act on the IL-2 promoter[53,54]. IL-2 production is controlled through several mechanisms, including an autoregulatory loop where IL-2 increases expression of BLIMP1, which in turn decreases IL-2 production[55]. IL-2 production is also controlled through uptake and degradation of IL-2 through the IL-2 receptor[56,57].

The IL-2 receptor exists in two forms, the low affinity dimeric form, composed of CD122 (also known as IL-2Rβ) and CD132 (the common cytokine receptor γ chain), and the high affinity trimeric form, composed of CD122, CD132, and CD25 (also known as IL-2Rα)[57]. Naïve T cells have low expression of the IL-2 receptor and T cell activation increases expression of all three subunits, while memory T cells and NK cells express the dimeric IL-2 receptor, and memory T cell induce CD25 expression upon activation [52,58]. Regulatory T cells constitutively express the high affinity trimeric IL-2 receptor, and depend on IL-2 signaling for survival[59,60]. Cells expressing the dimeric IL-2 receptor respond to high levels of IL-2, but are presumed to be unresponsive to physiological low levels of IL-2[58]. CD25 is commonly used experimentally as a marker of T cell activation, as it is rapidly upregulated in naïve and memory T cells upon T cell stimulation.

IL-2 supports the proliferation and differentiation of CD4 and CD8 T cells[61]. The strength and duration of IL-2 signaling has been linked to T cells differentiation into long lived memory cells or short lived effector cells, with stronger IL-2 signaling associated with effector cell formation[62,63]. IL-2 also supports CD4 T cell differentiation into various T cell subsets, though the role of IL-2 in differentiation is complex. Generally, IL-2 supports Treg survival and function and inhibits Th17 cell generation, although once Th17 cells have been established Tregs can support Th17 function[58,64]. IL-2 can also influence Th1/Th2 differentiation, and has been shown to support both Th1

differentiation by enhancing IFNγ production, and Th2 differentiation by inducing the IL-4 receptor and by maintaining the accessibility of the IL-4 gene[65,66]. As such, IL-2 is both a marker of T cell activation, and is important to support T cell proliferation and function[57]. Its role is complex, as it both stimulates effector CD4 and CD8 T cell function, but also is critical for the survival and function of Tregs.

IFNγ

IFNγ is a cytokine with pleotropic effects in the immune system that is important for antimicrobial and antiviral defenses. It is produced by several cells of the immune system, mainly NK cells, NKT cells, CD8 T cells, and CD4 T cells, though B cells and APCs can also produce IFNγ[67]. IFNγ is the signature cytokine produced by Th1 CD4 T cells, and coordinates the immune response directed by Th1 cells. IFNγ is induced upon T cell activation at low levels in CD4 T cells, and production increases in CD4 T cells that differentiate into Th1 cells, and is suppressed in the other CD4 T cell subsets[68]. Transcriptional regulation of IFNγ is complex, and varies depending on cell type and differentiation status[69]. Induction of IFNγ upon T cell activation is regulated by NFAT, AP-1, and NF-κB binding to regulatory regions of the IFNγ gene[70,71]. IFNγ production in CD4 T cells is also induced by cytokines, including IL-12, and production in differentiated Th1 cells is supported by transcription factors including T-bet, STAT4, Runx3, and epigenetic modifications in regulatory regions of the IFNγ gene[17,72–74].

IFNγ upregulates the antiviral response in cells, including upregulation of antigen presentation by MHC I and MHC II, induction of immunoproteasome subunits, and induction of antiviral enzymes[67,75–78]. It also activates antimicrobial responses in macrophages and neutrophils, by increasing phagocytosis and killing ability through

upregulation of genes such as NADPH oxidase and inducible nitric oxide synthase (iNOS)[67,79–81]. IFNγ also has anti-proliferative and pro-apoptotic effects, depending on context[67,82]. IFNγ promotes B cell class switching to IgG2a in mice and IgG1 in human cells, and upregulates chemokines and adhesion molecules to regulate leukocyte trafficking to inflamed areas[67,83,84]. As such, IFNγ is involved in antiviral and antimicrobial immune responses, and is produced upon T cell activation, and in a more tightly regulated manner, during CD4 T cell differentiation, as it is important mediator of Th1-specific immune responses.

GM-CSF

GM-CSF is a cytokine produced by a number of cell types, including T cells, B cells, monocytes, macrophages, neutrophils, fibroblasts, endothelial and epithelial cells, and tumor cells. GM-CSF was named for its ability to induce the growth of macrophage and granulocytes colonies *in vitro*. However, mice lacking GM-CSF develop normally, except for a defect seen in the maturation of alveolar macrophages leading to lung pathology, and changes in levels of some dendritic cell subsets[85]. The GM-CSF-null mice are more susceptible to infection and show defects in host defense, indicating a role for GM-CSF in inflammation and host defense, but not in steady-state myelopoiesis[86,87]. GM-CSF has since been shown to play a role in tissue inflammation and recruitment and function of myeloid cells.

The GM-CSF receptor is a heterodimer composed of an α chain and a β chain, and binding of GM-CSF induces signaling through JAK2- STAT-5 and PI3K[88,89]. T cells are a major source of GM-CSF, with GM-CSF production transiently upregulated upon T cell activation, and in some effector CD4 T cells. Upon T cell stimulation, GM-CSF

expression is induced by a proximal promoter and an upstream enhancer, and requires the coordination of NFAT, NF-κB, and AP-1 transcription factors, along with constitutively expressed factors such as Ets1 for transcription[90–92].

It has been shown that GM-CSF is involved in the pathology of a number of inflammatory conditions, including rheumatoid arthritis and EAE, a mouse model of multiple sclerosis[89,93,94]. However, the differentiated T cell subsets responsible for GM-CSF production and the molecular mechanisms regulating GM-CSF expression remains to be elucidated. Various Th cell subsets have been shown to produce GM-CSF, and several proteins have been shown to be involved in GM-CSF production in CD4 T cells, including IL-7 induced STAT5 signaling, induction of the transcription factor DEC1 (also known as Bhlhe40), and IL-23 signaling[89,93,95–97]. GM-CSF is produced by several cell types including CD4 T cells, both upon activation and in differentiated T cells, and plays a role in host defense and in a number of inflammatory conditions.

TNFα

TNFα is a cytokine with pleiotropic effects in the body involved in immunity, inflammation, cell death, and proliferation. It is produced by a number of immune cell types, including macrophages, mast cells, NK cells, B cells, and T cells[98,99]. Regulation of TNFα is tightly controlled and is both cell type and signal specific. TNFα is induced by a number of different stimuli, including pattern-recognition receptor agonists such as lipopolysaccharide, viral infection, certain cytokines, and others, and is complex and stimulus and cell type specific[99]. In T cells, TNFα is produced upon T cell activation and at varying levels by different effector CD4 T cell subsets[8,100]. During T cell activation, TNFα gene transcription is induced by NFAT and c-Jun/ATF-2 in a cooperative

manner[101,102]. The role of NF-κB in TNFα regulation is a subject of debate, as NF-κB has been shown to play a role in TNFα regulation in other cell types, such as macrophages, but appears to be dispensable for TNFα induction upon T cell activation[100].

TNFα acts through two receptors, TNFR1 and TNFR2. TNFR1 is ubiquitously expressed, and binding of TNFα to TNFR1 either leads to an inflammatory response through activation of NF-κB and stimulation of MAPK signaling cascades, or to cell death[103]. TNFR2 is only expressed on specific cell types, including immune cells, endothelial cells, and neurons, and signaling through TNFR2 results in stimulation of MAPK and AKT signaling cascades and activation of NF-κB expression, and cell survival and tissue regeneration[104]. TNFα production and signaling is tightly regulated as TNFα has a complex role in inflammation, disease, and immunity throughout the body, and T cells are an important source of TNFα.

CD69 induction upon T cell activation

CD69 is a c-type lectin protein expressed on the cell surface that is upregulated upon activation in many immune cell types, including T cells. While the exact functions of CD69 remain under investigation, it is thought to be involved in lymphocyte trafficking, but not in T cell co-stimulation or proliferation[105–107]. CD69 is induced rapidly and transiently upon T cell activation and is commonly used experimentally as a marker of T cell activation, but the molecular mechanisms underlying induction have only been partially determined. Pharmacological inhibition of MEK1 or calcineurin each partially inhibits CD69 induction upon T cell activation, and regulatory elements for NF-κB, AP-1, CREB, and early growth response (EGR) proteins have been identified in the CD69

promoter region and can influence CD69 transcription[8,108–110]. Other regulatory regions for CD69 have also been identified as well, and overall several of the transcription factors induced upon T cell activation, including NFAT, NF-κB, and AP-1, all play a role in CD69 induction[111].

T cell introduction summary

In summary, T cells are a critical part of the adaptive immune response, and CD4 T cells coordinate the immune response to protect against a wide range of potential pathogens. CD4 T cell responses are highly regulated, as they must be effective enough to eliminate the pathogen, but not cause irreparable damage to the surrounding tissue, or be directed against a self-antigen. T cell activation is the first step in the process by which T cells mediate these responses, involving a highly branched signaling cascade initiated by ligation of the TCR and a co-stimulatory receptor. While extensive work has been done to elucidate this cascade as well as the mechanisms that underlie T cell differentiation, there are still many unknowns, and new factors involved that can modulate this process are still being determined. Of interest to our lab, cellular stress is one such factor.

Introduction to Nrf2

The ability to respond to stressors that arise either from extracellular sources or as byproducts of cellular processes is an important cellular defense mechanism. These can include things such as reactive oxygen species (ROS), reactive nitrogen species, xenobiotic compounds, hypoxia, and others. Over time, cells have developed several strategies to respond to these stressors. One such response is the Nrf2-ARE pathway. In response to electrophilic stimuli such as reactive oxygen species, toxic metals, and

electrophilic xenobiotics, the Nrf2-ARE pathway upregulates cytoprotective genes that protect the cell against insult.

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) was initially identified as a protein capable of binding hypersensitive site 2 in the β-globin locus control region, and as a homolog of another protein known to bind this site, NF-E2 (nuclear factor erythroid 2)[112,113]. The function of Nrf2 was initially unknown, as Nrf2 is widely expressed in cell types beyond those involved in erythropoiesis, and initial investigations showed mice with disrupted Nrf2 genes had no overt phenotype[112,114]. Shortly thereafter, Nrf2 was found to bind to antioxidant response elements (AREs) and regulate the activity of downstream genes[115,116].

The ARE was characterized as a response element in the promoter region of the glutathione-s-transferase Ya subunit (GSTYa) and NAD(P)H:Quinone oxidoreductase (NQO1) genes, and is involved in induction of these genes by hydrogen peroxide and electrophilic compounds, though the transcription factors involved were initially unknown[117,118]. AREs were found in the promoters of a number of phase II detoxification genes, and Nrf2, along with binding partners, was found to upregulate these genes[115,116,119]. Following these discoveries, Nrf2 was studied as a transcription factor with a role in regulating antioxidant and detoxification defenses in response to oxidative stressors. Canonically, Nrf2 is regarded a cytoprotective transcription factor that regulates a cell stress pathway. Induction of electrophilic or oxidative stress induces Nrf2, which upregulates antioxidant and detoxification genes to protect the cell against these types of stressors. The study of Nrf2 expanded from this base.

Structure of Nrf2

Nrf2 is a basic leucine zipper (bZIP) transcription factor comprised of 605 amino acids in humans, and 597 amino acids in the mouse and rat[120]. It is divided into 6 regions called Nrf2-ECH homology (Neh) domains based on homology between species, and a 7th Neh domain that has been added more recently (Fig. 3). Starting from the N-terminus of the protein, the Neh2 domain is important for degradation and regulation of Nrf2. It contains two sites, the DLG and ETGE, that mediate binding to Keap1, which is a repressor protein that facilitates the cytoplasmic localization and proteasome-mediated degradation of Nrf2[121]. The next two domains, Neh4 and Neh5, are transactivation domains[122]. The Neh6 domain also negatively regulates Nrf2, and controls Keap1-independent degradation of Nrf2[123]. It contains two degrons that are recognized by β-TrCP, which also facilitates the ubiquitination and degradation of Nrf2[124]. The Neh1 domain contains the CNC-bZIP region, and is needed for heterodimerization with other transcription factors and for the activity of Nrf2 as a transcription factor[112]. The c-terminal Neh3 domain has a role in transactivation of target genes and is important for the transcriptional activity of Nrf2[125]. The recently described Neh7 domain is between the Neh5 and Neh6 domains, and is involved in protein-protein interactions. It is needed for the interaction between Nrf2 and retinoic X receptor alpha (RXRα), and the region has yet to be fully characterized[126].

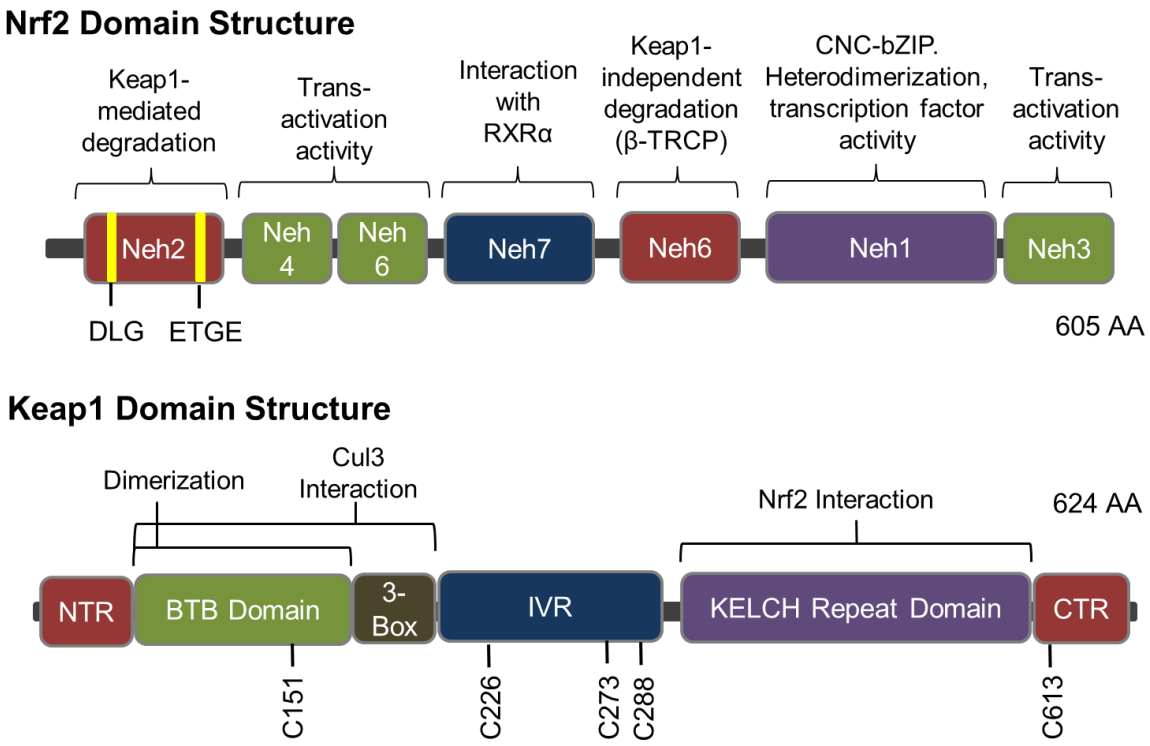

Figure 3. Domain structure of Nrf2 and Keap1 proteins.

Nrf2-regulated genes

Nrf2 regulates gene transcription via binding to cis-acting response elements in the regulatory regions of genes. The consensus binding sequence for Nrf2, known as the antioxidant response element (ARE), or sometimes referred to as the electrophile response element (EpRE), is 5'-TGA$^{C}/_{G}$NNNGC($^{A}/_{G}$)-3'[120,127,128]. The ARE was identified as the cis-acting element in the promoter of the mouse glutathione s-transferase Ya subunit gene and the rat NAD(P)H Quinone oxidoreductase gene that conferred the ability of these genes to be upregulated by electrophilic compounds[118,129,130]. The reported ARE sequence was similar to the sequence bound by NF-E2, and as a NF-E2 related protein, Nrf2 was tested and shown to be a factor that mediated the induction of these genes by electrophilic compounds through the ARE[115,116].

Subsequently, Nrf2 was found to regulate a number of genes involved in cellular defense against oxidative stressors and xenobiotic compounds, including the glutamine-cysteine ligase catalytic and regulatory subunits (GCLC and GCLM), heme oxygenase-1 (HMOX1), and others[119,131,132]. Microarray studies done on cell lines in which Nrf2 was activated either by treatment with pharmacological Nrf2 activators or by genetic activation of Nrf2 demonstrated that Nrf2 activation upregulates genes involved in NADPH generation, xenobiotic metabolism, glutathione synthesis, iron metabolism, and antioxidant activity[133]. Subsequent follow-up experiments either focused on regulation of specific genes or were done using techniques such as ChIP-seq have shown that Nrf2 directly regulates expression of genes involved in these processes[127,134,135]. As the number of identified target genes expanded, it was demonstrated that Nrf2 activation had effects beyond these cytoprotective pathways, and regulated genes involved in lipid metabolism, carbohydrate metabolism, and cell signaling, among others[136]. In addition, some of these genes were downregulated with Nrf2 activation, whereas previously identified Nrf2 targets were all upregulated with Nrf2 activation. Generally, it is recognized that Nrf2 also plays a role in lipid and carbohydrate metabolism, in addition to induction of cytoprotective genes[137,138].

Regulation of Nrf2

Nrf2 protein is continually produced, and under basal conditions, is quickly degraded by the 26s proteasome. In the presence of oxidative or electrophilic stress, the half-life of Nrf2 increases, and Nrf2 translocates to the nucleus and regulates transcription of its target genes (Fig. 4). The best characterized mechanism of Nrf2 regulation is by Kelch-like ECH-associated protein 1 (Keap1). Keap1 is an adaptor

protein that facilitates the ubiquitination and subsequent degradation of Nrf2, and also functions as a redox sensor. Keap1 is divided into five domains (Fig. 3). The c-terminal region along with the double glycine repeat domain (also called the Kelch repeat domain) forms the structure that interacts with Nrf2. The broad complex, tram track, brick-a-brac (BTB) domain near the N-terminus of Keap1 mediates homodimerization of Keap1 and interacts with cullin-3 to enable formation of the Cul3 RING-box 1E3 ubiquitin ligase complex (Fig. 3). Keap1 also binds the cytoskeleton and resides principally in the cytosol, allowing it to retain Nrf2 in the cytosol as well.

Two sites in the Neh2 domain of Nrf2 interact with Keap1: the low affinity DLG motif and the high affinity ETGE motif. In the “hinge and latch” model, binding of the high affinity ETGE motif to one Keap1 subunit in the homodimer is followed by binding of the low affinity DLG motif to the other Keap1, which positions Nrf2 for ubiquitination. In the presence of oxidative or electrophilic stress, reactive cysteine molecules on Keap1 are modified. This alters the conformation of Keap1, inhibiting the ability of this complex to ubiquitinate Nrf2. As a result, Nrf2 protein accumulates and eventually saturates Keap1, allowing Nrf2 to enter the nucleus.

Several reactive cysteine residues on Keap1 are sensors for electrophilic and oxidative stress. Modification of these residues leads to stabilization of Nrf2 and increased transcription of ARE-mediated gene expression. So far, three different sets of residues have been identified based on activation by different compounds. One group of compounds interacts mainly with cysteine 151, including *tert*-butylhydroquinone (tBHQ), sulforaphane, and nitric oxide[139]. Another group of compounds, including 15d-PGJ2, interacts primarily with cysteines 288 and 273, and sometimes with cysteine 151 as

well[140]. The effects of CDDO-Im have been reported to both preferentially depend on cysteine 151, or on cysteine 288 and 273, indicating the need for clarification on this for certain compounds[139,140]. Finally, induction by metals including zinc, cadmium, and arsenic is independent of cysteines 151, 288 and 273, and is thought to involve cysteines 226 and 613[141]. Modification of these residues tracks with induction of Nrf2, and mutation of these residues abolishes Nrf2 induction upon treatment with these compounds.

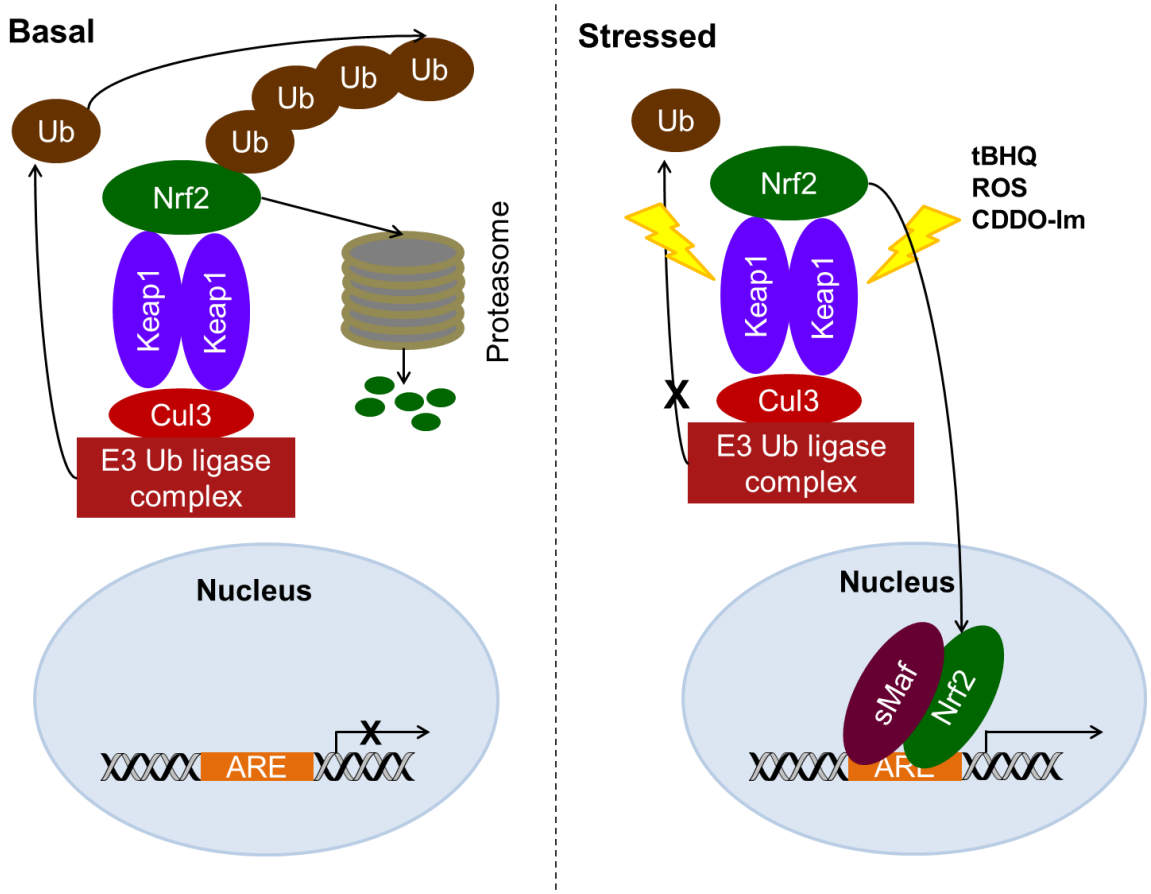

Figure 4. Regulation of Nrf2.
Under basal conditions, Nrf2 is continually produced but is not active, as Nrf2 is bound in the cytosol by Keap1, which facilitates the ubiquitination and proteasomal degradation of Nrf2. Under stressed conditions, the ubiquitination and subsequent degradation of Nrf2 is disrupted, allowing Nrf2 to translocate to the nucleus and regulate transcription of genes with an antioxidant response element as a heterodimer with small Maf proteins.

Keap1interacts with proteins besides Nrf2, and these interactions can be another mechanism through which Nrf2 is regulated. Keap1 binds p62/SQSTM1 (p62), a protein involved in autophagy that can serve as an adaptor to target ubiquitinated and aggregated proteins for autophagic degradation[142]. p62 interacts with the same sites on Keap1 as Nrf2, and can compete with Keap1 for Nrf2 binding[143,144]. p62 can also target Keap1 for degradation by autophagy, indirectly increasing levels of Nrf2[145,146]. The promoter of p62 itself contains a functional ARE and is a target gene of Nrf2[143]. Altogether, this could indicate a potential mechanism for activation of Nrf2 during conditions of cell stress in addition to short-term electrophilic or oxidative stress. In a similar scenario, the protein $p21^{Cip1/WAF1}$, which is involved in several cellular processes including promoting cell survival in response to oxidative stress, can activate Nrf2 through binding to DLG and ETGE sites on Nrf2, competing for the Keap1 interaction and activating Nrf2[147].

The Keap1-facilitated ubiquitination and proteasomal degradation of Nrf2 via interaction with the Neh2 domain is the main mechanism of Nrf2 regulation under homeostatic conditions, and serves as a redox sensor for Nrf2 activation. However, other mechanisms for regulating Nrf2 protein levels have been shown. The Neh6 domain of Nrf2 also contains a degron, which is redox-insensitive and contributes to Nrf2 degradation during conditions of cell stress[121]. It has been shown that glycogen synthase kinase 3β (GSK-3β) can phosphorylate Nrf2, and this attenuates Nrf2 transcriptional activity and translocation of Nrf2 to the nucleus induced by cellular stress or in cells expressing a Nrf2 protein lacking the Keap1-binding ETGE[148,149].

GSK-3β phosphorylates serine residues in the Neh6 domain that overlap with a motif that matches a known SCF/β-TrCP destruction motif[123]. β-TrCP is another E3 ubiquitin ligase adaptor, and targets Nrf2 for degradation when the β-TrCP motif is phosphorylated by GSK-3β[150]. Subsequently, another β-TrCP binding site was identified in the Neh6 domain of Nrf2, and it was demonstrated that inhibition of GSK-3β increases levels of Nrf2 and Nrf2 target genes[124,150]. This mechanisms provides a second layer of control for Nrf2, allowing for the cell to turn off Nrf2 upon induction by oxidative stress, and for the cell to fine-tune Nrf2 levels by regulation of GSK-3β[151]. GSK-3β is basally active, and is regulated by several pathways, such as PI3K/AKT, in which activation of PI3K inhibits GSK-3β[152]. This indicates that GSK-3β contributes to the low basal activity of Nrf2, and provides a way for other signaling cascades to interact with Nrf2. There is evidence that PI3K/AKT can regulate Nrf2, as inhibition of PI3K or Akt1 reduces Nrf2 activation, and this is likely through effects on GSK-3β[153].

Similarly to β-TRCP, other E3 ubiquitin ligase adaptors that can bind to Nrf2 have been identified. In conditions of hypoxia, Siah2, an E3 ubiquitin ligase known to be active in hypoxic conditions, interacts with and downregulates Nrf2 in a proteasome-dependent manner[154]. In conditions of liver cirrhosis, Hrd1, an E3 ubiquitin ligase involved in the ER stress response, can interact with and inhibit Nrf2 through ubiquitination and degradation of Nrf2[155]. CR6-interacting Factor 1 (CRIF1) is not known as a ubiquitin ligase or as an adaptor for ubiquitin ligases, but has also been demonstrated to bind Nrf2 and promote the ubiquitination and proteasomal degradation of Nrf2 in cell lines, though how this occurs remains unknown[156].

DJ-1, a protein linked to Parkinson's disease with roles in antioxidant defenses during oxidative insult, has also been linked to the stabilization and activation of Nrf2. Initially, work done in mouse embryonic fibroblasts and a liver cell line showed that DJ-1 stabilized Nrf2 and was needed for Nrf2 induction[157]. Subsequent papers demonstrated that in primary cortical neuron and astrocyte cultures, loss of DJ-1 did not affect Nrf2 levels or Nrf2 induction, and in a human corneal endothelial cell line, loss of DJ-1 decreased basal Nrf2 levels without altering the Keap1-Nrf2 interaction[158,159]. Taken together, these studies highlight the tissue and context specific regulation of Nrf2 by many proteins.

There is also evidence that stimuli in addition to oxidative stress can modulate Nrf2 activity. Nrf2 can be phosphorylated by a number of kinases. Various MAP kinases, including JNK, ERK, and p38, have been shown to be involved in induction of Nrf2 activity by various Nrf2 activators by use of pharmacological inhibitors, with inhibitors of ERK or JNK generally decreasing the induction seen with Nrf2 activators[160–162]. Inhibition of p38 has a mixed effect, sometimes showing inhibition, and other times enhancement, of Nrf2 induction[163–166]. Which MAPKs are activated and regulate Nrf2 varies across studies and is dependent on specific Nrf2 activators and experimental conditions. In some studies, different MAPK pathway inhibitors show effects and others do not, indicating that the role of MAPKs is cell type and activator specific[160,161,166,167]. It has been shown that MAP kinases, including JNK, ERK, and p38, have the ability to phosphorylate Nrf2, however, mutation of the amino acids phosphorylated by MAPKs has only a slight impact on Nrf2 protein levels or ARE-luciferase reporter activity induced by various Nrf2 activators in cell lines[162,168,169]. This indicates that the direct

impact of MAPKs on Nrf2 may be modest, and the effects of MAPKs are likely to be more modulatory in nature or act on Nrf2 via indirect mechanisms.

Published studies indicate that protein kinase C (PKC) can phosphorylate Nrf2 *in vitro*, and that this phosphorylation decreases Nrf2 binding to Keap1, but not to MafG or the ARE[170,171]. In cells, PKC inhibitors can decrease induction of Nrf2 by various activators, indicating that PKC phosphorylation contributes to Nrf2 activation *in vivo*[167,172,173]. This is thought to occur by decreasing the binding of Nrf2 to Keap1, and investigation into the mechanism by which this occurs, as well as the contribution of PKC in Nrf2 activation in physiological models, is ongoing[171,174–176]. Other kinases such as protein kinase CK2 and PERK can also phosphorylate Nrf2, and increase Nrf2 activation as well[177–179]. Finally, Src family kinases such as Fyn have a role in regulation of Nrf2[180]. Increases in nuclear Fyn levels correlate with inhibition of Nrf2. This is thought to occur by phosphorylation of Nrf2, which in this case, increases Nrf2 export from the nucleus[181–183]. Interestingly, it seems that serine/threonine phosphorylation of Nrf2 is often stimulatory, and tyrosine phosphorylation is inhibitory. How all of these signals interact and are integrated by Nrf2 remains under investigation.

Nrf2 can also be acetylated by p300/CBP, primarily in the Neh1 domain[184]. Acetylation of Nrf2 increases induction of ARE-reporter plasmids, and de-acetylation decreases Nrf2 activity[185]. The mechanisms underlying this remain under investigation. Acetylation of Nrf2 has been shown to increase ARE binding and gene transcription through specific promoters, and alternatively, to regulate the subcellular localization of Nrf2[184,185].

Though primarily regulated post-translationally, Nrf2 is also regulated at the transcriptional level. The Nrf2 gene contains two ARE elements in the promoter region that allow Nrf2 to upregulate its own expression, and thereby increases the sensitivity of Nrf2 as a sensor of reactive xenobiotics[186]. Nrf2 mRNA expression can also be induced by ligands for the aryl hydrocarbon receptor (AhR), as the AhR has been shown to bind to xenobiotic response elements (XREs) in the promoter region of Nrf2 and increase Nrf2 transcription in response to AhR ligands[187]. Nrf2 expression is transcriptionally upregulated in some cancers, and this can occur through oncogenic KRAS induction of an AP-1 element in the Nrf2 promoter, or through NF-κB driven upregulation[188–190].

Binding partners of Nrf2

Nrf2 functions as a heterodimer with small Maf proteins (MafG, MafK, or MafF) to regulate gene transcription[191]. Upon translocation to the nucleus, Nrf2 dimerizes with a small Maf protein and binds to AREs in regulatory regions of its target genes[116,192]. The small Maf proteins are widely expressed bZIP transcription factor-type proteins that lack intrinsic transactivational activity[193]. Fibroblasts lacking all three small Mafs have a severely impaired Nrf2 response to oxidative stress, indicating that the small Maf proteins are important for Nrf2 upregulation of antioxidant responses[194]. The cellular levels of small Mafs are important and likely closely regulated, as overexpression of small Mafs inhibits induction of Nrf2-target genes, likely through formation of small Maf homodimers, which inhibit transcription[195].

Less well established is the ability of Nrf2 to heterodimerize with other bZIP proteins, such as c-Jun and ATF4[196]. The Jun proteins and c-Fos can bind the ARE sequence *in vitro*, though the *in vivo* significance of this is still being determined[197]. In

cell lines transfected with ARE-reporter plasmids, co-transfection of Jun proteins with c-Fos or Fra1 inhibited activity of the reporter plasmid, but co-transfection with Nrf2 increased reporter activity[115,198]. Nrf2 and c-Jun can be co-immunoprecipitated, and may coordinately regulate Nrf2 target genes in some cell types, indicating that Nrf2 may dimerize with other bZIP binding partners under certain conditions[199].

Nrf2 cross-talk with other pathways

Nrf2 interacts with a number of other cellular pathways, including the AhR, NF-κB, Notch signaling, and RXRα. The AhR is a ligand-activated transcription factor that upregulates gene expression through the xenobiotic response elements (XREs)[200]. Nrf2 can upregulate transcription of the AhR, and the AhR can upregulate transcription of Nrf2, through XRE and ARE elements in the promoters of Nrf2 and AhR, respectively[187,201,202]. Several Nrf2 target genes, such as NQO1, are also regulated by both the AhR and Nrf2, and there is a report of interaction between the two transcription factors[203,204].

Keap1-Nrf2-ARE signaling also interacts with the NF-κB signaling pathway. Nrf2-null mice exposed to stimuli that activate NF-κB have greater NF-κB signaling compared to wild type mice, and Nrf2 activation has been shown to decrease NF-κB activation[205–209]. IKKβ, a kinase that activates NF-κB family members by phosphorylating and causing the degradation of IκB, interacts with Keap1, and potentially competes with Nrf2 for binding to Keap1[210,211]. Keap1 promotes the degradation of IKKβ through increases in ubiquitination and autophagic degradation, decreasing NF-κB signaling[210,212]. NF-κB family members can also affect Nrf2 signaling. Increases in p65 suppress Nrf2-dependent transcription of target genes through several mechanisms. Nrf2 and p65

both interact with CBP, an acetyl transferase that increases transcription of target genes, and p65 can compete with Nrf2 for CBP binding, inhibiting Nrf2 promoter activity[213]. p65 also helps recruit histone deacetylase 3 to the ARE via MafK, further repressing Nrf2 gene transactivation[213]. Finally, p65 also interacts with Keap1, and promotes Keap1 nuclear localization, where it interacts with and inhibits Nrf2[214]. At the transcriptional level, NF-κB increases Nrf2 mRNA levels via a κB response element in the Nrf2 promoter[190]. In addition, NF-κB can interact with Nrf2 target genes, such as HMOX1, which can inhibit NF-κB activation[215,216]. More generally, NF-κB is also regulated by oxidative stress, meaning that changes in the redox state of the cell that occur as a result of Nrf2 activation-induced increases in antioxidant proteins can influence NF-κB activity[217].

Nrf2 also interacts with the Notch signaling pathway, which is a signaling pathway involved in the determination of cell fate, including cell differentiation, proliferation, and survival[218]. Microarray studies done on wild-type or Nrf2-null cells showed down regulation of Notch1 and Notch target gene mRNA in the Nrf2-null cells. This was followed up in mouse embryonic fibroblasts (MEFs) and in mouse livers, where it was demonstrated that Nrf2 increased Notch1 expression through AREs located in the Notch1 promoter, and that loss of Nrf2 decreased Notch1 levels and expression of Notch target genes in both fibroblasts and in an *in vivo* model of liver regeneration[219]. Nrf2 regulation of Notch has also been demonstrated in hematopoietic stem progenitor cells, where pharmacologic Nrf2 activation increased Notch signaling[220]. Notch signaling can also upregulate Nrf2, as it has been demonstrated that Notch signaling increases Nrf2 transcription through a functional binding site in the

Nrf2 promoter for recombination signal binding protein for the immunoglobulin kappa J region (RBPJ), which is the binding partner for the Notch intracellular signaling domain[221].

Activation of RXRα inhibits Nrf2 activity. Initially, it was shown that retinoic acid receptor alpha (RARα) agonists decreased the induction of Nrf2 target genes by electrophilic compounds such as tBHQ and sulforaphane[222]. This was followed with studies demonstrating that RXRα physically interacts with Nrf2 through the Neh7 domain and downregulates Nrf2 signaling[126,223]. RXRα was identified as a target gene upregulated by Nrf2 in a ChIP-seq study, indicating a potential negative feedback loop on Nrf2 activity[224].

Nrf2 Activators

Several different Nrf2 activators are commonly used experimentally, such as tert-butylhydroquinone (tBHQ), 1[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), Sulforaphane (SFN), cadmium, arsenic, and others.

Of particular interest in our lab is tBHQ. In addition to being a commonly used Nrf2 activator, tBHQ is a food preservative. It is a GRAS (generally recognized as safe) compound, allowed for use in food “as an antioxidant alone or in combination with butylated hydroxyanisol (BHA) and/or butylated hydroxytoluene (BHT)” and can be used in fat-containing foods with the stipulation that “the total antioxidant content of a food containing the additive will not exceed 0.02 percent of the oil or fat content of the food” (21 CFR§172.185)[225]. tBHQ acts as an antioxidant to prevent the rancidification of fats, and can be used in oils or fats up to 200 mg tBHQ per kg fat. Because of this, tBHQ is a common human exposure, and of interest from a toxicological perspective. The

acceptable daily intake (ADI) for tBHQ is 0-0.7 mg/kg body weight per day, which was set by the World Health Organization's Joint FAO/WHO Expert Committee on Food Additives, based on a no-observed effect level of 70 mg/kg-body weight/day in dogs and a safety factor of 100[226]. The same committee also undertook an evaluation of tBHQ intake in five countries including the USA, using estimates derived from diet surveys, model diets, and poundage of tBHQ used. Based on poundage, intake ranged from 0.004-0.14 mg/kg-body weight/day, well below the ADI, but based on model diets, the mean intake of tBHQ was 0.37 mg/kg-body weight/day. For high consumers, the daily intake based on model diets was above the ADI of 0.7 mg/kg body weight/d[227]. Altogether, this indicates that tBHQ is a potentially significant exposure for a portion of the population. Studies on humans are limited, but indicate that tBHQ is readily absorbed from the gastrointestinal tract, metabolized by conjugation, and rapidly excreted in the urine, with tBHQ almost completely excreted within 48 h of administration of a single lump dose to volunteers[228]. In the same volunteers, serum tBHQ levels after administration of 100-150 mg dose of tBHQ reached the high micromolar range[226]. In our studies, concentrations of tBHQ used experimentally *in vitro* are often in the nanomolar to low micromolar range, indicating that people may consume levels of tBHQ high enough to achieve serum concentrations that correspond to concentrations used experimentally.

Another commonly used Nrf2 activator is CDDO-Im, an imidazolide derivative of the triterpenoid CDDO. CDDO-Im is a potent Nrf2 activator, effective in the nanomolar concentration range, but targets other pathways as well at micromolar concentrations[229,230]. At these higher concentrations, CDDO-Im also inhibits proliferation

and induces apoptosis[231,232]. CDDO-Im and other, related, CDDO compounds were initially developed as cancer therapeutics, as they inhibit proliferation of cancer cell lines[233,234]. They have also been investigated as potential therapeutics for other diseases, such as kidney disease, following the observation that CDDO compounds induce Nrf2 and inhibit inflammatory gene induction in macrophage cell lines[232,235,236]. CDDO-Im and related compounds are frequently used experimentally as Nrf2 activators as well.

Nrf2 and the immune system

Although Nrf2 was discovered as cytoprotective transcription factor, it was quickly shown to play a role in inflammation and the immune system. Nrf2-null mice are viable with no overt phenotype, but are more sensitive to cellular stressors such as carcinogens[114,237,238]. However, the Nrf2-null mice develop pathology as they age, with the older female mice developing a condition similar to systemic lupus erythematosus[239]. The autoimmune disease in the older female Nrf2-null mice is characterized by glomerular nephritis, immunoglobulin deposits in the kidneys, antibodies against double-stranded DNA, and death[239–241]. The onset of the disease varies by mouse strain, with the 129SVJ mice developing pathology at 30 weeks of age, and other strains from a year to a year and a half[239–241]. Subsequent investigation demonstrated that Nrf2-null mice are susceptible to a range of inflammatory stimuli, indicating a role for Nrf2 in regulating inflammation and the immune response.

Nrf2-null mice are more sensitive to lung inflammation and injury, showing a greater response to ovalbumin, carrageenan, bleomycin, or hyperoxia challenge[242–245]. In these models, the Nrf2-null mice have greater lung injury, as shown by increased

lung pathology, and greater numbers of infiltrating immune cells in the lung. They also have higher production of inflammatory cytokines, such as TNFα, MIP-2, and IL-6[244,245], and an increased NF-κB response upon injury[242]. One study also shows increases in the cytokines IL-4 and IL-13, which are indicative of a Th2 helper T cell response, a specific subset of CD4 T cells, without an increase in IFNγ in the Nrf2-null mice[245]. Treatment with the Nrf2 activator sulforaphane reduces viral titers and lung inflammation in wild-type but not Nrf2-null mice infected with respiratory syncytial virus[246]. Nrf2-null mice have increased damage in ConA-induced liver injury, and Nrf2 activation by hepatocyte specific Keap1-deletion helps protect against injury[247]. Mice lacking Nrf2 are also more sensitive to arthritis[248].

The Nrf2-null mice are also more sensitive in models of sepsis and endotoxemia, either from cecal-ligation and puncture (CLP), or injection of lipopolysaccharide (LPS), and show increased inflammatory cytokine production in these models[205,249,250]. The Nrf2-null mice produce more TNFα, IL-6, and have greater NF-κB activation in these models[205,251]. Myeloid-specific deletion of Nrf2 enhances susceptibility to sepsis, and myeloid-specific activation of Nrf2 by deletion of Keap1 protects against sepsis in CLP[250]. With CLP, myeloid deletion of Nrf2 increases production of many cytokines, including IL-6, IL-10, IL-1β, IFNγ, and IL-4, and myeloid deletion of Keap1 decreased cytokine production, showing effects on a broad range of immune cell types and functions in this model[250].

Nrf2-null mice are also more sensitive to experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis[252]. In EAE, mice are immunized with a myelin-like antigenic peptide, which induces an immune response

against myelin that results in demyelination and neuronal death mimicking multiple sclerosis. Nrf2-null mice have increased severity of EAE, characterized by increased clinical signs, increased disease incidence, increased infiltration of CD4 T cells, B cells, and neutrophils in the spinal cord, and increased inflammatory enzyme and cytokine gene expression in the spinal cord[252,253]. Treatment with compounds that activate Nrf2, including CDDO-TFEA or sulforaphane, protects wild-type mice from EAE, as evidenced by decreased disease severity (as determined by clinical scoring), and decreases levels of inflammatory cytokines including IL-17 and IFNγ[254–256].

In kidney injury, loss of Nrf2 is generally deleterious, and increases in Nrf2 have generally been shown to be protective [257]. There is some evidence that the role of Nrf2 may be more complicated, depending on model. In the lpr mouse model of lupus, in which mice are deficient in Fas-mediated apoptosis and develop autoimmunity through the survival of autoreactive T cells, the role of Nrf2 is less clear. In one study, loss of Nrf2 is protective, with the Nrf2-null mice showing increased survival and lower levels of kidney damage compared to Nrf2-expressing lpr mice[258], a confounding result given that Nrf2-null mice develop a similar autoimmune disease spontaneously. Another group later showed that loss of Nrf2 is not protective in the lpr lupus model, with the Nrf2-null/lpr mice showing decreased survival and increased kidney pathology compared to both Nrf2-null and lpr control mice[259]. This discrepancy could be due to differences in the mouse backgrounds used in these two studies. In a model of lupus induced by pristane treatment, the Nrf2-null mice develop greater kidney damage and antibody deposits, but also have increased basal levels of kidney damage, oxidative stress, and antibody deposits, such that the increase with pristane is similar between

the two genotypes[206]. These studies indicate that the role for Nrf2 in these multifaceted disease conditions is complicated and dependent on numerous factors, some of which have yet to be identified.

The role of Nrf2 in host defense against bacterial or viral pathogens is a subject of ongoing research. Nrf2-null mice have an increased inflammatory response and increased lung injury to chronic infection with nontypeable *Haemophilus influenzae*, which was used in this study to mimic exacerbations in chronic obstructive pulmonary disease [260]. Further work showed that increasing levels of Nrf2 via a Nrf2 activator or Keap1 knockout increases the ability of macrophages to phagocytose bacteria in culture[261]. When bacteria are given to a whole animal, the Nrf2-null animals have a slightly lower ability to phagocytose bacteria and a higher bacterial burden when exposed to cigarette smoke, but similar bacterial burden to the WT mice without the cigarette smoke exposure[261]. The response to pneumonia from infection with *Streptococcus pneumoniae* in mice is mixed. At lower numbers of instilled bacteria, the Nrf2-null mice have increased bacterial clearance, with lower levels of bacteria in the lungs. They initially have lower levels of neutrophils, and over time accumulate slightly more neutrophils in the lung. However, with a greater number of instilled bacteria, the Nrf2-null mice show decreased survival, with increased bacterial burden in the lungs. The Nrf2-null mice have increased disease severity as determined by scoring of clinical signs, but lose less weight and do not have increased levels of damage-associated molecular pattern molecules in the brochoalveolar lavage fluid (BALF). In addition, only levels of the cytokines/chemokines IL-12p70, TNF, and KC are increased in the BALF, with many other cytokines unchanged or showing only a modest trend towards an

increase[262]. Interestingly, this potentially indicates a differential role for Nrf2 in regulating immune cell processes such as bacterial clearance and production of inflammatory cytokines.

The Nrf2-null mice are also more sensitive to lung infection with respiratory syncytial virus, with increased viral titers, increased lung pathology, increased numbers of immune cells in the lungs, and increased lung levels of the cytokines IL-6, IL-18, IL-10, and IL-13[246]. Upon infection with the influenza A virus, Nrf2-null mice have a worsened response as compared to the wild type mice only if they were first exposed to cigarette smoke[263]. There is also evidence that infection regulates Nrf2 activation with several different viruses. One example is the Marburg virus, where a viral protein directly activates Nrf2 via interaction with Keap1, and Nrf2-null mice are protected against infection[264]. Overall, there is a trend towards loss of Nrf2 as deleterious and activation of Nrf2 as protective, with evidence that the role of Nrf2 is complex and is context-and-disease specific.

Other conditions with an inflammatory component have a more ambiguous role for Nrf2. Nrf2-null mice have been shown to be both protected from and more sensitive to high fat diet induced obesity and insulin resistance[265–268]. Likewise, there are differential responses of Nrf2-null mice to contact sensitizers, which are small molecules that induce an inflammatory immune response upon skin exposure. The Nrf2-null mice have a greater ear swelling response upon repeated exposure to two sensitizers[269], but a decreased ear swelling response to two other sensitizers as compared to wild type mice[270].

In addition to the numerous whole animal studies that have focused on Nrf2, the role of Nrf2 in isolated cells has also been investigated in vitro. As many diseases have a significant inflammatory component, much of the research in isolated immune cells has focused on the role of Nrf2 in regulating inflammatory target genes and functions of innate immune cells (mainly macrophages and dendritic cells).

In bone marrow-derived dendritic cells (BMDC) from wild type or Nrf2-null mice, the cells from the Nrf2-null mice show a more mature phenotype. They express higher levels of costimulatory molecules, have lower phagocytic capacity at baseline, and induce increased T cell proliferation in culture[271–274]. At the cytokine level, Nrf2-null dendritic cells produce more inflammatory cytokines, including IL-6, IL-12, and TNF when exposed to stimuli such as ragweed extract or particulate matter[271,272]. Interestingly, Nrf2-null dendritic cells also make more IL-10, which is generally considered to be immunosuppressive, upon stimulation with particulate matter or LPS [271,274]. However, basal differences in cytokine production are inconsistent, with reports of both increased and unchanged cytokine levels between unstimulated wild type and Nrf2-null cells[271,272]. Treatment of wild-type dendritic cells with sulforaphane does not change the expression of the cell surface proteins CD80, CD86, or MHC II, the expression of which is typically associated with the mature phenotype. However, sulforaphane does inhibit induction of IL-12p70 and IL-23 by LPS in wild-type dendritic cells[256].

Published studies also indicate a role for Nrf2 in the activity of transcription factors that modulate gene expression in dendritic cells. One study demonstrated impaired activation of NF-kB by LPS in dendritic cells derived from Nrf2-null mice.

Another study showed increased basal levels of phosphorylated p38 and phosphorylated CREB in Nrf2-null dendritic cells[273,274]. Finally, sulforaphane treatment inhibits NF-kB activation by LPS in wild type dendritic cells[256]. Together, these data indicate that Nrf2 may have differential effects on different dendritic cell functions.

Because Nrf2 induces antioxidant gene expression, and ROS/GSH levels have been shown to play a role in dendritic cell function[275], several studies have investigated the effect of antioxidant intervention with respect to dendritic cell function. Treatment with N-acetyl cysteine (NAC) reduces the induction of cell surface markers and cytokines by particulate matter or ragweed extract[271,272]. With respect to other antioxidants, inhibition of glutathione synthesis in wild-type dendritic cells did not change phagocytic capacity, cell surface marker expression, or ability to induce T cell proliferation. Likewise, treatment of Nrf2-null dendritic cells with vitamins to reduce ROS levels did not change basal differences in cell surface markers or ability to induce T cell proliferation[273,274]. Overall, Nrf2 impacts dendritic cell function, though the mechanisms by which this occurs are not fully characterized. Nrf2 may impact dendritic cell function through changes in the redox status of the cell, but could also function through direct effects on transcription factors or gene regulation.

The role of Nrf2 in macrophages and neutrophils has also been studied. In peritoneal neutrophils derived from wild-type or Nrf2-null mice, stimulation of the Nrf2-null neutrophils with LPS induces greater expression of the cytokines/chemokines TNFα, IL-6, MCP-1, and MIP-2. NAC reduces LPS induction of cytokines in both genotypes, and CDDO-Im reduces this induction only in neutrophils from wild-type mice[249]. Similar results were observed in peritoneal macrophages from Nrf2-null mice

stimulated with LPS. LPS treatment of peritoneal macrophages from Nrf2-null mice results in greater NF-κB activity, though basal activity is similar[205]. An increase in LPS-induced cytokine production in macrophages isolated from Nrf2-null mice has been reported several times[250,251,276]. There is also evidence for a role for Nrf2 in human myeloid cells, as treatment with CDDO-IM inhibits IL-6 and TNFα induction by LPS in human peripheral blood mononuclear cells[277]. Likewise, knockdown of Nrf2 by siRNA in a human monocyte cell line increases IL-1β, IL-6, MCP-1, and TNFα levels at baseline and with PMA activation, and also increases levels of nuclear p50 and phosphorylated ERK1/2 with PMA activation[278]. Treatment with Nrf2 activators, such as sulforaphane or CDDO-IM, reduces the cytokine induction seen with LPS, though depending on the model and compound this is sometimes only partially Nrf2 dependent[249,276,279].

Macrophages that are both Nrf2-null and deficient in NADPH oxidase, a main source of ROS in macrophages, have reduced cytokine induction with LPS as compared to Nrf2-null cells, and in fact, express levels similar to wild-type cells[251]. This indicates that this effect of Nrf2 in this case may be due to changes in the redox status of the macrophage. However, Nrf2 also directly regulates targets in macrophages. Nrf2 has been shown to directly upregulate CD36 and MARCO, a scavenger receptor involved in bacterial uptake expressed on macrophages through AREs in the promoters of these genes[134,261]. In addition, Nrf2 activation increases bacterial phagocytosis by macrophages *in vitro*[261]. Loss of Nrf2 decreases the ability of macrophages to stimulate T cell activation in culture due to reduced GSH and cysteine levels in the Nrf2-null cells[280]. Nrf2 also directly down-regulates IL-6 and IL-1β production through binding to their promoters (though this effect was ARE-independent)[135]. In summary, in

macrophages Nrf2 activation generally down-regulates inflammatory cytokines and genes, and deletion of Nrf2 up-regulates these targets. Nrf2 also impacts the ability of macrophages to phagocytose bacteria, and to stimulate other cells. Some of these effects are due to direct interactions between Nrf2 and the regulatory regions of genes, whereas other effects are due to changes in the redox status of the cell and changes in levels and activity of other transcription factors that regulate inflammation, including NF-κB and various MAPKs.

Innate immune cells are only part of the immune response; the adaptive immune response also plays a significant role in many of the disease models and conditions for which the Nrf2-null mice are more sensitive. The role of Nrf2 in individual cells of the adaptive immune response is less well studied. B cells from Nrf2-null mice activated *in vitro* with LPS show similar levels of proliferation and cell death, and slightly less IgM production as compared to wild-type B cells[281].

Our lab has shown that activation of CD4 T cells from Nrf2-null mice results in significantly decreased induction of the Th2 cytokines IL-4, IL-5, and IL-13, and increased induction of the Th1 cytokine IFNγ. In addition, treatment of activated wild-type CD4 T cells with a Nrf2 activator increases expression of the Th2 cytokines and DNA binding of the transcription factor GATA3, while concurrently decreasing expression of IFNγ and the DNA binding of the Th1 transcription factor T-bet. These effects were observed in CD4 T cells derived from wild type, but not Nrf2-null mice, indicating that Nrf2 is skewing CD4 T cell differentiation in mice[282].

Studies in cultured splenocytes treated with Nrf2 activators prior to stimulation with the mitogen concanavalin A (ConA) or αCD3/αCD38 show that the Nrf2 activators

inhibit proliferation and production of cytokines such as IL-2 and IFNγ, but the role of Nrf2 in these effects remains undetermined[283,284]. A study from a different group investigating the effect of trivalent arsenic on primary human T cells found that Nrf2 nuclear accumulation increases upon T cell activation, and that arsenic III inhibits induction of IL-2, IFNγ, and IL-17. Although this group attempted to knockdown Nrf2 in primary human T cells, they were only able to reduce Nrf2 expression by around 40% and thus the role of Nrf2 in the inhibitory effects of arsenic III remained unclear in this study[285]. Nrf2 activation by Keap1 deletion in T cells helps protect against kidney injury in a mouse model of acute kidney injury. In addition to reduced kidney damage, decreased IFNγ expression, increased IL-10 and IL-17 production, and greater numbers of regulatory T cells were observed in the kidneys of the T cell-specific Keap1-null mice.[286]. Splenocytes from sulforaphane-treated wild type or Nrf2-null mice with EAE activated *in vitro* showed decreases in IL-17 and increases in IL-10, suggesting that Nrf2 activation potentially decreased Th17 differentiation and increased Treg differentiation[255].

A role for Nrf2 in the proliferation and differentiation of hematopoietic stem cells (HSCs) has also been demonstrated. HSCs from the Nrf2-null mice proliferate more quickly, showing increased turnover and progression through the cell cycle, and Nrf2-null cells have a lower ability to repopulate the bone marrow after transplant[287,288]. One of the studies suggests that this could be due in part to a deficiency in homing to the bone marrow after transplantation in the Nrf2-null cells due to lower levels of CXCR4[288]. Keap1-deletion in HSCs affects the cell fate of HSCs, as increased granulocyte precursors and decreased lymphocyte and erythrocyte precursors were found in the

bone marrow of mice with conditional deletion of Keap1 in HSCs [289]. HSC-specific deletion of Keap1 also influences HSC proliferation, with increased numbers of cells undergoing proliferation in the bone marrow of these mice. Once again there are no increases in the overall numbers of cells, and the Keap1-null cells are less effective at reconstituting the bone marrow after transplant, potentially due to stem cell exhaustion from the increased proliferative levels[290]. These studies indicate a role for Nrf2 beyond regulation of antioxidant levels, with both loss and activation of Nrf2 influencing cell proliferation and cell fate determination in immune cell precursors.

Overall, Nrf2 has demonstrated effects on the immune system. Generally, loss of Nrf2 leads to exacerbated injury and inflammation, especially in an acute setting, and Nrf2 activation is protective in these conditions. Nrf2 also modulates the response of specific immune cell types, such as macrophages and dendritic cells. There are several ways by which Nrf2 can induce these effects, which are not mutually exclusive. Nrf2 activation and Nrf2 loss change the levels of antioxidant and metabolizing enzymes in the cell, which modulates the cellular levels of ROS and GSH, and can thereby impact immune cell responses. Nrf2 can also directly regulate immune genes such as cytokines through effects on transcription mediated by AREs in the regulatory regions of genes. Finally, cross talk between Nrf2 and Nrf2 target genes with other transcription factors in the cell such as NF-κB can alter the cellular response to stimuli. Although T cells are a key part of the immune response, the role of Nrf2 in T cell activation and function remains unclear and is the focus of this dissertation. We hypothesize that Nrf2 modulates events of CD4 T cell activation in both mouse and human primary CD4 T

cells, and that the effects of the food additive tBHQ on primary CD4 T cell activation are mediated through Nrf2.

Chapter 2

Differential effects of the Nrf2 activators tBHQ and CDDO-Im on the early events of T cell activation

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*These authors contributed equally to this work

Abstract

We previously demonstrated that activation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) promotes CD4+ Th2 differentiation. In the current study, we assessed the role of Nrf2 in early events following T cell activation. The Nrf2 activators, tBHQ (*tert*-butylhydroquinone) and CDDO-Im (the imidazolide derivative of the triterpenoid CDDO), were used in conjunction with splenocytes derived from wild-type and Nrf2-null mice to distinguish between Nrf2-specific and off-target effects. CDDO-Im inhibited early IFNγ and TNFα production in a largely Nrf2-dependent manner. In contrast, tBHQ and CDDO-Im had little effect on expression of CD25 or CD69. Furthermore, tBHQ inhibited GM-CSF and IL-2 production in both wild-type and Nrf2-null T cells, suggesting this effect is Nrf2-independent. Conversely, CDDO-Im caused a concentration-dependent increase in IL-2 secretion in wild-type, but not Nrf2-null, splenocytes, suggesting that Nrf2 promotes IL-2 production. Interestingly, both compounds inhibit NFκB DNA binding, where the suppression by tBHQ is Nrf2-independent and CDDO-Im is Nrf2-dependent. Surprisingly, as compared to wild-type splenocytes, Nrf2-null splenocytes showed lower nuclear accumulation of c-Jun, a member of the AP-1 family of transcription factors, which have been shown to drive multiple immune genes, including IL-2. Both Nrf2 activators caused a Nrf2-dependent trend towards increased nuclear accumulation of c-Jun. These data suggest that modulation of cytokine secretion by tBHQ likely involves multiple pathways, including AP-1, NFκB, and Nrf2. Overall, the data suggest that Nrf2 activation inhibits secretion of the Th1 cytokine IFNγ, and increases early production of IL-2, which has been shown to promote Th2 differentiation, and may support the later occurrence of Th2 polarization.

Introduction

Nrf2 (Nuclear factor erythroid 2-related factor 2) is a transcription factor that is activated by various types of cellular stress, including oxidative stress and electrophilic insult[114,291]. In the absence of cell stress, Nrf2 is bound to its repressor protein, Keap1 (Kelch-like ECH-associated protein 1), in the cytosol and is subsequently ubiquitinated and degraded by the proteasome[292,293]. Upon introduction of cell stress, Nrf2 is no longer degraded and translocates to the nucleus where it induces transcription of its target genes[115]. Nrf2 regulates a battery of target genes that perform a variety of functions, including detoxification and metabolism of reactive compounds, transport of xenobiotics, antioxidant functions, and upregulation of the proteasomal subunits[116,294].

Nrf2 is activated by numerous compounds, including heavy metals, phenols, reactive oxygen species, and many others[294]. In addition, there are numerous activators that are commonly used experimentally to activate Nrf2, which include 1[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), *tert*-butylhydroquinone (tBHQ), butylated hydroxyanisole, and sulforaphane. CDDO-Im is one of the most potent known activators of Nrf2, active at nanomolar concentrations, but it also activates other pathways at micromolar concentrations[230,232,295]. Although both compounds activate Nrf2 by modification of key cysteine residues on Keap1, there are distinct potency differences, as 0.1-1 μM tBHQ was used vs. 0.001-0.1 μM CDDO-Im in our experiments. The regulation of the Nrf2 pathway by CDDO-Im is also dependent on cell type, as is likely true for tBHQ as well[232]. At the concentrations of CDDO-Im used in the current study (nM range), no decrease in viability was observed. In addition to CDDO-Im, tBHQ was also used in these studies as we have determined previously that

it is a potent activator of Nrf2 in T cells and does not cause cytotoxicity at the concentrations used in our studies[282]. Interestingly, tBHQ is also a commonly used food preservative found in a variety of foods, including cereals, crackers, and fast food items, among others[296].

In addition to its functions in detoxification and the antioxidant response, Nrf2 also has anti-inflammatory activity, which has been reported in numerous inflammatory models. For instance, Nrf2 appears to play a protective role in experimental autoimmune encephalomyelitis, experimental sepsis from cecal ligation and puncture or lipopolysaccharide administration, traumatic brain injury, endotoxemia-induced vascular inflammation, and experimental autoimmune hepatitis[205,297–299]. In addition, Nrf2 seems to be particularly important in lung inflammation. Nrf2-null mice have increased susceptibility to ovalbumin-induced asthma, hyperoxia-induced acute lung injury, pulmonary fibrosis, allergic airway inflammation, and *Staphylococcus aureus* pneumonia[242,245,300–302]. It is also notable that aged female Nrf2-null mice develop an autoimmune disease that resembles systemic lupus erythematosus in humans, which suggests an endogenous role for Nrf2 in immune regulation[239–241].

T cell activation occurs upon ligation of the T cell receptor and a costimulatory receptor by their respective agonists. Experimentally, T cells can be activated by treatment with monoclonal antibodies directed against the T cell receptor (CD3) and a costimulatory receptor, such as CD28. Activation of T cells by anti-CD3/anti-CD28 triggers a signaling cascade that ultimately results in a number of cellular events, such as activation of NFκB and AP-1, as well as other transcription factors, and induction of early cytokines, such as IL-2, GM-CSF, TNFα, and IFNγ[303]. In addition, a number of

cell surface proteins are also induced, including CD25, which is the high-affinity IL-2 receptor, and CD69, which is a C-type lectin receptor. Although the exact function of CD69 is not fully known, both CD25 and CD69 are highly expressed after T cell stimulation and serve as markers of activation.

Our previous studies have demonstrated that activation of Nrf2 skews $CD4^+$ T cells toward Th2 differentiation, whereas absence of Nrf2 skews $CD4^+$ T cells toward Th1 differentiation[282]. However, little is known about the role of Nrf2 in the early events following T cell activation, which can influence polarization. Whereas our previous studies necessarily focused on events occurring 4 to 6 days after T cell activation (which is the time needed for $CD4^+$ T cell differentiation), the purpose of the present studies is to investigate a potential role for Nrf2 in T cell activation within 24 h after CD3/CD28 ligation. It has been previously reported that the early events, such as cytokine release, post T cell stimulation are integral in determination of $CD4^+$ T cell effector function and polarization.

Materials and methods

Materials

CDDO-Im was synthesized as previously described at >95% purity [25,26]. tBHQ, and all other reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

Nrf2-null mice

Nrf2-null mice on a mixed C57BL/6 and AKR background were generated as described previously and received from Dr. Jefferson Chan[114]. The mice were subsequently back-crossed 8 generations onto the C57BL/6 background and are 99% congenic (analysis performed by Jackson Laboratories, Bar Harbor, ME). Female mice were used for the current studies. Age-matched wild-type female C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were given food and water *ad libitum*. All animal studies were conducted in accordance with the Guide for the Care and Use of Animals as adopted by the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University.

Cell culture

Splenocyte isolation: Single-cell suspensions from spleens were washed, filtered, counted and cell density adjusted to 5×10^6 c/ml, unless otherwise noted. Cells were cultured in DMEM (with L-glutamine, sodium bicarbonate and D-glucose)

supplemented with 100 units penicillin/ml, 100 units streptomycin/ml, 50 µM 2-mercaptoethanol, and 10% fetal bovine serum (FBS). For most studies, cells were treated with vehicle, tBHQ, or CDDO-Im at the indicated concentrations for 30 min prior to T cell activation. 24 h post T cell receptor/co-receptor ligation, supernatants and cells were harvested and analyzed as noted in the figure legends. T cells were activated with purified hamster anti-mouse CD3ε (500A2, 1.5 µg/ml), purified hamster anti-mouse CD28 (37.51, 1.5 µg/ml), and an $F(ab')_2$ fragment specific for anti-Syrian hamster IgG that was used to cross-link CD3 and CD28 to enhance activation. Anti-CD3 and anti-CD28 were purchased from E-Biosciences (San Diego, CA), and the $F(ab')_2$ cross-linker was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cytokine protein quantification: ELISA

IFNγ, IL-2, GM-CSF, and TNFα protein was quantified by sandwich ELISAs using commercially-available kits following the manufacturer's protocol (For GM-CSF, TNFα, and IFNγ: Biolegend, San Diego, CA; for IL-2: E-Bioscience, San Diego, CA).

Measurement of CD25 and CD69 expression by flow cytometry

Freshly-isolated splenocytes were washed and resuspended in FACS buffer (PBS, 1% FBS). The cells were then incubated with anti-CD4/FITC, anti-CD25/APC, and/or anti-CD69/PE-Cy7 for 30 min at 4° C, after which the cells were washed and resuspended in FACS buffer. The fluorescence was then detected and quantified with a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). The data were analyzed using CFlow software (BD Accuri, San Jose, CA). The CD4 antibody was purchased

from E-Bioscience (San Diego, CA) and the CD25 and CD69 antibodies were purchased from Biolegend, (San Diego, CA).

ELISA-based DNA binding assay

Four hours after activation by anti-CD3/anti-CD28, nuclear protein was extracted from 1×10^7 wild-type and Nrf2-null splenocytes, using a commercially available kit (Active Motif, Carlsbad, CA). After extraction, nuclear protein was quantified via Bradford assay (Bio-Rad, Hercules CA). Five micrograms of nuclear protein was used to quantify NFκB DNA binding using a commercially available ELISA-based DNA binding assay (Active Motif). Assays were performed per the manufacturer's protocol.

Nuclear protein isolation

Wild-type and Nrf2-null mouse splenocytes were collected and resuspended at $5x10^6$ c/mL. 3 h 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). Cell lysates were then pelleted by centrifugation. The resulting nuclear pellets were then 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 incubated on ice for 1 h, vortexing every 10 min. After 1 h samples were spun down and nuclear protein was quantified. Nuclear protein was quantified via Bradford assay (Bio-Rad, Hercules CA).

Western analysis

After protein quantification, samples were diluted in Laemmli Sample Buffer (Bio-Rad) containing 2.5% 2-mercaptoethanol. Samples were subjected to SDS-PAGE and subsequently transferred to a PVDF membrane using 10 µg of each sample. Membranes were blocked with 5% BSA (Bovine Serum Albumin) or NFDM (nonfat dry milk) in PBS containing 0.05% Tween 20 (PBST). Histone H3 (7074S) and c-Jun (Cell Signaling) primary antibodies were diluted 1:1000 in PBST containing 5% NFDM and 5% BSA, respectively. The H3 primary antibody was purchased from Cell Signaling (Danvers, MA). c-Jun antibodies were purchased from Santa-Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling. Anti-rabbit IgG HRP-linked secondary antibody was diluted 1:2000 in 0.05% PBST containing 2% of the blocking protein and was purchased form 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 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

Activation of Nrf2 by CDDO-Im inhibits early IFNγ production by activated T cells

We previously demonstrated that activation of Nrf2 by tBHQ skews $CD4^+$ T cells toward Th2 differentiation, whereas absence of Nrf2 results in Th1-skewing[282]. $CD4^+$ T cell differentiation is a process that generally occurs several days after T cell activation and little is known about the role of Nrf2 on early T cell activation. Thus, the purpose of the present studies was to determine the role of Nrf2 in early events following T cell activation. The current studies demonstrate that CDDO-Im markedly inhibits IFNγ secretion in anti-CD3/anti-CD28-activated T cells from wild-type mice (Fig. 5), similar to the effects of tBHQ on IFNγ reported in our previous studies[282]. Also similar to previous observations, activated T cells derived from Nrf2-null mice produced significantly more IFNγ than those derived from wild-type mice at early time-points. The highest concentration of CDDO-Im caused a modest, though statistically significant, inhibition of IFNγ secretion. Collectively, the current studies suggest that activation of Nrf2 inhibits early IFNγ production following T cell stimulation.

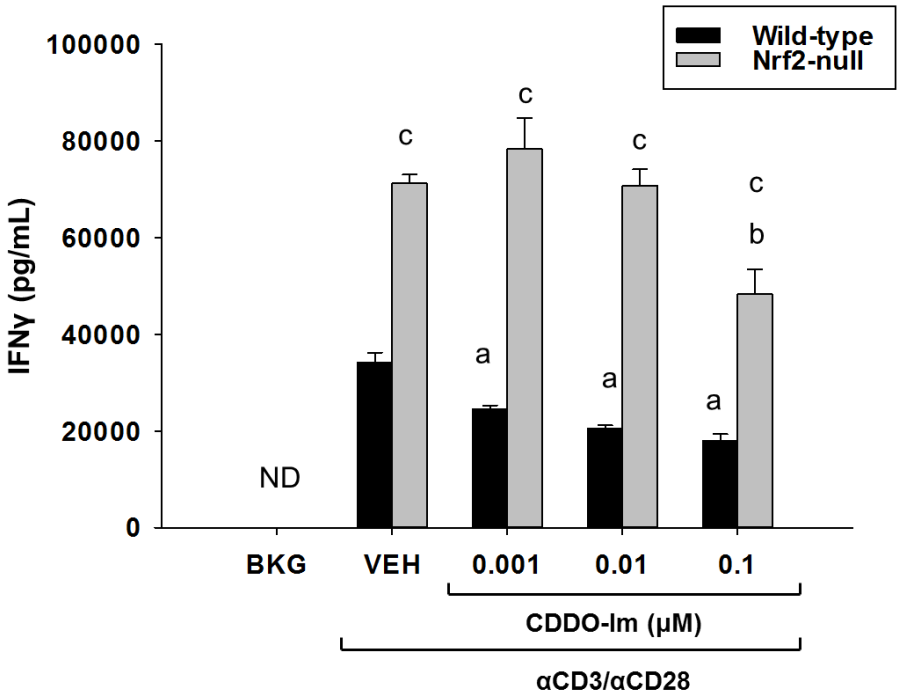

Figure 5. The Nrf2 activator CDDO-Im inhibits IFNγ secretion in activated mouse T cells.
Splenocytes from Nrf2-null and wild-type mice were isolated from whole spleens and were either left untreated (BKG) or pretreated with either vehicle (VEH, 0.01% DMSO) or CDDO-Im for 30 min prior to activation with the T cell activator, anti-CD3/anti-CD28. After 24 h, the cell supernatants were collected and IFNγ production was quantified by sandwich ELISA. 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 synthetic food preservative tBHQ inhibits IL-2 secretion independently of Nrf2 while the triterpenoid CDDO-Im promotes IL-2 secretion in a Nrf2-dependent manner

Because activation of Nrf2 by tBHQ and CDDO-Im inhibited early IFNγ production by activated T cells, we investigated the effects of both Nrf2 activators on IL-2, which is also produced at early time-points after T cell activation. Similar to IFNγ, tBHQ significantly inhibited IL-2 secretion by wild-type anti-CD3/anti-CD28-activated T cells (Fig. 6a). The decrease in IL-2 production by tBHQ was also observed in Nrf2-null

T cells however, suggesting that the effect is independent of Nrf2. In contrast, wild-type, but not Nrf2-null, T cells treated with CDDO-Im showed a concentration-dependent increase in IL-2 secretion at 24 h (Fig. 6b). Overall, the data demonstrate that activation of Nrf2 promotes IL-2 production.

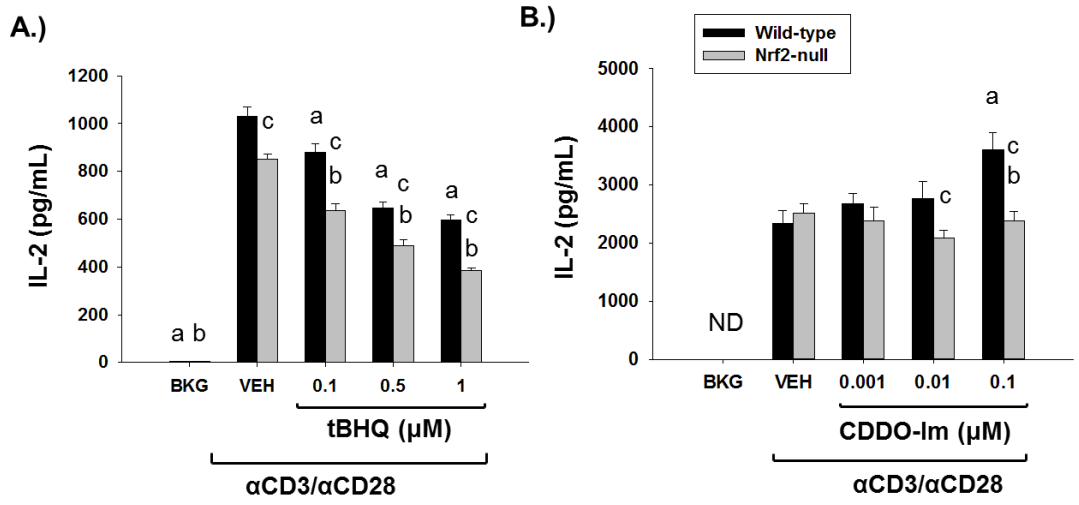

Figure 6. The Nrf2 activators CDDO-Im and tBHQ have differential effects on IL-2 secretion.
Freshly isolated splenocytes from Nrf2-null and wild-type mice were either left untreated (BKG) or were pretreated with vehicle (VEH, 0.01% ethanol for tBHQ or 0.01% DMSO for CDDO-Im), tBHQ, or CDDO-Im for 30 min prior to activation with anti-CD3/anti-CD28. After 24 h, the cell supernatants from A.) tBHQ treated splenocytes or B.) CDDO-Im treated splenocytes was collected and IL-2 was quantified by sandwich ELISA 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.

tBHQ inhibits, and CDDO-Im promotes, GM-CSF secretion in a Nrf2-independent manner

Although not known for driving the polarization of CD4 T cells toward a Th2 phenotype, the early cytokine GM-CSF has been shown to be highly proinflammatory. Therefore, we investigated the effect of the Nrf2-activators CDDO-Im and tBHQ in the

regulation of GM-CSF, as Nrf2 has been shown to be important in regulating inflammation. Treatment of wild-type and Nrf2-null splenocytes with tBHQ resulted in a Nrf2-independent suppression of GM-CSF, as both genotypes appear to be equally suppressed (Fig. 7a). Conversely, treatment of splenocytes with the synthetic triterpenoid CDDO-Im, resulted in a modest Nrf2-independent increase in the secretion of GM-CSF (Fig. 7b). These data further demonstrate the differential effects of tBHQ and CDDO-Im on immune endpoints.

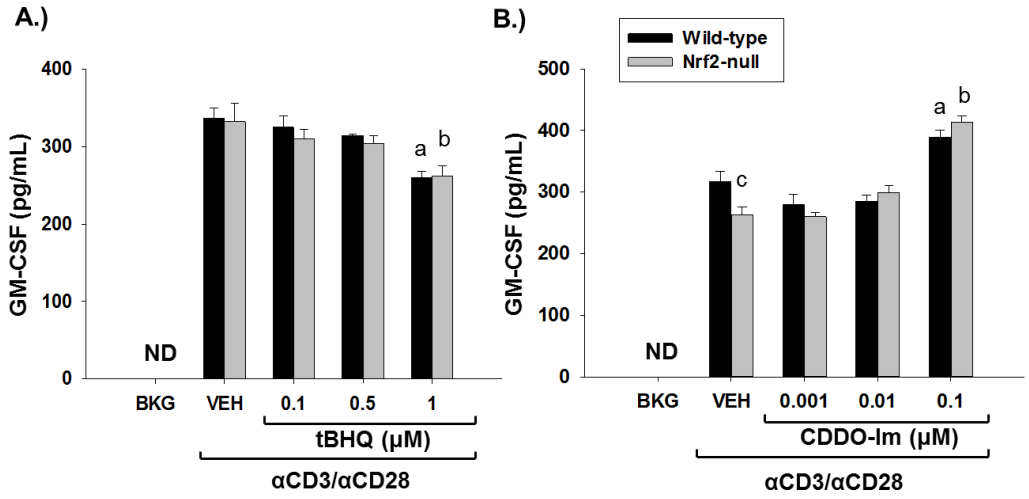

Figure 7. The Nrf2 activators CDDO-Im and tBHQ have differential effects on GM-CSF secretion.
Freshly isolated splenocytes from Nrf2-null and wild-type mice were either left untreated (BKG) or were pretreated with vehicle (VEH, 0.01% ethanol for tBHQ or 0.01% DMSO for CDDO-Im), tBHQ, or CDDO-Im for 30 min prior to activation with anti-CD3/anti-CD28. After 24 h, the cell supernatants from A.) tBHQ-treated splenocytes or B.) CDDO-Im treated splenocytes were collected and GM-CSF was quantified by sandwich ELISA. 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.

CDDO-Im induces TNFα secretion independently of Nrf2, whereas tBHQ inhibits TNFα secretion in a Nrf2-dependent manner

To further characterize the role of Nrf2 in early cytokine production, we assessed the effects of tBHQ and CDDO-Im on TNFα induction. Similar to the effects upon GM-CSF, tBHQ and CDDO-Im had differential effects on TNFα production. Treatment of wild-type and Nrf2-null splenocytes with CDDO-Im resulted in a modest Nrf2-independent induction of TNFα secretion (Fig. 8b). In contrast, treatment of splenocytes with tBHQ resulted in a Nrf2-dependent suppression of TNFα secretion (Fig. 8a). Furthermore, Nrf2-null splenocytes produced more TNFα than wild type cells, which is consistent with what has been previously reported[304]. Overall, these studies demonstrate that Nrf2 inhibits TNFα secretion.

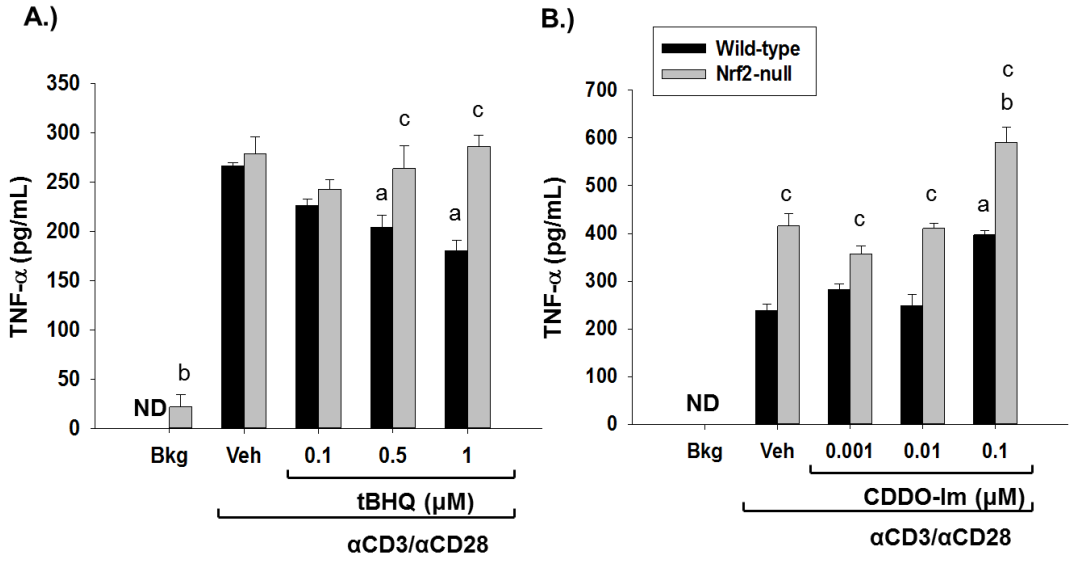

Figure 8. The Nrf2 activators CDDO-Im and tBHQ have differential effects on TNFα secretion.
Freshly isolated splenocytes from Nrf2-null and wild-type mice were either left untreated (BKG) or were pretreated with vehicle (VEH, 0.01% ethanol for tBHQ or 0.01% DMSO for CDDO-Im), tBHQ, or CDDO-Im for 30 min prior to activation with anti-CD3/anti-CD28. After 24 h, the cell supernatants from A.) tBHQ-treated splenocytes or B.)

Figure 8 (cont'd)
CDDO-Im treated splenocytes were collected and TNFα was quantified by sandwich ELISA. 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 Nrf2 activators tBHQ and CDDO-Im have little effect on the induction of CD25 and CD69 in activated mouse splenocytes

The differential effects of Nrf2 on early cytokine production by activated T cells led us to investigate the role of Nrf2 in other early events associated with T cell activation. In addition to increased cytokine production, T cells rapidly upregulate expression of the cell surface proteins CD25 and CD69 upon activation. In contrast to cytokine production, tBHQ and CDDO-Im had no effect on expression of CD25 or CD69 (Figs. 9-10). Collectively, the data demonstrate that Nrf2 plays a negligible role in the regulation of expression of the early T cell activation markers CD25 and CD69.

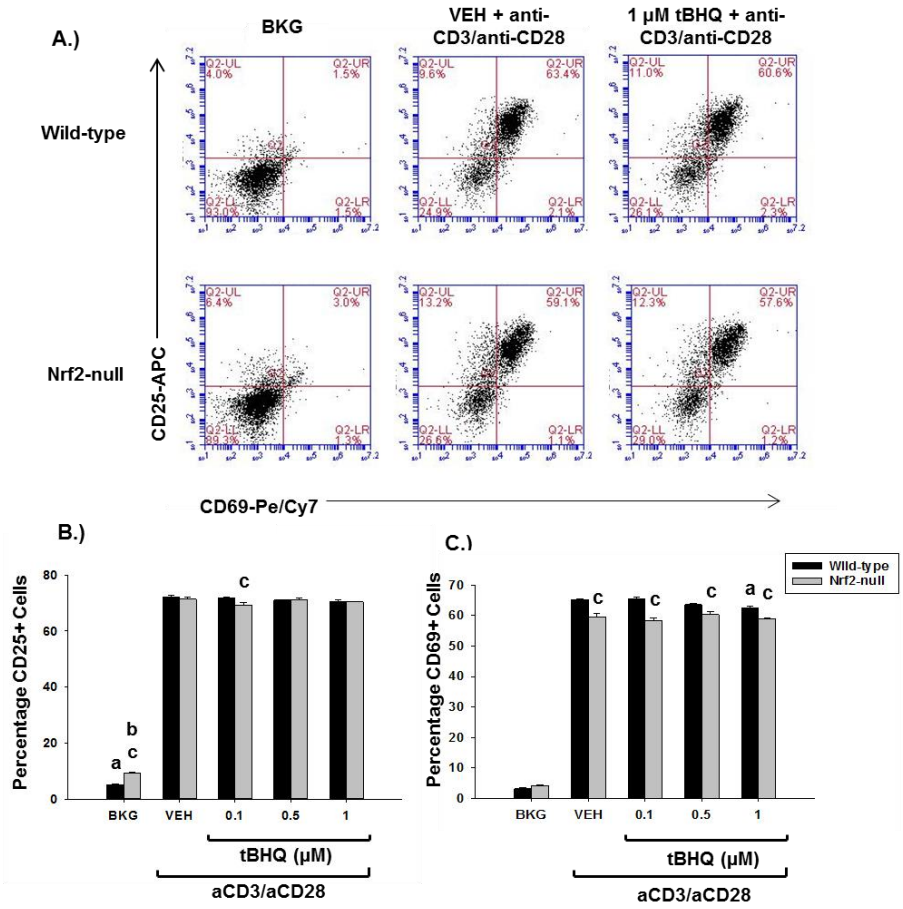

Figure 9. The Nrf2 activator tBHQ has little effect on CD25/CD69 expression in activated T cells.

Freshly isolated splenocytes from wild-type or Nrf2-null mice were left untreated (BKG) or pretreated with either vehicle (VEH, 0.01% ethanol) or tBHQ (0.1-1 µM) for 30 min prior to activation with anti-CD3/anti-CD28. A.) 24 h after activation, cells were collected, labeled with anti-CD4/FITC, anti-CD69/PECy7 and anti-CD25/APC and analyzed by flow cytometry. CD25/CD69 fluorescence was quantified in the gated $CD4^+$ T cell population. Six representative dot plots are shown. B.) Graphical representation of CD25 expression. C.) Graphical representation of CD69 expression. a = $p<0.05$ versus wild-type VEH, b = $p<0.05$ versus Nrf2-null VEH, c = $p<0.05$ between the wild-type and Nrf2-null genotypes.

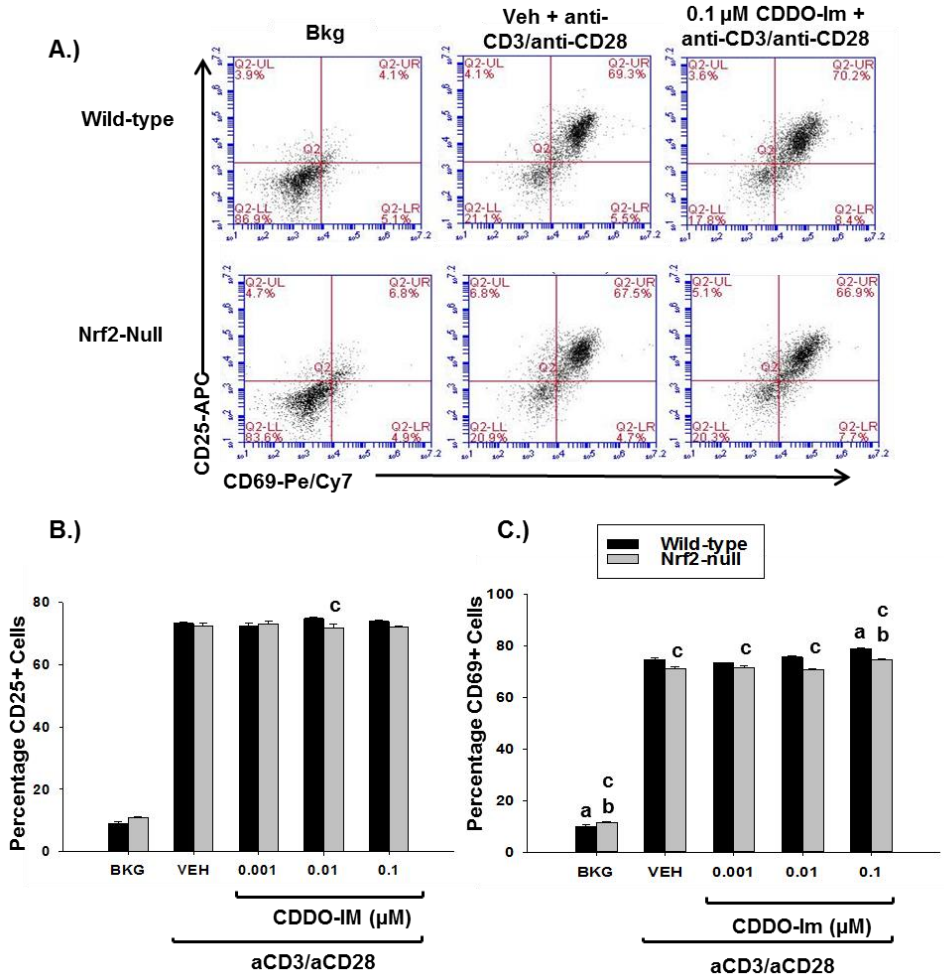

Figure 10. The Nrf2 activator CDDO-Im has little effect on CD25/CD69 expression in activated T cells.

Freshly isolated splenocytes from wild-type or Nrf2-null mice were left untreated (BKG) or pretreated with either vehicle (VEH, 0.01% DMSO) or CDDO-Im (0.001-0.1 µM) for 30 min prior to activation with anti-CD3/anti-CD28. A.) 24 h after activation, cells were collected, labeled with anti-CD4/FITC, anti-CD69/PECy7 and anti-CD25/APC and analyzed by flow cytometry. CD25/CD69 fluorescence was quantified in the gated $CD4^+$ T cell population. Six representative dot plots are shown. B.) Graphical representation of CD25 expression by flow cytometry. C.) Graphical representation of CD69 expression by flow cytometry. 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

Inhibition of NFκB DNA binding by CDDO-Im and tBHQ is Nrf2-dependent and – independent, respectively

Previous studies have shown cross-talk between the Nrf2 and the NFκB signaling pathways, whereby activation of NFκB is inhibited by Nrf2 through suppression of its activating kinase, inhibitor of kappa B kinase. In Jurkat T cells, we have previously shown that the Nrf2 activator tBHQ suppresses NFκB luciferase activity. Accordingly, we assessed the effects of Nrf2 activation by tBHQ and CDDO-Im in the DNA binding activity of p65, a major component of the NFκB signaling complex. tBHQ inhibited NFκB p65 DNA binding activity in both wild-type and Nrf2-null primary splenocytes, suggesting the effect is Nrf2-independent (Fig. 11a). The Nrf2-independent suppression of NFκB by tBHQ was consistent with its effect on IL-2 and GM-CSF secretion. In contrast, CDDO-Im inhibited p65 DNA binding in wild-type, but not Nrf2-null, splenocytes, suggesting that the effect of CDDO-Im is Nrf2-dependent. Taken together, these data suggest that although both compounds are Nrf2 activators, they are likely exerting their effects by different mechanisms (Fig. 11b).

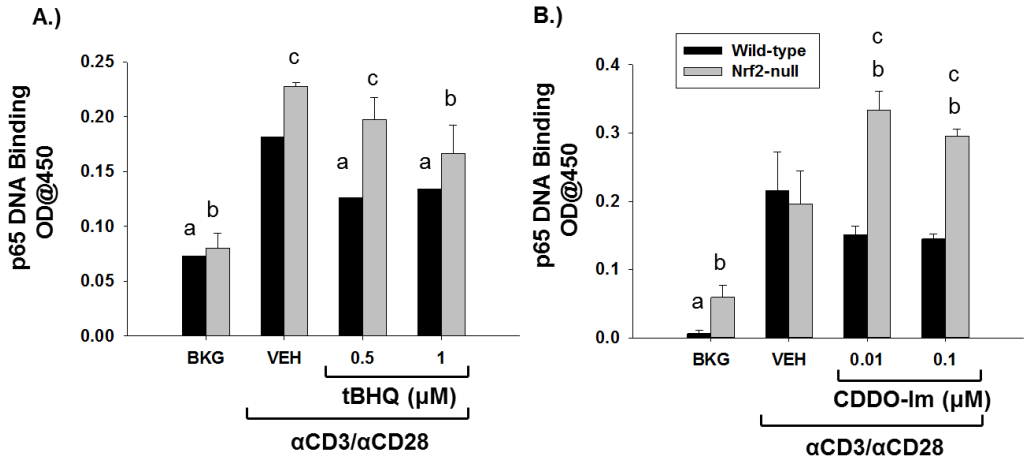

Figure 11 (cont'd)

Figure 11. Suppression of p65 DNA binding by tBHQ is Nrf2-independent whereas suppression by CDDO-Im is Nrf2-dependent.

Freshly isolated splenocytes isolated from wild-type and Nrf2-null mice were either left untreated (BKG) or were pretreated with vehicle (VEH, 0.01% ethanol for tBHQ or 0.01% DMSO for CDDO-Im), tBHQ, or CDDO-Im for 30 min prior to activation with anti-CD3/anti-CD28. After 4 h, nuclear proteins were extracted from either A.) tBHQ-treated splenocytes or B.) CDDO-Im treated splenocytes and p65 DNA binding was quantified by a commercially-available kit. 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 Nrf2 activators tBHQ and CDDO-Im increase c-Jun nuclear accumulation in a Nrf2-dependent manner

It has been previously shown that Nrf2 can partner with and potentiate AP-1 signaling in some models, which has potential implications in immune endpoints, as AP-1 is known to drive many immune functions, including the transcription of IL-2[303,305]. We therefore analyzed the nuclear accumulation of c-Jun, an AP-1 family member, in splenocytes that were treated with either tBHQ or CDDO-Im by PAGE and Western blot analysis. The resulting blots showed that activated splenocytes derived from Nrf2-null mice had markedly less nuclear accumulation of c-Jun as compared to wild-type (Fig. 12, Fig. 13). Furthermore, treatment of splenocytes with tBHQ resulted in a trend towards a Nrf2-dependent increase in c-Jun nuclear accumulation (Fig. 12). Similarly, treatment of wild-type and Nrf2-null splenocytes with CDDO-Im also resulted in a trend towards a Nrf2-dependent increase in nuclear accumulation of c-Jun (Fig. 13). Taken together, these data suggest that Nrf2 activity promotes c-Jun nuclear accumulation in activated T cells, as loss of Nrf2 reduces nuclear c-Jun accumulation and activation of Nrf2 results in a trend towards increased nuclear c-Jun.

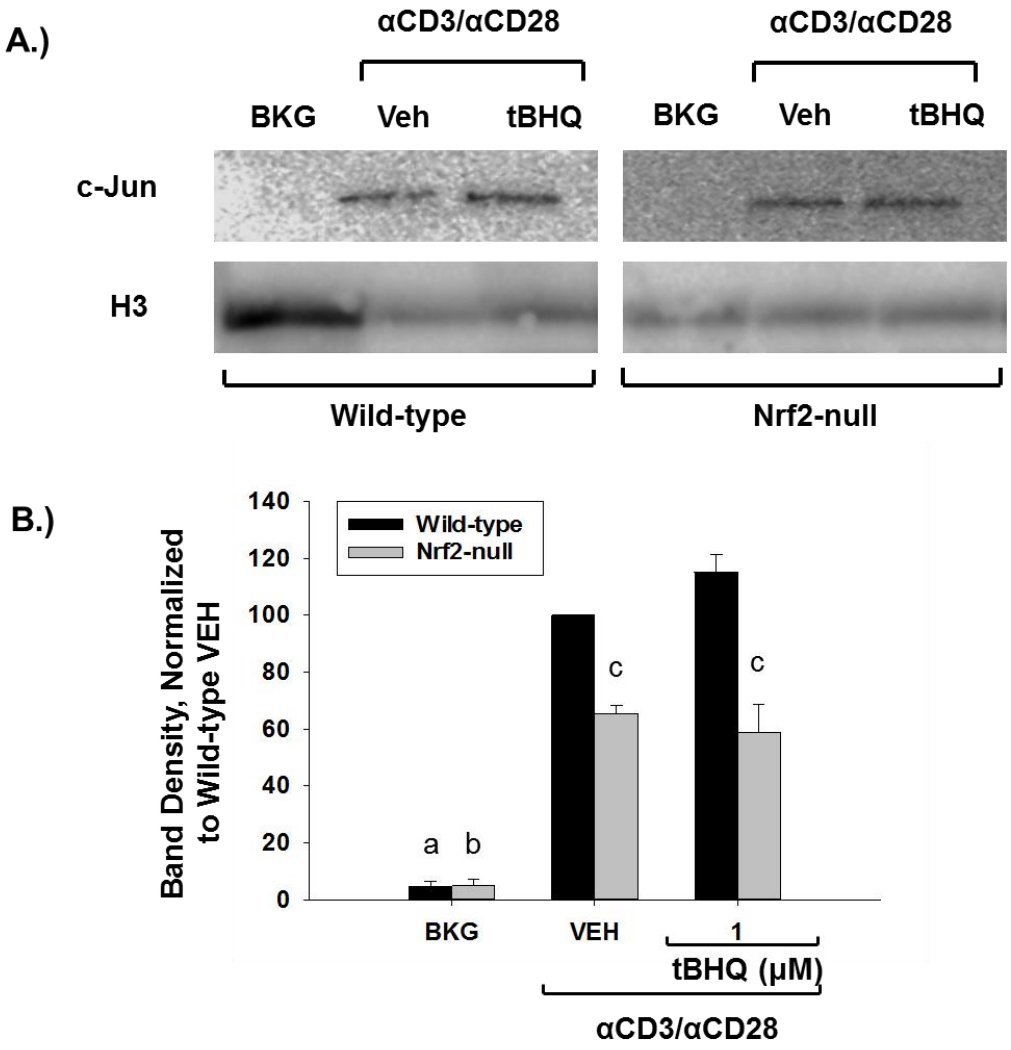

Figure 12. tBHQ increases nuclear accumulation of c-Jun in wild-type, but not Nrf2-null, primary mouse splenocytes.

Wild-type and Nrf2-null mouse splenocytes were left untreated (BKG) or were pretreated with vehicle (VEH, 0.01% ethanol) or tBHQ (1 μM) for 30 min prior to activation with anti-CD3/anti-CD28. After 4 h nuclear protein was then isolated and expression of c-Jun and the housekeeper histone H3 was probed by western analysis. (A) Representative western blot. Wild-type and Nrf2-null samples are from the same blot, and have been separated for clarity. (B) Densitometry of the resulting western blots was calculated as a ratio of the c-Jun band to the H3 band, and then normalized for each replicate as a percent of the wild-type vehicle value. Results are depicted as averages of the normalized values from four experiments. a = $p<0.05$ versus wild-type VEH, b = $p<0.05$ versus Nrf2-null VEH, c = $p<0.05$ between the wild-type and Nrf2-null genotypes.

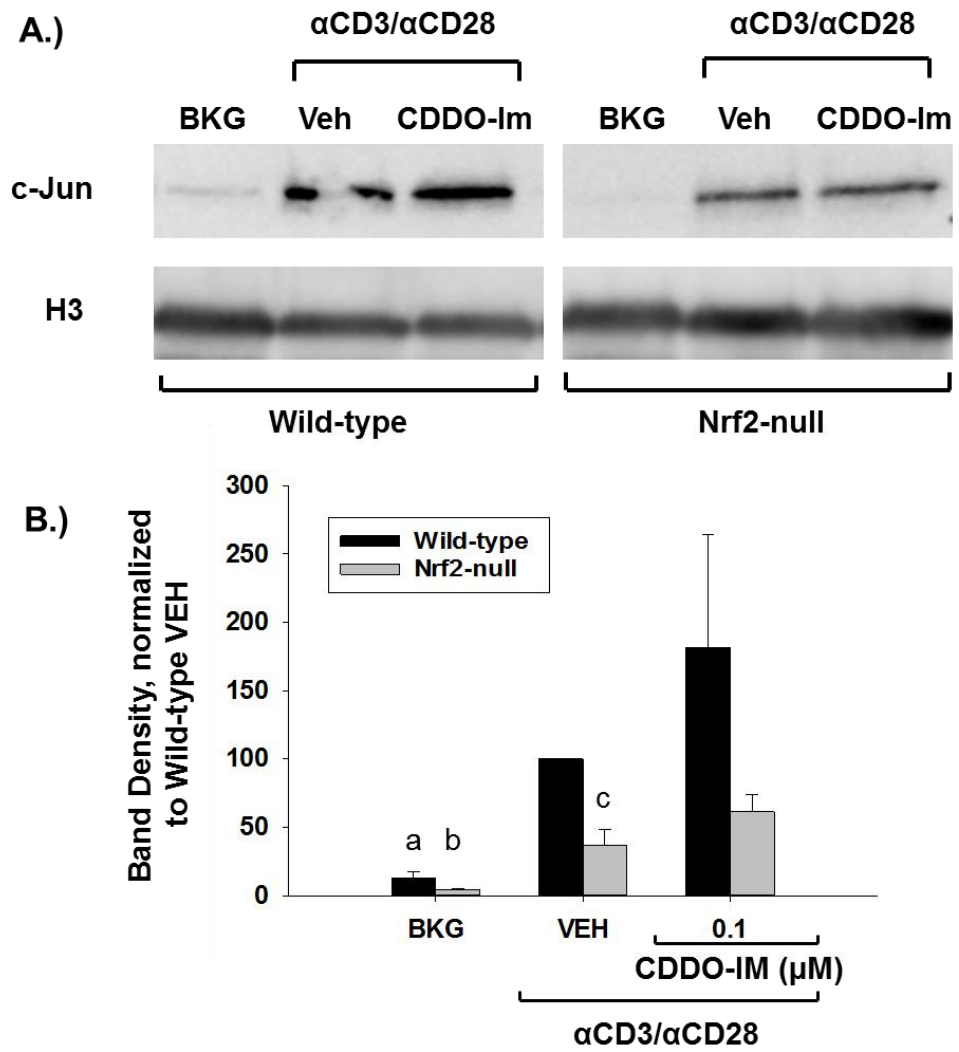

Figure 13. CDDO-Im increases nuclear accumulation of c-Jun in wild-type, but not Nrf2-null, primary mouse splenocytes.

Wild-type and Nrf2-null mouse splenocytes were left untreated (BKG) or were pretreated with vehicle (VEH, 0.01% DMSO) or CDDO-Im (0.1 μM) for 30 min prior to activation with anti-CD3/anti-CD28. After 4 h nuclear protein was then isolated and expression of c-Jun and the housekeeper histone H3 was probed by western analysis. (A) Representative western blot. Wild-type and Nrf2-null samples are from the same blot, and have been separated for clarity. (B) Densitometry of the resulting western blots was calculated as a ratio of the c-Jun band to the H3 band, and then normalized for each replicate as a percent of the wild-type vehicle value. Results are depicted as averages of the normalized values from four experiments. 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

Our previous studies demonstrated that activation of Nrf2 results in skewing of $CD4^+$ T cells toward Th2 differentiation[282]. However, these studies did not address the role of Nrf2 in early T cell activation. Thus, the purpose of the present studies was to determine the role of Nrf2 in early events following T cell activation. The data demonstrate that Nrf2 inhibits early IFNγ production, but has no effect on activation-induced CD25 or CD69 expression. Treatment of wild-type and Nrf2-null splenocytes with tBHQ decreased IL-2 production in both genotypes, suggesting the effect of the synthetic food preservative tBHQ on IL-2 secretion is independent of Nrf2. Interestingly, the triterpenoid, CDDO-Im, caused a concentration-dependent increase in IL-2 secretion in wild-type, but not Nrf2-null, splenocytes. Thus, the differential effects of CDDO-Im and tBHQ upon the secretion of IL-2 reflect differences in mechanism of action. Indeed, tBHQ and CDDO-Im had differential effects on the induction of all the early cytokines we analyzed, with the exception of IFNγ, which was decreased by both Nrf2 activators. The differential effects of tBHQ and CDDO-Im on cytokine production underscores the capacity of these Nrf2 activators for off-target effects in activated T cells. In general, tBHQ decreased early cytokine production, whereas CDDO-Im tended to increase cytokine production with the exception of IFNγ, which was decreased.

Overall, the data suggest that rather than simply turning T cell activation on or off, Nrf2 plays a more complex role in modulating T cell function. Nrf2 has no effect on the expression of CD25 or CD69, inhibits secretion of the Th1 cytokines, IFNγ and TNFα, and promotes early production of IL-2, which has been shown to promote Th2 differentiation[65,306]. In addition, other studies have implicated Nrf2 in the promotion of

Th2 differentiation in both mouse and human models[282,307]. For example, the Nrf2 activator dimethyl fumarate, which is used to treat multiple sclerosis, has been shown to promote Th2 cytokine production[308,309]. Taken together, the findings of the current study suggest that the effect of Nrf2 activation on the early events following T cell activation support the later occurrence of Th2 polarization.

The transcription factor AP-1 plays a critical role in driving transcription of many immune endpoints, including the proinflammatory cytokines IFNγ, TNFα, and GM-CSF. Interestingly, nuclear accumulation of c-Jun was decreased in Nrf2-null splenocytes as compared to wild-type splenocytes, and treatment of with both tBHQ and CDDO-Im resulted in a Nrf2-dependent trend towards an increase in the nuclear accumulation of c-Jun, a key member of the AP-1 family of proteins. Although it seems logical that an increase in c-Jun nuclear accumulation should result in a subsequent increase in AP-1 signaling, this may not be the case. It has been previously shown that some AP-1 family members, such as Fra-1, can partner with activated Nrf2 and serve to inhibit Nrf2 signaling through the ARE, after being bound by Nrf2[115]. Knowing this, it is plausible that a similar mechanism may be functioning on AP-1 binding sites, resulting in the suppression of target gene transcription.

Similar to AP-1, NFκB is a transcription factor that drives gene transcription through the IL-2 promoter. Previous studies have shown cross-talk between the Nrf2 and NFκB signaling pathways, which results in a Nrf2-dependent suppression of NFκB activation, by inhibition of inhibitor of kappa B kinase[214,310] Therefore, the present studies investigated the effects of the Nrf2 activators CDDO-Im and tBHQ upon NFκB DNA binding. The results demonstrate a Nrf2-independent suppression of NFκB DNA

binding by tBHQ. This finding was unexpected, as the literature has shown activation of Nrf2 to suppress NFκB function. Given the electrophilic properties of tBHQ, it likely has a complex mechanism of action. In addition to activating Nrf2, it is possible that tBHQ directly interacts with NFκB and thereby suppresses it, as has been shown with a similar antioxidant, BHA[311]. In contrast to tBHQ, CDDO-Im-induced suppression of NFκB is Nrf2-dependent. These findings, coupled with the Nrf2-dependent increase in the secretion of IL-2, are interesting, as NFκB is an important positive regulator of the IL-2 promoter. According to sequence analysis by the ECR Browser (www.ecrbrowser.dcode.org), the promoter region of the mouse IL-2 gene contains at least one Nrf2 binding site. It is possible that the increase in IL-2 secretion may be due to Nrf2 directly binding to the IL-2 promoter. The partial inhibition of NFκB by CDDO-Im may not be a robust enough suppression to negatively impact IL-2 induction. Taken together, the data show that the mechanism of action of CDDO-Im and tBHQ is complex and operates through multiple signaling pathways, including NFκB, c-Jun, and Nrf2.

The increase in IL-2 production by Nrf2 may appear to conflict with the notion that Nrf2 plays a protective inhibitory role within the immune system, preventing inappropriate induction of pro-inflammatory immune responses. However, unlike IFNγ and IL-17, IL-2 does not necessarily play a pro-inflammatory role in most immune responses. Whereas IFNγ, for example, is most notably associated with Th1 responses, which are often pro-inflammatory, IL-2 plays a complex role in T cell activity. IL-2 is a T cell growth factor that contributes to T cell proliferation following activation and is key to the induction of an effective T cell response[312]. However, IL-2 is also vital for the differentiation and function of Treg cells. Treg cells are $CD4^+$ T cells that

function to control and/or suppress the proliferation and function of effector T cells, which are responsible for launching and maintaining immune responses. Treg cells, therefore, play a protective regulatory role within the immune system to maintain peripheral tolerance and prevent the development of autoimmunity. Thus, promotion of IL-2 production by Nrf2 is consistent with a protective, anti-inflammatory role. Furthermore, the literature shows that an early increase in IL-2 production promotes the development of Th2 cells. Taken together, the effects of Nrf2 on early cytokine production further support a role for Nrf2 in Th2 polarization[65,313].

Overall, this study shows Nrf2 has differential effects on the early events of T cell activation. Whereas Nrf2 inhibits early TNFα and IFNγ production, it promotes IL-2 production, and has no effect on induction of CD25 or CD69. Importantly, this is the first paper to report that activation of Nrf2 increases early IL-2 production by activated T cells. Furthermore, the Nrf2-mediated inhibition of the Th1 cytokine, IFNγ (which suppresses Th2 differentiation), coupled with enhanced early production of IL-2, which is known to promote Th2 differentiation, suggest that the effects of Nrf2 on Th2 differentiation may occur earlier during polarization than previously thought. Collectively, these studies suggest an important and novel role for Nrf2 in regulating T cell activation, differentiation and cytokine production.

Chapter 3

The Nrf2 activator tBHQ inhibits T cell activation of primary human CD4 T cells

The work presented in the chapter has been published as:

Turley, A. E. *et al*. The Nrf2 activator tBHQ inhibits T cell activation of primary human CD4 T cells. Cytokine **71**, 289–295 (2015).

Abstract

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) regulates a battery of antioxidant, detoxification, and cell stress genes. It is activated by oxidative stress and a number of exogenous compounds, including *tert*-butylhydroquinone (tBHQ), a widely used food preservative, and 1[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im). Nrf2 modulates immune responses in numerous rodent models of inflammation, but its effects on human immune cells are not well characterized. The purpose of these studies was to evaluate the effects of the Nrf2 activators tBHQ and CDDO-Im on early events of T cell activation in primary human cells. Treatment with tBHQ induced mRNA expression of the Nrf2 target genes HMOX-1, GCLC, and NQO1, and also increased NRF2 mRNA expression, albeit to a lesser extent than the other target genes. tBHQ decreased production of the cytokines IL-2 and IFN-γ at both the protein and mRNA levels after stimulation with anti-CD3/anti-CD28 in human peripheral blood mononuclear cells and to an even greater extent in isolated CD4 T cells. Likewise, tBHQ decreased induction of CD25 and CD69 in peripheral blood mononuclear cells and this decrease was even more marked in isolated CD4 T cells. CDDO-Im also inhibited production of IL-2 and IFNγ, and inhibited induction of CD25 and CD69 in isolated CD4 T cells. In addition, tBHQ inhibited induction of NFκB DNA binding in anti-CD3/anti-CD28-activated PBMCs. Collectively, these data suggest that tBHQ and CDDO-Im inhibit activation of primary human CD4 T cells, which correlates with activation of Nrf2 and inhibition of NFκB DNA binding. Although these studies suggest the food additive tBHQ negatively impacts T cell

activation, further studies will be needed to fully elucidate the effect of tBHQ on human immune response.

Introduction

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that acts as a sensor for oxidative stress. Under basal conditions, Nrf2 is tethered in the cytosol to its repressor protein, Kelch-like ECH-associated protein 1 (Keap1), which facilitates the ubiquitination and subsequent proteasomal degradation of Nrf2[292]. After stimulation by reactive oxygen species or electrophilic stimuli, Nrf2 ubiquitination is disrupted so that Nrf2 translocates to the nucleus. Upon heterodimerizing with small Maf proteins or other binding partners, Nrf2 binds to antioxidant response elements to regulate the transcription of a number of detoxification, antioxidant, and cell stress-related genes[116,314]. One activator of Nrf2 is *tert*-butylhydroquinone (tBHQ), a commonly used food additive found in a number of processed foods[227,291,315]. Another Nrf2 activator commonly used experimentally is 1[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), which activates Nrf2 at nanomolar concentrations, but also activates other pathways at micromolar concentrations[232].

Nrf2 activation has been shown to have anti-inflammatory effects, and conversely, Nrf2 deletion has been shown to have pro-inflammatory effects[276,316,317]. Nrf2-null mice develop a lupus-like autoimmune disease, and have increased sensitivity to inflammation and infection in models such as sepsis and lung injury[205,239,241,242,252]. Recently, our laboratory demonstrated that Nrf2 modulates T cell responses in primary mouse CD4+ T cells and Jurkat T cells[282,318]. Collectively, this indicates that Nrf2

modulates immune responses in a variety of different models and cell types. However, the role of Nrf2 in the activation of primary human T cells remains unclear.

T cells are a critical part of the adaptive immune response. Helper ($CD4^+$) T cells direct the immune response to different pathogens; thus T cell activation is critical for an effective adaptive immune response. T cell activation is characterized by a number of early events which have important downstream effects, including production of early cytokines, such as IL-2 and IFNγ, and upregulation of CD25 and CD69. IL-2 acts in an autocrine/paracrine fashion to help drive the proliferation and clonal expansion of naïve T cells, among other functions[303]. IFNγ is the signature cytokine produced by Th1 cells and is important in driving cell-mediated immunity[319]. CD25 is the high affinity subunit of the IL-2 receptor, whereas CD69 is a C-type lectin protein. Both CD25 and CD69 are considered cell surface markers of T cell activation[320]. Several transcription factors have been shown to be important in T cell activation, including NFAT, NFκB, and AP-1[303,321]. Our previous studies demonstrated that the Nrf2 activator tBHQ inhibits IL-2 secretion, CD25 expression, and NFκB induction in activated Jurkat cells[318]. The purpose of the present studies was to determine the effects of the Nrf2 activator tBHQ on the early events following T cell activation in primary human cells.

Materials and Methods

Peripheral Blood Mononuclear Cell (PBMC) and CD4 T Cell Isolation

Whole human blood was purchased from Innovative Research (Novi, MI). PBMCs were isolated using Lymphocyte Separation Medium following the manufacturer's protocol (MP Biomedicals, Santa Ana, CA). CD4 T cells were isolated

from PBMCs by positive selection using commercially available magnetic bead separation (Miltenyi Biotec, Auburn, CA). Cell treatments are described in figure legends. T cells were activated with purified hamster anti-human CD3e (clone UCHT1, 1.5 µg/ml), purified hamster anti-human CD28 (clone CD28.2, 1.5 µg/ml), and an $F(ab')_2$ fragment specific for anti-Syrian hamster IgG that was used to cross-link CD3 and CD28. Anti-CD3 and anti-CD28 were purchased from Affymetrix/E-Bioscience (San Diego, CA), and the $F(ab')_2$ cross-linker from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cytokine analysis

Concentrations of IL-2 or IFN-γ were quantified in cell supernatants using commercially available human IL-2 or IFN-γ ELISA kits following the manufacturer's protocol (Biolegend, San Diego CA). Absorbance was quantified on a Bio-Tek µQuant microplate reader (Highland Park, VT).

mRNA quantification

Total RNA was isolated using TRIzol (Ambion, Life Technologies, Grand Island, NY) extraction according to the manufacture's protocol. RNA was reverse transcribed to cDNA after which quantitative-real time PCR was performed using Sybr green analysis (Applied Biosystems, Life Technologies). Fluorescence was detected by a Life Technologies/Applied Biosystems Sequence Detection System 7500, and relative transcript levels were quantified using the ΔΔCT method, comparing the target genes to ribosomal protein L13a. Primer sequences were acquired from qPrimerDepot

(http://primerdepot.nci.nih.gov/) and are as follows: RPL13A forward primer, 5'GTTGATGCCTTCACAGCGTA-3' and reverse primer, 5'-AGATGGCGGAGGTGCAG-3'; IL-2 forward primer, 5'-GCACTTCCTCCAGAGGTTTG-3' and reverse primer 5'-TCACCAGGATGCTCACATTT-3'; IFN-γ forward primer 5'-TCAGCCATCACTTGGATGAG-3' and reverse primer 5'-CGAGATGACTTCGAAAAGCTG-3'; CD69 forward primer, 5'-ACAGGAACTTGGAAGGACCC-3' and reverse primer, 5'-AGAACAGCTCTTTGCATCCG-3'; CD25 forward primer, 5'-TAGGCCATGGCTTTGAATGT-3' and reverse primer, 5'-ATACCTGCTGATGTGGGGAC-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'; GCLC forward primer, 5'-CTTTCTCCCCAGACAGGACC-3' and GCLC reverse primer 5'-CAAGGACGTTCTCAAGTGGG-3' All primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Flow Cytometry

PBMCs were labeled with CD4-FITC (Affymetrix/E-Bioscience), CD69-PE/Cy7 (Biolegend), and CD25-APC (Affymetrix/E-Bioscience) for 30 min in the presence of an FcR blocking reagent (Miltenyi Biotec, Auburn, CA) and then washed. Fluorescence

was detected by a C6 BD Accuri flow cytometer (BD Accuri, San Jose, CA). Fluorescence was quantified using CFlow software (BD Accuri).

ELISA-based DNA binding assay

PBMCs or CD4 T cells were treated with tBHQ (1-5 µM) or vehicle, and then activated with anti-CD3/anti-CD28 30 min later. Three hours after activation, nuclear protein was extracted from $1x10^7$ cells using a commercially available kit (Active Motif, Carlsbad, CA). After extraction, nuclear protein was quantified via the Bradford assay (BioRad). NFκB DNA binding was quantified from 10 µg of nuclear protein, by a commercially available ELISA-based DNA binding assay (Active Motif). Assays were performed per manufacturer's protocol.

Statistical analysis

All data were analyzed using SigmaPlot 12.3 (Systat, Chicago, IL). Data were analyzed by one-way ANOVA followed by a Dunnett's two-tailed post-hoc test. Data are expressed as mean ± standard error. A p-value of less than 0.05 was considered statistically significant.

Results

The food additive tBHQ induces expression of the Nrf2 target genes HMOX-1, NQO1, and GCLC in human peripheral blood mononuclear cells (PBMCs)

tBHQ is a known activator of Nrf2, and accordingly we investigated the ability of our tBHQ treatment (0.1-5 µM) to induce expression of Nrf2 target genes. Consistent

with other cell types, tBHQ treatment increased expression of the Nrf2 target genes, heme oxygenase 1 (HMOX-1), NAD(P)H quinone oxidoreductase 1 (NQO1), and glutamate-cysteine ligase, catalytic subunit (GCLC), in human peripheral blood mononuclear cells (Fig. 14)[282,318]. In addition, tBHQ caused a modest induction of NRF2 mRNA expression itself, which has also been observed previously in primary mouse T cells and suggests that NRF2 upregulates itself. Taken together, these data suggest that the tBHQ concentrations used in this study activate Nrf2 in primary human PBMCs.

The Nrf2 activator tBHQ inhibits production of IL-2 in human PBMCs and isolated CD4 T cells activated with anti-CD3/anti-CD28

Our previous studies showed the Nrf2 activator tBHQ inhibited IL-2 production in activated Jurkat T cells, but the effect of tBHQ on primary human PBMCs has not been fully characterized[318]. Therefore, we determined the effect of tBHQ treatment on IL-2 production by PBMCs activated with anti-CD3/anti-CD28, a T cell specific activator. IL-2 protein and mRNA levels were significantly decreased by tBHQ in PBMCs activated with anti-CD3/anti-CD28 (Fig. 15A, B). Interestingly, tBHQ caused a more marked inhibition of IL-2 production in isolated CD4 T cells activated with anti-CD3/anti-CD28, suggesting that isolated T cells are more sensitive to tBHQ than T cells in the mixed PBMC population (Fig. 15C). However, tBHQ did not affect cell viability at these concentrations (data not shown). These studies demonstrate that tBHQ inhibits IL-2 secretion in primary human PBMCs and CD4 T cells, consistent with previous studies in Jurkat cells.

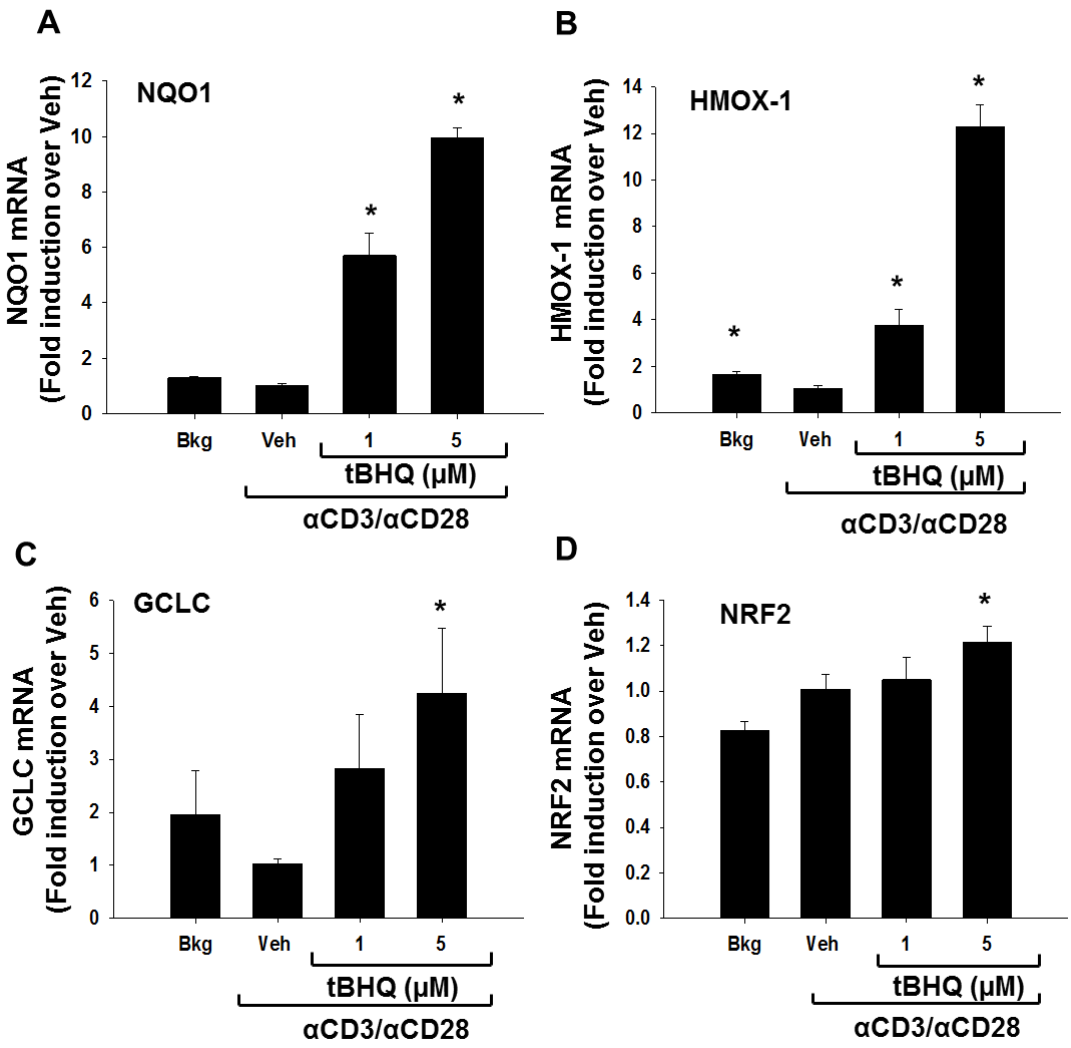

Figure 14. **tBHQ treatment induces expression of the Nrf2 target genes NQO1, GCLC, and HMOX as well as NRF2 expression in PBMCs.**
PBMCs were either left untreated (Bkg), or pretreated with vehicle (Veh, 0.01% ethanol) or tBHQ (1-5 μM) and then activated 30 min later with anti-CD3/anti-CD28. Total RNA was isolated after 6h and mRNA expressions of (A) NQO1, (B) GCLC, (C) HMOX, and (D) NRF2 were quantified by quantitative RT-PCR. * indicates $p<0.05$ compared to the Veh group.

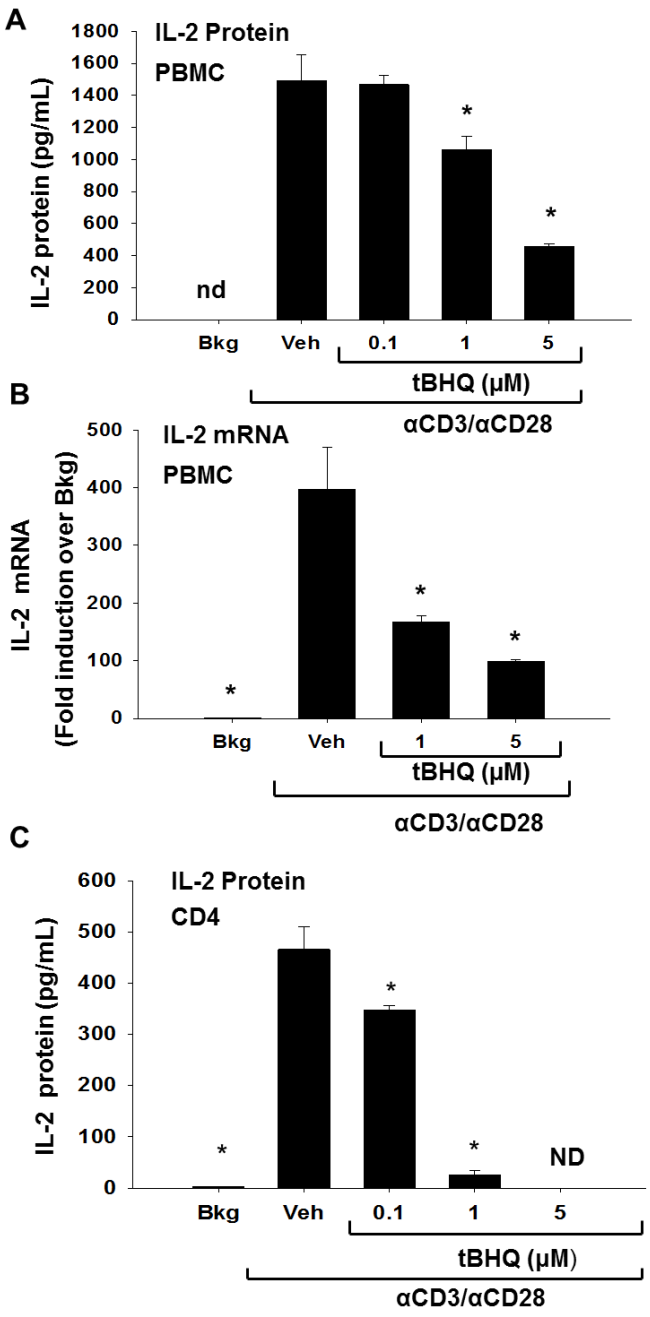

Figure 15. tBHQ treatment inhibits production of the early cytokine IL-2.
(A-B) PBMCs or (C) CD4 cells were either left untreated (Bkg), or pretreated with vehicle (Veh, 0.01% ethanol) or tBHQ (0.1-5 μM) and then activated 30 min later with anti-CD3/anti-CD28. (A, C) Supernatants were collected after 24h and analyzed for IL-2 by ELISA. (B) Total RNA was isolated from PBMCs after 6h and mRNA expression of IL-2 was quantified by quantitative RT-PCR. * indicates $p<0.05$ compared to the Veh group, ND indicates samples below the limit of detection.

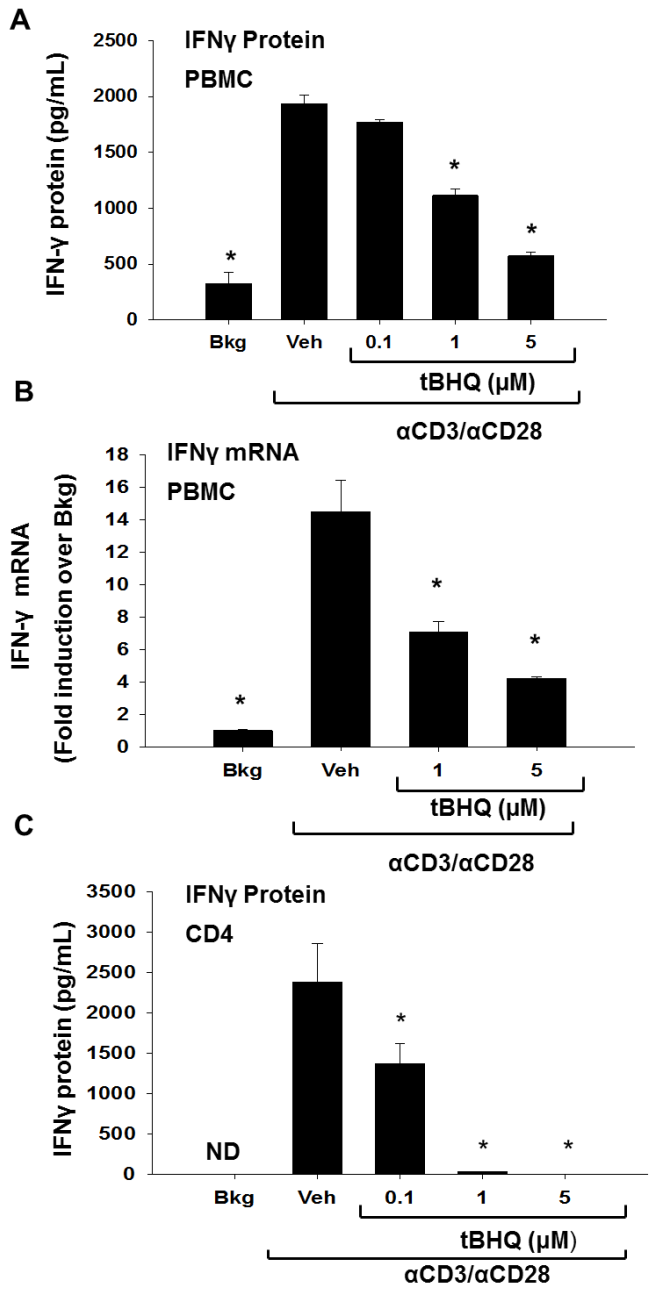

Figure 16. tBHQ treatment inhibits production of the cytokine IFNγ.
A-B) PBMCs or (C) CD4 cells were either left untreated (Bkg), or pretreated with vehicle (Veh, 0.01% ethanol) or tBHQ (0.1-5 μM) and then activated 30 min later with anti-CD3/anti-CD28. (A, C) Supernatants were collected after 24h and analyzed for IFNγ by ELISA. (B) Total RNA was isolated from PBMCs after 6h and mRNA expression IFNγ was quantified by quantitative RT-PCR. * indicates $p<0.05$ compared to the Veh group, ND indicates samples below the limit of detection.

The Nrf2 activator tBHQ inhibits production of IFNγ in human PBMCs and isolated CD4 T cells activated with anti-CD3/anti-CD28

The inhibition of IL-2 production with tBHQ treatment prompted us to investigate the effect of tBHQ on production of IFNγ, which, like IL-2, is rapidly induced in T cells in response to stimulation with anti-CD3/anti-CD28. Similar to its effect on IL-2, tBHQ significantly inhibited IFNγ protein and mRNA production in activated PBMCs (Fig. 16 A, B). Likewise, tBHQ inhibited IFNγ production to an even greater extent in isolated CD4 T cells as compared to T cells in the mixed PBMC population (Fig. 16 C). Collectively, these studies demonstrate that tBHQ inhibits IL-2 and IFNγ secretion in primary human PBMCs and isolated CD4 T cells.

The food additive tBHQ inhibits the expression of CD25 and CD69 in PBMCs and primary CD4 T cells

The inhibition of IL-2 and IFN-γ by tBHQ in anti-CD3/anti-CD28-activated PBMCs prompted us to investigate the effect of tBHQ on other early events following T cell activation. Shortly after T cell activation, the cell surface proteins CD25 and CD69 are induced, so we next quantified CD25 and CD69 expression. CD25 protein expression was significantly decreased compared to vehicle at 1 µM and at 5 µM tBHQ in the PBMCs and at 0.1 µM, 1 µM, and 5 µM in the CD4 T cells (Fig. 17 A and B, and Fig. 18, A and B). Similarly, CD69 protein expression was also decreased by tBHQ in both (Fig. 17 A and C, Fig. 18, A and C). At the mRNA level, CD25 expression was inhibited upon tBHQ treatment in PBMCs (Fig. 17 D). CD69 mRNA expression in the PBMCs showed a trend toward a decrease with tBHQ treatment, but was not statistically significant (Fig.

17 E). Isolated CD4 T cells were more sensitive to the effects of tBHQ on CD25 and CD69 than CD4 T cells cultured in the mixed PBMC population. Overall, these studies indicate that the Nrf2 activator tBHQ inhibits multiple events associated with early T cell activation.

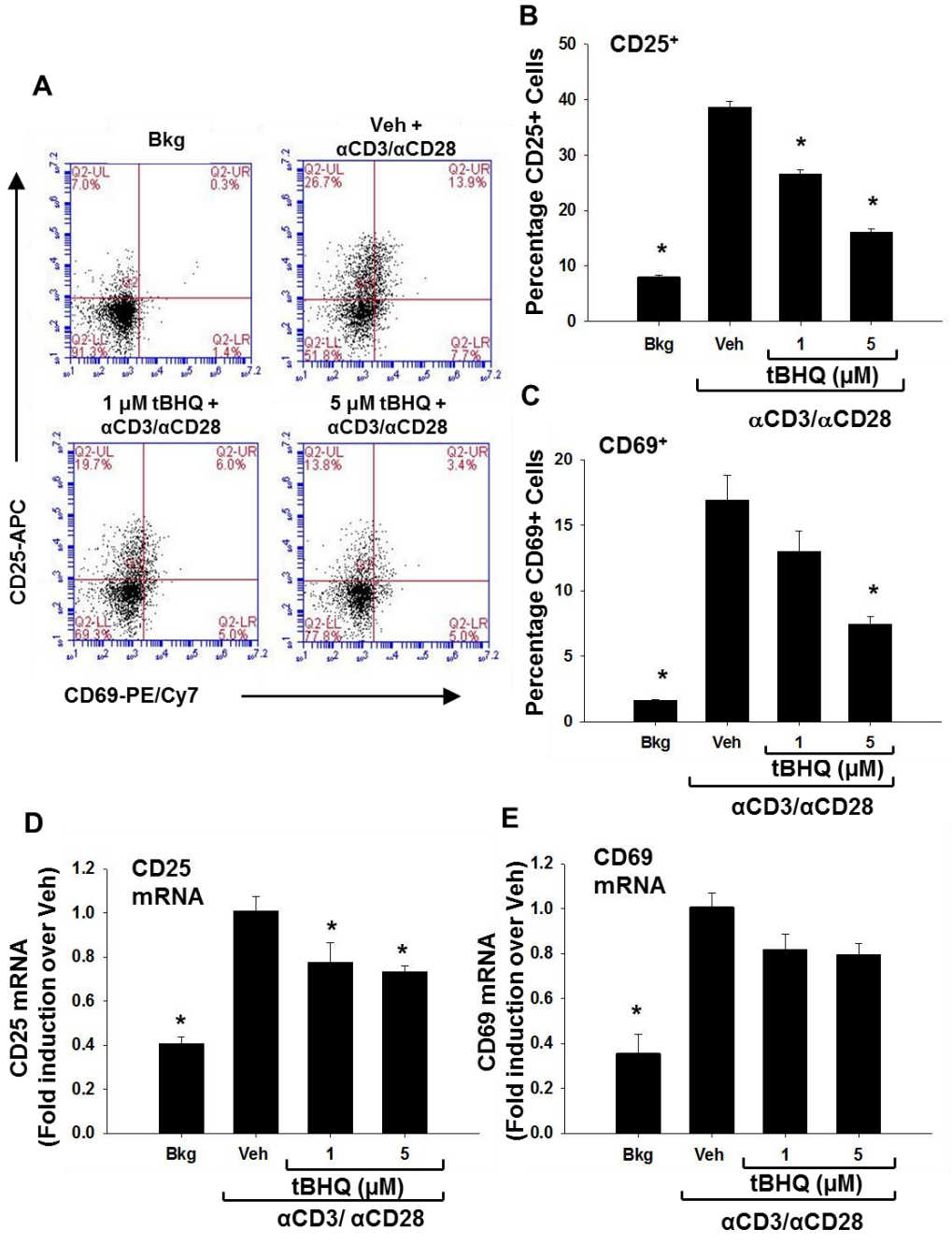

Figure 17 (cont'd)

Figure 17. tBHQ treatment inhibits the expression of the early cell surface proteins CD25 and CD69 in CD4+ T cells within the PBMC cell population.

PBMCs (A, B, C, D, and E) were either left untreated (Bkg), or pretreated with vehicle (Veh, 0.01% ethanol) or tBHQ (0.1-5 µM) and then activated 30 min later with anti-CD3/anti-CD28. Cells were collected after 24h and labeled with fluorochrome-conjugated antibodies against CD25 and CD69. CD4+ cells were gated prior to analysis of CD25 and CD69 expression. A) Representative dot plots from a PBMC experiment, and quantification of (B) CD25 and (C) CD69 protein expression. Total RNA was isolated from PBMCs 6h after activation, and mRNA expressions of (D) CD25 and (E) CD69 were quantified by q-RT-PCR. * indicates $p<0.05$ compared to the Veh group.

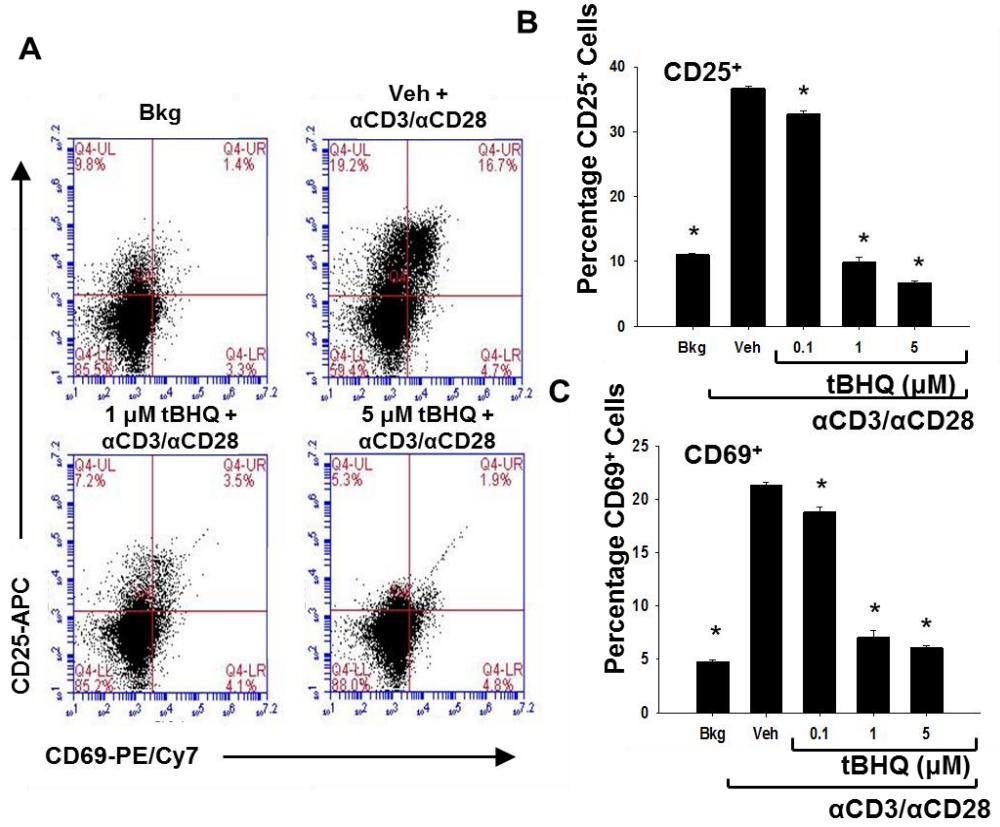

Figure 18. tBHQ treatment inhibits the expression of the early cell surface proteins CD25 and CD69 in isolated CD4+ T cells.

CD4 T cells were either left untreated (Bkg), or pretreated with vehicle (Veh, 0.01% ethanol) or tBHQ (0.1-5 µM) and then activated 30 min later with anti-CD3/anti-CD28. Cells were collected after 24h and labeled with fluorochrome-conjugated antibodies against CD25 and CD69. CD4+ cells were gated prior to analysis of CD25 and CD69 expression. A) Representative dot plots from an isolated CD4 T cell experiment, and

Figure 18 (cont'd)
quantification of (B) CD25 and (C) CD69 protein expression. * indicates $p<0.05$ compared to the Veh group.

The Nrf2 activator tBHQ inhibits NFκB DNA binding in PBMCs

We see a decrease in multiple events following early T cell activation with tBHQ treatment. Several transcription factors have been shown to be involved in T cell activation, including NFAT, AP-1, and NFκB, all of which regulate IL-2 expression, among other genes. In addition, previous studies have shown that tBHQ treatment decreases NFκB activation in other cell types[318,322]. Accordingly, we next investigated the effect of tBHQ treatment on NFκB activation. 3h after activation with anti-CD3/anti-CD28, we observed a decrease in NFκB DNA binding in PBMCs treated with 1 μM tBHQ (Fig. 19). Taken together, these studies indicate that tBHQ treatment inhibits multiple events of T cell activation, which correlates with inhibition of NFκB activity.

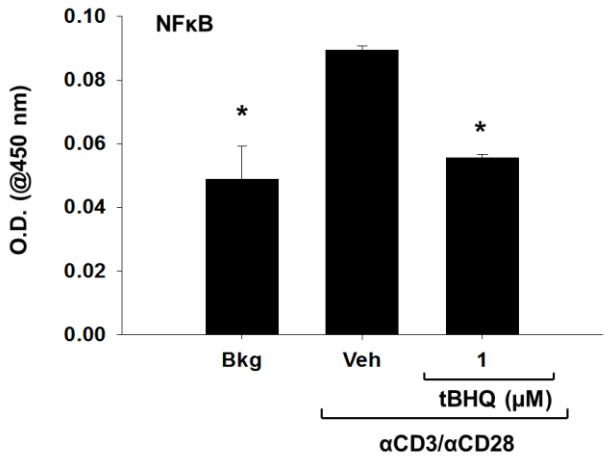

Figure 19. tBHQ treatment inhibits NFκB activation in PBMCs.
PBMCs were left untreated (Bkg), or pretreated with vehicle (Veh, 0.01% ethanol) or tBHQ (1 μM), then activated 30 min later with anti-CD3/anti-CD28. 3h after activation, cells were collected and nuclear protein was isolated. p65 NFκB DNA binding activity was quantified by a commercially available DNA binding ELISA as per manufacturer's protocol (Active Motif).

The Nrf2 activator CDDO-IM also inhibits events of T cell activation in primary human CD4 T cells

As we had seen Nrf2-independent effects in primary mouse CD4 T cells with tBHQ, and differential effects with different Nrf2 activators, we utilized another Nrf2 activator, CDDO-IM, to look at effects on activation in primary human T cells. Similar to tBHQ, pretreatment for 30 min with CDDO-IM (0.001-0.1 µM) prior to T cell activation inhibited production of IL-2 by primary human CD4 T cells (Fig 20. A). Treatment with CDDO-IM also inhibited production of IFNγ in primary human CD4 T cells (Fig 20. B). As inhibition of cytokine production was seen with CDDO-IM treatment, the effect of CDDO-IM treatment on cell surface protein expression was investigated. Treatment with CDDO-IM (0.001-0.1 µM) moderately inhibited induction of CD25 and CD69 protein expression upon T cell activation (Fig. 21 A-C).

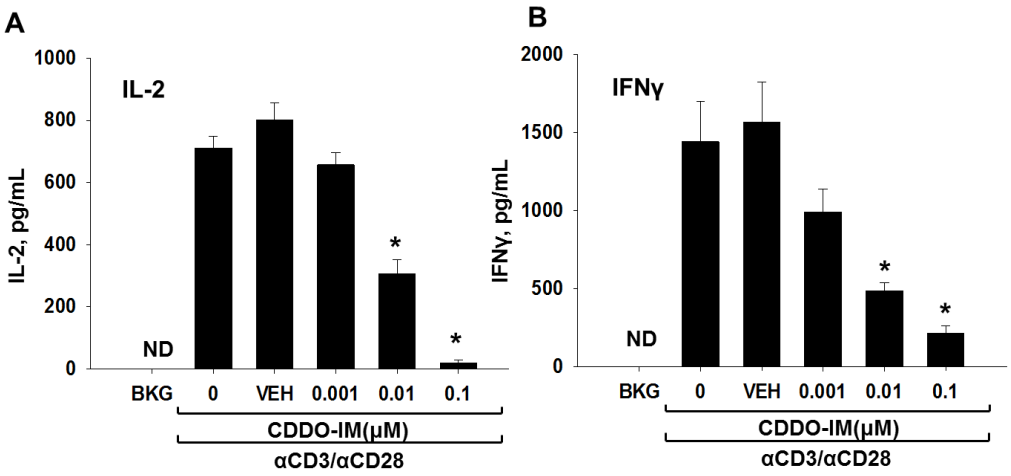

Figure 20. CDDO-IM treatment inhibits production of the cytokines IL-2 and IFNγ in isolated CD4+ T cells.
CD4 cells were either left untreated (Bkg), or pretreated with vehicle (Veh, 0.01% DMSO) or CDDO-IM (0.001-0.1 µM) and then activated 30 min later with anti-CD3/anti-CD28. Supernatants were collected after 24h and analyzed for A) IL-2 or B) IFNγ by

Figure 20 (cont'd)
ELISA. * indicates p<0.05 compared to the Veh group, ND indicates samples below the limit of detection.

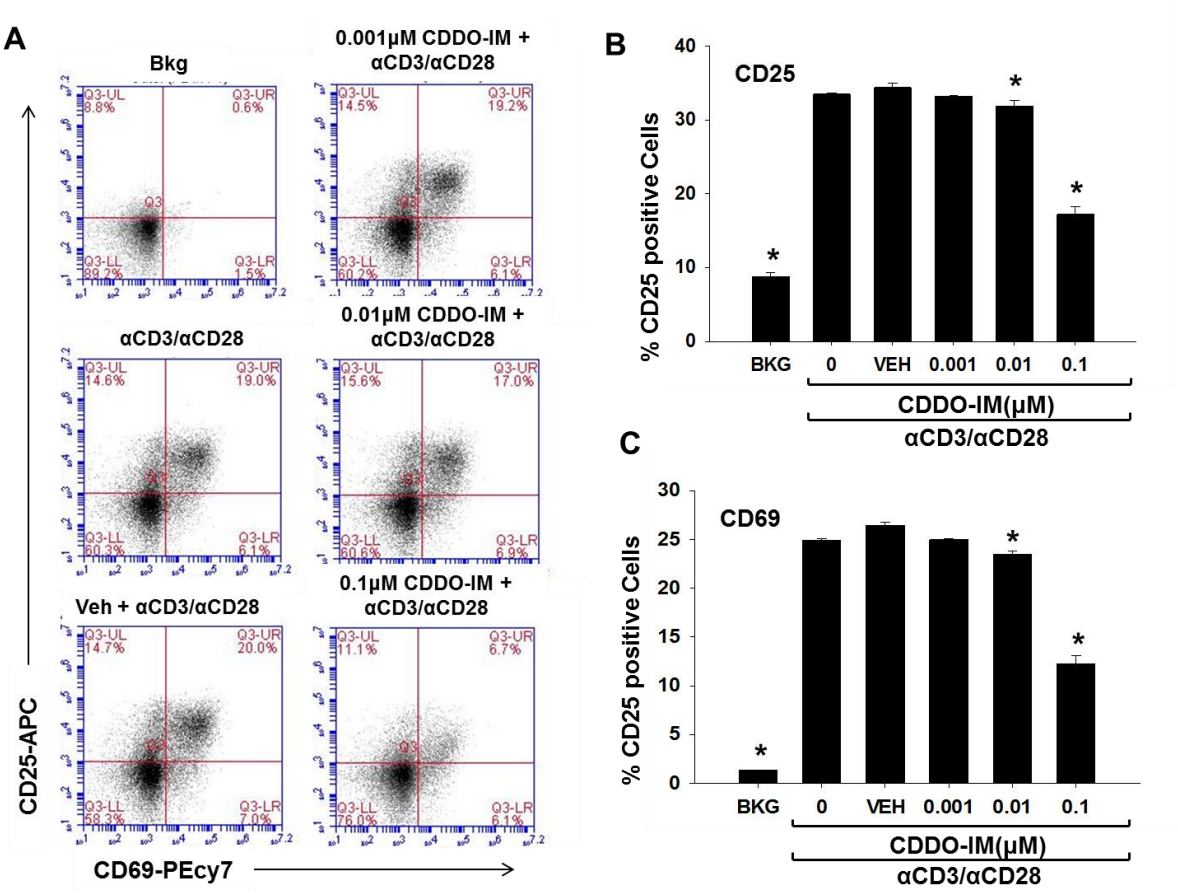

Figure 21. CDDO-IM treatment inhibits expression of the cell surface proteins CD25 and CD69 in isolated CD4+ T cells.
CD4 cells were either left untreated (Bkg), or pretreated with vehicle (Veh, 0.01% DMSO) or CDDO-IM (0.001-0.1 μM) and then activated 30 min later with anti-CD3/anti-CD28. Cells were collected after 24h and labeled with fluorochrome-conjugated antibodies against CD4, CD25 and CD69. CD4+ cells were gated prior to analysis of CD25 and CD69 expression. A) Representative dot plots from an isolated CD4 T cell experiment, and quantification of (B) CD25 and (C) CD69 protein expression. * indicates p<0.05 compared to the Veh group.

Discussion

The current studies are the first to demonstrate that the Nrf2 activator tBHQ inhibits many of the early events associated with T cell activation in primary human T cells. We have shown that tBHQ treatment upregulates the Nrf2 target genes HMOX, GCLC, and NQO1, which strongly suggests Nrf2 is activated at the concentrations of tBHQ used in these studies. This is consistent with what was seen by Morzadec et. al., where treatment of human CD4 T cells with tBHQ induced NQO1 mRNA expression[285]. We also show that tBHQ markedly inhibits production of IFNγ and IL-2 in anti-CD3/anti-CD28-activated PBMCs and to an even greater extent in isolated human CD4 T cells. Similarly, tBHQ significantly suppressed the induction of CD25 and CD69 in CD4 T cells. Finally, tBHQ treatment decreases NFκB DNA binding activity in PBMCs. Collectively, these studies suggest that tBHQ inhibits activation of primary human T cells, as evidenced by decreased cytokine production and decreased expression of CD25 and CD69. The Nrf2 activator CDDO-Im also suppressed production of IL-2, and IFNγ, and expression of CD25 and CD69 in primary human CD4 T cells.

Interestingly, the isolated CD4 T cells were more sensitive to tBHQ than the PBMCs, with a greater decrease in IFNγ and IL-2 protein production and a greater reduction in CD25 and CD69 cell surface upregulation at the same concentration of tBHQ. In addition, tBHQ caused a decrease in cytokine production at lower concentrations in isolated CD4 T cells compared to PBMCs. This difference is likely due to the presence of other cells types in the PBMC population which seemingly exert a protective effect. For example, the presence of other immune cell types in the PBMC

population likely increases the costimulatory signals received by the T cell, which may be protective against the negative effect of tBHQ on T cell activation.

The U.S. Food and Drug Administration (FDA) set a maximum limit for tBHQ of 200 mg/kg in foods, which is lower than the international standard for certain food types[225]. Because tBHQ is most often used to prevent rancidification of oils, this limit is typically applied to cooking oils, but is also applicable to many other types of food as well. Although the acceptable daily intake (ADI) for tBHQ is currently 0.7 mg/kg/day, actual intake is difficult to determine. A variety of different methods have been used to estimate the daily intake of tBHQ in the U.S. and other countries. Estimation by poundage in the U.S. yielded values 80-90% below the ADI. However, estimation based upon model diets yielded much higher values in which the mean intake was roughly half the ADI, whereas intake of individuals in the 90th percentile was estimated to exceed the ADI (0.74 mg/kg/day). Estimates based upon the international standards were considerably higher (1.2 mg/kg/day), as were estimates of daily tBHQ intake in other countries, such as the U.K. and Australia/New Zealand (0.7 – 6.9 mg/kg/day)[227]. Notably, it has been reported that healthy volunteers who consumed 100 – 150 mg of tBHQ had serum concentrations in the high micromolar range[228]. Because many of the estimated daily intakes in the U.S. and other countries approach or even surpass 100 – 150 mg/day of tBHQ, this suggests that the concentrations of tBHQ used in the current studies are relevant and applicable to humans.

Several transcription factors have been shown to be important in T cell activation, including NFAT, AP-1, and NF-κB, all of which regulate IL-2 as well as other genes that are induced upon T cell activation[303]. An important signaling event in T cell activation is

calcium influx, which ultimately leads to NFAT activation[303]. Although our recent studies showed that calcium influx was delayed and decreased by tBHQ in activated Jurkat cells, NFAT activation was not affected by tBHQ. However, these studies demonstrated that tBHQ inhibited NFκB p65 transcriptional activity in activated Jurkat T cells[318]. Previous studies have also shown that Nrf2 inhibits NF-κB signaling in various cell types, making this a likely mechanism for the effects seen in the current studies[205]. Indeed, we see that tBHQ treatment decreases NFκB DNA binding activity in nuclear extracts from PBMCs.

Activation of T cells is an important event for the initiation of a variety of adaptive immune responses. Such responses are critical for host defense against a number of bacterial and viral pathogens as well as fungi and parasites. The current studies are the first to demonstrate that the food preservative tBHQ impairs primary human T cell activation as evidenced by decreased expression of CD25 and CD69 in CD4 T cells and suppressed production of the cytokines IFNγ and IL-2. Further studies will be needed to determine the effect of tBHQ on human host defense, however. A second Nrf2 activator, CDDO-Im, had similar effects on primary human T cell activation, and overall, these studies suggest that Nrf2 represents a novel regulatory pathway in primary human T cells.

Chapter 4

Development of a Nrf2 knockdown model in primary human CD4 T cells to determine the role of Nrf2 in T cell activation

Abstract

The transcription factor nuclear factor erythroid-derived 2 like-2 (Nrf2) is activated by a number of cellular stressors, including electrophilic compounds and reactive oxygen species. One such Nrf2 activator is *tert*-butylhydroquinone (tBHQ), a widely used food preservative. When activated, Nrf2 upregulates the transcription of a large number of cytoprotective genes, including genes involved in antioxidant defense, detoxification, and phase II metabolism. Nrf2 has been shown to play a role in the immune system, especially in the regulation of inflammation. Studies have also indicated cell-type specific roles for Nrf2 in dendritic cells, macrophages, and T cells, among other cell types. In murine helper (CD4) T cells, Nrf2 activation skews CD4 T cell differentiation towards a Th2 (allergy-like) phenotype. However, the role of Nrf2 in T cell activation and in human CD4 T cells remains unclear. A prior study from our lab demonstrated that the Nrf2 activator tBHQ inhibits events of primary human CD4 T cell activation, but the role of Nrf2 in these effects was not determined. The present study addresses this by developing a Nrf2-knockdown model in primary human CD4 T cells using siRNA. This model was then used to test the role of Nrf2 in tBHQ-mediated effects on early T cell activation. tBHQ treatment inhibited production of IL-2, IFNγ, GM-CSF, and TNFα, and inhibited induction of CD25 and CD69 in both scrambled control and Nrf2-deficient CD4 T cells. However, RNA-seq analysis of transfected, activated cells identified differentially expressed genes between the scrambled control and Nrf2-deficient CD4 T cells, suggesting there are genotype differences in early T cell activation. In addition, the RNA-seq analysis showed Nrf2 independent and dependent effects of tBHQ at the RNA level. This indicates that there is a potential role for Nrf2 in

primary human T cell activation and differentiation, but there were no differences in early cytokine production between genotypes in this model.

Introduction

Nuclear factor erythroid-derived 2 like-2 (Nrf2) is a cell-stress responsive transcription factor that regulates a battery of antioxidant, detoxification, and metabolizing genes[116]. Nrf2 is activated by stimuli such as reactive oxygen species, electrophilic xenobiotics, and metals like cadmium and arsenic. Under basal conditions, Nrf2 remains in the cytosol bound to its repressor protein Keap1, which facilitates the ubiquitination and subsequent degradation of Nrf2[292,323]. Upon exposure to activating cellular stressors, ubiquitination is disrupted and Nrf2 translocates to the nucleus, where it heterodimerizes with binding partners such as small Maf proteins and binds to antioxidant response elements (AREs), which are the Nrf2 DNA binding sequences[116]. Nrf2 upregulates the transcription of a number of genes, including antioxidant genes, and those involved in phase II metabolism, such as NAD(P)H:Quinone Oxidoreductase 1 (NQO1), Glutamate-cysteine ligase regulatory subunit (GCLM), Glutamate-cysteine ligase catalytic subunit (GCLC), Heme oxygenase 1 (HMOX), and others[324].

Nrf2 has an established cytoprotective function, and Nrf2 has also been implicated in immune modulation. Female Nrf2-null mice develop a disease similar to systemic lupus erythematosus as they age, characterized by glomerular nephritis and antibodies against double-stranded DNA[239–241]. Nrf2-null mice are more sensitive to a number of inflammatory conditions, including cecal-ligation and puncture, experimental autoimmune encephalomyelitis, and acute lung inflammation, while Nrf2 activators help

protect against these conditions[205,242,245,249,252,254,279]. Nrf2 also has demonstrated effects in isolated cell types, including dendritic cells and macrophages[250,270–272]. However, the role of Nrf2 in human T cell function remains unclear. Prior studies in mice have demonstrated that Nrf2 influences T cell differentiation, where Nrf2 activation skews CD4 T cell differentiation towards the Th2 subtype[282]. Additionally, our lab has shown that the Nrf2 activator tBHQ, a commonly used food preservative, inhibits T cell activation in primary human CD4 T cells[325]. However, the role of Nrf2 in these effects remains unknown. However, a recent study demonstrated that some of the effects of tBHQ on T cell activation are Nrf2-independent in Jurkat T cells, a human T cell line, highlighting the need to determine the role of Nrf2 in primary human CD4 T cell activation[326].

CD4 T cells are a critical part of adaptive immunity, coordinating the immune response to immunogenic stimuli. Prior to antigen exposure, mature CD4 T cells circulate in a quiescent state. T cell activation requires two stimuli, one from stimulation of the T cell receptor, and another from stimulation of co-stimulatory receptors such as CD28[303]. This triggers signaling cascades that results in the activation of transcription factors including NFκB, NFAT, and AP-1 that subsequently turn on a program of transcription for T cell activation[6]. Early events of T cell activation include production of cytokines including interleukin-2 (IL-2), interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and granulocyte-macrophage colony stimulating factor (GM-CSF), and upregulation of cell surface proteins including CD25 and CD69[58,92,319,320,327]. These initial events set up the T cell to proliferate and eventually differentiate into functional

helper T cell subsets tailored to the immunogenic stimuli. As such, effects on events of T cell activation are crucial for the development of an appropriate immune response.

The purpose of the current studies is to determine the role of Nrf2 in primary human CD4 T cell activation. To do this, we have developed a Nrf2 knockdown model in primary human CD4 T cells. We tested several methods of gene knockdown, including use of short hairpin RNA (shRNA), small interfering RNA (siRNA), and CRISPR/Cas9 gene editing before moving forward with siRNA in these studies. siRNA and shRNA both utilize RNA interference, wherein small double stranded RNA molecules guide the destruction of complementary mRNAs through RNA-induced silencing complexes. In these experiments, the shRNA sequences are encoded on a plasmid and transcribed in the transfected cell as short hairpin structures that induce silencing after processing by cellular machinery into the small double stranded RNA molecules. In siRNA, the small double stranded RNAs are directly transfected into the cell to induce silencing[328,329]. In contrast, the CRISPR/Cas9 system utilizes an endonuclease (Cas9) that is targeted by a guide RNA (gRNA) to a specific location on the genome by sequence complementarity, resulting in a double strand break. This is repaired by error-prone non-homologous end joining, which frequently introduces insertions or deletions at the cut site, potentially disrupting the targeted gene and resulting in a non-functional gene product and gene knockout in the cell[330–333].

We also utilized different transfection strategies, including use of lipofectamine, before settling on nucleofection, which is a type of electroporation. Lipofectamine is a lipid-based transfection system, where cationic lipids form a liposome containing the nucleic acid. This complex fuses with the plasma membrane of cells, delivering the

contained DNA or RNA into the cell[334,335]. Electroporation uses an electrical field to permeabilize the cell to larger molecules, allowing the cell to take up materials such as plasmids and RNA. Nucleofection is an electroporation system that is commercially available from Lonza with buffers and electroporation settings for specific cell types that results in good transfection efficiency and cell survival after transfection[336–338]. Ultimately, nucleofection with siRNA was utilized to knockdown Nrf2 expression in primary human CD4 T cells. The resulting scrambled control, and Nrf2-deficient, human CD4 T cells were then used to investigate the role of Nrf2 in the effects of tBHQ on T cell activation.

Materials and Methods

Materials

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

Isolation of primary human CD4 T cells

Leukopaks (apheresis product enriched in white blood cells) were purchased from Gulf Coast Regional Blood Center (Houston, TX), and peripheral blood mononuclear cells (PBMCs) were isolated using lymphocyte separation medium (MP Biomedicals, Santa Ana, CA) as per the manufacturer's protocol. PBMCs were washed twice with Dulbecco's phosphate buffered saline, then resuspended in DMEM (with L-glutamine, sodium bicarbonate and D-glucose) supplemented with 100 units

penicillin/ml, 100 units streptomycin/ml, 50 µM 2-mercaptoethanol, 25 mM HEPES, 10 mM nonessential amino acids, and 10% fetal bovine serum (FBS), and counted. CD4 T cells were isolated from PBMCs using a magnetic bead separation (human CD4 T cell isolation kit, Miltenyi Biotec, Auburn CA) as per the manufacturer's protocol. T cells were activated using purified hamster anti-human CD3ε (clone UCHT1, 1.5 µg/ml) and purified hamster anti-human CD28 (clone CD28.2, 1.5 µg/ml) crosslinked with an $F(ab')_2$ fragment specific for anti-mouse IgG. Anti-CD3 and anti-CD28 were purchased from Affymetrix/ E-Bioscience (San Diego, CA), and the $F(ab')_2$ cross-linker from Jackson ImmunoResearch Laboratories (West Grove, PA).

Jurkat T cell culture and transfection

Human Jurkat E6-1 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were then cultured in RPMI 1640 medium, with the addition of 10% fetal bovine serum (Biowest LLC, Kansas City, MO), 25 mM HEPES, 1 mM sodium pyruvate, 10 mM nonessential amino acids, 100 units/ml penicillin, and 100 units/ml streptomycin. Human Jurkat T cells were reverse transfected with 1.5 mg of plasmid co-expressing Green Fluorescent Protein (GFP) and control or Nrf2 shRNA (GeneCopoeia, Rockville, MD) and 3 ml of Lipofectamine 2000 (Life Technologies, Grand Island, NY) per 5×10^5 cells for 12 hours in complete medium. After 12 hours, the cells were washed and resuspended at a concentration of 5×10^5 cells/mL in complete medium. 48h after transfection, the cells were sorted by GFP expression using a BD Influx flow cytometer (BD Biosciences, San Jose, CA). After FACS sorting, cells were plated and treated with 5 µM tBHQ or Veh prior to cell collection and RNA isolation.

Transfection of primary human CD4 T cells

Isolated human CD4 T cells were nucleofected using the primary human CD4 T cell kit from Lonza (Anaheim, CA) according to the manufacture's protocol. Briefly, approximately 1×10^7 cells per nucleofection reaction were used ($0.8\text{-}1.2\times10^{\wedge7}$ cells). Cells were spun down at 100×g for 10 min, and the supernatant removed. Cells were resuspended in 100 uL of the nucleofection reagent with supplement added, followed by addition of 100 picomoles of siRNA or 5 µg of plasmid. Cells were transferred to a nucleofection cuvette, then nucleofected using the human T cell-high viability protocol for unstimulated T cells. Immediately after, 0.5 mL of warm medium was added to the cuvette and cells were gently transferred into 2 mL of warm medium in a 12 well plate. The cuvette was washed twice with 0.5 mL warm medium and added into the same well. 6 h after nucleofection, the culture medium was replaced. Cells were collected and used at various time points post transfection. For shRNA knockdown, cells were nucleofected with scrambled control or Nrf2 shRNA plasmids co-expressing GFP from Genecopoeia (Rockville, MD) or Sigma (St. Louis, MO). Cells were collected and FACS sorted based on GFP expression 24, 48, or 72 h after transfection using a BD Influx flow cytometer (BD Biosciences, San Jose, CA). The cells were then cultured and treated as described in figure legends. For Nrf2 knockdown using CRISPR/Cas9, cells were nucleofected with CRISPR/Cas9 plasmid co-expressing GFP targeting Nrf2, or control plasmid that did not contain gRNA sequences generated as described previously[326]. Cells were analyzed for GFP expression at various time points after transfection on a BD Accuri C6 flow cytometer (BD Biosciences). For analysis of Nrf2 protein expression, 16 h after transfection, cells were collected and FACS sorted based on GFP expression

using a Synergy Head Cell sorter (iCyt, Champaign, IL). Sorted cells were treated with 10 µM tBHQ or vehicle (0.01% ethanol) for 3 h, prior to cell collection and nuclear protein isolation. For siRNA knockdown, cells were nucleofected with 100 picomoles of siRNA targeting Nrf2 or scrambled control siRNA (Santa Cruz Biotechnologies, Santa Cruz, CA). 12 h after nucleofection, cells were collected, counted, and treated as indicated in the figure legends.

Western Blot

For nuclear protein isolation, cells were collected 3 h after treatment and nuclear and cytosolic protein fractions were collected using a Nuclear Extract Kit from Active Motif as per the manufacturer's protocol. For whole cell protein levels, 6 h after treatment with 10 µM MG132 (a proteasome inhibitor) or vehicle (0.1% DMSO) cells were collected in RIPA buffer (phosphate buffered saline (PBS) supplemented with 1% octylphenoxypolyethoxyethanol (IGEPAL), 0.5% sodium deoxycholate, 0.5% SDS, 1× Halt protease inhibitor cocktail (Thermo Scientific, Waltham, MA), and 0.5 mM dithiothreitol (dTT))[339]. Protein levels were quantified using the Bradford reagent (Bio-Rad, Hercules CA) for nuclear protein samples, and by BCA assay (Thermo Fisher Scientific, Waltham, Ma) for whole cell protein samples. After quantification, protein was diluted in Laemmli Sample Buffer (Bio-Rad) supplemented with 5% 2-mercaptoethanol so that each well contained 5-10 µg of protein, depending on the target. Samples were subjected to SDS-PAGE and subsequently transferred to a polyvinylidene difluoride membrane overnight. Membranes were blocked with 5% nonfat dehydrated milk (NFDM) in phosphate- buffered saline containing 0.05% Tween 20 (PBST). Membranes

were incubated overnight with primary antibodies targeting Nrf2, Histone H3, or β-actin in 5% NFDM in PBST. The histone H3 (7074S) primary antibody was purchased from Cell Signaling Technology (Danvers, MA), and the β-actin antibody from Abcam (Cambridge, MA). The Nrf2 primary antibodies were obtained from Santa Cruz Biotechnologies (H-300 and C-20), Cell Signaling Technology (D1Z9C), Abcam (AB92946, AB31163, and AB62352), and Novus Biologicals (Littleton, CO) (NBP1-32822), and are indicated in the figure legends. Membranes were incubated with anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Cell Signaling Technology) diluted 1:2000 in 2% NFDM in PBST. Blots were developed using Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL) per the manufacturer's protocol. The bands were visualized and analyzed by the LI-COR Odyssey FC infrared imaging system (Lincoln, NE).

Quantitative Real time PCR

Cells were treated with tBHQ and activated after transfection as indicated in the figure legends. 6h after activation, cells were collected and RNA was isolated by TRIzol (Ambion, Life Technologies, Grand Island NY) extraction as per the manufacturer's protocol. RNA was reverse transcribed to cDNA, followed by quantitative-real time PCR using Sybr green analysis (Applied Biosystems, Life Technologies). Fluorescence was detected by a Life Technologies/Applied Biosystems Sequence Detection System 7500, and relative transcript levels were quantified using the ΔΔCT method, by comparing the target genes to ribosomal protein L13a. Primer sequences were acquired from

qPrimerDepot (http://primerdepot.nci.nih.gov/) and are listed in Table 1. All primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Table 1. Primer sequences used in qRT-PCR

Gene Target	Forward Primer	Reverse Primer
RPL13A	5'-GTTGATGCCTTCACAGCGTA-3'	5'-AGATGGCGGAGGTGCAG-3'
IL-2	5'-GCACTTCCTCCAGAGGTTTG-3'	5'-TCACC AGGATGCTCACATTT-3'
IFNγ	5'-TCAGCCATCACT TGGATGAG-3'	5'-CGAGATGACTTCGAAAAGCT G-3'
CD25	5'-TAGGCCATGGCTTTGAATGT-3'	5'-A TACCTGCTGATGTGGGGAC-3'
CD69	5'-ACAGGAACTTGGAAGGACCC-3'	5'-AGAACAGCTCTTTGCATCCG-3'
TNFα	5'-AGATGATCTGACTGCCTGGG-3'	5'-CTGCTGCACTTTGGAGTGAT
GMCSF	5'-AAAGGGGATGACAAGCAGAA-3'	5'-ACTACAAGCAGCACTGCCCT-3'
NRF2	5'-TCTTGCCT CCAAAGTATGTCAA-3'	5'-CACGGTCCA CAGCTCATC-3'
NQO1	5'-TCCTTTCTTCAAAGCCG- 3'	5'-GGACTGCACCAGAGCCAT-3'
GCLC	5'-CTTTCTCCCCAGACAGGACC-3'	5'-CAAGGACGTTCTCAAGTGGG-3'
GCLM	5'-GCTTCTTGGAAACTTGCTTCA-3'	5'-CTGTGTGATGCCACCAGATT-3'
HMOX	5'-GGCTTCCCTCTGGGAGTCT-3'	5'-AGCTGCTGACCCATGACAC-3'
KEAP1	5'-AAGAACTCCTCTTGCTTGGC-3'	5'-CCAACTTCGCTGAGCAGATT-3'

Cytokine analysis

Cytokine levels in the supernatant were analyzed 24 h after activation with αCD3/αCD28 using commercially available ELISA kits for IL-2, IFNγ, TNFα, and GM-CSF (Biolegend, San Diego, CA). Kits were run according to the manufacturer's protocols, and absorbance was quantified on a Tecan Infinite M1000 Pro Microplate Reader (Tecan, San Jose, CA).

Flow Cytometry

24 h after activation with αCD3/ αCD28, cells were collected and split into three groups, and each group was labeled with one of three panels. One group was labeled for viability using the CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit (ThermoFischer Scientific, Waltham, MA) as per the manufacturer's protocol (but with

smaller labeling volumes) . The remaining cells were treated with an FcR blocking reagent (Miltenyi Biotec, Auburn, CA), and then labeled either with: 1) CD4-FITC, CD25-APC, CD69-PECy7, CD45RA-PE, CD45RO-PerCP, CD44-AF700, and CD62L-APCCy7 or with 2) CD4-FITC, CD40-AF700, CD40L-APCCy7, CD80-PE, CD86-PECy7, and CD122-PerCPCy5.5. Fluorescence was detected using an Attune NxT Flow Cytometer from Life Technologies (ThermoFischer Scientific, Waltham, MA), and fluorescence was analyzed using the Attune software.

RNA quantification and qualification for RNA-seq

12 h after nucleofection with control siRNA or Nrf2 siRNA, cells were collected and treated with vehicle (0.001% ethanol) or 1 μM tBHQ. 30 min after tBHQ treatment, cells were activated with 1.5 μg/mL crosslinked αCD3/αCD28. 6 h after activation, RNA was collected using an RNeasy plus universal miniprep kit (Quiagen, Germantown, MD). Sequencing and bioinformatic analysis was conducted by Novogene (Chula Vista, CA). RNA degradation and contamination was monitored on 1% agarose gels, RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA), and RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Library preparation and sequencing

A total amount of 3 μg RNA per sample was used as input material for the RNA sample preparation. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads, then fragmented under elevated temperature using divalent cations in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primers and M-MuLV Reverse Transcriptase (RNase H-), and second strand cDNA was synthesized using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. The 3' ends of DNA fragments were adenylated and ligated with hairpin loop structures using NEBNext Adaptors to prepare for hybridization. In order to preferentially select cDNA fragments of 150~200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). The size-selected, adaptor-ligated cDNA was treated with 3 μL USER Enzyme (NEB, USA) at 37°C for 15 min followed by 5 min at 95 °C before PCR. PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer, PCR products were purified using the AMPure XP system, and library quality was assessed on the Agilent Bioanalyzer 2100 system. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using HiSeq PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and 150 bp paired-end reads were generated.

Data Analysis

Raw fastq files were initially processed through in-house perl scripts at Novogene to remove reads containing adapters, reads containing more than 10% uncertain nucleotides, and low quality reads from raw data. At the same time, Q20, Q30 and GC content were calculated. All downstream analyses were based on high-quality clean data. Reference genome and gene model annotation files were directly downloaded from the genome website, and the index of the reference genome was built using Bowtie v2.2.3[340]. Paired-end clean reads were aligned to the reference genome using TopHat v2.0.12[341]. TopHat was selected due to the ability to generate a database of splice junctions based on the gene model annotation files and thus yield a better mapping result than other non-splice mapping tools.

Quantification of gene expression level and differential expression analysis

HTSeq v0.6.1 was used to count the reads numbers mapped to each gene. The fragments per kilobase of transcript sequence per million base pairs sequenced (FPKM) for each gene was calculated based on the number of read counts mapped to the gene, gene length, and sequencing depth[342]. Differential expression analysis of two conditions/groups (three biological replicates per condition) was performed using the DESeq R package (1.18.0)[343–345]. DESeq provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted P-value <0.05 found by DESeq were assigned as differentially expressed.

Statistical analysis

All data except for the RNA-seq experiment were analyzed using SigmaPlot 12.3 (Systat, Chicago, IL). Data were analyzed by two-way ANOVA followed by a Holmes-sidak post-hoc test. Data are expressed as mean ± standard error. A p-value of less than 0.05 was considered statistically significant.

Results

Expression of a Nrf2-shRNA plasmid co-expressing GFP in primary human CD4 T cells does not substantially knockdown Nrf2 levels

We have shown that the Nrf2 activators tBHQ and CDDO-Im inhibit events of primary human CD4 T cell activation. However, the role of Nrf2 in these events was undetermined. Both tBHQ and CDDO-Im are thiol-reactive compounds that can have other effects in the cell in addition to Nrf2 activation, and both had Nrf2-independent and -dependent effects in primary mouse CD4 T cells. To determine the role of Nrf2 in the effects seen in primary human CD4 T cells, we developed a Nrf2 knockdown model. As primary human CD4 T cells are not well suited for experimentally induced clonal expansion from single cells cultured *in vitro*, a transient transfection approach using GFP-selectable plasmids expressing shRNA against Nrf2 was utilized. These plasmids were first tested in Jurkat T cells, a human T cell line. Commercially available Nrf2-shRNA or control shRNA plasmids co-expressing GFP were transfected into Jurkat T cells using lipofection. 48 h after transfection, the GFP-expressing cells were FACS-sorted and treated with vehicle or 5 µM tBHQ for 6 h. The mRNA expression of Nrf2

and its target genes was then quantified by qPCR. In Jurkat cells, expression of the Nrf2-shRNA plasmid substantially reduced mRNA levels of Nrf2, and abolished induction of the Nrf2 target gene NQO1 by tBHQ (Fig. 22 A, B). This indicated that the Nrf2 shRNA plasmid has the capacity to knockdown Nrf2 in human cells, so we next tested this plasmid in primary human CD4 T cells.

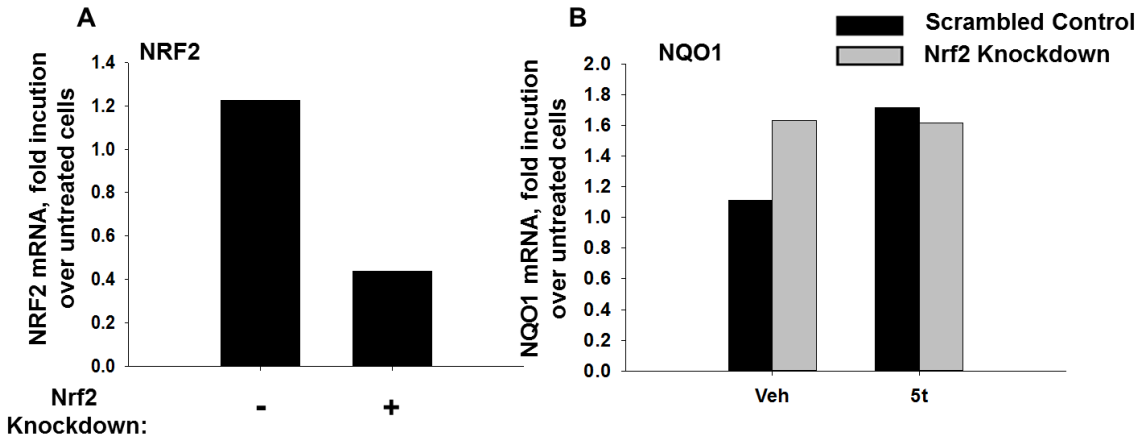

Figure 22. Transfection with the Nrf2 shRNA expression plasmid decreases Nrf2 mRNA and inhibits the induction of the Nrf2 target gene NQO1 in Jurkat T cells. Jurkat T cells were transfected with the Nrf2 shRNA plasmid using lipofection, sorted by GFP expression 48h after transfection, then activated with 5 μM tBHQ or Veh (0.01% ethanol). After 6 h, RNA was collected and mRNA levels of A) NRF2 and B) NQO1 were quantified by qRT-PCR.

To do this, it was necessary to transfect primary human T cells. Nucleofection, a commercially-available, cell type-specific, transfection system, was utilized. First, primary human CD4 T cells were transfected with the Nrf2-targeted shRNA plasmid, or the scrambled control shRNA plasmid. The efficiency of this transfection was 16%, when assessed 24 h after nucleofection (Fig. 23). From this, it was determined that nucleofection was a practical method of transfection in primary human CD4 T cells for the purpose of Nrf2 knockdown. After having demonstrated the feasibility of transfecting

shRNA expression plasmids into primary human CD4 T cells, the ability of these plasmids to knockdown Nrf2 was determined. 48 h after nucleofection, cells were sorted into GFP positive and GFP negative populations by FACS, treated with vehicle (0.01% ethanol) or 5 μM tBHQ for 6 h, and the mRNA expression of Nrf2 and Nrf2 target genes was investigated. Although transfection of the Nrf2-targeted shRNA plasmid did not impact Nrf2 mRNA levels, it is possible the primers amplified fragments of the degraded Nrf2 transcript. Notably, the Nrf2-targeted shRNA plasmid reduced the tBHQ-mediated induction of the Nrf2 target genes NQO1 (Fig. 24). GCLC induction by tBHQ was also diminished by the Nrf2-targeted shRNA plasmid; however the induction of GCLC by tBHQ was also somewhat modest in the group transfected with the control plasmid. Taken together, the results were potentially promising, but analysis of protein expression of Nrf2 was needed. For this, cells were sorted 72 h after nucleofection based on GFP expression, treated with 10 μM MG132 for 6 h to block proteasomal degradation of Nrf2, and Nrf2 protein levels were quantified in whole cell lysates by western blot analysis. There was an unexpected increase in the ratio of Nrf2 to β-actin expression in the group transfected with the shRNA control plasmid relative to the groups that were exposed to nucleofection, but negative for GFP (Fig. 25). This observation, in combination with the variability in the expression of the housekeeper, β-actin, cast some uncertainty on the level of Nrf2 knockdown at the protein level.

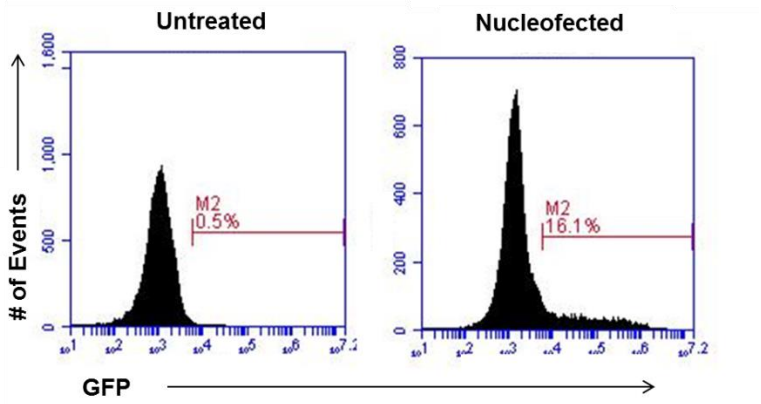

Figure 23. Nucleofected primary human CD4 T cells express the Nrf2 shRNA plasmid.

CD4 T cells were nucleofected with a Nrf2 shRNA expression plasmid containing a GFP selectable marker. 24 h after nucleofection, untreated or transfected cells were collected and analyzed for GFP expression using an Accuri C6 flow cytometer. A) Representative histograms for GFP expression.

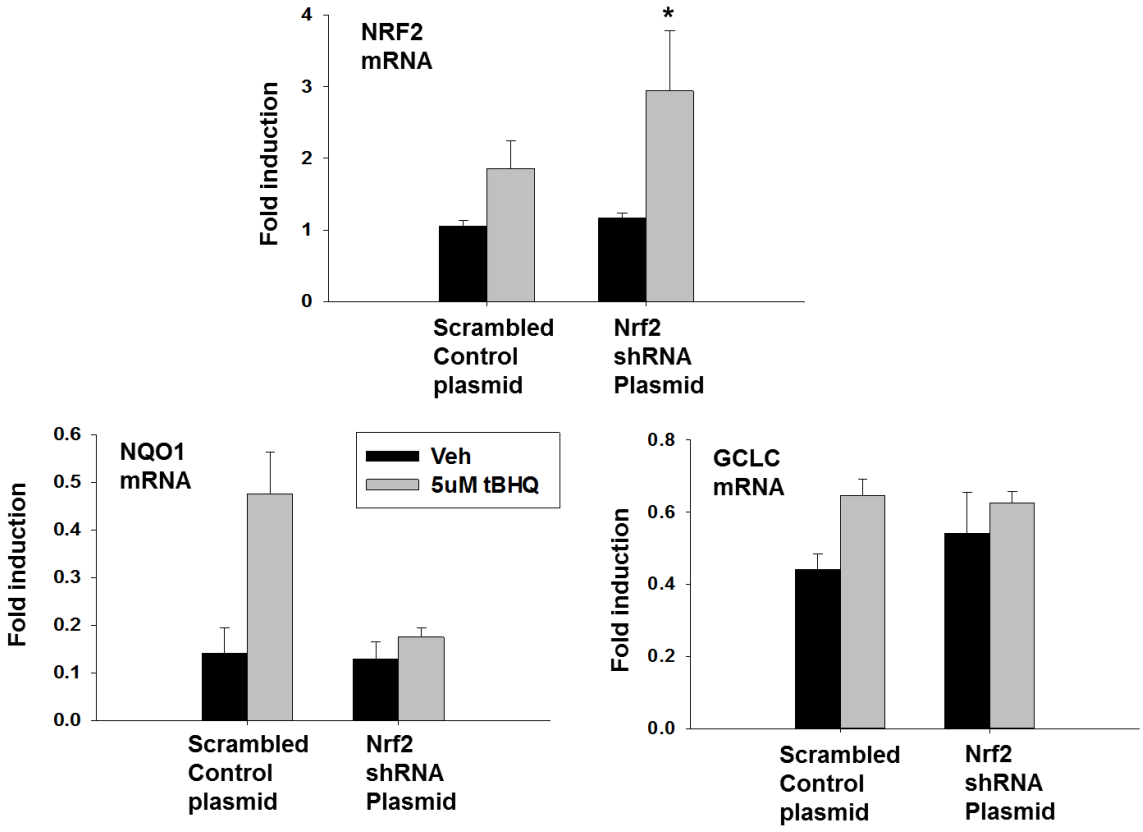

Figure 24 (cont'd)

Figure 24. Expression of the Nrf2 shRNA plasmid in primary human CD4 T cells does not reduce Nrf2 mRNA levels, and moderately inhibits the induction of Nrf2 target genes.

Primary human CD4 T cells were nucleofected with Nrf2 shRNA plasmid, or with a control shRNA plasmid. Cells were FACS sorted 48 h after nucleofection based on GFP expression (using a BD influx cell sorter). Cells were treated with vehicle (0.01% ethanol) or 5 µM tBHQ and total mRNA was collected 6 h after treatment. Levels of A) NRF2, B) NQO1, and C) GCLC mRNA were quantified by q-RT-PCR. * indicates a difference between Veh and 5 µM tBHQ treatment within the sample treatment.

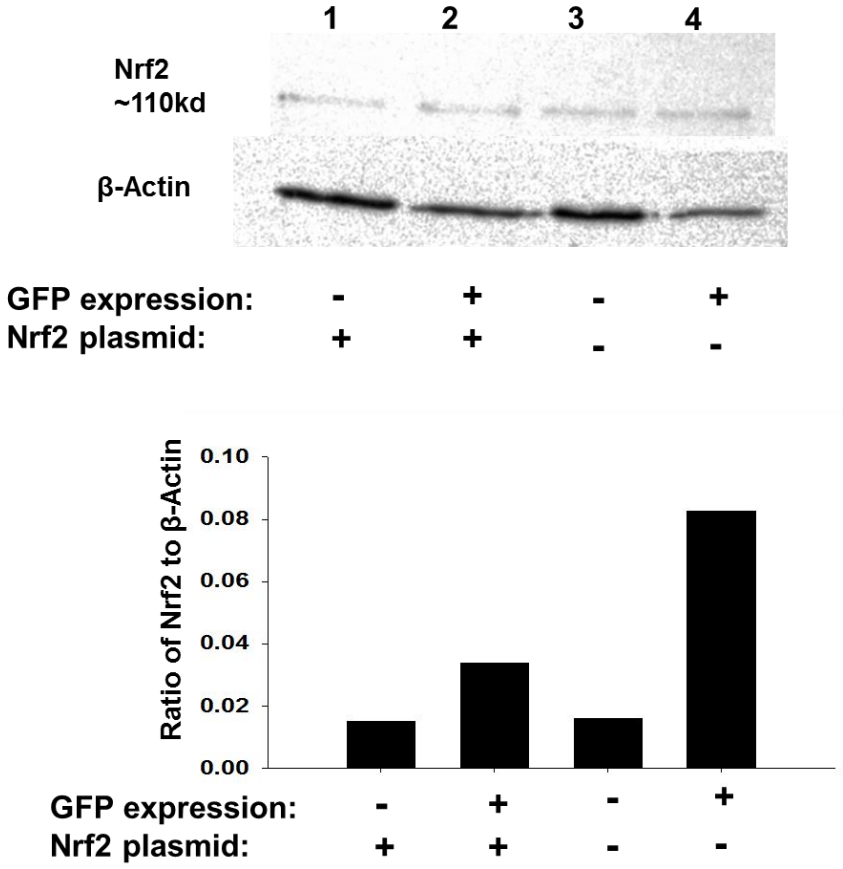

Figure 25. Expression of the Nrf2 shRNA plasmid in primary human CD4 T cells does not substantially reduce Nrf2 protein levels.

Primary human CD4 T cells were nucleofected with Nrf2 shRNA plasmid, or with a control shRNA plasmid. Cells were FACS sorted 72 h after transfection based on GFP expression (using a BD influx cell sorter) and treated with 10 µM MG132 for 6 h to block proteasomal protein degradation. Whole cell protein levels were analyzed by western blot for levels of Nrf2 and β-actin. Densitometry was conducted and the relative ratios of Nrf2 to β-actin are reported.

Comparison of Nrf2 antibodies for use in western blot

While developing a Nrf2 knock down model in the primary human CD4 T cells, we determined that the Nrf2 antibody (H-300, Santa Cruz) we were initially using was unreliable with high variability in our ability to detect the Nrf2 band. Because of this, we screened several commercially-available Nrf2 antibodies in primary human CD4 T cells, and found that the NBP1-32822 Nrf2 antibody (Novus biologicals) was reliable for the detection of Nrf2 protein in isolated human CD4 T cells (Fig. 26). Prior to this, we primarily tested antibodies in PBMCs, a mixed cell preparation that includes neutrophils, monocytes and lymphocytes. However for these studies, we tested the antibodies in both isolated CD4 T cells and in PBMCs. Interestingly, the banding patterns were different between the isolated CD4 T cells and the PBMCs (Fig. 26). This was an unexpected finding, and could help explain our prior difficulty in reliably detecting Nrf2, as the initial antibody we used first worked in PBMCs, and then no longer worked in isolated CD4 T cells.

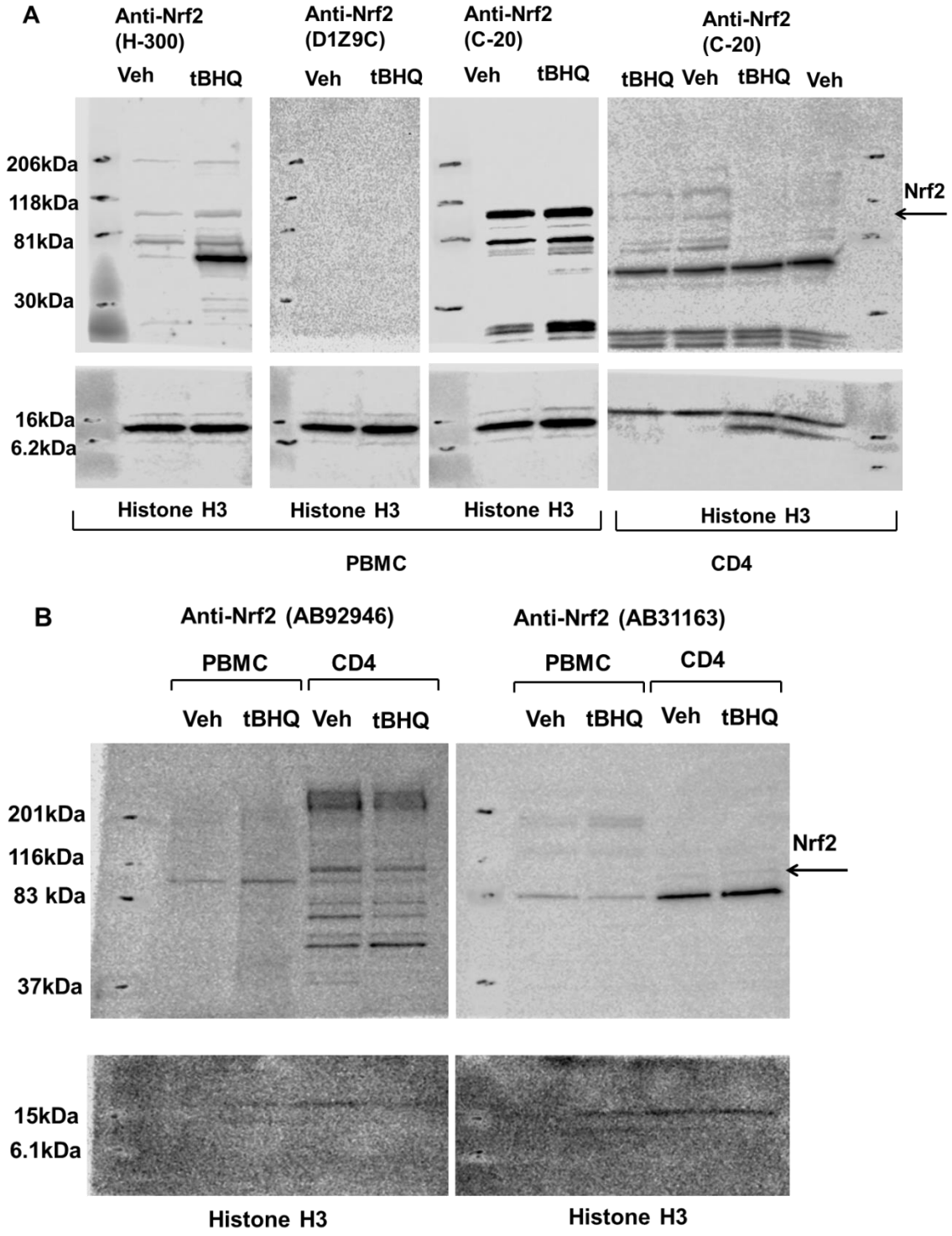
A
Anti-Nrf2 (H-300)
Anti-Nrf2 (D1Z9C)
Anti-Nrf2 (C-20)
Anti-Nrf2 (C-20)
Veh
tBHQ
206kDa
118kDa
81kDa
30kDa
Nrf2
16kDa
6.2kDa
Histone H3
PBMC
CD4
B
Anti-Nrf2 (AB92946)
Anti-Nrf2 (AB31163)
201kDa
116kDa
83 kDa
37kDa
15kDa
6.1kDa

Figure 26 (cont'd)

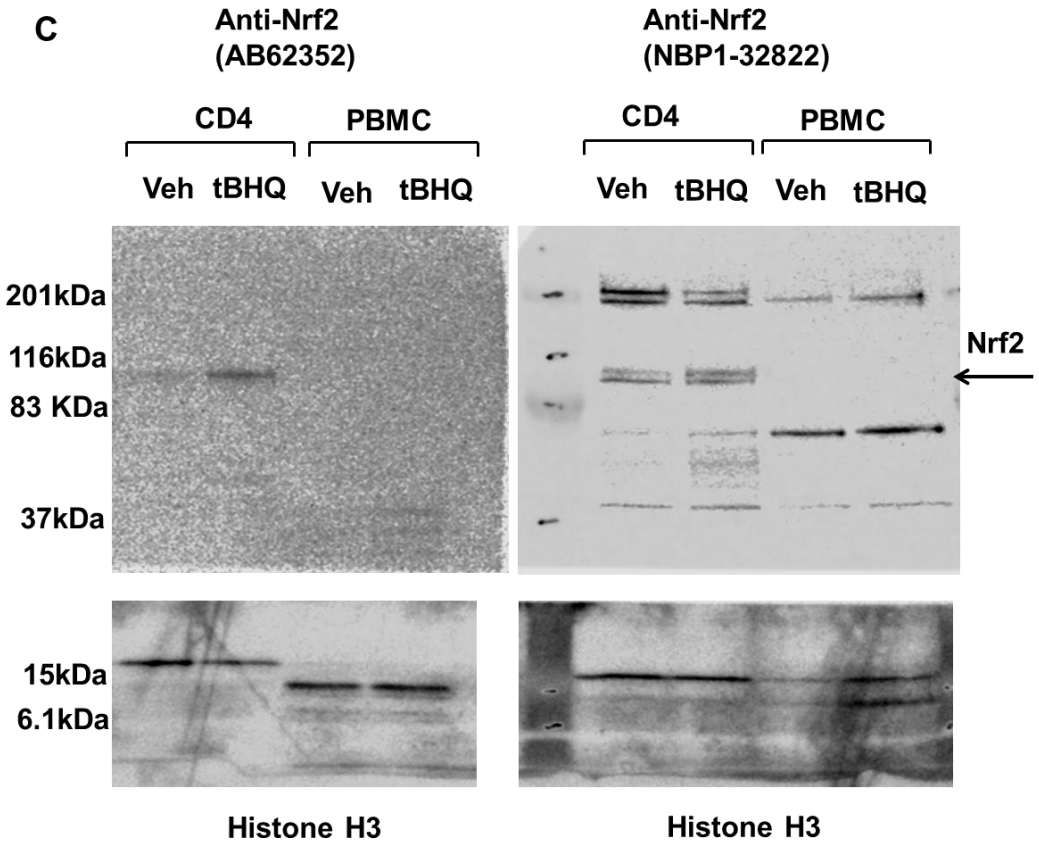

Figure 26. Detection of Nrf2 protein by western blot.
Primary human PBMCs or CD4 T cells were treated with Veh (0.01% ethanol) or 10 µM tBHQ. 3 h after treatment nuclear protein was isolated and quantified by Bradford assay, then analyzed by western blot for levels of Nrf2 and Histone H3 using the indicated antibodies in panels A, B, and C. Location of the Nrf2 band is indicated by the arrows.

Nrf2 knockdown in primary human CD4 T cells using CRISPR/Cas9

After determining that transfection with the Nrf2 shRNA expression plasmid did not substantially knock down Nrf2 levels, other means of Nrf2 knockdown were investigated. Two potential methods include using siRNA, or using CRISPR/Cas9 gene editing. Previously, our lab had cloned guide RNA sequences against Nrf2 into a CRISPR/Cas9 plasmid co-expressing GFP[326]. Transfection with this plasmid induces double strand cuts in the Nrf2 gene in cells that express the plasmid, potentially leading

to insertions and deletions that disrupt the Nrf2 gene and lead to loss of Nrf2 protein. In the absence of single cell cloning (which is technically challenging in unactivated, primary T cells), this approach will not result In 100% knock out, but will create a Nrf2 knockdown in the overall pool of cells based on the efficiency at which the CRISPR/Cas9 system causes insertions and deletions in the Nrf2 gene. This plasmid has been successfully used to knockout Nrf2 in Jurkat T cells, and demonstrated a relatively high rate of mutagenesis in cells expressing the plasmid[326]. To test this in primary human CD4 T cells, primary human CD4 T cells were transfected with the CRISPR/Cas9 plasmid targeting Nrf2 co-expressing GFP, and GFP expression was tracked over 4 days. GFP expression peaked 12h post transfection, and slowly decreased over time, indicating that cells could be successfully transfected with this plasmid (Fig. 27). Cells were transfected with the CRISPR/Cas9 plasmid targeting Nrf2 or with an empty control CRISPR/Cas9 plasmid prior to cell sorting based on GFP expression 16 h post-transfection. Analysis of nuclear levels of Nrf2 protein in the transfected cells after a 3 h treatment with 10 μM tBHQ demonstrated a 72% decrease in Nrf2 protein levels (Fig. 28).

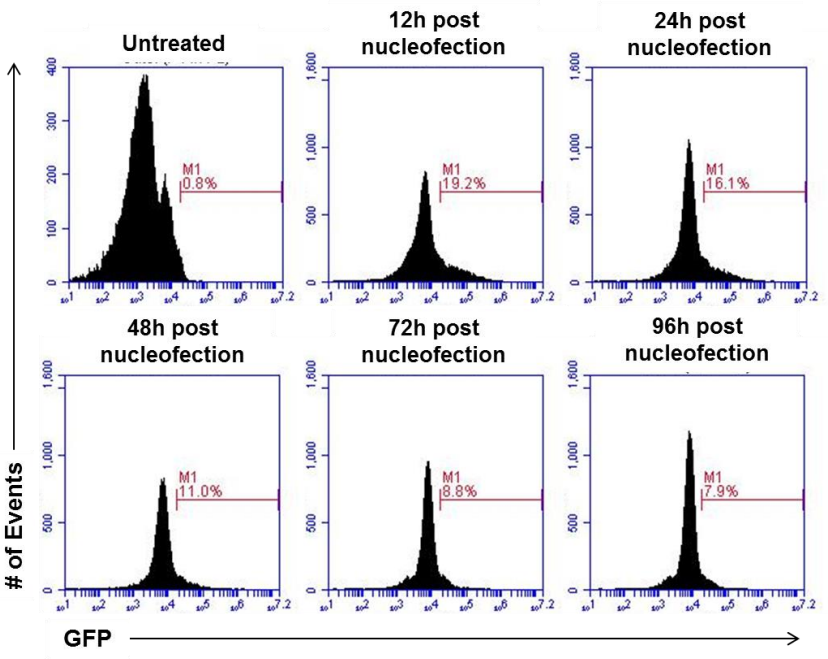

Figure 27. Nucleofected primary human CD4 T cells express CRISPR/Cas9 plasmid coexpressing GFP targeting Nrf2.

CD4 T cells were nucleofected with a CRISPR/Cas9 plasmid coexpressing GFP containing a guide RNA targeting Nrf2. 12 h, 24 h, 48 h, 72 h, and 96 h after nucleofection, untreated or transfected cells were collected and analyzed for GFP expression using an Accuri C6 flow cytometer. Data are presented as representative histograms for GFP expression.

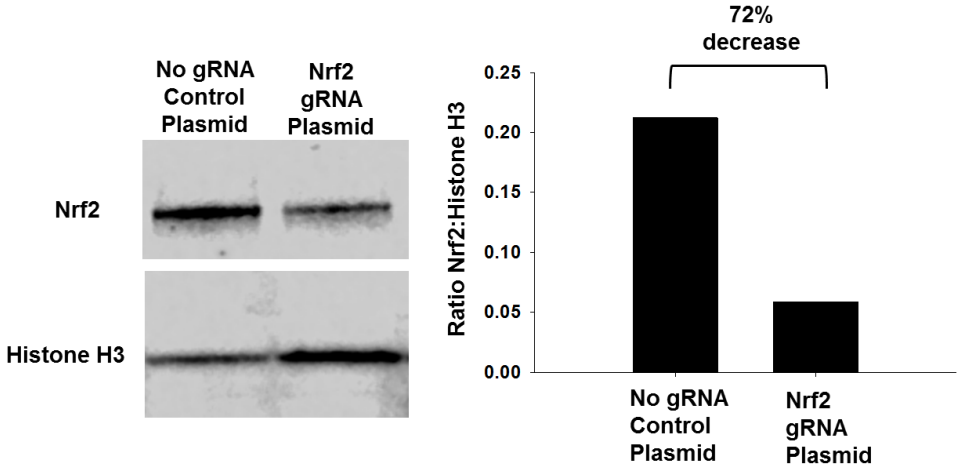

Figure 28. Transfection with a CRISPR/Cas9 plasmid targeting Nrf2 co-expressing GFP knocks down Nrf2 in primary human CD4 T Cells.

CD4 T cells were nucleofected with a CRISPR/Cas9 plasmid targeting Nrf2 coexpressing GFP or a control CRISPR/Cas9 GFP plasmid with no guide RNA. 16 h after nucleofection, cells were FACS sorted based on GFP expression using a synergy

Figure 28 (cont'd)
flow cytometer. Cells were treated with 10 μM tBHQ for 3 h, and then nuclear protein was collected. Protein levels were quantified by Bradford, and expression of Nrf2 and histone H3 were analyzed by western blot. Densitometry was conducted on the Nrf2 and histone H3 bands, and data are reported as a ratio of the Nrf2 band to the histone H3 band.

Nrf2 knockdown in primary human CD4 T cells using siRNA

Concurrent with testing the CRISPR/Cas9 plasmids, we conducted experiments to determine the feasibility of using siRNA directed against Nrf2 to knock down Nrf2 expression in primary human CD4 T cells. Commercially available siRNAs against Nrf2 or scrambled control siRNAs from Santa Cruz Biotechnologies were used. Based on the studies using the CRISPR-Cas9 plasmid showing that GFP expression peaked 12h after nucleofection, levels of Nrf2 knockdown were investigated at 12 h, 24 h, and 36 h after nucleofection with scrambled control or Nrf2 siRNA. Analysis of Nrf2 protein levels in the nuclear fraction after 3 h of treatment with 10 μM tBHQ indicate that Nrf2 protein expression is significantly knocked down at 12 h, demonstrating an average knockdown of 90% (Fig. 29 A, B). Knockdown persists to 36 h after nucleofection, showing approximately 80% knockdown at 24 h and 70% knockdown at 36 h (data not shown). Viability of the remaining cells after nucleofection varied from donor to donor, averaging around 75-80% viability.

Furthermore, expression of Nrf2 target gene mRNA was analyzed 12 h after nucleofection with Nrf2-targeted or scrambled control siRNA. Treatment with tBHQ (0.1-1 μM) for 6 h induced expression of NQO1, GCLC, and GCLM in scrambled control siRNA transfected cells, but not in Nrf2 siRNA transfected cells (Fig. 29 C, D, E). Taken

together, these data indicate that transfection with Nrf2 siRNA significantly reduces Nrf2 protein expression in primary human CD4 T cells.

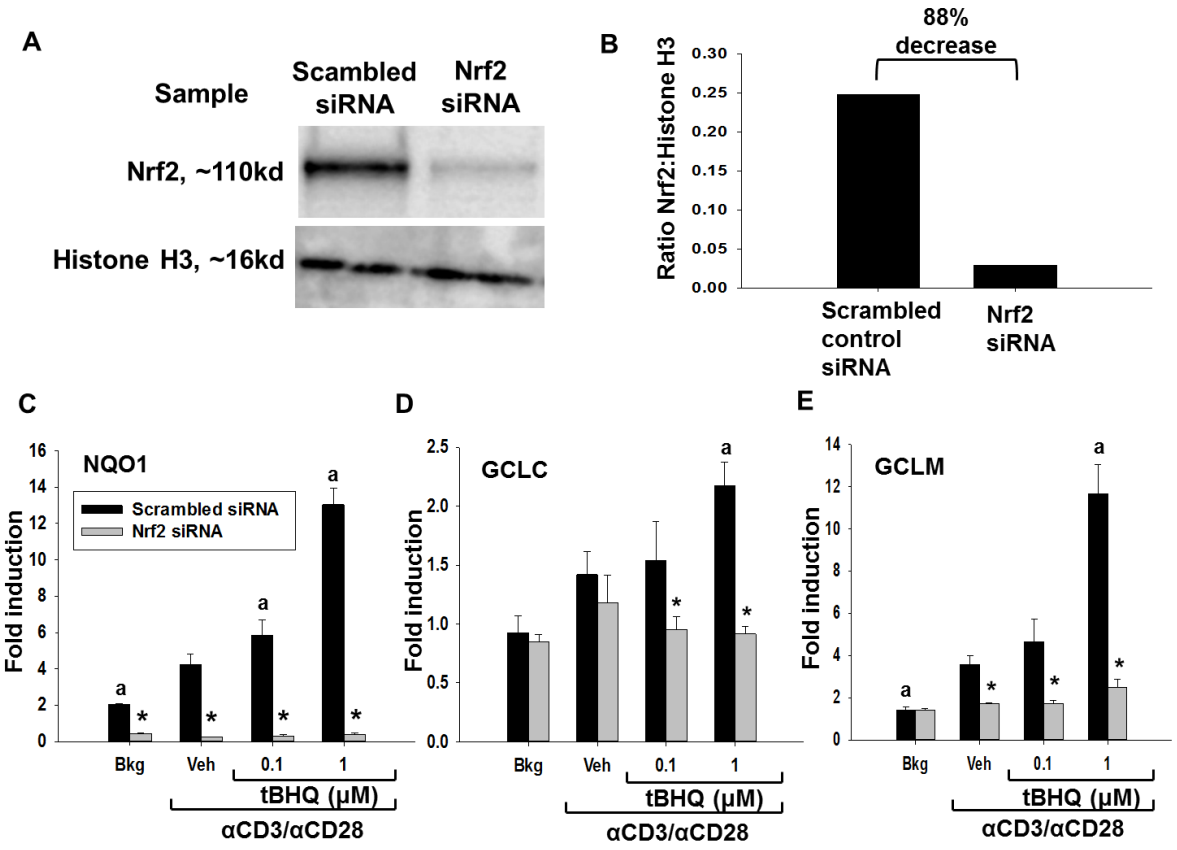

Figure 29. Transfection with siRNA against Nrf2 knocks down Nrf2 in primary human CD4 T cells.

Primary human CD4 T cells were nucleofected with either scrambled control siRNA or siRNA against Nrf2. A, B) 12 h after nucleofection cells were treated with 10 μM tBHQ, and 3 h after treatment, nuclear protein was collected. Protein levels were quantified and analyzed for levels of Nrf2 and histone H3 by western blot. A) A representative western blot. B) Densitometry of the scrambled control and Nrf2 siRNA samples from (A) shown as a ratio of the Nrf2 band to the histone H3 band. C-E) 12 h after nucleofection cells were treated with Veh (0.001% ethanol) or tBHQ (0.1-1 μM), followed by activation with αCD3/αCD28, or left untreated (Bkg). Total RNA was isolated from PBMCs 6 h after activation, and mRNA expression of C) NQO1, D) GCLC, and E) GCLM was analyzed by q-rtPCR. a indicates $p < 0.05$ as compared to the scrambled siRNA Veh group. * indicates $P < 0.05$ between the scrambled siRNA and Nrf2 siRNA groups of the same tBHQ (or Veh) treatment.

Inhibition of IFNγ, IL-2, TNFα, and GM-CSF production by tBHQ in primary human CD4 T cells occurs both in cells transfected with Nrf2-targeted or with scrambled control siRNA

After characterizing the Nrf2 knockdown using siRNA in primary human CD4 T cells, this model was utilized to determine the role of Nrf2 in the effects of tBHQ on early events following T cell activation. 12 h after nucleofection, cells were treated with tBHQ (0.01-1 μM) or vehicle (0.001% ethanol) followed by activation with αCD3/αCD28 for 6 h (mRNA) or 24 h (protein). tBHQ treatment inhibited IL-2 production in cells transfected with scrambled control siRNA as well as in cells transfected with siRNA targeting Nrf2 at both the protein and the mRNA level (Fig 30. A, B). tBHQ also inhibited IFNγ production at the protein and mRNA level in cells transfected with both scrambled control and siRNA targeting Nrf2 (Fig. 30 C, D).

The inhibition of IL-2 and IFNγ induction by tBHQ in primary human CD4 T cells prompted us to investigate the impact of tBHQ on two other cytokines produced rapidly upon T cell activation, TNFα and GM-CSF. tBHQ inhibited both TNFα and GM-CSF production at the protein and the mRNA level in scrambled control and Nrf2-deficient CD4 T cells (Fig. 31). Collectively, these data suggest that in primary human CD4 T cells, inhibition of early cytokine production by tBHQ occurs regardless of Nrf2 expression level.

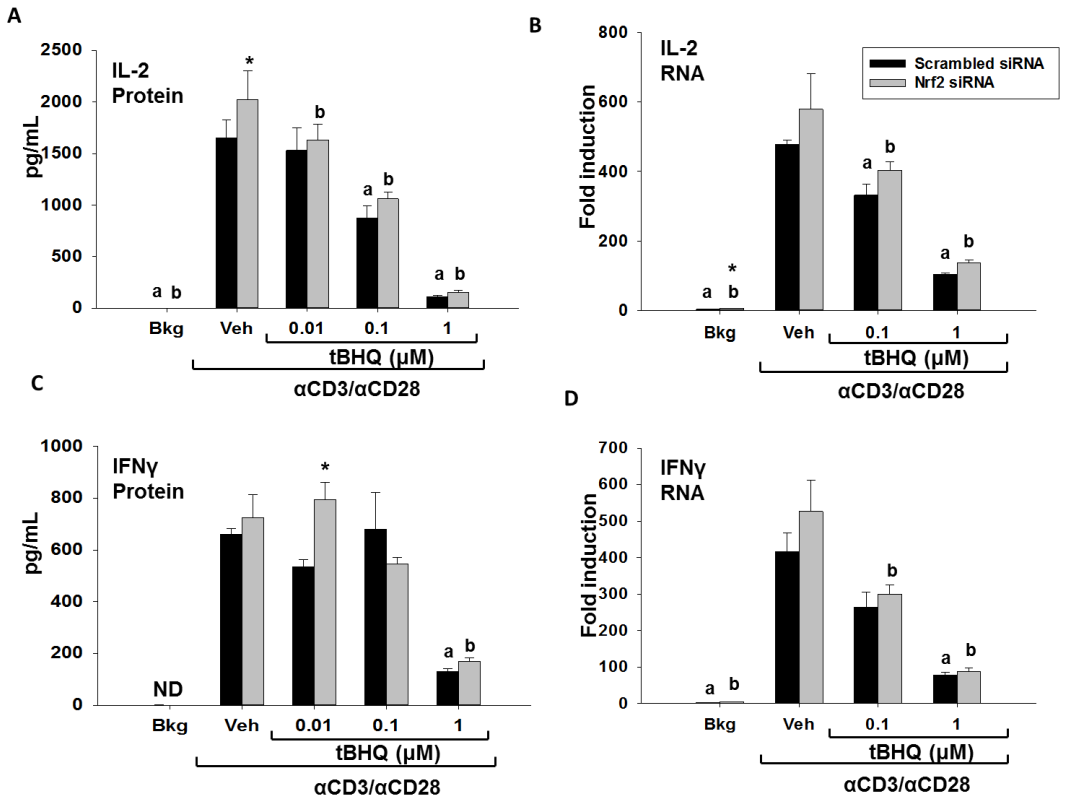

Figure 30. tBHQ inhibits induction of the cytokines IL-2 and IFNγ in both scrambled control and Nrf2-deficient CD4 T cells.

Isolated human CD4 T cells were nucleofected with scrambled control siRNA or Nrf2 siRNA. 12 h after nucleofection, cells were collected, counted, then treated with vehicle (Veh, 0.001% ethanol) or 0.01-1 μM tBHQ (protein) or 0.1-1 μM tBHQ (mRNA). 30 min later cells were activated with αCD3/αCD28 or left untreated (Bkg). 6 h after activation cells were collected, RNA isolated, and relative levels of B) IL-2 and D) IFNγ mRNA were quantified. 24 h after activation cells were collected and levels of A) IL-2 and C) IFNγ protein in the supernatant were quantified by ELISA as per the manufacturer's protocol. Data are presented as mean ± standard error. a indicates $p < 0.05$ as compared to the scrambled siRNA Veh group. b indicates $p < 0.05$ as compared to the Nrf2 siRNA Veh group. * indicates $P < 0.05$ between the scrambled siRNA and Nrf2 siRNA groups of the same tBHQ (or Veh) treatment. ND indicates samples below the limit of detection.

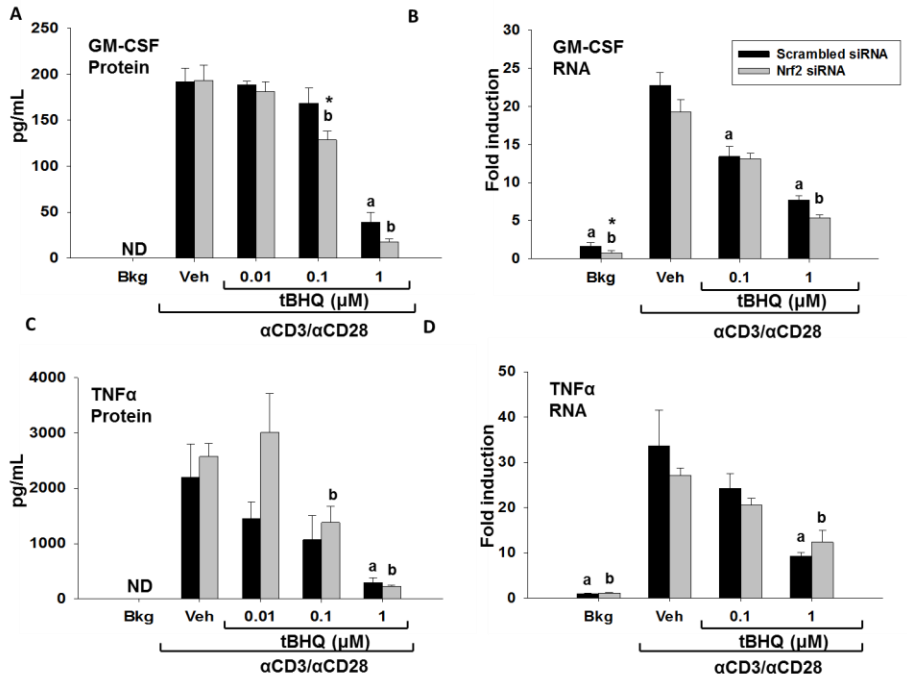

Figure 31. tBHQ inhibits induction of the cytokines GM-CSF and TNFα in both scrambled control and Nrf2-deficient CD4 T cells.
Isolated human CD4 T cells were nucleofected with scrambled control or Nrf2 siRNA. 12 h after nucleofection, cells were collected, counted, then treated with vehicle (Veh) or 0.01-1 μM tBHQ (protein) or 0.1-1 μM tBHQ (mRNA). 30 min later cells were activated with αCD3/αCD28, or left untreated (Bkg). 6 h after activation cells were collected, RNA isolated, and relative levels of B) GM-CSF and D) TNFα mRNA were quantified. 24h after activation cells were collected and levels of A) GM-CSF and C) TNFα protein in the supernatant were quantified by ELISA as per the manufacturer's protocol. Data are presented as mean ± standard error. a indicates $p < 0.05$ as compared to the scrambled siRNA Veh group. b indicates $p < 0.05$ as compared to the Nrf2 siRNA Veh group. * indicates $P < 0.05$ between the scrambled siRNA and Nrf2 siRNA groups of the same tBHQ (or Veh) treatment. ND indicates samples below the limit of detection.

Inhibition of the cell surface proteins CD25 and CD69 with tBHQ occurs in both scrambled control and Nrf2-deficient CD4 T cells

In addition to cytokine production, we also investigated other early events following T cell activation. Expression of the cell surface molecules, CD25 and CD69, is rapidly upregulated upon T cell activation, and prior work has shown that tBHQ inhibits expression of these cell surface proteins[346]. tBHQ inhibited CD25 expression in

scrambled control and in Nrf2-deficient cells, at both the protein and the mRNA level (Fig. 32). For CD69, tBHQ slightly inhibited protein levels of CD69. CD69 induction was decreased by tBHQ in both scrambled control and Nrf2-deficient CD4 T cells at the mRNA level as well (Fig. 33). Overall, CD69 expression was less sensitive to tBHQ treatment than other endpoints, and inhibition of CD25 and CD69 appears to be Nrf2-independent.

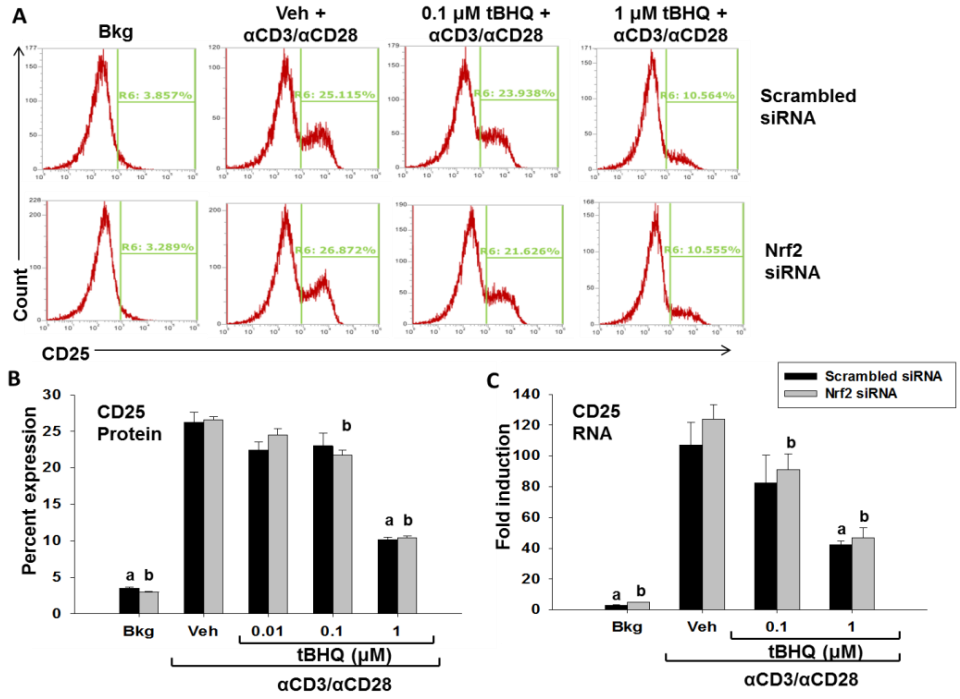

Figure 32. tBHQ inhibits induction of the cell surface protein CD25 in both scrambled control and Nrf2-deficient CD4 T cells.
Isolated human CD4 T cells were nucleofected with scrambled control or Nrf2 siRNA. 12 h after nucleofection, cells were collected, counted, then treated with vehicle (Veh) or 0.01-1 μM tBHQ (protein) or 0.1-1 μM tBHQ (mRNA). 30 min later cells were activated with αCD3/αCD28 or left untreated (Bkg). A, B) 24 h after activation cells were collected, labeled with fluorochrome-conjugated antibodies against CD25, and quantified by flow cytometry. A) Representative histograms and B) quantification of CD25 expression. C) 6 h after activation cells were collected, RNA isolated, and relative levels of CD25 mRNA were quantified. Data are presented as mean ± standard error. a indicates $p < 0.05$ as compared to the scrambled siRNA Veh group. b indicates $p < 0.05$

Figure 32 (cont'd)
as compared to the Nrf2 siRNA Veh group. * indicates P < 0.05 between the scrambled siRNA and Nrf2 siRNA groups of the same tBHQ (or Veh) treatment.

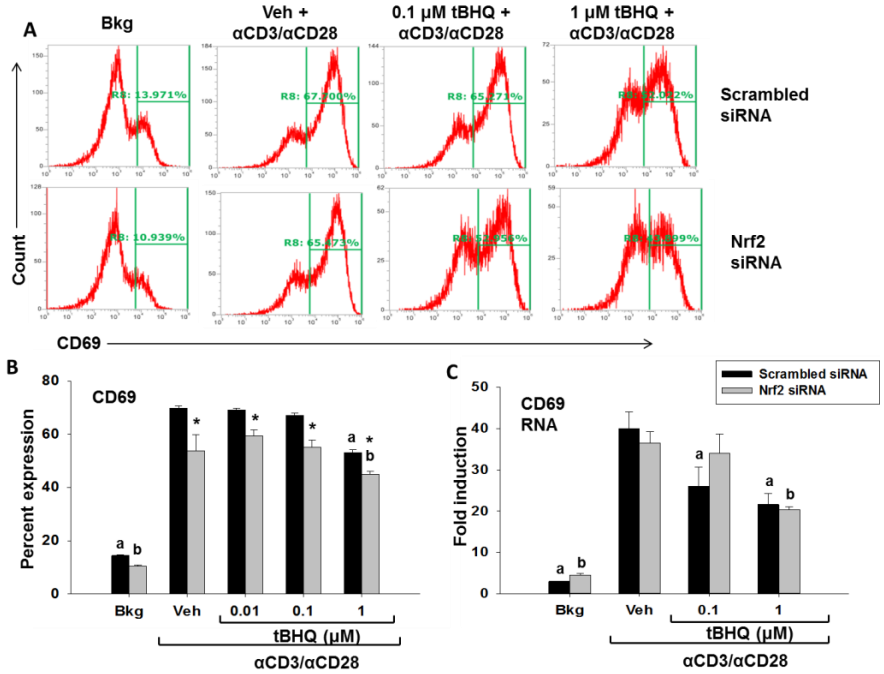

Figure 33. tBHQ inhibits induction of the cell surface protein CD69 in both scrambled control and Nrf2-deficient CD4 T cells.
Isolated human CD4 T cells were nucleofected with scrambled control or Nrf2 siRNA. 12 h after nucleofection, cells were collected, counted, then treated with vehicle (Veh) or 0.01-1 μM tBHQ (protein) or 0.1-1 μM tBHQ (mRNA). 30 min later cells were activated with αCD3/αCD28 or left untreated (Bkg). A, B) 24 h after activation cells were collected, labeled with fluorochrome-conjugated antibodies against CD69, and quantified by flow cytometry. A) Representative histograms and B) quantification of CD69 expression. C) 6 h after activation cells were collected, RNA isolated, and relative levels of CD69 mRNA were quantified. Data are presented as mean ± standard error. a indicates p < 0.05 as compared to the scrambled siRNA Veh group. b indicates p < 0.05 as compared to the Nrf2 siRNA Veh group. * indicates P < 0.05 between the scrambled siRNA and Nrf2 siRNA groups of the same tBHQ (or Veh) treatment.

Analysis of differential gene expression by RNA-seq

The effects of tBHQ on T cell activation investigated so far occur regardless of Nrf2 expression in this model, but only include a small number of potentially affected genes in these cells. Accordingly, global gene changes in scrambled control and Nrf2-

deficient T cells with or without tBHQ treatment prior to T cell activation were investigated by RNA-seq. Cluster analysis of gene expression in each of the six treatment groups demonstrates that overall changes in gene expression are primarily driven by T cell activation, followed by tBHQ treatment (Fig. 34). Within each treatment group, there were also changes between the scrambled control and Nrf2-deficient CD4 T cells, suggesting that the expression of certain genes is dependent on Nrf2 (Fig. 34). Differentially expressed genes (DEGs) between specific groups were analyzed, and gene changes that were unique or similar between different comparison groups are shown as Venn diagrams (Fig. 35). A large number of genes are differentially expressed upon T cell activation, with the majority of these genes (7050 genes) shared between the scrambled control and Nrf2-deficient CD4 T cells (Fig. 35 A). tBHQ treatment causes changes in a smaller number of genes (1276). More of the genes differentially expressed between the vehicle and tBHQ treatment groups are specific to the scrambled control siRNA transfection group (1720 genes), indicating that tBHQ has a number of Nrf2-specific effects, though there are also a number of DEGs shared between the scrambled control and Nrf2-deficient groups (Fig. 35 B). Interestingly, there are 499 DEGs with tBHQ treatment in the Nrf2-deficient CD4 T cells, suggesting that the absence of Nrf2 may also make the cells more sensitive to electrophilic exposure (Fig. 35 B).

There are many DEGs between the scrambled control and Nrf2-deficient CD4 T cells (Fig. 35 C). The largest number of DEGs is found in the unactivated cells, suggesting significant differences in basal gene expression based on Nrf2 expression in human CD4 T cells. 118 genes are differentially regulated between scrambled control

and Nrf2-deficient CD4 T cells treated with tBHQ prior to activation with αCD3/αCD38, indicating that these are genes regulated by tBHQ in activated CD4 T cells in a Nrf2-dependent manner. 73 of the genes that differ between the scrambled control and Nrf2-deficient CD4 T cells are consistently differentially expressed regardless of T cell activation or tBHQ treatment, and are listed in tables 2 and 3. Genes down regulated with Nrf2-knockdown by siRNA regardless of treatment group include a number of canonical Nrf2 target genes, including SLC7A11 (cystine/glutamate transporter xCT), NQO1, GCLM, TXNRD1 (thioredoxin), FTL and FTH1 (ferritin light and heavy chains), and AKR1C1 (aldo-keto reductase family 1 member C1). As expected, this group also included Nrf2 (NFE2L2) itself, further indicating successful Nrf2 knockdown. Also included are genes involved in the pentose phosphate pathway (G6PD, TALDO1), which has previously been shown to be regulated by Nrf2 in other cell types. Other DEGs included PANX2 (pannexin-2), ARHGEF26 (Rho guanine nucleotide exchange factor 26), PRKCB (protein kinase C beta type), CUEDC1 (CUE domain-containing protein 1), and AIFM2 (apoptosis-inducing factor 2) (Table 2). Fitting with the general view of Nrf2 as a transcriptional activator, fewer genes were consistently upregulated with Nrf2-knockdown. These included TNFAIP2 (tumor necrosis factor alpha-induced protein 2), FSCN1 (fascin), EID3 (EP300-interacting inhibitor of differentiation 3), CCL22, and LAD1 (Ladinin-1) (Table 3).

The most highly DEGs between the scrambled control and Nrf2-deficient CD4 T cells for each treatment group are listed in tables 4-8. Downregulated genes in the Nrf2-deficient CD4 T cells as compared to the scrambled control T cells for each treatment group are similar to the list of genes downregulated in all three treatment groups (Table

3), and include a number of Nrf2 target genes such as NQO1 and the cysteine/glutamate transporter (SLC7A11), as well as other genes such as pannexin-2 (Tables 4-6). In the activated cells treated with tBHQ, Nrf2-deficient cells express lower levels of the hemoglobin alpha 1, alpha 2, and beta subunits (HBA1, HBA2, HBB), which are known to be controlled by nuclear factor-erythroid 2 (NF-E2), a bZIP transcription factor closely related to Nrf2 (Table 6)[112,347]. Interestingly, genes that are upregulated in the Nrf2-deficient CD4 T cells as compared to scrambled control T cells in both un-activated and activated cells treated with tBHQ include a number of structural and extracellular matrix proteins, including collagen genes (COL3A1, COL1A2, COL1A1) and fibronectin 1 (FN1) (Tables 7 and 8).

There are numerous genes that are either upregulated or downregulated with tBHQ treatment in activated T cells in the Nrf2-deficient or scrambled control T cells. It is notable that several of the genes most strongly down-regulated by tBHQ treatment are shared between the scrambled control and Nrf2-deficient CD4 T cells, and include several genes with immune-cell specific functions, including CLC (galectin-10), IFNγ, IL-2, CCL4, and CXCL10 (Tables 9 and 10). This seems to indicate that many of the immunomodulatory early gene expression changes seen with tBHQ tend to occur regardless of siRNA transfection group. Interestingly, many of the genes increased with tBHQ treatment in the scrambled control siRNA transfected cells are not increased in the Nrf2 siRNA transfected cells, even at a lower fold change, indicating that genes upregulated by tBHQ tend to be more Nrf2 specific than the downregulated genes. Genes upregulated by tBHQ in the scrambled control siRNA group include hemoglobin genes, as well as nestin (NES), neurocan (NCAN), phospholipase D1 (PLD1), and a

tyrosine protein phosphatase (PTPRZ1) (Table 11). In the Nrf2-deficient CD4 T cells, many of the genes upregulated by tBHQ were also upregulated in the scrambled control siRNA transfected cells at a lower fold change, including the heat shock proteins (HSPA6 and HSPA1A), HMOX1, WNT9A, LYNX1, ARC, FOSB, CDKN1C, and GAS2L1 (Table 12). Other genes were induced by tBHQ only in the Nrf2-deficient CD4 T cells, including the zinc finger proteins SNAI3 and ZNF467, LMNTD2, the pseudogenes ETF1P1 and HSPA7, and antisense RNAs RP11-361D15.2 and KRTAP5-AS1 (Table 12).

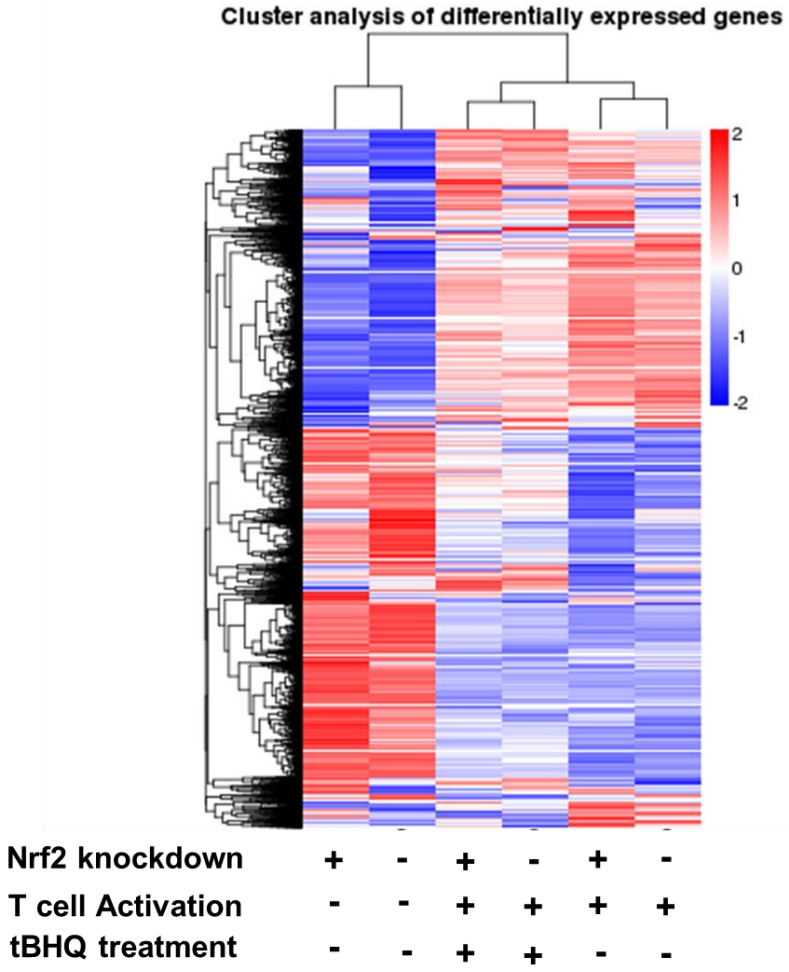

Figure 34 (cont'd)

Figure 34. Heatmap of differentially expressed genes in RNA-seq samples. Cluster analysis was done using log10(FPKM+1) values. Red denotes genes with high expression levels, and blue denotes genes with low expression levels.

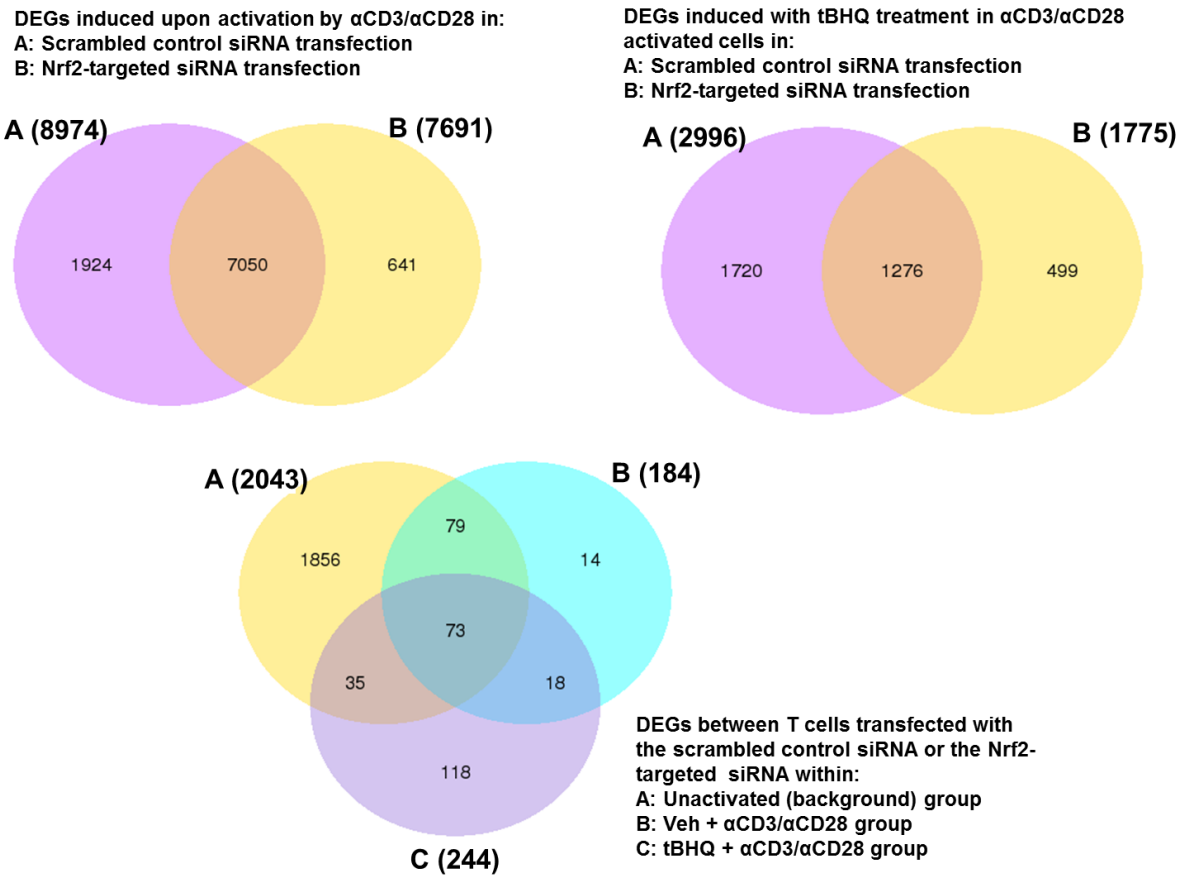

Figure 35. Numbers of differentially expressed genes in RNA-seq samples. Differentially expressed genes (DEGs) between the indicated treatment groups were identified as indicated in the methods. Numbers of DEGs shared between different comparison groups are shown in the overlapping area, and DEGs identified only in one comparison group are shown in each circle. Total numbers of DEGs for each comparison are listed in parenthesis.

Table 2. Genes down-regulated in Nrf2-deficient CD4 T cells as compared to scrambled control CD4 T cells regardless of treatment group.

Ensembl Gene ID	Gene Symbol	UniProt Protein Name	Log2 Fold Change in Untreated Groups	Log2 Fold Change in Veh + αCD3/αCD28 Groups	Log2 Fold Change in tBHQ +αCD3/αCD28 Groups
ENSG00000073150	PANX2	Pannexin-2	-4.1156	-4.4681	-3.8493
ENSG00000151012	SLC7A11	Cystine/glutamate transporter	-2.2935	-2.3963	-3.54
ENSG00000181019	NQO1	NAD(P)H dehydrogenase [quinone] 1	-1.911	-2.3242	-3.2024
ENSG00000116044	NFE2L2	Nuclear factor erythroid 2-related factor 2	-2.0445	-2.1947	-2.3225
ENSG00000140961	OSGIN1	Oxidative stress-induced growth inhibitor 1	-2.048	-2.0642	-4.607
ENSG00000198431	TXNRD1	Thioredoxin reductase 1, cytoplasmic	-1.9726	-2.0351	-3.1066
ENSG00000087086	FTL	Ferritin light chain	-1.4599	-2.0285	-2.4582
ENSG00000114790	ARHGEF26	Rho guanine nucleotide exchange factor 26	-1.3208	-1.9765	-3.1403
ENSG00000160211	G6PD	Glucose-6-phosphate 1-dehydrogenase	-1.6302	-1.8824	-2.0323
ENSG00000248323	LUCAT1	Lung cancer associated transcript 1 (non-protein coding)	-1.4326	-1.8677	-3.1834
ENSG00000187134	AKR1C1	Aldo-keto reductase family 1 member C1	-1.2043	-1.8544	-3.4768
ENSG00000166501	PRKCB	Protein kinase C beta type	-1.4303	-1.6258	-2.5313
ENSG00000177156	TALDO1	Transaldolase	-1.2144	-1.4258	-2.2733
ENSG00000180891	CUEDC1	CUE domain-containing protein 1	-1.3242	-1.3863	-2.0181
ENSG00000136810	TXN	Thioredoxin	-1.0352	-1.199	-1.9316
ENSG00000023909	GCLM	Glutamate--cysteine ligase regulatory subunit	-0.65363	-1.1682	-1.6934
ENSG00000042286	AIFM2	Apoptosis-inducing factor 2	-0.44288	-1.0239	-1.0439
ENSG00000167996	FTH1	Ferritin heavy chain; Ferritin	-0.99373	-1.0022	-1.6726
ENSG00000100439	ABHD4	Protein ABHD4 (abhydrolase domain containing 4)	-0.61906	-0.85192	-1.2868
ENSG00000104687	GSR	Glutathione reductase, mitochondrial	-0.52814	-0.80357	-1.6476
ENSG00000162616	DNAJB4	DnaJ homolog subfamily B member 4	-0.70572	-0.76809	-1.6463
ENSG00000105245	NUMBL	Numb-like protein	-0.6655	-0.7579	-1.0748
ENSG00000125089	SH3TC1	SH3 domain and tetratricopeptide repeat-containing protein 1	-0.94756	-0.74148	-0.96948
ENSG00000144040	SFXN5	Sideroflexin-5	-0.57304	-0.66289	-0.59955
ENSG00000149781	FERMT3	Fermitin family homolog 3	-0.66682	-0.64071	-0.9375
ENSG00000139112	GABARAPL1	Gamma-aminobutyric acid receptor-associated protein-like 1	-0.53256	-0.6041	-0.87371
ENSG00000102393	GLA	Alpha-galactosidase A	-0.2696	-0.60207	-1.1255
ENSG00000068323	TFE3	Transcription factor E3;	-0.58951	-0.56885	-0.63171
ENSG00000225783	MIAT	Myocardial infarction associated transcript (non-protein coding)	-0.48383	-0.56033	-0.69651
ENSG00000176463	SLCO3A1	Solute carrier organic anion transporter family member 3A1	-0.52454	-0.5455	-0.62613
ENSG00000109854	HTATIP2	Oxidoreductase HTATIP2	-0.65962	-0.52349	-0.87065
ENSG00000161011	SQSTM1	Sequestosome-1	-0.48986	-0.52248	-0.58159
ENSG00000177169	ULK1	Serine/threonine-protein kinase ULK1	-0.60695	-0.51148	-0.49952
ENSG00000181045	SLC26A11	Sodium-independent sulfate anion transporter	-0.49822	-0.50732	-0.53745
ENSG00000084207	GSTP1	Glutathione S-transferase P	-0.54475	-0.49939	-0.56002
ENSG00000070882	OSBPL3	Oxysterol-binding protein-related protein 3	-0.36117	-0.48256	-0.63793
ENSG00000127824	TUBA4A	Tubulin alpha-4A chain	-0.54527	-0.47033	-0.76394
ENSG00000184014	DENND5A	DENN domain-containing protein 5A	-0.24941	-0.4673	-0.76041
ENSG00000157193	LRP8	Low-density lipoprotein receptor-related protein 8	-0.71201	-0.46187	-0.88895
ENSG00000079999	KEAP1	Kelch-like ECH-associated protein 1	-0.4187	-0.45424	-0.60521
ENSG00000197063	MAFG	Transcription factor MafG	-0.48461	-0.43387	-0.58377
ENSG00000165271	NOL6	Nucleolar protein 6	-0.4964	-0.41536	-0.33457
ENSG00000107959	PITRM1	Presequence protease, mitochondrial	-0.26093	-0.41498	-0.63207
ENSG00000001084	GCLC	Glutamate--cysteine ligase catalytic subunit	-0.50931	-0.404	-0.96932
ENSG00000203485	INF2	Inverted formin-2	-0.5504	-0.38246	-0.28506
ENSG00000162413	KLHL21	Kelch-like protein 21	-0.64847	-0.37224	-0.57641
ENSG00000160185	UBASH3A	Ubiquitin-associated and SH3 domain-containing protein A	-0.27627	-0.35637	-0.31524
ENSG00000152270	PDE3B	Phosphodiesterase 3B	-0.33418	-0.35054	-0.3119
ENSG00000067057	PFKP	ATP-dependent 6-phosphofructokinase, platelet type	-0.46834	-0.33932	-0.41663
ENSG00000099204	ABLIM1	Actin-binding LIM protein 1	-0.16986	-0.33349	-0.7393
ENSG00000111885	MAN1A1	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	-0.18305	-0.3061	-0.3841
ENSG00000100297	MCM5	DNA replication licensing factor MCM5; DNA helicase	-0.31645	-0.30211	-0.5046
ENSG00000064393	HIPK2	Homeodomain-interacting protein kinase 2	-0.2038	-0.28931	-0.49012
ENSG00000117450	PRDX1	Peroxiredoxin-1	-0.36021	-0.28665	-0.66866
ENSG00000011275	RNF216	E3 ubiquitin-protein ligase RNF216	-0.34795	-0.28642	-0.38352
ENSG00000101849	TBL1X	F-box-like/WD repeat-containing protein TBL1X	-0.25263	-0.27736	-0.34194
ENSG00000142657	PGD	6-phosphogluconate dehydrogenase, decarboxylating	-0.33567	-0.2583	-0.7442
ENSG00000105220	GPI	Glucose-6-phosphate isomerase	-0.30433	-0.23928	-0.29848
ENSG00000067225	PKM	Pyruvate kinase; Pyruvate kinase PKM	-0.23593	-0.22352	-0.30238
ENSG00000171658	-	NmrA like redox sensor 2, pseudogene	-4.167	Undetected in Nrf2	-6.5981

Table 3. Genes up-regulated in Nrf2-deficient CD4 T cells as compared to scrambled control CD4 T cells regardless of treatment group.

Ensembl Gene ID	Gene Symbol	UniProt Protein Name	Log2 Fold Change in Untreated Groups	Log2 Fold Change in Veh + αCD3/αCD28 Groups	Log2 Fold Change in tBHQ +αCD3/αCD28 Groups
ENSG00000159166	LAD1	Ladinin-1	1.4851	1.4356	1.3902
ENSG00000102962	CCL22	C-C motif chemokine 22	1.3846	1.3458	1.3218
ENSG00000255150	EID3	EP300-interacting inhibitor of differentiation 3;	0.80547	0.8008	0.51743
ENSG00000075618	FSCN1	Fascin	0.44754	0.65967	1.0292
ENSG00000185215	TNFAIP2	Tumor necrosis factor alpha-induced protein 2	0.77793	0.59504	0.72575
ENSG00000167460	TPM4	Tropomyosin alpha-4 chain;	0.52608	0.54903	0.38984
ENSG00000123131	PRDX4	Peroxiredoxin-4	0.63431	0.48803	0.44538
ENSG00000119922	IFIT2	Interferon-induced protein with tetratricopeptide repeats 2	0.56186	0.43062	0.42473
ENSG00000196141	SPATS2L	SPATS2-like protein	0.36102	0.42512	0.42223
ENSG00000096968	JAK2	Tyrosine-protein kinase JAK2; Tyrosine-protein kinase	0.37904	0.31308	0.38902
ENSG00000140968	IRF8	Interferon regulatory factor 8;	0.68924	0.30852	0.58952
ENSG00000166197	NOLC1	Nucleolar and coiled-body phosphoprotein 1	0.29077	0.29842	0.26509
ENSG00000118971	CCND2	G1/S-specific cyclin-D2	0.20416	0.2505	0.36127

Table 4. Genes down-regulated in Nrf2-deficient CD4 T cells as compared to scrambled control CD4 T cells in the unactivated (background) group.
The 20 genes with the largest fold change values are displayed, provided that absolute value of the Log2FoldChange was greater than1.5.

Ensembl Gene ID	Gene Symbol	Description	log2 FoldChange	Adjusted P value
ENSG00000171658	RP11-443P15.2	pseudogene	-4.167	0.00026962
ENSG00000073150	PANX2	Pannexin-2	-4.1156	9.89E-24
ENSG00000135454	B4GALNT1	Beta-1,4 N-acetylgalactosaminyltransferase 1	-3.4005	0.0054942
ENSG00000106178	CCL24	C-C motif chemokine ligand 24	-2.7336	0.049528
ENSG00000271303	SRXN1	Sulfiredoxin-1	-2.3323	0.018672
ENSG00000087842	PIR	Pirin (iron-binding nuclear protein)	-2.3099	0.03701
ENSG00000151012	SLC7A11	Cystine/glutamate transporter	-2.2935	9.48E-68
ENSG00000249846	RP11-77P16.4	processed transcript	-2.078	0.022251
ENSG00000140961	OSGIN1	Oxidative stress-induced growth inhibitor 1	-2.048	0.030805
ENSG00000116044	NFE2L2	Nuclear factor erythroid 2-related factor 2	-2.0445	2.24E-169
ENSG00000198431	TXNRD1	Thioredoxin reductase 1, cytoplasmic	-1.9726	2.34E-72
ENSG00000181019	NQO1	NAD(P)H dehydrogenase [quinone] 1	-1.911	5.43E-11
ENSG00000160211	G6PD	Glucose-6-phosphate 1-dehydrogenase	-1.6302	8.35E-65
ENSG00000198853	RUSC2	RUN and SH3 domain containing 2, Iporin	-1.6209	3.87E-10

Table 5. Genes down-regulated in the Nrf2-deficient CD4 T cells as compared to scrambled control CD4 T cells in the vehicle + αCD3/αCD28 treatment group.
The 20 genes with the largest fold change values are displayed, provided that absolute value of the Log2FoldChange was greater than1.5.

Ensembl Gene ID	Gene Symbol	Description	log2 FoldChange	Adjusted P value
ENSG00000073150	PANX2	Pannexin-2; Pannexin;	-4.4681	2.11E-09
ENSG00000154277	UCHL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	-2.8286	0.042614
ENSG00000100292	HMOX1	Heme oxygenase 1	-2.6365	0.00048746
ENSG00000215267	AKR1C7P	aldo-keto reductase family 1, member C7, pseudogene	-2.5669	0.0039522
ENSG00000151012	SLC7A11	Cystine/glutamate transporter	-2.3963	4.37E-59
ENSG00000181019	NQO1	NAD(P)H dehydrogenase [quinone] 1	-2.3242	7.86E-17
ENSG00000116044	NFE2L2	Nuclear factor erythroid 2-related factor 2	-2.1947	1.56E-157
ENSG00000140961	OSGIN1	Oxidative stress-induced growth inhibitor 1	-2.0642	0.0081315
ENSG00000198431	TXNRD1	Thioredoxin reductase 1, cytoplasmic	-2.0351	7.87E-199
ENSG00000087086	FTL	Ferritin light chain	-2.0285	1.04E-219
ENSG00000114790	ARHGEF26	Rho guanine nucleotide exchange factor 26	-1.9765	0.01529
ENSG00000160211	G6PD	Glucose-6-phosphate 1-dehydrogenase	-1.8824	7.64E-119
ENSG00000248323	LUCAT1	lung cancer associated transcript 1 (non-protein coding)	-1.8677	5.87E-08
ENSG00000187134	AKR1C1	Aldo-keto reductase family 1 member C1	-1.8544	0.0013744
ENSG00000166501	PRKCB	Protein kinase C beta type	-1.6258	1.48E-74
ENSG00000171658	RP11-443P15.2	pseudogene	Undetected in Nrf2	4.78E-09

Table 6. Genes down-regulated in the Nrf2-deficient CD4 T cells as compared to scrambled control CD4 T cells in the tBHQ + αCD3/αCD28 treatment group.
The 20 genes with the largest fold change values are displayed, provided that absolute value of the Log2FoldChange was greater than1.5.

Ensembl Gene ID	Gene Symbol	Description	log2 FoldChange	Adjusted P value
ENSG00000188536	HBA2	Hemoglobin subunit alpha 2	-9.4617	4.11E-112
ENSG00000244734	HBB	Hemoglobin subunit beta	-7.4862	6.92E-139
ENSG00000206172	HBA1	Hemoglobin subunit alpha 1	-6.7307	1.22E-49
ENSG00000171658	RP11-443P15.2	Pseudogene	-6.5981	4.27E-31
ENSG00000162873	KLHDC8A	Kelch domain-containing protein 8A	-4.7379	0.009305
ENSG00000130287	NCAN	Neurocan core protein	-4.636	0.017119
ENSG00000140961	OSGIN1	Oxidative stress-induced growth inhibitor 1	-4.607	2.05E-32
ENSG00000100292	HMOX1	Heme oxygenase 1	-4.6041	6.33E-12
ENSG00000073150	PANX2	Pannexin-2	-3.8493	6.52E-24
ENSG00000196326	AKR1CL1	Aldo-keto reductase family 1, member C-like 1	-3.7393	0.041164
ENSG00000151012	SLC7A11	Cystine/glutamate transporter	-3.54	7.06E-193
ENSG00000087842	PIR	Pirin (iron-binding nuclear protein)	-3.522	4.57E-10
ENSG00000187134	AKR1C1	Aldo-keto reductase family 1 member C1	-3.4768	9.25E-35
ENSG00000250033	SLC7A11-AS1	SLC7A11 antisense RNA 1	-3.4255	5.45E-10
ENSG00000154277	UCHL1	Ubiquitin carboxyl-terminal hydrolase L1	-3.241	0.014234
ENSG00000196139	AKR1C3	Aldo-keto reductase family 1 member C3;	-3.2382	3.42E-27
ENSG00000181019	NQO1	NAD(P)H dehydrogenase [quinone] 1;	-3.2024	3.75E-17
ENSG00000248323	RP11-213H15.3	processed_transcript	-3.1834	7.36E-43
ENSG00000114790	ARHGEF26	Rho guanine nucleotide exchange factor 26;	-3.1403	1.09E-19
ENSG00000215267	AKR1C7P	Aldo-keto reductase family 1, member C7, pseudogene	-3.1088	5.00E-16

Table 7. Genes up-regulated in the Nrf2-deficient CD4 T cells as compared to scrambled control CD4 T cells in the unactivated (background) group.
The 20 genes with the largest fold change values are displayed, provided that absolute value of the Log2FoldChange was greater than1.5.

Ensembl Gene ID	Gene Symbol	Description	log2 FoldChange	Adjusted P value
ENSG00000091986	CCDC80	Coiled-coil domain-containing protein 80	None Detected in Ctrl	0.039337
ENSG00000113721	PDGFRB	Platelet-derived growth factor receptor beta	None Detected in Ctrl	0.026588
ENSG00000117009	KMO	Kynurenine 3-monooxygenase	None Detected in Ctrl	0.04045
ENSG00000168542	COL3A1	Collagen alpha-1(III) chain	None Detected in Ctrl	1.92E-05
ENSG00000164692	COL1A2	Collagen alpha-2(I) chain	7.4746	1.77E-05
ENSG00000113140	SPARC	secreted protein acidic and cysteine rich (osteonectin)	6.1508	0.046143
ENSG00000108821	COL1A1	Collagen alpha-1(I) chain	4.5593	0.0016528
ENSG00000120708	TGFBI	Transforming growth factor beta-induced	3.5334	0.0023275
ENSG00000182389	CACNB4	Voltage-dependent L-type calcium channel subunit beta-4	3.4708	0.012157
ENSG00000129277	CCL4	C-C motif chemokine ligand 4	2.7238	0.024038
ENSG00000225091	SNORA71A	Processed transcript	2.1522	0.036224
ENSG00000185433	LINC00158	Long intergenic non-protein coding RNA 158	2.1029	0.027544
ENSG00000105855	ITGB8	Integrin beta 8	1.5659	0.0022869

Table 8. Genes up-regulated in the Nrf2-deficient CD4 T cells as compared to scrambled control CD4 T cells in the tBHQ + αCD3/αCD28 treatment group.
The 20 genes with the largest fold change values are displayed, provided that absolute value of the Log2FoldChange was greater than1.5.

Ensembl Gene ID	Gene Symbol	Description	log2 FoldChange	Adjusted P value
ENSG00000138829	FBN2	Fibrillin-2	None Detected in Ctrl	0.015165
ENSG00000168542	COL3A1	Collagen alpha-1(III) chain	None Detected in Ctrl	1.02E-07
ENSG00000164692	COL1A2	Collagen alpha-2(I) chain	7.7653	8.56E-32
ENSG00000108821	COL1A1	Collagen alpha-1(I) chain	3.7674	2.96E-13
ENSG00000115414	FN1	Fibronectin 1	2.1453	2.87E-05
ENSG00000171992	SYNPO	Synaptopodin	1.8963	1.02E-07
ENSG00000128917	DLL4	Delta-like protein 4	1.8782	0.029801

Table 9. Genes down-regulated with tBHQ + αCD3/αCD28 treatment as compared to vehicle + αCD3/αCD28 treatment in the scrambled control CD4 T cells.
The 20 genes with the largest fold change values are displayed, provided that absolute value of the Log2FoldChange was greater than1.5.

Ensembl Gene ID	Gene Symbol	Description	log2 FoldChange	Adjusted P value
ENSG00000244065	RP11-221E20.5	Pseudogene	-4.3853	0.015795
ENSG00000105205	CLC	Galectin-10, Charcot-Leyden crystal protein	-3.0301	3.44E-07
ENSG00000196917	HCAR1	Hydroxycarboxylic acid receptor 1	-2.2272	2.90E-05
ENSG00000213018	AL590762.11	Pseudogene	-2.0637	0.041105
ENSG00000109471	IL2	Interleukin-2	-1.8527	2.84E-42
ENSG00000224940	PRRT4	Proline-rich transmembrane protein 4	-1.804	0.036012
ENSG00000111537	IFNG	Interferon gamma	-1.788	1.79E-34
ENSG00000143061	IGSF3	Immunoglobulin superfamily member 3	-1.7731	3.87E-18
ENSG00000127124	HIVEP3	Transcription factor HIVEP3	-1.6372	2.01E-94
ENSG00000129277	CCL4	Chemokine (C-C motif) ligand 4	-1.6149	6.28E-05
ENSG00000236254	MTND4P14	Pseudogene	-1.561	0.022614
ENSG00000169248	CXCL11	Chemokine (C-X-C motif) ligand 11	-1.5312	1.04E-55
ENSG00000000971	CFH	Complement factor H	-1.5269	0.036314
ENSG00000234285	GAPDHP49	Glyceraldehyde 3 phosphate dehydrogenase pseudogene 49	Undetected in tBHQ grp.	0.0064628
ENSG00000258620	RP11-362L22.1	sense_intronic	Undetected in tBHQ grp.	0.033092

Table 10. Genes down-regulated with tBHQ + αCD3/αCD28 treatment as compared to vehicle + αCD3/αCD28 treatment in the Nrf2-deficient CD4 T cells.
The 20 genes with the largest fold change values are displayed, provided that absolute value of the Log2FoldChange was greater than1.5.

Ensembl Gene ID	Gene Symbol	Description	log2 FoldChange	Adjusted P value
ENSG00000105205	CLC	Galectin-10, Charcot-Leyden crystal protein	-2.1596	0.011149
ENSG00000250274	CTB-114C7.4	lincRNA	-2.0089	0.023884
ENSG00000111537	IFNG	Interferon gamma	-1.8313	1.31E-24
ENSG00000196917	HCAR1	Hydroxycarboxylic acid receptor 1	-1.8222	1.74E-06
ENSG00000109471	IL2	Interleukin-2	-1.7994	3.16E-51
ENSG00000129277	CCL4	C-C motif chemokineligand 4	-1.7168	0.0056549
ENSG00000138755	CXCL9	C-X-C motif chemokine ligand 9	-1.6844	8.64E-43
ENSG00000169248	CXCL11	C-X-C motif chemokine ligand 11	-1.6658	2.12E-36
ENSG00000169245	CXCL10	C-X-C motif chemokine ligand 10	-1.6618	3.20E-42
ENSG00000226979	LTA	Lymphotoxin-alpha	-1.512	1.12E-17

Table 11. Genes up-regulated with tBHQ + αCD3/αCD28 treatment as compared to vehicle + αCD3/αCD28 treatment in the scrambled control CD4 T cells.
The 20 genes with the largest fold change values are displayed, provided that absolute value of the Log2FoldChange was greater than1.5.

Ensembl Gene ID	Gene Symbol	Description	log2 FoldChange	Adjusted P value
ENSG00000188536	HBA2	Hemoglobin subunit alpha 2	9.8223	2.06E-142
ENSG00000206172	HBA1	Hemoglobin subunit alpha 1	7.1672	1.60E-63
ENSG00000244734	HBB	Hemoglobin subunit beta	6.7388	2.77E-142
ENSG00000100292	HMOX1	Heme oxygenase 1	5.4489	1.26E-15
ENSG00000162873	KLHDC8A	Kelch domain-containing protein 8A	5.223	0.00012991
ENSG00000115461	IGFBP5	Insulin-like growth factor-binding protein 5	4.8802	0.0030304
ENSG00000134323	MYCN	N-myc proto-oncogene protein	4.6966	0.0091788
ENSG00000164434	FABP7	Fatty acid-binding protein 7, brain	4.2721	0.00016266
ENSG00000130287	NCAN	Neurocan	4.1712	0.0010483
ENSG00000234292	RP11-213H15.1	lincRNA	3.7495	0.018324
ENSG00000006468	ETV1	ETS translocation variant 1	3.7254	0.01272
ENSG00000265778	RP11-17M16.2	Antisense	3.5935	0.035865
ENSG00000106278	PTPRZ1	Receptor-type tyrosine-protein phosphatase zeta;	3.4307	0.04834
ENSG00000115457	IGFBP2	Insulin-like growth factor-binding protein 2	3.1945	0.00091962
ENSG00000132688	NES	Nestin	3.1908	0.00075383
ENSG00000188163	FAM166A	Protein FAM166A (Family with sequence similarity 166, member	2.9056	1.22E-06
ENSG00000075651	PLD1	Phospholipase D1	2.6017	0.0040418
ENSG00000173110	HSPA6	Heat shock 70 kDa protein 6	2.2646	1.05E-09
ENSG00000171658	RP11-443P15.2	Pseudogene	2.1696	1.44E-11
ENSG00000196326	AKR1CL1	Aldo-keto reductase family 1, member C-like 1	2.1658	0.045919

Table 12. Genes up-regulated with tBHQ + αCD3/αCD28 treatment as compared to vehicle + αCD3/αCD28 treatment in the Nrf2-deficient CD4 T cells.
The 20 genes with the largest fold change values are displayed, provided that absolute value of the Log2FoldChange was greater than1.5.

Ensembl Gene ID	Gene Symbol	Description	log2 FoldChange	Adjusted P value
ENSG00000232757	ETF1P1	pseudogene (eukaryotic translation termination factor 1 pseudogene 1)	None Detected in Nrf2 Veh	0.047959
ENSG00000225217	HSPA7	pseudogene (heat shock 70kDa protein 7 (HSP70B))	4.6387	0.01094
ENSG00000259238	RP11-361D15.2	Antisense	4.3661	0.035702
ENSG00000100292	HMOX1	Heme oxygenase 1	3.4748	7.22E-08
ENSG00000188163	FAM166A	Family with sequence similarity 166, member A	3.0966	0.003546
ENSG00000143816	WNT9A	Protein Wnt; Protein Wnt-9a	2.8997	0.0021294
ENSG00000173110	HSPA6	Heat shock 70 kDa protein 6	2.4257	2.01E-31
ENSG00000204389	HSPA1A	Heat shock 70 kDa protein 1A	2.2267	1.05E-06
ENSG00000233930	KRTAP5-AS1	Antisense RNA to KRTAP5-1/KRTAP5-2	2.1857	0.012956
ENSG00000197768	STPG3	Protein STPG3 (sperm-tail PG-rich repeat containing 3)	2.0568	0.049253
ENSG00000185669	SNAI3	Zinc finger protein SNAI3	1.7594	0.0076906
ENSG00000180155	LYNX1	Ly-6/neurotoxin 1	1.6491	1.80E-07
ENSG00000129757	CDKN1C	Cyclin-dependent kinase inhibitor 1C	1.6435	0.0072963
ENSG00000185340	GAS2L1	Growth arrest-specific 2 like 1	1.6212	0.00049876
ENSG00000198576	ARC	Activity-regulated cytoskeleton-associated protein	1.6148	0.0006783
ENSG00000142694	EVA1B	Protein eva-1 homolog B	1.5412	0.030794
ENSG00000181444	ZNF467	Zinc finger protein 467	1.5346	0.0089111
ENSG00000125740	FOSB	FosB Proto-Oncogene, AP-1 Transcription Factor Subunit	1.5268	2.86E-11
ENSG00000185522	LMNTD2	Lamin tail domain-containing protein 2	1.5036	0.0022019

Discussion

These studies detail the development of a Nrf2 knockdown model in primary human CD4 T cells. Three methods of generating a knockdown were tested: transfection with an shRNA expression plasmid, CRISPR/Cas9 gene editing, and transfection with siRNA. The most successful method was siRNA, which was used for further experiments. Using nucleofection to transfect primary CD4 T cells with siRNA directed against Nrf2, we achieved an average knockdown of Nrf2 protein levels of ~90% at 12h. The Nrf2-deficient CD4 T cells generated in this manner were then used to assess the role of Nrf2 in tBHQ-mediated inhibition of multiple immune parameters associated with early time points following activation of primary human CD4 T cells. tBHQ inhibited production of the cytokines IL-2, IFNγ, GM-CSF, and TNFα independently of Nrf2 expression level. tBHQ treatment also inhibited induction of the cell surface proteins CD25 and CD69 in both scrambled control and Nrf2-deficient CD4 T cells. Together, these data suggest that the effects of tBHQ on early events following

CD4 T cell activation may be independent of Nrf2, which is consistent with our previously published observations in primary mouse CD4 T cells.

To our knowledge, there is one other report of Nrf2 knockdown in primary human CD4 T cells, which showed a knockdown of roughly 40% of Nrf2 expression. With this level of Nrf2 knockdown, treatment with arsenic (III) inhibited IL-2, IFNγ, TNFα, and IL-17 induction in both the scrambled control and in T cells with reduced Nrf2 expression, which is consistent with our previously published studies with arsenic trioxide in primary T cells derived from wild-type and Nrf2-null mice[285]. The present studies demonstrate that tBHQ, commonly used as a Nrf2 activator, has a number of effects in primary human CD4 T cells that occur regardless of Nrf2 knockdown. As all of the Nrf2 activators that are currently used experimentally, including tBHQ and toxic metals such as arsenic, are known to have Nrf2-independent effects, these studies fit with established literature[199,232,348].

The potent and robust effects of tBHQ on immune parameters in activated T cells is of interest from a toxicological standpoint, as tBHQ is a widely used food additive and as such, people are commonly exposed to tBHQ. tBHQ is permitted by the FDA for use in food at concentrations at or below 200 mg/kg to prevent the rancidification of oils[225]. The acceptable daily intake of tBHQ set by the World Health Organization is 0.7 mg/kg body weight/day, and estimates calculated from model diets indicate that high consumers may consume close to or even exceed this level[227]. A study done with human volunteers given a single dose of 100-150 mg tBHQ saw serum concentrations of tBHQ in the high micromolar range, well above the concentrations used in these

studies[226]. Thus, exposure to tBHQ by humans could be considerable depending on diet, and the concentrations used in the current studies are physiologically relevant.

There are numerous mechanisms by which tBHQ could mediate immune effects independently of Nrf2. tBHQ functions as an antioxidant by scavenging ROS, but it is also capable of redox cycling. Furthermore, tBHQ is an electrophilic compound that can interact with cysteine residues and change the redox status of the cell[315,349,350]. It is known that the redox state of a T cell modulates T cell activation, though specific effects and mechanisms are still being determined[351]. ROS are generated upon T cell activation, and contribute to signaling downstream of the T cell receptor, however, high levels of ROS contribute to activation induced cell death in T cells. tBHQ may shift the redox state of the T cells in a manner that disrupts T cell activation. In addition, tBHQ could also interact with signaling pathways important for T cell activation independently of Nrf2. AP-1 is a dimeric transcription factor involved in T cell activation, and tBHQ has been shown to induce AP-1 family members, including c-Jun and Fra1[352]. Increases in some AP-1 family members do not necessarily induce AP-1 signaling overall, as some family members, such as Fra-1, lack a transactivational domain and can decrease AP-1 signaling under certain conditions[353,354]. Furthermore, T cell signaling is tightly regulated, and an imbalance in the levels of AP-1relative to other transcription factors, or activation at the wrong time, could interfere with the coordination of TCR signaling, leading to decreased activation[355]. tBHQ has also been shown to decrease NF-κB, which is another transcription factor crucial for T cell activation and is also a redox-regulated protein[217,322,326]. There is crosstalk between Nrf2 and NF-κB, but there is also a potential for a Nrf2-independent effect of tBHQ on NF-κB activation[310].

RNA-seq analysis of scrambled control and Nrf2-deficient CD4 T cells led to the identification of numerous genes that appear to be regulated by Nrf2. Although many of the genes that were identified are known Nrf2 target genes, we also identified a number of other genes that do not have an established link to Nrf2. This indicates that there is a potential role for Nrf2 in CD4 T cell activation, the nature of which remains to be elucidated. In addition, the modulation of some of these genes at early time points following T cell activation could potentially affect the differentiation of CD4 T cells at later time points, which could ultimately impact effector function.

Several of the genes that were differentially expressed between scrambled control and Nrf2-deficient CD4 T cells are involved in cellular metabolism and/or in cellular signaling pathways, indicating that these processes may be impacted by Nrf2 expression. Several of these DEGs, such as G6PD and TALDO1, are involved in the pentose phosphate pathway, which is important for the synthesis of NADPH. NADPH plays a key role in the response to oxidative stress as a reducing agent, and thus is also important in cytoprotective responses[356]. T cells undergo shifts in metabolism upon activation, which are needed to support the energy expenditure and cytoskeletal changes associated with activation, proliferation, and, at later time points, differentiation. Thus, the effect of Nrf2 expression on genes associated with cell metabolism may represent a potential mechanism by which Nrf2 could influence T cell differentiation and function[50]. Regulation of metabolism is necessary to support production of molecules needed for growth and proliferation. Several metabolic programs have been shown to influence T cell differentiation, and the effects of these changes would be more likely seen at later, rather than early, time points following T cell activation[11]. Other DEGs do

not have an obvious link to T cell activation and function, and indicate potential areas for further investigation, including pannexin-2, AHRGEF26, Ladinin-1, neurocan, collagen, and others. These studies also identified a number of DEGs which do not have established functions, such as CUEDC1, EID3, and several of the pseudogenes and antisense RNAs.

Pannexin-2 is one of the most highly downregulated genes in the Nrf2-deficient T cells as compared to the scrambled control T cells regardless of treatment group. Pannexin-2 belongs to a family of proteins including pannexin-1 and pannexin-3 that form small-molecule conducting single-membrane channels[357]. While a role of pannexin-2 in T cells has not been described, pannexin-1 has been shown to be able to contribute to T cell activation through the release of ATP and subsequent activation of P2X receptors upon T cell activation[358]. It has been proposed that pannexin-2 may also release ATP similarly to pannexin-1, but as pannexin-2 is primarily cytosolic, it may also be involved in molecular transport between organelles, play a role in Ca^{2+} release from the endoplasmic reticulum, or interact with endosomes[357,359–361]. All of these functions intersect with events of T cell activation, and could indicate a potential role for pannexin-2 in T cell activation.

Also consistently down-regulated in Nrf2-deficient T cells as compared to the scrambled control cells were protein kinase C beta (PKCβ) and ARHGEF26, a Rho guanine nucleotide exchange factor also known as SGEF. SGEF activates RhoG, a GTPase expressed in many cell types that in T cells has been proposed to have roles in actin cytoskeletal rearrangement and receptor internalization, providing a potential link between SGEF and T cell activation[362–365]. While PKC theta is a critical component of

signaling downstream of the T cell receptor and crucial for T cell activation, a role for PKCβ in T cells is possible but has not been clearly established[366,367].

Interestingly, tBHQ down regulated several genes known to be involved in T cell activation, such as IL-2, IFNγ, and CCL4, in both the scrambled control and Nrf2-deficient CD4 T cells, indicating that the immunosuppressive effects on T cell activation mediated by tBHQ in primary human CD4 T cells may not be Nrf2-dependent. Taken together, this supports the hypothesis that the role of Nrf2 in primary human CD4 T cells is likely to be in the regulation of CD4 T cell proliferation and differentiation, with lesser effects on early events of T cell activation. tBHQ does have a role in early T cell activation, but many of the immune effects of tBHQ occur in both the scrambled control and Nrf2-deficient CD4 T cells. The impact of tBHQ on later events following activation of primary human CD4 T cells was not examined in this study and will be the focus of future investigation.

T cells are a critical part of adaptive immunity, and are important for host defense against a variety of pathogens as well as in immune mediated diseases such as autoimmunity as allergy. Nrf2 activation is a common response in cells to a wide variety of toxicants and cellular stressors; however, Nrf2 plays a critical role in modulating immune and inflammatory responses. Accordingly, identifying the role of Nrf2 in T cells is crucial to understanding the immunomodulatory effects of Nrf2 in autoimmune and inflammatory diseases. These studies detail the development of a model with which to study the role of Nrf2 in T cell activation and function, and start to elucidate the effects of tBHQ and the role of Nrf2 in T cell activation. They indicate that Nrf2 may not be involved in tBHQ-mediated immunosuppression of cytokine production and cell surface

protein expression during T cell activation, but also indicate a potential role for Nrf2 in other events of T cell activation.

Chapter 5

Summary, Significance, and Future Directions

Summary and Significance

These studies have investigated the role of Nrf2 in T cell activation, in both mouse and human primary T cells. These studies focus on the effects of the Nrf2 activator tBHQ, a widely-used food additive to which humans are commonly exposed, on T cell activation. In murine CD4 T cells, tBHQ suppresses production of the cytokines IL-2, IFNγ, GM-CSF, and TNFα, but does not affect the induction of the cell surface proteins CD25 and CD69. In addition, tBHQ inhibits p65 NF-κB DNA binding, but modestly increases c-Jun nuclear translocation, in activated murine T cells. However, many of these effects occur in both wild-type and Nrf2-null cells, indicating that not all of these effects are Nrf2-dependent. Specifically, the suppression of TNFα and the increase in c-Jun are Nrf2-dependent and the suppression of IFNγ is partially Nrf2-dependent, but the effects on IL-2, GM-CSF, and NF-κB are Nrf2-independent. Because of this, a second Nrf2 activator, CDDO-Im, was also utilized. CDDO-Im inhibited IFNγ in a partially Nrf2-dependent manner and induced IL-2 and c-Jun nuclear translocation in a Nrf2-dependent manner. CDDO-Im increased GM-CSF and TNFα independently of Nrf2, but had no effect on CD25 and CD69 expression. Furthermore, CDDO-Im decreased p65 DNA binding activity in wild-type murine T cells, but not in Nrf2-null murine T cells, in which it actually increased p65 DNA binding activity. There are also genotype differences between the wild-type and Nrf2-null mice. Nrf2-null splenocytes activated with a T cell specific activator have increased NF-κB DNA binding, increased IFNγ and TNFα production, and decreased c-Jun translocation compared to wild type mouse splenocytes activated with a T cell specific activator.

Taken together, these data indicate that in mouse T cell activation, Nrf2 increases IL-2 production and c-Jun nuclear translocation, decreases IFNγ and TNFα production and p65 NF-κB DNA binding, and has no effect on CD25 and CD69 expression. This differential role for Nrf2 in murine T cell activation fits with the effects previously seen on CD4 T cell differentiation, where Nrf2 activation with tBHQ skews differentiation towards a Th2 phenotype and away from a Th1 phenotype in wild-type but not Nrf2-null cells. IFNγ is the signature cytokine produced by Th1 cells, and IL-2 can support Th2 differentiation, so the differential expression of these cytokines upon activation would help set up alterations in differentiation seen at later time points. These studies identify a role for Nrf2 in regulating cytokine production in primary mouse T cell activation. Nrf2 has been shown to regulate various cytokines in both whole animal models and in other cell types, and our lab had previously identified a role for Nrf2 in CD4 T cell differentiation, but effects on T cell activation were unknown prior to these studies. These studies also start to identify the molecular mechanisms underlying these changes, by demonstrating effects on two transcription factors involved in T cell activation.

Since differential effects on murine T cell activation were seen, the role of Nrf2 in primary human T cell activation was also investigated. First, we determined whether tBHQ modulates early events following primary human CD4 T cell activation. tBHQ inhibits IL-2 and IFNγ production, CD25 and CD69 expression, and p65 NF-κB DNA binding activity in primary human T cells, indicating an overall inhibition of T cell activation. However, the role of Nrf2 in these events was undetermined. Because tBHQ

has demonstrated Nrf2-independent effects in mice, further studies were needed to clarify the role of Nrf2.

To assess the role of Nrf2 in the inhibitory effects of tBHQ on human CD4 T cell activation, a Nrf2-knockdown model in primary human CD4 T cells was developed. Several methods were tested, including the use of shRNA or CRISPR/Cas9 gene editing, before successfully using siRNA to knockdown Nrf2. Using this model, we were able to assess the role of Nrf2 in the inhibitory effects of tBHQ on primary human CD4 T cell activation. tBHQ treatment inhibits production of IL-2, IFNγ, GM-CSF, and TNFα in both scrambled control and Nrf2-deficient primary human CD4 T cells. tBHQ also inhibits CD25 and CD69 expression in both scrambled control and Nrf2-deficient human CD4 T cells, though to a lesser degree than the cytokines. These data indicate that tBHQ has immunosuppressive effects on the activation of primary human CD4 T cells, but these effects appear to be Nrf2-independent. These studies also demonstrate that there are species differences in the effects of tBHQ between murine and human CD4 T cells, notably in the regulation of CD25 and CD69 expression.

RNA-seq analysis was performed to further investigate the role of Nrf2 in human CD4 T cell activation. RNA-seq verified that cytokine expression was downregulated in both scrambled control and Nrf2-deficient CD4 T cells, but also identified a number differentially regulated genes between scrambled control and Nrf2-targeted siRNA groups. These included canonical Nrf2 target genes, several genes involved in the pentose phosphate pathway, and several other genes of interest, including pannexin-2, PKCβ, and RHOGEF 26. This indicates that there is potentially a specific role for Nrf2 in T cell activation, perhaps involving metabolic changes accompanying T cell activation.

These studies establish a Nrf2-knockdown model with which to investigate Nrf2 function in primary human CD4 T cells, and provide evidence that the effects of tBHQ on early events following T cell activation occur in both scrambled control and Nrf2-deficient CD4 T cells. The Nrf2-knockdown achieved in these studies is around 90%, which is a significant improvement over the only other primary human T cell knockdown of Nrf2 reported, which showed around a 40% knockdown of Nrf2[285]. RNA-seq analysis of scrambled control and Nrf2-deficient T cells indicates numerous differentially regulated genes, indicating directions for further research to characterize Nrf2-dependent effects in primary human CD4 T cells. These studies also demonstrate that the commonly used food additive tBHQ inhibits events of CD4 T cell activation at concentrations relevant to human exposure. Given that tBHQ is a chemical to which humans are commonly exposed, this research suggests that tBHQ may exert immunomodulatory effects in people.

Future Directions

The results of the studies described in this dissertation represent a significant step forward with respect to characterizing the effect of tBHQ and the role of Nrf2 in the early events following T cell activation in mice and humans. However, these data also point to a number of new potential areas of research. One potential direction is to investigate how tBHQ is mediating the Nrf2-independent effects observed in primary human CD4 T cells. One possibility is that tBHQ is changing the redox state of the T cell, and thus impacting T cell activation. tBHQ functions as an antioxidant, but has also been shown to redox cycle[315,349]. T cells have increased levels of ROS produced upon T

cell activation, and it has been proposed that these molecules have cell signaling functions important in modulating T cell activation and function[368,369]. It has been shown that treatment with antioxidants inhibits cytokine production and T cell proliferation, but also that increased ROS levels lead to increased cell death, indicating that some level of ROS are necessary for T cell activation and differentiation, but excessive ROS levels are detrimental[351].

Recently, mitochondrial/intracellular ROS levels were shown to impact IL-2 production and T cell proliferation, but extracellular ROS levels were shown not to impact cytokine production upon T cell activation or T cell proliferation[368,369]. In the cells, ROS can inhibit phosphatases through oxidative modification, and have been shown to modulate NF-κB and AP-1 activity[370]. Altogether, it has been demonstrated that ROS can impact T cell activation, proliferation, and function, but the specific effects, mechanisms, and *in vivo* relevance of these functions remains unclear and a focus of current investigation[351]. Therefore, it is plausible that tBHQ could modulate T cell activation through direct changes in ROS levels. ROS levels could be determined in our cells upon treatment using intracellular fluorescent dyes to determine the effect of tBHQ treatment. ROS levels could also be manipulated using an antioxidant such as NAC, or treatment with ROS-generating compounds to see if this ameliorates or increases the effect seen with tBHQ.

There is also evidence that tBHQ can impact signaling cascades in addition to Keap1-Nrf2-ARE signaling, including increasing activation of Erk, PI3K, and Akt[348,352,353,371]. All of these are involved in T cell activation, and it is possible that shifts in the relative amounts of these transcription factors and/or mis-timed signaling through

different signaling cascades could disrupt T cell activation. This could be investigated by determining relative levels of these proteins in T cell activation with and without tBHQ treatment.

Finally, the RNA-seq data generated identified several genes that are differentially expressed by tBHQ in both control and Nrf2 siRNA transfected groups, including some of the heat shock proteins, FosB, growth arrest-specific 2 like 1, and cyclin-dependent kinase inhibitor 1C. Any of these genes could be investigated further to determine the molecular mechanisms underlying the effect of tBHQ.

The effects of tBHQ are seen at concentrations potentially relevant to human exposure, but human exposure to tBHQ remains understudied. Studies to determine human exposure to tBHQ, such as measurement of levels in human serum samples and in various foods, would be valuable. As many of the effects of tBHQ on cytokines and cell surface protein expression occur independent of genotype in these studies, these studies also highlight the need for the development of better pharmacologic tools with which to investigate Nrf2. In both human and mouse, tBHQ, which is commonly used experimentally as a Nrf2 activator, had significant off-target effects. A second Nrf2 activator, CDDO-Im, also had Nrf2-independent effects in murine T cells. However, many drug-discovery based labs are working toward this goal, so this may not be the best direction for our lab to pursue.

Another potential future direction for these studies is to focus on the role of Nrf2 in primary human T cell activation. We have developed a Nrf2 knockdown model and have used this to identify a number of actions of tBHQ that occur in both scrambled control and Nrf2-deficient human CD4 T cells. Further studies could be done to

determine events of T cell activation that are differentially regulated between the genotypes. Several interesting differentially expressed genes between Nrf2-deficient and scrambled control human CD4 T cells were identified by RNA-seq and could be investigated. Several of these genes are involved in the pentose phosphate pathway, so a follow-up study could be to investigate metabolic changes that occur upon T cell activation in scrambled control and Nrf2-deficient CD4 T cells. Upon activation, T cells increase glucose metabolism, mitochondrial ATP production, lipid synthesis, and protein synthesis in order to support the increased protein production and support cellular proliferation[50,372]. This process is regulated by T cell receptor and co-stimulatory receptor signaling, through proteins such as AKT, mTOR, and Myc[373–375]. Nrf2 has been shown to help direct metabolic changes in cancer cells, so it is possible that Nrf2 is influencing the metabolic changes in T cells[138,376]. This could be investigated by determining changes in glycolytic flux and mitochondrial oxygen consumption, changes in pentose phosphate pathway intermediates levels, or by investigating changes in AKT and mTOR phosphorylation and levels of downstream target genes in these cells.

The gene most highly downregulated in the Nrf2-deficient CD4 T cells as compared to the scrambled control T cells in all three treatment groups is pannexin-2. Pannexin-2 is one of a group of three pannexin genes, which code for a family of proteins that form single membrane channels that allow passage of small molecules[357]. Pannexin-1 is the best-studied of the group, and can mediate the release of ATP into the extracellular space to facilitate purinergic signaling in response to various stimuli. While a role for pannexin-2 in T cells has not been described, pannexin-1 has been shown to be able to contribute to T cell activation through the release of ATP and

subsequent activation of P2X receptors upon T cell activation[358]. The functional role of pannexin-2 is unresolved, and it has been proposed that pannexin-2 may also release ATP similarly to pannexin-1, but also that pannexin-2 may be involved in molecular transport between organelles, function in Ca^{2+} release from the endoplasmic reticulum, or interact with endosomes as it has been shown that pannexin-2 is primarily localized to the cytosol[357,359–361]. Any of these proposed functions could potentially impact T cell activation, indicating that investigation of the expression levels and function of pannexin-2 is a potential future direction from these studies.

Transfection is highly stressful to T cells, so it is possible that potential genotype differences are currently masked by the changes occurring due to the stress of transfection. In this case, one solution would be to give the cells more time to recover after transfection. As siRNA knockdown is transient, it might be necessary to further develop the CRISPR/Cas9 Nrf2 knockdown for use in these studies. Another method would be to use the CRISPR/Cas9 system to knockdown Nrf2 in induced pluripotent stem cells (iPSC), which could then be grown up and differentiated into CD4 T cells and used to determine effects of Nrf2 on CD4 T cell activation and differentiation. iPSC-derived cells could also be useful if further genetic manipulations, such as knockdown of genes in addition to Nrf2, are necessary.

Another future direction is to determine the role of Nrf2 and tBHQ in primary human T cell differentiation. These studies have focused on events occurring relatively early after T cell activation, and a logical next step is to investigate events that happen at later time points, including proliferation and differentiation. The lab has previously shown that Nrf2 plays a role in mouse CD4 T cell differentiation, and preliminary data in

primary human CD4 T cells indicates that tBHQ treatment can modulate cytokine production at later time points, indicating a potential role in T cell differentiation. These effects need to be clarified, expanded upon, and the role of Nrf2 determined. This could involve characterizing the effects of Nrf2 activators on T cell proliferation, cytokine production, and expression of transcription factors associated with the different Th cell subsets in scrambled control and Nrf2-deficient CD4 T cells. This could be initially approached by using the siRNA knockdown model, but as siRNA knockdown is not permanent, further development of the CRISPR/Cas9 Nrf2 knockdown for use in these studies would likely be required.

Conclusion

Altogether, these studies investigate the role of Nrf2 in primary CD4 T cell activation. They demonstrate Nrf2-dependent and independent effects of commonly used Nrf2 activators in murine and human CD4 T cells. The characterization of the role of Nrf2 in human T cells required the development of a Nrf2-knockdown model in primary human CD4 T cells using siRNA. Nrf2-knockout and –knockdown models are of critical importance to determine the role of Nrf2 in various processes, particularly with the number of off-target effects that occur with the currently available pharmacological tools. Our Nrf2-knockdown model is the first report of substantial levels of Nrf2-knockdown achieved in primary human CD4 T cells, and will be of great use in determining the function of Nrf2 in human CD4 T cells. CD4 T cells are a critical part of the adaptive immune response, and proper function of these cells is integral in the successful clearance of pathogens. CD4 T cell function must be tightly controlled to

assure that pathogens are successfully cleared, but also that diseases such as asthma, allergy, and autoimmunity do not occur. As the prevalence of such immune-mediated diseases has been increasing in the past decade, investigation into the control of T cell activation and differentiation is crucial for the understanding and treatment of these diseases.

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