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Th2 Skewing by Activation of Nrf2 in CD4$^+$ T Cells

Cheryl E. Rockwell,*[,1] Mingcai Zhang,[†] Patrick E. Fields,[†] and Curtis D. Klaassen*

NF erythroid 2-related factor 2 (Nrf2) is a transcription factor that mediates the upregulation of a battery of cytoprotective genes in response to cell stress. Recent studies showed that Nrf2 also modulates immune responses and exhibits anti-inflammatory activity. In this article, we demonstrate that a common food preservative, *tert*-butylhydroquinone, can activate Nrf2 in T cells, as evidenced by Nrf2 binding to the antioxidant response element and the subsequent upregulation of Nrf2 target genes. The activation of Nrf2 suppresses IFN-γ production, while inducing the production of the Th2 cytokines IL-4, IL-5, and IL-13. Nrf2 activation also suppresses T-bet DNA binding and promotes GATA-binding protein 3 DNA binding. Collectively, the present studies suggested that Nrf2 activation skews CD4$^+$ T cells toward Th2 differentiation and, thus, represents a novel regulatory mechanism in CD4$^+$ T cells. Further studies are needed to determine whether the commercial use of Nrf2 activators as food preservatives promotes food allergies in humans. ***The Journal of Immunology*****, 2012, 188: 1630–1637.**

T helper cell 1/Th2 differentiation of CD4$^+$ T cells is a critical process in tailoring an adaptive immune response to a specific pathogen, which allows for flexibility in T cell function and downstream immune activity. However, there is also an inherent potential danger in pathological responses that are due to Th1/Th2 imbalance. Th1 responses are vital in cell-mediated immunity, which is important in host defense against numerous bacterial and viral pathogens (1). Conversely, Th2 responses are thought to be important in host defense against larger parasites, such as helminths. Th2 responses also play a major pathological role in the development of allergy and asthma (1, 2). Although the incidence of allergy and asthma has sharply increased in the United States and worldwide over the last several decades, the underlying cause is unknown (3–5).

NF erythroid 2-related factor 2 (Nrf2) is a basic leucine zipper transcription factor that is activated by cell stress from various sources, including oxidative and electrophilic stimuli (6, 7). In the absence of oxidants and electrophiles, Nrf2 is sequestered in the cytosol by its repressor protein, Kelch ECH associating protein 1 (Keap1), and is subsequently ubiquitinated and degraded (8, 9). During cell stress, ubiquitination of Nrf2 by Keap1 is disrupted, allowing Nrf2 to translocate to the nucleus and upregulate genes containing an antioxidant response element (ARE) in their promoter regions (10–12). Nrf2-regulated genes facilitate a variety of functions, including, but not limited to, antioxidative activity, detoxification and transport of xenobiotics, and glutathione homeostasis (13–15). In immune cells, Nrf2 was reported to upregulate numerous genes, such as Hmox1, Nqo1, and Gclc. Therefore, these genes are useful markers of Nrf2 activation in leukocytes (16, 17).

The synthetic antioxidants, *tert*-butylhydroquinone (tBHQ) and butylated hydroxyanisole (BHA), are widely used experimentally as Nrf2 activators. Specifically, it was shown that tBHQ modifies the thiol groups of cysteine-273 and cysteine-288 on the Keap1 protein, which ultimately inhibits the repression of Nrf2 by Keap1 and facilitates Nrf2 signaling (18). With respect to commercial use, both tBHQ and BHA are food preservatives used to stabilize oils and fats against oxidative deterioration (19). Although banned for use in food in Japan and other countries, tBHQ and BHA are used as preservatives in numerous types of food in the United States, including cereals, crackers, potato chips, noodles, grains, vegetable oils, margarine, and others.

Recent studies demonstrated anti-inflammatory effects of Nrf2 activation and conversely, the proinflammatory effects of Nrf2 deletion, in a number of models. For instance, Nrf2-null mice were shown to have increased mortality in models of sepsis (20), more severe pathology in experimental autoimmune encephalomyelitis (21), increased production of inflammatory mediators in LPS-induced neuroinflammation (22), and worsened injury in a model of T cell-mediated hepatitis (23). With respect to lung inflammation, Nrf2-null mice have increased susceptibility to OVA-induced asthma (24), allergic airway inflammation (25), and hyperoxia-induced acute lung injury (26). Accordingly, there has been considerable interest in developing anti-inflammatory therapeutics targeting Nrf2 (16).

Further evidence for an important role for Nrf2 in immune regulation is derived from the development of autoimmune disease in Nrf2-null mice. Several articles have characterized the development of multiorgan autoimmune inflammation in Nrf2-null mice, which resembles systemic lupus erythematosus (SLE) in humans (14, 27, 28). Like SLE in humans, the autoimmune disease in Nrf2-null mice is found predominantly in adult females, progresses with age, is characterized by the production of Abs against dsDNA, and ultimately results in glomerulonephritis. Moreover, it was reported that, in addition to the SLE-like disease, 14-mo-old Nrf2-null female mice develop hemolytic anemia, which often occurs in human SLE patients (29). Notably, a recently identified

*Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS 66160; and [†]Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, Kansas City, KS 66160

[1]Current address: Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI.

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Address correspondence and reprint requests to Dr. Curtis D. Klaassen, Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, 3901 Rainbow Boulevard, MS1018, Kansas City, KS 66160. E-mail address: cklaasse@kumc.edu

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Abbreviations used in this article: ARE, antioxidant response element; BAC, bacterial artificial chromosome; BHA, butylated hydroxyanisole; Fluc, firefly luciferase; GATA-3, GATA-binding protein 3; Keap1, Kelch ECH associating protein 1; Nrf2, NF erythroid 2-related factor 2; Rluc, *Renilla* luciferase; SLE, systemic lupus erythematosus; tBHQ, *tert*-butylhydroquinone.

NRF2 polymorphism was shown to be associated with autoimmune nephritis in female SLE patients (30).

In addition to its general anti-inflammatory effects, a number of studies suggested that Nrf2 activation may alter Th1/Th2 balance specifically. Expression of IL-4 and IL-13 is elevated in lungs of Nrf2-null mice in models of asthma and pulmonary fibrosis (24, 31). In addition, Nrf2-deficient lung dendritic cells were shown to have heightened oxidative stress that confers a Th2-like immunoresponsiveness upon stimulation with ambient particulate matter (32). With respect to Th1 responses, activation of Nrf2 by sulforaphane was shown to restore the age-related decrease in dendritic cell responses in a model of contact hypersensitivity (33). Collectively, these studies initiated the investigation of Nrf2 in Th1/Th2 responses and served as the basis for the current studies.

The present studies investigated the role of Nrf2 in T cell function and differentiation. These studies indicated that Nrf2 is activated by tBHQ in T cells, as evidenced by the binding of Nrf2 to its response element, ARE, and subsequent upregulation of Nrf2 target genes. The current studies also demonstrated that Nrf2 activation inhibits Th1 cytokine production and T-bet activity, while promoting Th2 cytokine production and GATA-binding protein 3 (GATA-3) binding activity. In addition, the Nrf2 activator, tBHQ, induced *Il4*-regulated firefly luciferase (Fluc) activity and *Il13*-regulated *Renilla* luciferase (Rluc) activity in $CD4^+$ T cells isolated from *Il4*-Fluc/*Il13*-Rluc transgenic mice, suggesting that Nrf2 promotes transcriptional activity in both the *Il4* and *Il13* genes.

Materials and Methods

Materials

BHA, 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 at the University of California San Francisco, San Francisco, CA (6). The mice were subsequently back-crossed for eight generations onto the C57BL/6 background and were 99% congenic (analysis performed by The Jackson Laboratory, Bar Harbor, ME). Female mice, 8–14 wk of age, were used for the current studies. Wild-type female C57BL/6 mice, 8 wk of age, 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 at the University of Kansas Medical Center.

T cell isolation

Single-cell suspensions from spleens and lymph nodes were washed and filtered, after which $CD3^+$ or $CD4^+$ T cells were isolated with magnetic beads by negative selection, following the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). The total number of cells isolated varied, depending on the specific experiment. Cellular viability was >90%, as determined by trypan blue exclusion. The purity of the isolated cell populations was >90%, as determined by flow cytometric analyses.

Cell culture

Cells were cultured in DMEM (with L-glutamine, sodium bicarbonate, and D-glucose), supplemented with 100 U penicillin/ml, 100 U streptomycin/ml, 50 μM 2-ME, and 10% FCS. For most studies, cells were treated with either tBHQ or BHA at various concentrations for 30 min prior to T cell activation. The duration of T cell activation ranged from 6 h (mRNA quantification of Nrf2 target genes) to 96 h or more (cytokine analysis), depending on the specific end point measured (noted in the figure legends). T cells were activated with purified hamster anti-mouse CD3e (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 BD Biosciences (San Jose, CA), and the $F(ab')_2$ cross-linker was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

mRNA quantification by branched DNA assay

For studies not restricted by limited amounts of total RNA, branched DNA analysis was performed for detection of mRNA. Cells were treated, as described, after which they were lysed with diluted lysis buffer. The cell lysis and branched DNA assays were performed using a commercially available kit (Quantigene 1.0; Panomics, Fremont, CA), following the manufacturer's protocol, as described previously (34). The specific oligonucleotide probe sets used for the quantification of *Ifng* are shown in Supplemental Table I. The probe sets were designed using ProbeDesigner software (Bayer, Diagnostics Division, Tarrytown, NY), as described previously (35). Probe sets were synthesized by Integrated DNA Technologies (Coralville, IA).

Cytokine protein quantification: IFN-γ ELISA

For single-plex analysis, IFN-γ protein was quantified by a sandwich ELISA method, using a commercially available kit and following the manufacturer's protocol (eBioscience, San Diego, CA).

mRNA quantification: real-time PCR

For studies for which limited total RNA was available, real-time PCR was used for mRNA quantification. Total RNA was isolated using TRI Reagent (Sigma-Aldrich), following the manufacturer's protocol. The isolated RNA was then reverse transcribed, after which relative expression levels of the target genes were determined by SYBR Green real-time PCR. Relative mRNA expression for the target genes was normalized to ribosomal protein L13a and calculated using the DDCt method, as previously described (36). Primers for rpL13a were designed using Primer3 software (http://primer3.sourceforge.net) and were synthesized by Integrated DNA Technologies: rpL13a forward primer, 5′-ACAAGAAAAAGCGGATGGTG-3′ and rpL13a reverse primer, 5′-TTCTCCTCCAGAGTGGCTGT-3′. The primers for *Nfe2l2* (Nrf2), *Hmox1*, *Nqo1*, and *Gclc* have been published (28, 37, 38).

Nuclear protein isolation

Nuclear protein was isolated using the Nuclear Extract kit (Active Motif, Carlsbad, CA), following the manufacturer's protocol. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA), which is based on the Bradford method of protein quantification.

ELISA-based DNA/protein-binding assay

Nrf2 (ARE)-, T-bet–, and GATA-3–binding activities were quantified using TransAM assay kits (Active Motif), following the manufacturer's protocol. The wild-type oligonucleotide (provided by the manufacturer) is an unlabeled free-consensus oligonucleotide that competes with the plate-bound oligonucleotide to confirm specificity. In addition to the wild-type oligonucleotide, a mutated oligonucleotide was used as a control. The Cos-7 (Nrf2 transfected), Cos-7 (T-bet transfected), and Jurkat nuclear extracts served as positive controls for the Nrf2-, T-bet–, and GATA-3–binding assays, respectively.

Cytokine protein quantification: multiplex suspension array

For multiplex cytokine analysis, cytokine concentrations were determined using a MilliPlex mouse cytokine kit for the detection of IFN-γ, TNF-α, IL-4, IL-5, IL-13, and IL-10, following the manufacturer's protocol (Millipore, Billerica, MA). The cytokine concentrations were quantified using a Bio-Plex 200 System (Bio-Rad). PE and xMAP bead fluorescence was detected by a dual-laser detector (532 and 635 nm) and quantified by Bio-Plex Manager 5.0 software (Bio-Rad).

Generation of the Il4-*Fluc*/Il13-*Rluc transgenic mouse*

A 184-kb bacterial artificial chromosome (BAC) clone (RP24-259D13) that includes both the *Il4* and *Il13* genes from chromosome 5 was selected from a BAC library and used to generate the Il4-*Fluc*/Il13-*Rluc* transgenic mouse (manuscript in preparation). In addition to the *Il4* and *Il13* genes, the BAC clone includes the *Il5* gene, as well as the proximal regulatory regions of *Il5*; thus, it includes the majority of the Th2 locus (it is missing distal regulatory regions for the *Il5* gene, however). The first exon of the *Il4* gene was replaced with Fluc, and the first exon of the *Il13* gene was replaced with Rluc by homologous recombination. The purified BAC DNA was then introduced into fertilized murine oocytes by pronuclear micro-

injection. The fertilized oocytes were then transferred into pseudopregnant foster mothers. The copy number of the transgene in the pups was determined by quantitative real-time PCR, as previously described (39). A detailed description of the generation of *Il4*-Fluc/*Il13*-Rluc transgenic mice is being prepared for publication.

Statistical analysis

The mean ± SE was determined for each treatment group in the individual experiments. Homogeneous data were evaluated by one-way or two-way parametric ANOVA. When significant differences were observed, either the Dunnett (for one-way ANOVA) or the Holm–Sidak (for two-way ANOVA) post hoc test was used to compare treatment groups to the vehicle control using SigmaStat 3.01a software from Systat Software (Chicago, IL).

Results

Inhibition of IFN-γ production by the Nrf2 activator tBHQ

Although Nrf2 has been described as anti-inflammatory in various in vivo models of inflammation, its role in T cells remains largely uncharacterized. Accordingly, the effects of Nrf2 activation on T cell activity were investigated. In our initial studies, a mixed splenocyte population was used in combination with a T cell-specific activator, anti-CD3/anti-CD28. A marked concentration-dependent inhibition of IFN-γ production by the Nrf2 activator tBHQ was observed in CD3/CD28-activated splenocytes (Fig. 1*A*). It is notable that even low concentrations of tBHQ (0.01 μM) substantially decreased IFN-γ secretion (58%), whereas higher concentrations completely abolished IFN-γ production. Treatment with another Nrf2 activator, BHA, also significantly reduced IFN-γ production by T cells, causing a marked decrease in IFN-γ secretion at 1 μM (data not shown). Neither tBHQ nor BHA significantly affected viability (data not shown). Consistent with its effect on protein secretion, tBHQ also inhibited IFN-γ mRNA expression in wild-type splenocytes, whereas splenocytes from Nrf2-null mice were relatively refractory to this effect (Fig. 1*B*). In addition, markedly higher mRNA expression of IFN-γ was observed in anti-CD3/anti-CD28–activated splenocytes derived from Nrf2-null mice relative to wild-type mice (Fig. 1*C*), suggesting Nrf2 plays an endogenous role in the regulation of IFN-γ. There was no difference in the ratios of $CD4^+$, $CD8^+$, $CD19^+$, $CD4^+CD25^+CD62L^{hi}CD44^{lo}$ (naive), $CD4^+CD25^+CD62L^{lo}CD44^{hi}$ (effector memory), or $CD4^+CD25^+$ populations (Supplemental Figs. 1, 2). These studies demonstrated that activation of Nrf2 can inhibit IFN-γ production by activated T cells and that Nrf2 plays a critical role in the endogenous regulation of IFN-γ.

Upregulation of Nrf2 and its target genes in $CD3^+$ cells by the Nrf2 activator tBHQ

Because activation of Nrf2 inhibited IFN-γ production by anti-CD3/anti-CD28–activated splenocytes (Fig. 1), the expression of Nrf2 and its ability to be activated was assessed in the pan T cell population. Similar to other cell types, Nrf2 expression in T cells is upregulated after treatment with the Nrf2 activator tBHQ (40), suggesting that Nrf2 upregulates its own expression (Fig. 2*A*). In addition, treatment of wild-type T cells with tBHQ resulted in increased binding of Nrf2 to the ARE, whereas decreased binding of Nrf2 to the ARE was observed in T cells derived from Nrf2-null mice (Fig. 2*B*). The increased binding of Nrf2 to the ARE correlated with increased mRNA expression of the Nrf2-target genes, Hmox1, Nqo1, and Gclc, in tBHQ-treated wild-type T cells. In contrast, treatment of Nrf2-null T cells with tBHQ did not upregulate Nqo1 or Gclc and only modestly induced Hmox1 expression (Fig. 2*C–E*). Collectively, these data indicated that Nrf2 is expressed, inducible, and capable of gene transactivation in wild-type T cells, as evidenced by the detection and induction of Nrf2 mRNA levels, binding of Nrf2 to the ARE, as well as the Nrf2-dependent upregulation of the target genes Hmox1, Nqo1, and Gclc.

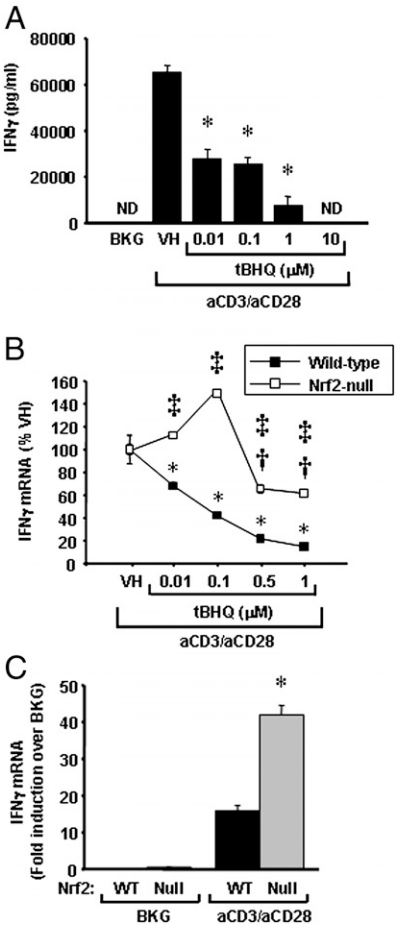

FIGURE 1. Nrf2 inhibits IFN-γ mRNA expression and protein secretion in splenocytes activated with anti-CD3/anti-CD28. Freshly isolated splenocytes were pretreated with vehicle (VH) or tBHQ for 30 min prior to activation with anti-CD3/anti-CD28. *A*, The cell supernatants were collected after 24 h, and cytokines were analyzed. *B*, Total RNA was isolated 6 h after activation, and IFN-γ mRNA expression was quantified by real-time PCR. *C*, Fold difference in IFN-γ mRNA levels between wild-type and Nrf2-null splenocytes 6 h after activation. $*p < 0.05$ versus wild-type control, $^{\dagger}p < 0.05$ versus Nrf2-null control, $^{\ddagger}p < 0.05$ wild-type versus Nrf2-null genotypes. BKG, background.

Nrf2 activation skews $CD4^+$ T cells toward Th2 differentiation

The early effects of tBHQ on IFN-γ production prompted an investigation into the effects of Nrf2 activation in the $CD4^+$ T cell polarization. Similar to the early effects shown in Fig. 1, treatment of $CD4^+$ T cells with tBHQ over 96 h resulted in inhibition of IFN-γ secretion, whereas substantially increased IFN-γ production was observed in $CD4^+$ T cells from Nrf2-null mice (Fig. 3*A*). In correlation, tBHQ treatment increased IL-4 secretion in wild-type $CD4^+$ T cells, whereas decreased IL-4 production was observed in $CD4^+$ T cells from Nrf2-null mice (Fig. 3*B*). Treatment with tBHQ also increased IL-5 and IL-13 secretion by $CD4^+$ T cells, and, accordingly, the absence of the Nrf2 gene resulted in decreased IL-5 and IL-13 production (Fig. 3*C*, 3*D*). The decreased IFN-γ production and increased IL-4, IL-5, and IL-13 production by tBHQ were also observed in $CD4^+$ T cells from AhR^d mice, which have a defective aryl hydrocarbon receptor, suggesting the effects are independent of the aryl hydrocarbon receptor (Supplemental Fig. 3). There was little difference in TNF-α and IL-10 secretion between the Nrf2-null and wild-type genotypes, sug-

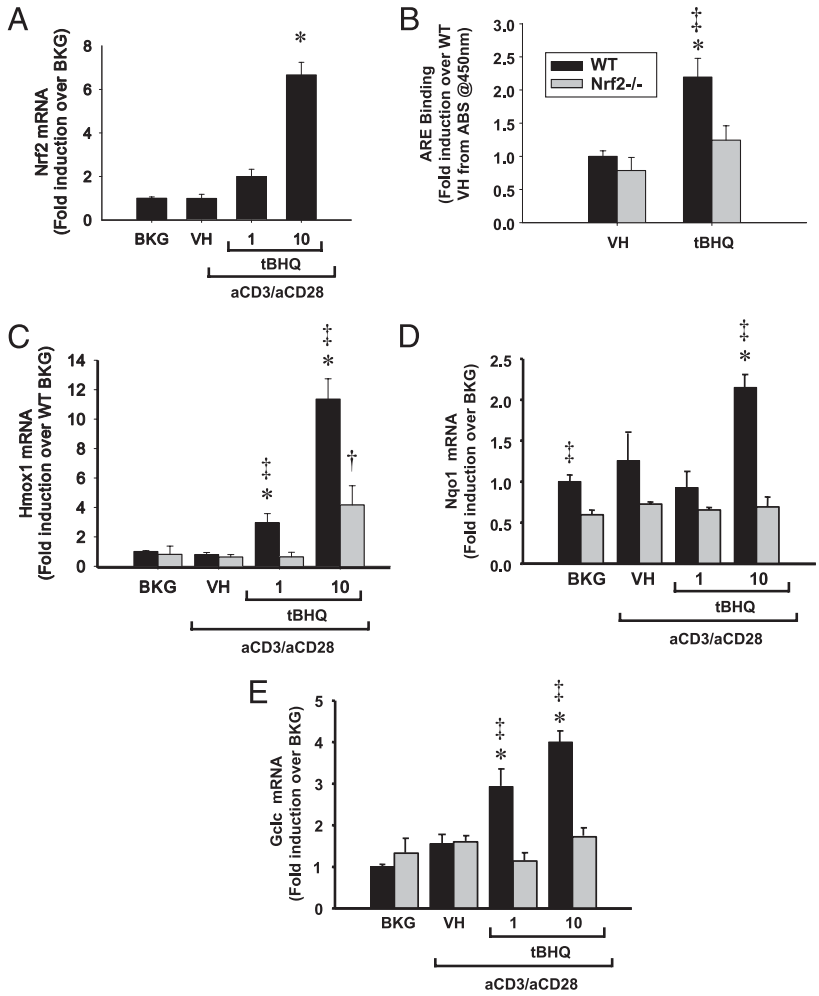

FIGURE 2. Activation of Nrf2 by tBHQ in isolated T cells. Magnetically isolated $CD3^+$ T cells were left untreated (BKG) or were pretreated with tBHQ or vehicle (VH) for 30 min prior to activation with anti-CD3 and anti-CD28. Cells were cultured for either 6 h (mRNA analysis; *A*, *C–E*) or 4 h (ARE binding; *B*). *A*, Nrf2 mRNA was detected by real-time PCR from total RNA. *B*, ARE binding was determined from nuclear protein extracts and quantified spectrophotometrically by measuring absorbance at 450 nm. Hmox1 (*C*), Nqo1 (*D*), and Gclc (*E*) mRNA expression was detected by real-time PCR from total RNA. $*p < 0.05$ versus wild-type VH, $^{\dagger}p < 0.05$ versus Nrf2-null VH, $^{\ddagger}p < 0.05$ wild-type versus Nrf2-null T cells.

gesting that the effects of Nrf2 activation are relatively selective for Th1/Th2 cytokines (Fig. 3*E*, 3*F*). Furthermore, the effects of Nrf2 activation at 96 h were also observed in restimulated $CD4^+$ T cells in which wild-type $CD4^+$ T cells treated with tBHQ exhibited decreased IFN-γ production and increased IL-4, IL-5, and IL-13 secretion (Fig. 4). Conversely, IL-4, IL-5, and IL-13 levels were low in restimulated $CD4^+$ T cells from Nrf2-null mice. Collectively, the data suggested that activation of Nrf2 promotes Th2 differentiation of $CD4^+$ T cells, while inhibiting Th1 differentiation.

Nrf2 activation potentiates GATA-3 DNA binding and concurrently suppresses T-bet DNA binding

Because cytokine analyses suggested that tBHQ promotes Th2 differentiation, the effect of Nrf2 activation on transcription factors involved in Th1/Th2 differentiation (T-bet and GATA-3) was assessed. Consistent with the cytokine analyses, activation of Nrf2 suppressed binding of T-bet to its consensus sequence and concurrently potentiated GATA-3 binding to its consensus sequence (Fig. 5). Furthermore, $CD4^+$ T cells from Nrf2-null mice exhibited significantly increased T-bet DNA binding and suppressed GATA-3 DNA binding. Collectively, these studies provided further evidence that Nrf2 positively regulates Th2 differentiation, which is associated with enhanced GATA-3–binding activity.

tBHQ drives IL-4–regulated and IL-13–regulated luciferase activity in a transgenic mouse model

Recently, we engineered a transgenic mouse model with which to study the molecular events that occur during Th2 differentiation (manuscript in preparation). A 184-kb BAC clone (RP24-259D13) from mouse chromosome 5 with *Il4* and *Il13* at center and flanked by *kif3a* and *rad50* genes was used to generate BAC transgenic mice. The BAC clone was modified by excising the first exon of the *Il4* gene and replacing it with the Fluc gene. In addition, the first exon of the *Il13* gene was replaced with the Rluc gene. The modified Th2 locus was then inserted into the mouse genome to create the transgenic mouse. This unique model allows for focused investigation into the molecular modifications that occur in the Th2 locus during $CD4^+$ T cell differentiation. Treatment of $CD4^+$ T cells from this transgenic mouse with tBHQ caused a robust increase in both Fluc and Rluc activities, suggesting increased transcriptional activity in both the *Il4* and *Il13* genes (Fig. 6).

Discussion

Previously published studies demonstrated that Nrf2 plays a significant immunomodulatory role in a number of models of inflammation (20, 22, 24). Although many of these studies suggested that Nrf2 can affect various aspects of T cell biology, its specific role in T cell function is incompletely understood (17, 21, 23, 24, 33). Accordingly, the purpose of the current study was to determine the role of Nrf2 in peripheral T cell differentiation.

Our data demonstrated that Nrf2 is expressed and is able to be activated by tBHQ in T cells, as determined by binding of Nrf2 to the ARE and induction of Nrf2 target genes. Importantly, these studies indicated that activation of Nrf2 inhibits production of the Th1 cytokine IFN-γ, while concurrently promoting the secretion of Th2 cytokines by wild-type $CD4^+$ T cells during both primary

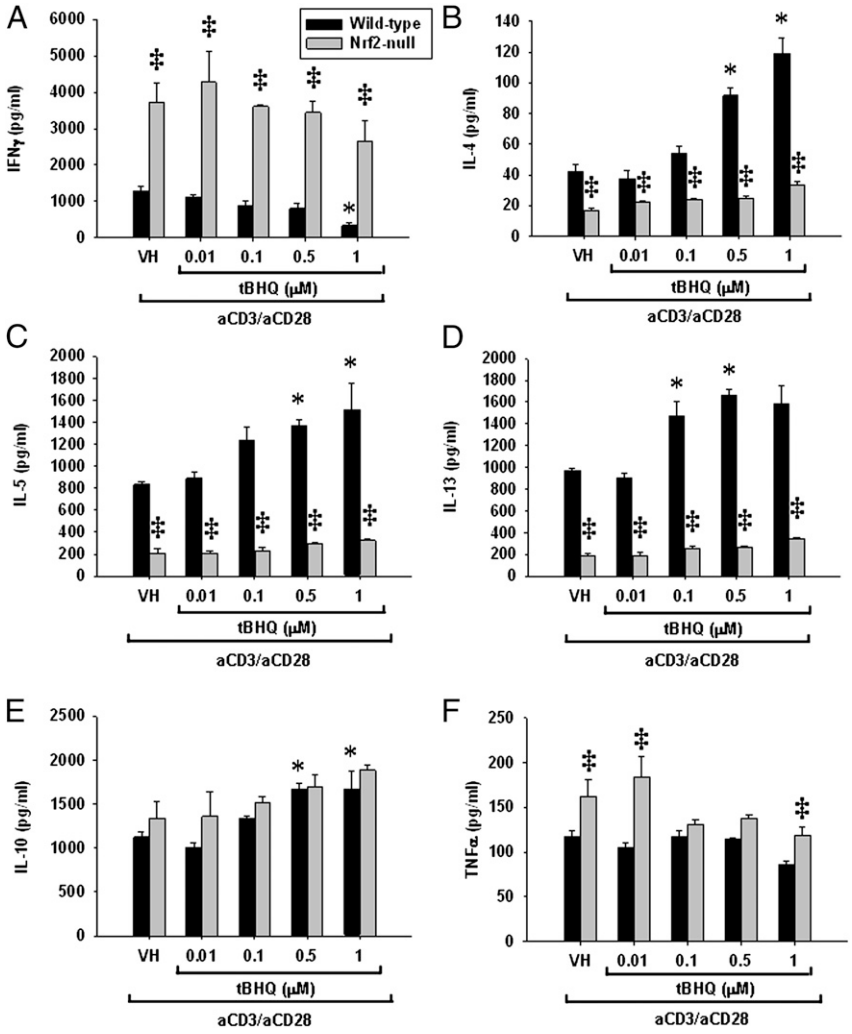

FIGURE 3. Effect of Nrf2 activation on cytokine production in activated CD4+ cells. Magnetically isolated CD4+ cells from Nrf2-null and wild-type mice were pretreated with vehicle (VH) or tBHQ for 30 min prior to activation with anti-CD3/anti-CD28. The cells were then cultured for 96 h, after which the cell supernatants were harvested. Cytokine concentrations of IFN-γ (*A*), IL-4 (*B*), IL-5 (*C*), IL-13 (*D*), IL-10 (*E*), and TNF-α (*F*) in the supernatants were quantified by multiplex suspension assay. *$p < 0.05$ versus wild-type control, ‡$p < 0.05$ wild-type versus Nrf2-null genotypes.

and secondary activation. Conversely, CD4+ T cells from Nrf2-null mice produce increased IFN-γ and decreased IL-4, IL-5, and IL-13 compared with wild-type T cells. In addition, the present studies demonstrated that tBHQ suppressed T-bet DNA binding and enhanced GATA-3 DNA binding in wild-type, but not Nrf2-null, CD4+ T cells. In *Il4/Il13*-regulated luciferase transgenic mice, tBHQ treatment induced Fluc, regulated by the *Il4* gene, as well as Rluc, regulated by the *Il13* gene. Collectively, these

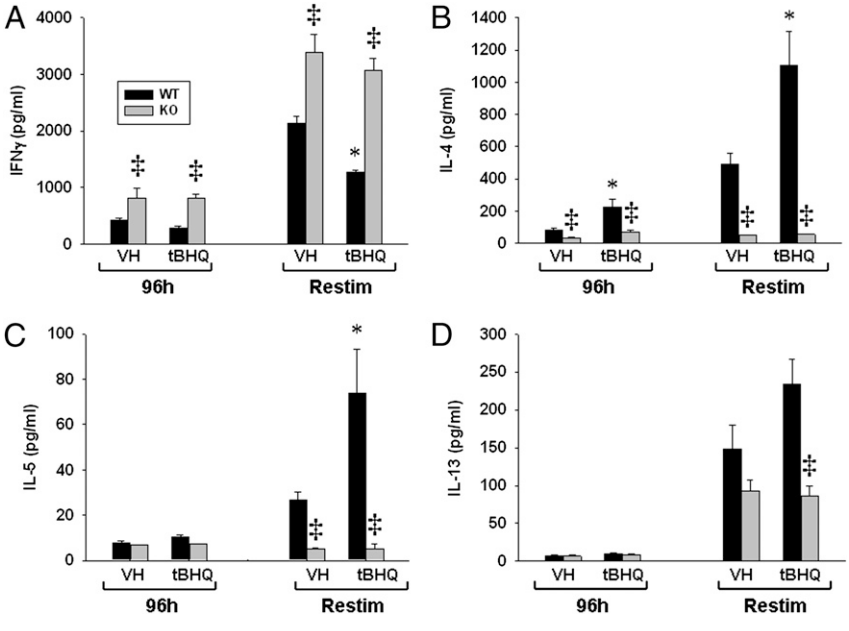

FIGURE 4. Effect of Nrf2 activation on cytokine production in restimulated CD4+ cells. Magnetically isolated CD4+ cells were treated as in Fig. 3 and then cultured for 5 d, after which the cells were harvested, resuspended, and restimulated. The supernatants were harvested 24 h after restimulation, and IFN-γ (*A*), IL-4 (*B*), IL-5 (*C*), and IL-13 (*D*) were quantified. *$p < 0.05$ versus wild-type control, ‡$p < 0.05$ wild-type versus Nrf2-null genotypes. VH, vehicle.

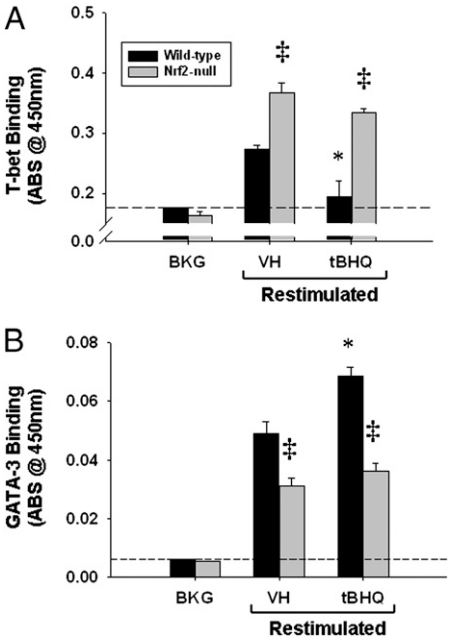

FIGURE 5. Effect of Nrf2 activation on T-bet– and GATA-3–binding activity in restimulated $CD4^+$ cells. Magnetically isolated $CD4^+$ cells were treated and restimulated as in Fig. 4. The cells were harvested 24 h after restimulation. Nuclear protein was then isolated, and T-bet– (*A*) and GATA-3– (*B*) binding activity was quantified. $^*p < 0.05$ versus wild-type control, $^‡p < 0.05$ wild-type versus Nrf2-null genotypes. VH, vehicle.

studies indicated that Nrf2 activation in $CD4^+$ T cells results in Th2 skewing, whereas lack of functional Nrf2 results in Th1 skewing. Overall, these studies suggested that the Nrf2-signaling pathway represents a novel regulatory mechanism in Th1/Th2 differentiation that warrants further investigation.

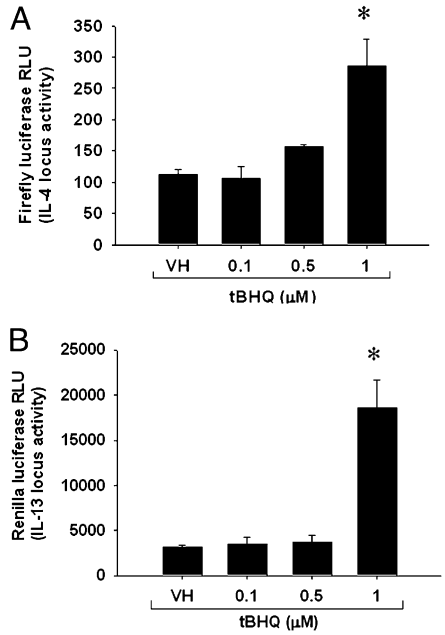

FIGURE 6. Effect of Nrf2 activation by tBHQ on *Il4*/*Il13*-regulated luciferase activity in restimulated $CD4^+$ cells. $CD4^+$ cells were isolated from *Il4*-Fluc/*Il13*-Rluc transgenic mice, after which the cells were treated and restimulated as in Fig. 5. The cells were harvested 24 h after restimulation, lysed, substrate was added, and chemiluminescence was quantified. Quantification of *Il4*-regulated Fluc activity (*A*) and *Il13*-regulated Rluc activity (*B*). $^*p < 0.05$ versus vehicle (VH) control.

It is interesting to note that Nrf2-null female mice develop an autoimmune disease that resembles SLE in humans (27, 28). The autoimmunity in the Nrf2-null mice is characterized by production of autoantibodies against dsDNA; deposition of IgM, IgG, and C3 in the glomeruli; and the development of glomerulonephritis. Although autoantibody production in SLE may suggest a Th2 response, there is considerable evidence to indicate that IFN-γ contributes to the pathology of lupus nephritis in both humans and mice (41–48). Neither IFN-$\gamma^{-/-}$ MRL/lpr nor IFN$\gamma^{+/-}$ MRL/lpr mice develop the overt renal disease that is typically associated with the MRL/lpr model of SLE (41). In addition, SLE patients with diffuse proliferative lupus nephritis have increased numbers of IFN-γ–producing cells (42, 43). Increased IFN-γ sera concentrations were also reported in SLE patients (44, 45). A higher incidence of single nucleotide polymorphism mutations in both IFN-γR1 and IFN-γR2 has been found in SLE patients (46). Importantly, patients who were treated with IFN-γ for rheumatoid arthritis or myeloproliferative disorder developed a severe SLE-like disease, suggesting that IFN-γ plays a causative role in SLE in some patients (47, 48). Consistent with the aforementioned studies, our data demonstrated that $CD4^+$ T cells derived from the Nrf2-null mice, which develop an SLE-like autoimmunity, produced markedly increased amounts of IFN-γ compared with wild-type.

The food additive tBHQ belongs to a family of phenolic antioxidant preservatives, which also includes BHA and butylated hydroxytoluene, all of which are added to a variety of foods to avert and/or slow spoilage. Because there has been considerable concern that many of the phenolic antioxidants may cause cancer, the primary toxicological focus has been the carcinogenicity of these chemicals (19). In this regard, the phenolic antioxidants have been widely studied and appear to be noncarcinogenic to humans. In contrast, the immunotoxicity of the phenolic antioxidants is not well characterized. It was reported that BHA inhibits IL-2 production by T cells (49); however, to our knowledge, the current study is the first to report the mechanism by which this may occur. This is also, to our knowledge, the first report of the effects of tBHQ on T cell function and differentiation.

The effects of the food preservatives tBHQ and BHA on $CD4^+$ T cell function occur at concentrations that are of physiological relevance to humans. Concentrations of tBHQ as low as 10 nM produce significant inhibition of IFN-γ secretion, whereas BHA inhibits IFN-γ at 1 μM (Fig. 1, data not shown). Notably, human volunteers administered tBHQ orally (100–150 mg) had serum concentrations of tBHQ ranging from 24 to 222.5 μM, concentrations ≥2–20-fold higher than those used in the current study (50). Thus, the modulation of Th1/Th2 differentiation by tBHQ occurs at concentrations to which humans are likely exposed. Given that humans are exposed to tBHQ by ingestion, and the present studies suggested that tBHQ can enhance production of Th2 cytokines by $CD4^+$ T cells, it seems possible that ingestion of tBHQ and potentially other phenolic antioxidants may create an environment in the gut that is permissive to food allergy or other Th2-skewed imbalances. Further studies are needed to investigate the role of tBHQ in oral tolerance and atopic responses.

Inhibition of IFN-γ transcription by tBHQ appears to be largely Nrf2 dependent. However, at higher concentrations, tBHQ can also suppress IFN-γ production in a Nrf2-independent fashion (Fig. 1*B*). This suppressive activity may be due to impaired calcium influx, as was described to occur with di-tBHQ, a structurally related congener of tBHQ (51).

The current studies suggested that activation of Nrf2 in CD3/CD28-stimulated $CD4^+$ T cells skews differentiation toward a Th2 response. This effect was unexpected, because previous

studies suggested that Nrf2 may serve to limit or regulate Th2 responses in models of asthma (24, 32, 52). The reason for the differential effects of Nrf2 on Th2 cytokine responses is unclear, but it may due to a number of factors. The current studies assessed the effects of Nrf2 activation on isolated CD3/CD28-stimulated $CD4^+$ T cells, whereas asthma and experimental models of asthma are complex, with numerous cell types involved. In addition, the environment of the lung itself appears to be particularly conducive to Th2 responses, whereas the present studies are likely to be more neutral with respect to Th1/Th2 differentiation.

An important implication of these studies is that activation of Nrf2 by food additives, such as tBHQ and BHA, may compromise cell-mediated immunity by impairing the production of the signature Th1 cytokine, IFN-γ. Furthermore, induction of the Th2 cytokines, IL-4, IL-5, and IL-13, may create an environment that is conducive for the development of atopy or other Th2-mediated conditions. Because humans are exposed to tBHQ through ingestion of food, the development of food allergies may be of particular concern. Notably, there has been an increase in reports of food allergy that seems to correlate with the increased use of tBHQ and other phenolic antioxidants as food preservatives (53–55). Collectively, the current findings suggested that tBHQ and other food preservatives may modulate immune responses, which warrants further investigation.

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Disclosures

The authors have no financial conflicts of interest.

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