Original article

Nrf2 activation by sulforaphane restores the age-related decrease of T_H1 immunity: Role of dendritic cells

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Background: The decrease in cellular immunity with aging is of considerable public health importance. Recent studies suggest that the redox equilibrium of dendritic cells (DCs) is a key factor in maintaining protective cellular immunity and that a disturbance of this homeostatic mechanism could contribute to immune senescence.

Objectives: We sought (1) to elucidate the role of DC redox equilibrium in the decrease of contact hypersensitivity (CHS) and T_H1 immunity during aging and (2) to determine how restoration of glutathione (GSH) levels by the Nrf2-mediated antioxidant defense pathway affects this decrease.

Methods: We assessed the effect of Nrf2 deficiency and boosting of GSH levels by the Nrf2 agonist sulforaphane or the thiol precursor N-acetyl cysteine (NAC) on the CHS response to contact antigens in old mice. We studied the effect of SFN and NAC on restoring T_H1 immunity by treating DCs *ex vivo* before adoptive transfer and *in vivo* challenge.

Results: Aging was associated with a decreased CHS response that was accentuated by Nrf2 deficiency. Systemic SFN treatment reversed this decrease through Nrf2-mediated antioxidant enzyme expression and GSH synthesis. Adoptive transfer of DCs from old animals induced a weakened CHS response in recipient animals. Treatment of DCs from old animals with SFN or NAC *ex vivo* restored the *in vivo* challenge response.

Conclusion: SFN and NAC upregulate T_H1 immunity in aging through a restoration of redox equilibrium. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: *Aging, redox equilibrium, cellular immunity, dendritic cells, Nrf2, glutathione, N-acetyl cysteine, sulforaphane*

Abbreviations used

APC:	Antigen-presenting cell
ARE:	Antioxidant response element
BM-DC:	Bone marrow–derived dendritic cell
CHS:	Contact hypersensitivity
DC:	Dendritic cell
DNBS:	2,4-Dinitrobenzene sulfonic acid
DNFB:	2,4-Dinitro-1-fluorobenzene
γ-GCL:	γ-Glutamylcysteine ligase
γ-GCLR:	γ-GCL regulatory subunit
GPx:	Glutathione peroxidase
GSH:	Glutathione
GSSG:	Oxidized GSH
LC:	Langerhans cell
MBB:	Monobromobimane
NAC:	N-acetyl cysteine
NQO1:	Reduced nicotinamide adenine dinucleotide phosphate–quinone oxidoreductase
OXA:	Oxazolone
p2E:	Phase II enzyme
ROS:	Reactive oxygen species
SFN:	Sulforaphane

Immune senescence is an important topic from the perspective of aging demographics and the associated increase in infectious disease episodes. Although functional changes in cellular immunity, such as a decrease of naive T cells, aberrant signal transduction by lymphocyte antigen receptors, and a change in cytokine profiles, have been described,[1] an overarching molecular hypothesis to explain these findings is lacking. Harman's original free radical theory suggested that aging could be attributed to the deleterious effects of reactive oxygen species (ROS).[2] Although it is known that ROS can damage structural cellular components and can induce a state of oxidative stress by means of glutathione (GSH) depletion, it is not intuitive how disrupting redox equilibrium could induce immune effects. We are beginning to understand, however, that oxidative stress is not just confined to oxidant injury, but we also have to consider antioxidant defense mechanisms that could determine whether ROS will induce oxidant injury. In fact, the coordinated antioxidant defense that is initiated by the Nrf2 pathway is the most sensitive oxidative stress response.[3] Our hierarchic oxidative stress hypothesis posits that lower levels of oxidative stress induce a protective and adaptive antioxidant defense that allows oxidant injury to become manifest only when this defense is overcome by high levels of ROS production.[4]

Nrf2 regulates the transcriptional activation of more than 200 antioxidant and protective genes that constitute the so-called phase II response. Examples of phase II enzymes (p2Es) include the rate-limiting enzyme in the GSH synthesis pathway, γ-glutamylcysteine ligase (γ-GCL), as well as glutathione peroxidase (GPx), heme oxygenase 1, superoxide dismutase, glutathione S-transferase, and reduced nicotinamide adenine dinucleotide phosphate–quinone oxidoreductase (NQO1).[3] We propose that

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the dynamic equilibrium between the Nrf2 pathway and injurious oxidant stress responses could determine the effect of aging in the immune system. This is compatible with the tendency toward a generalized decrease in GSH levels and γ-GCL expression with aging.[5] Aging also leads to a decrease in Nrf2 activity and p2E expression in parallel with increased markers of oxidative stress.[6] Although the exact reason for decreased Nrf2 activity is unknown, aging leads to decreased binding of this transcription factor to the antioxidant response element (ARE), which regulates the transcriptional activation of p2E gene promoters.[5] Moreover, the decrease in antioxidant activity is exaggerated during aging of female *nrf2* knockout mice.[7] In spite of this decrease in Nrf2 activity, it is noteworthy that p2E expression and GSH production in old rats is correctable by the Nrf2 agonist, α-lipoic acid.[5] Thus the fact that the Nrf2 pathway remains responsive in old animals could also be of benefit to elderly human subjects. This could include the use of even more potent agonists, such as the broccoli chemical sulforaphane (SFN).[8]

Nrf2 protects memory T cells from age-related oxidant injury, including protection against the decrease in mitochondrial function and phenotypic changes in the T-cell compartment with aging.[9] There is increasing evidence that Nrf2 also regulates the function of the innate immune system. Knockout of this gene leads to exaggerated cytokine production by innate cellular elements.[10] This includes our own demonstration that Nrf2 is important in regulating the antigen-presenting cell (APC) activity of dendritic cells (DCs). Thus exposure of myeloid DCs to exogenous oxidative stress stimuli (eg, pro-oxidative chemicals) has been shown to interfere in IL-12 production and T_H1 immunity.[11] There is also growing evidence that the opposite might be true, namely that boosting of GSH levels at the APC level might favor T_H1 skewing of the immune response.[12,13]

We hypothesized that Nrf2 plays a critical role in the decrease of T_H1 immunity and contact hypersensitivity (CHS) during aging. Moreover, we propose that this effect is, in part, explicable by the effect of the Nrf2 pathway on DC function. We assessed the effect of Nrf2 deficiency and boosting of GSH levels by SFN on the CHS response to contact-sensitizing chemicals in old mice. We also made use of adoptive transfer of antigen-pulsed DCs, which were treated *ex vivo* with SFN to test CHS responses *in vivo*. We demonstrate that Nrf2 activation by *in vivo* or *ex vivo* SFN administration reverses the decrease of T_H1 immunity in aged mice.

METHODS

Mice

Young (2-4 months) and old (19-22 months) female C57BL/6 (B6) mice were obtained from the Jackson Laboratory and the National Institute of Aging colony (Bethesda, Md), respectively. *Nrf2*$^{+/+}$ and *nrf2*$^{-/-}$ mice, which were initially obtained from Dr Y. Kan,[14] were backcrossed onto a C57BL/6 background for 7 generations.

Reagents

For more information, see the Online Repository at www.jacionline.org.

CHS testing with contact-sensitizing agents

Oxazalone (OXA; 3%), dissolved in 100% ethanol, was applied on the shaved mouse abdomen on day 0. Control animals were exposed to vehicle alone. Six days after sensitization, mice were challenged on both sides of both ears by means of epicutaneous application of 20 μL of a 1% OXA solution.[15] 2,4-Dinitro-1-fluorobenzene (DNFB) sensitization was accomplished by the application of 0.5% of the chemical dissolved in 4:1 acetone/olive oil onto the shaved abdomen (days 0 and 1). On day 5, mice were challenged by means of epicutaneous application of 0.2% DNFB on both ears.[15] Ear thickness was measured before and 24 and 48 hours after challenge by using a dial thickness gauge (Mitutoyo, Japan). Mice were killed 48 hours after challenge, and ear tissues were removed for RNA extraction and cytokine message expression, as well as for hematoxylin and eosin staining.

SFN oral administration

SFN (9 μmol/d per mouse) in 0.2 mL of corn oil was administered by means of gavage on consecutive days. The control group received corn oil alone. Pretreatment with SFN or corn oil commenced 5 days before and was carried through until the performance of the antigen challenge (ie, 11 days total).

RNA isolation and real-time RT-PCR

For more information, see Table E1 in the Online Repository.

Generation of bone marrow–derived DCs

Bone marrow–derived DCs (BM-DCs) were prepared as previously described.[13] See the Online Repository for more details.

Surface staining, monobromobimane staining, and flow cytometry

Cells were surface stained with phycoerythrin-labeled anti-CD11c. Monobromobimane (MBB) was used to stain intracellular thiol, followed by conducting flow cytometry, as previously described.[13] See the Online Repository for more details.

Magnetic bead separation of CD11c$^+$ cells

Magnetic cell sorting was performed by using microbead-labeled anti-CD11c (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described.[9,11] See the Online Repository for more details.

Eliciting CHS responses by means of adoptive DC transfer

CHS was induced by *in vivo* inoculation of antigen-pulsed DCs.[13] Cultured BM-DCs were incubated with or without N-acetyl cysteine (NAC) (20 mM for 1 hour) or SFN (5 μM for 24 hours) and then washed and resuspended in PBS containing 100 μg/mL 2,4-dinitrobenzene sulfonic acid (DNBS) for 30 minutes. For sensitization (day 0), 0.5×10^6 DNBS-treated DCs were injected subcutaneously with 100 μL of saline into the flanks of recipient mice. Five days later, mice were challenged by means of DNFB application to the ear. Mice injected with the same number of unmodified DCs or mock treated and challenged with vehicle alone served as negative controls.

Hematoxylin and eosin staining

For more information, see the Online Repository.

Statistical analysis

Results were expressed as means ± SD and analyzed by using the Student *t* test. *P* values of less than .05 were considered significant.

RESULTS

SFN restores the age-related decrease in the CHS and T_H1 immunity

We have previously shown that aging leads to a decrease of the CHS to contact antigens placed on the skin.[13] Although a number

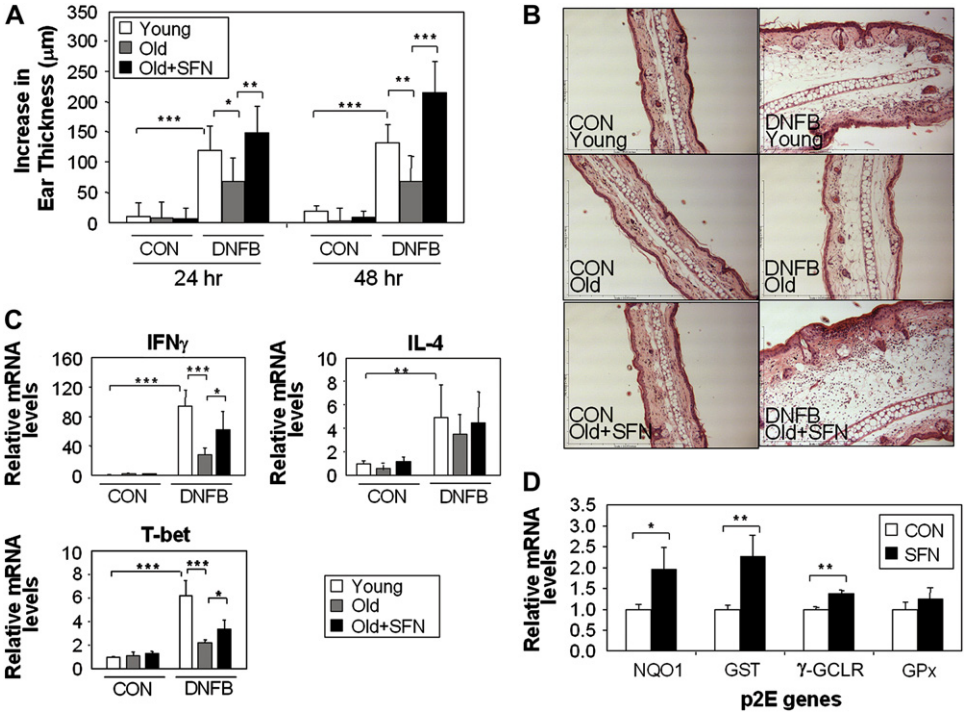

FIG 1. SFN reverses the age-related decrease in the CHS response. **A**, Ear-swelling response (mean ± SD). **B**, Hematoxylin and eosin staining of ear tissue. **C** and **D**, Real-time PCR for mRNA levels of genes in the ear (Fig 1, *C*) and liver (Fig 1, *D*). Results represent the fold increase (means ± SDs) compared with the CON-Young group (n = 6). $*P < .05$, $**P < .01$, and $***P < .001$. *CON*, Vehicle-treated control; *SFN*, SFN treated; *DFNB*, DNFB sensitized/challenged.

of mechanisms might explain the increase in oxidant stress during aging, it is important to consider the role of the Nrf2 pathway in the response outcome. Recent studies indicate that SFN significantly activates Nrf2-mediated p2E gene expression that is absent in Nrf2-deficient animals.[8] SFN administration can therefore be used to study the effect of the Nrf2 pathway on the decrease of T_H1 immunity in aging. To determine whether SFN gavage affects the CHS response, a previously determined effective dose (9 μmol/d per mouse) of the nutraceutical was delivered to 20- to 22-month-old mice before performance of the ear-swelling response.[8] Nontreated animals of similar age or 2- to 3-month-old mice were used as comparative control animals. Indeed, the ear-swelling response to DNFB challenge was significantly reduced in old compared with young animals. However, prior treatment of the old animals by means of daily SFN gavage before and during sensitization prevented the response decrease and could restore the CHS response to the levels seen in young animals (Fig 1, *A*). These response differences were maintained after 48 hours and were also reflected by histologic changes in the ear, which showed that the decrease in lymphocyte infiltration and intercellular edema in old animals could be reversed by means of SFN administration (Fig 1, *B*). SFN had no effect on nonsensitized (control) animals.

IFN-γ and IL-4 message levels were measured in the ear tissues that were taken 48 hours after challenge to determine whether the induction of the CHS response is accompanied by polarized T-cell differentiation. Quantitative RT-PCR showed that DNFB challenge induced the expression of the T_H1 cytokine IFN-γ, which was significantly suppressed in old compared with young animals; SFN treatment significantly increased IFN-γ expression (Fig 1, *C*). In contrast, the message level of a representative T_H2 cytokine, IL-4, was not significantly affected by aging or SFN administration (Fig 1, *C*). In addition to the cytokine changes, message levels for T-bet, a T_H1-specific transcription factor,[16] were significantly decreased in old versus young sensitized animals on DNFB challenge. However, SFN administration could prevent this decrease to a lesser but a significant degree (Fig 1, *C*). By contrast, aging or SFN treatment did not affect the expression of GATA-3, a T_H2-specific transcription factor (data not shown).[16]

The mRNA levels of NQO1, glutathione S-transferase, γ-GCL regulatory subunit (γ-GCLR), and GPx were determined by means of quantitative PCR to show that SFN affects p2E expression *in vivo* (Fig 1, *D*). Compared with the expression levels in the livers of control animals, message levels for 3 of the 4 genes were increased by SFN administration (Fig 1, *D*).

Nrf2 deficiency accentuates the CHS response decrease in old mice

Nrf2 deficiency affects the immune function of old mice.[7,17] To determine whether this includes an effect on T_H1 immunity and CHS, we compared the ear-swelling response of 22-month-old $nrf2^{-/-}$ mice with littermate control animals ($nrf2^{+/+}$ mice) during OXA sensitization and challenge. Nrf2-deficient mice showed a significant decrease in their ear-swelling response compared with that seen in wild-type control animals (Fig 2, *A*). The same effect was observed when mice were sensitized and challenged with DNFB (see Fig E1 in the Online Repository at www.jacionline.org), thereby indicating that the effect is not just limited to

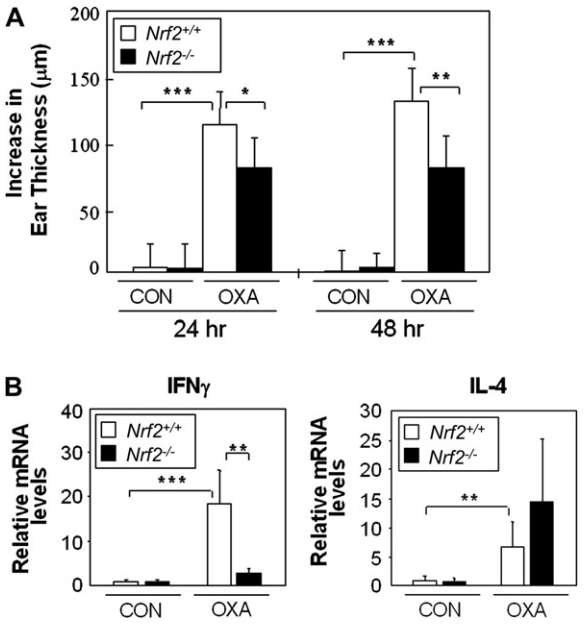

FIG 2. Nrf2 deficiency suppresses the CHS response. **A**, Ear-swelling response (means ± SDs). **B**, IFN-γ and IL-4 mRNA levels were measured by means of real-time PCR. Results represent the fold increase (means ± SDs) compared with the CON-*Nrf2*$^{+/+}$ group (n = 6). **P* < .05, ***P* < .01, and ****P* < .001. *CON*, Vehicle-treated control; *OXA*, OXA sensitized/challenged.

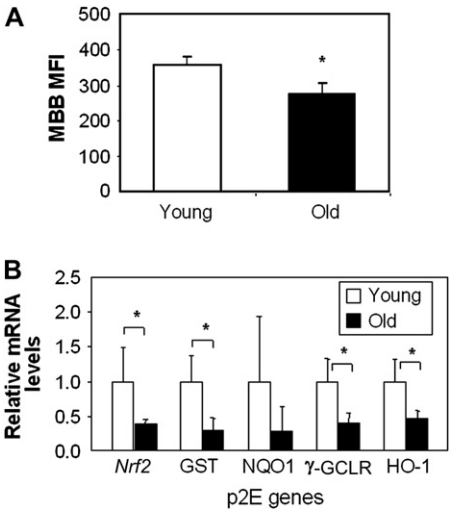

FIG 3. DCs from old mice have lower thiol levels and phase II mRNA expression. **A**, Mean fluorescence intensity for MBB staining in CD11c^{+} splenocytes from young and old mice (means ± SDs). **B**, Phase II mRNA expression in CD11c^{+} splenocytes from young and old mice. Results represent the fold increase (means ± SDs) of old compared with young mice (n = 4). **P* < .05.

a single contact antigen. This response reduction was accompanied by decreased IFN-γ mRNA expression, whereas IL-4 levels remain unaffected (Fig 2, *B*). Interestingly, when this experiment was repeated in younger (6-month-old) animals, there was no response reduction in Nrf2-deficient mice (not shown). These results suggest that cumulative oxidative stress during aging accentuates the effect of Nrf2 deficiency in the immune system. This is in accordance with previously published data.[7] All considered, the above data suggest that through its ability to maintain redox equilibrium in the immune system, Nrf2 plays an important role in regulating T_H1 immunity; this effect becomes particularly obvious under age-related oxidative stress conditions.

DCs from old mice contain lower levels of p2Es and a decreased thiol content

The CHS response involves several cell types in the skin, including helper T cells, cytotoxic T lymphocytes, and Langerhans cells (LCs). Although T-cell function is clearly affected by the oxidative stress events during aging,[9] increased ROS production also targets DCs.[11,13,18] Because it is not possible to obtain a sufficient number of LCs to study the effect of changes in redox status, we compared thiol levels from CD11c^{+} cells that were purified from the spleens of young and old mice. This showed a significant decrease in MBB mean fluorescence intensity in the CD11c^{+} populations from old animals (Fig 3, *A*). Please note that this decrease of 24% is highly significant from a homeostatic perspective because a small decrease in GSH content leads to a big decrease in the GSH/oxidized GSH (GSSG) ratio. This aspect is further discussed in the Online Repository. Similar observations were made when CD11c^{+} BM-DCs were compared in young and old mice (see below). RNA was also isolated from purified CD11c^{+} cells to perform quantitative PCR analysis to assess p2E message expression. Not only did we observe decreased mRNA expression of p2Es in cells from old animals, but we also demonstrated a decrease in *nrf2* message (Fig 3, *B*). All considered, these data indicate that aging leads to altered redox equilibrium in DCs. This could affect their APC function.

DC redox disequilibrium interferes in the CHS response, and this is reversed by the restoration of DC thiol levels

Through the use of an adoptive transfer protocol, we have previously demonstrated that it is possible to elicit a CHS response in recipient animals receiving antigen-pulsed BM-DCs from another donor.[13] Moreover, we have demonstrated that GSH depletion of these DCs at the time of antigen processing leads to a reduced ear-swelling response *in vivo*.[13] We hypothesized that a similar disturbance of the DC redox equilibrium by aging could affect the adoptive CHS. Thus BM-DCs from young and old mice were used for *ex vivo* pulsing with the water-soluble DNFB analogue DNBS before subcutaneous injection into recipient young naive mice.[13] Five days later, a CHS response was elicited by means of DNFB application to the ears of the recipient. This demonstrated a significant decrease in the ear-swelling response when DCs from old compared with DCs from young animals were used (Fig 4, *A*). Their differences were also reflected by the reduced inflammatory infiltrates in the ear tissue of old animals (not shown). No response was obtained in animals receiving naive DCs (control animals; Fig 4, *A*). Because the data suggest that altered redox equilibrium could be responsible for the decrease in DC function, we also performed MBB staining to look at BM-DC thiol levels (see Fig E2, A, in the Online Repository at www.jacionline.org). The small but significant decrease (14%, $P < .05$) of total thiol levels in DCs from old animals could be responsible for a significant change in the GSH/GSSG ratio.

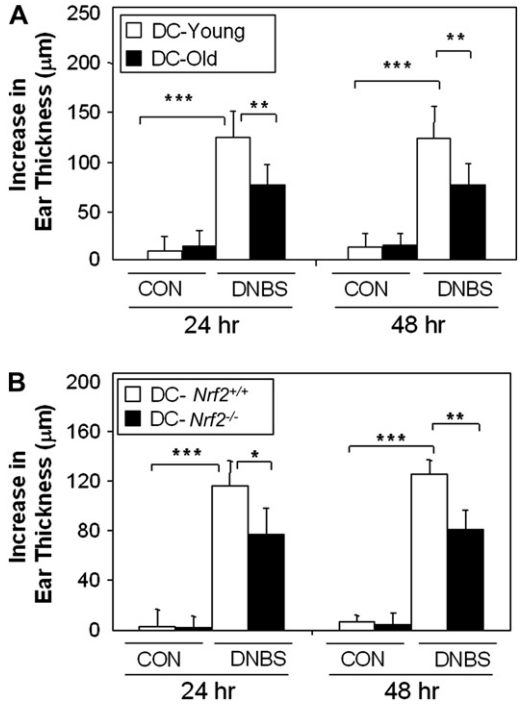

FIG 4. DC redox disequilibrium interferes in the CHS response on adoptive transfer. The ear-swelling response in recipient mice (3 months old) receiving DNBS-pulsed DCs from young versus old mice **(A)** or $nrf2^{+/+}$ versus $nrf2^{-/-}$ mice **(B)**, followed by DNFB challenge, is shown (n = 6). $*P < .05$, $**P < .01$, and $***P < .001$. *CON*, Vehicle-treated control; *DNBS*, DNBS pulsed.

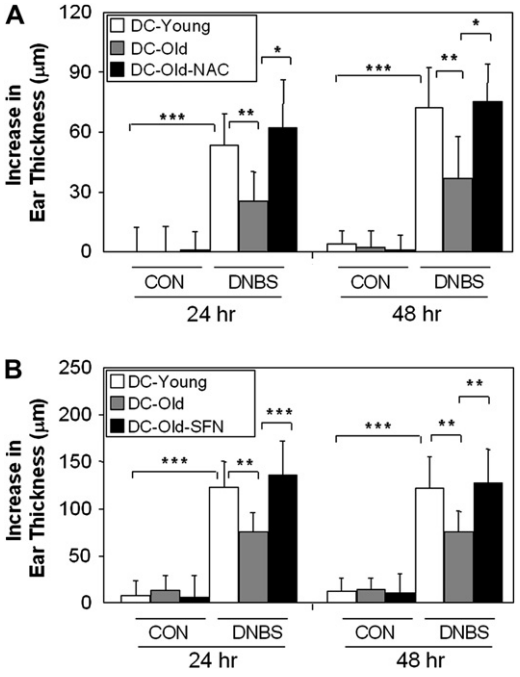

FIG 5. NAC or SFN treatment of DCs reverses the age-related decrease in the CHS response on adoptive transfer. NAC-treated **(A)** or SFN-treated **(B)** DCs were exposed to DNBS *ex vivo* and adoptively transferred into mice that were challenged with DNFB (mean ± SD, n = 6). $*P < .05$, $**P < .01$, and $***P < .001$. *CON*, Vehicle-treated control; *DNBS*, DNBS pulsed.

The same experiment was performed with DCs from old $nrf2^{+/+}$ and $nrf2^{-/-}$ mice. Fig 4, *C*, shows that there is a significant decrease in the ear-swelling response in mice receiving DNBS-pulsed DCs from $nrf2^{-/-}$ compared with $nrf2^{+/+}$ mice. This was accompanied by a significant reduction (13%, $P < .05$) in the thiol content of BM-DCs from $nrf2^{-/-}$ compared with $nrf2^{+/+}$ mice (see Fig E2, *B*, in the Online Repository).

To confirm that the age-related changes in DC redox equilibrium is important for the maintenance of T_H1 immunity, we used the adoptive transfer approach to determine whether *ex vivo* thiol repletion could restore the CHS response. First, we confirmed that the ear-swelling response of recipient mice injected with DNBS-pulsed DCs from old animals was significantly decreased compared with that of mice injected with DCs from young animals. Second, we showed that *ex vivo* treatment with NAC restored the CHS response in the recipients (Fig 5, *A*). MBB staining showed a 25% increase ($P < .05$) in cellular thiol content with NAC exposure (see Fig E3 in the Online Repository at www.jacionline.org). These data demonstrate that a perturbation of DC redox equilibrium as a result of aging, thiol repletion, or *nrf2* knockout affects APC activity *in vivo*.

SFN treatment in DCs reverses the age-related decrease of CHS response on adoptive transfer

BM-DCs from old mice were previously treated with SFN before DNBS pulsing and injection into young recipient mice to determine whether SFN could exert similar effects in the DC adoptive transfer model as during oral administration (Fig 1). Again, the CHS response to antigen-pulsed DCs from old animals was reduced compared with the response to cells from young animals. Second, the data demonstrate that *ex vivo* SFN exposure could restore the CHS response elicited by DCs from old animals (Fig 5, *B*). These data clearly indicate that activation of the Nrf2 pathway in DCs can reverse the age-related decrease in T_H1 immunity. As confirmation of upregulation of antioxidant enzyme expression in SFN-treated DCs, real-time PCR showed significant upregulation of the mRNA levels for NQO1 ($P < .001$), γ-GCLR ($P < .05$), and heme oxygenase 1 ($P < .05$; see Fig E4, A, in the Online Repository at www.jacionline.org). This was accompanied by a 15% increase ($P < .05$) in cellular thiol levels (see Fig E4, *B*).

DISCUSSION

In this study we demonstrate that manipulation of the Nrf2 pathway affects CHS and T_H1-mediated immune responses in old mice. Similar observations were made by using an adoptive transfer approach that uses *ex vivo* modification of DC redox status to study CHS responses *in vivo*. This is compatible with the growing recognition of the importance of the Nrf2 pathway on innate immunity.[10] We demonstrate that the oral administration of a potent Nrf2 agonist, SFN, reverses the decrease of CHS responses in old mice. This effect can be reproduced when antigen-pulsed DCs from old mice are treated with SFN *ex vivo* before adoptive transfer. This finding is compatible with decreased *nrf2* expression, decreased p2E expression, and lower thiol levels in DCs from old animals. GSH repletion by SFN and NAC restored the DC redox equilibrium, allowing DCs from old animals to function normally *in vivo*. Taken together, these data show that the state of redox equilibrium of DCs is important in the decrease of T_H1 immunity with aging.

A decrease in T_H1 immunity with aging is of particular importance in defense against viral and mycobacterial pathogens, as

well as for immune surveillance against cancer. Although a host of specific molecular and cellular events have been described in senescent immune cells,[1] it is not clear whether aging is responsible for a common mechanism of immune decrease. We propose, however, that a disturbance of the redox equilibrium of cells of the immune system might provide an overarching mechanism by which to dissect the functional decrease of the immune system during aging. This view shifts the free radical theory of aging to an adaptive multifactorial process that is determined by a dynamic interplay between pro-oxidant and antioxidant forces.[19] Such an evolution also allows us to postulate that persons with decreased antioxidant protection might be more prone to immune senescence and that dietary or therapeutic intervention to strengthen the effects of the antioxidant pathway could reverse the injurious effects of oxidative stress in the immune system. Our data clearly show that it is possible to reverse the age-related decrease in T_H1 immunity in old mice within days of restoring redox equilibrium in the immune system.

We have previously shown that the oxidative stress effects of aging might be involved in the loss of naive T cells and a decrease in T_H1 immunity.[9,13] Our focus on DCs originates from the demonstration that knockout of the *nrf2* gene has prominent effects on the innate immune system, including the function of macrophages and DCs.[10] Changes in DC function might contribute to effects on T-cell differentiation. Although a lot needs to be learned about the role of DC lineage and biology in determining the outcome of T-cell differentiation, a number of recent studies have emphasized the role of the redox equilibrium in these events. This is illustrated by the finding that *in vivo* GSH augmentation in APCs favors T_H1 development, whereas thiol depletion shifts the immune response from T_H1 to T_H2 dominance.[12] Moreover, we have shown that GSH depletion of antigen-pulsed DCs interferes in their ability to mount a CHS response on adoptive transfer *in vivo*.[13] Although there are a number of possible explanations of why redox equilibrium could determine DC function, one possible explanation is that oxidative stress decreases IL-12 and subsequent IFN-γ production in T cells.[20] This could involve a role for Nrf2 that, through its effects on GSH synthesis and p2E expression, could modify the signaling pathways that are required for DC maturation, cytokine production, and costimulatory receptor expression.[13,14,21] Another possible explanation is that DCs play an important role in neutralizing extracellular oxidative stress through the expression of surface thiol groups.[22,23] Not only does this allow the DCs to survive in an oxidative stress environment,[22] but it also contributes to the maintenance of thiol levels and viability in bystander T lymphocytes.[23] Although there is evidence that the redox homeostasis of DCs is important in shaping their activity under conditions of systemic oxidative stress, it has not been demonstrated previously that the redox equilibrium of DCs from old animals is important in their immune decrease. Here we report for the first time that DCs from old animals exhibit lower thiol levels and decreased expression of *nrf2* and phase II gene message levels than DCs from young animals. Thus the decrease in DC antioxidant capacity could contribute in a number of ways to the decrease in the ear-swelling response and T_H1 immunity in old animals (Fig 3).

Our focus on DCs is fortuitous from the perspective that relatively little work has been done on the cell type in immune senescence. Moreover, previous studies have yielded conflicting results.[24-27] For instance, some studies did not observe a difference in DC surface marker expression,[24] whereas others have shown decreased MHC class II and costimulatory receptor expression in aged individuals.[25] Other abnormalities reported in DCs with aging include abnormal recruitment to sites of immune pathology, decreased transportation of antigens to lymph node germinal centers, impaired capacity toward IFN-γ production, and interference in APC activity caused by a putative increase in IL-10 levels.[26]

Although it would have been ideal to have access to LCs to conduct this study, it was not logistically possible to purify enough LCs to conduct these studies. Instead, we settled for the use of myeloid DCs that are derived from the same precursors as LCs. Minimally, this has allowed us to provide proof of principle that DCs from old animals do not perform as well as DCs from young animals in the adoptive transfer model. Not only is this approach highly reproducible, but it also allowed us to show that manipulation of the DC redox status influences APC activity *in vivo*. This allowed us to demonstrate that either an increase or a decrease in Nrf2 activity exerts major effects on APC function. This is directly relevant to the study of aging, in which a decrease of Nrf2 levels has been reported previously.[5,6] Although the mechanism for this decrease is unknown, we know that Nrf2 function is regulated at multiple levels and that aging can target 1 or more of these events. This aspect is further discussed in the Online Repository for the interested reader.

In spite of the decrease in Nrf2 activity with aging, we show that SFN can effectively restore redox equilibrium in old animals in parallel with an improvement in CHS and T_H1 immunity. This finding could be of major significance in preventing or reversing the effects of immune senescence in elderly human subjects. Dietary antioxidants have been shown to have important effects on immune function, including improvement of CHS and vaccination responses in human subjects.[28] To this list we can now add broccoli and other cruciferous vegetables that are deserving of a human trial. In addition, the electrophilic chemistry that leads to Nrf2 release from its chaperone provides a platform for further drug discovery. Also of note, it has been demonstrated that treatment of old rats with α-lipoic acid can increase nuclear Nrf2 levels in parallel with increased γ-CLC expression and GSH production.[5] Finally, our study shows the potential for using DCs to conduct vaccination therapy as a means of restoring *in vivo* cellular immune function during aging.

Further studies are needed to delineate all the mechanisms and cell types that are involved in the improvement of T_H1 immunity during treatment with Nrf2 agonists. Although for the most part we focused on DCs in this communication, it is also possible that other cell types might contribute. It is quite possible that enhanced Nrf2 activity could have equally important effects on T cells and other cell types that participate in the CHS response. It is also important to mention that previous studies in mice have shown conflicting data with respect to the effect of NAC and N,N′-diacetyl-L-cystine on the CHS response to chemicals.[29] Although there could be a number of experimental reasons for these differences, it is possible that the thiol group in these antioxidants could covalently bind to the contact-sensitizing chemicals and interfere in their ability to generate the haptens that are required for antigen presentation.[30,31] This aspect is further discussed in the Online Repository.

In summary, intervention through the Nrf2 pathway provides a rational approach to improve cellular immune function during aging. In addition to the beneficial effects on specific immunity, it is possible that many of the chronic inflammatory changes that

develop in the elderly might originate in the innate immune system. It is possible that an age-related decrease in Nrf2 activity could lead to oxidative stress–mediated proinflammatory responses in cells from the innate immune system. It is possible that Nrf2 agonists could also intervene in this aspect of aging.

Clinical implications: Restoration of the redox equilibrium in the immune system could restore or delay the decrease of cellular immunity with aging.

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METHODS

Reagents

RPMI-1640 and FCS were obtained from Cellgro (Herndon, Va) and Irvine Scientific (Santa Ana, Calif), respectively. OXA and DNFB were purchased from Sigma (St Louis, Mo). DNBS was obtained from MP Biomedicals, Inc (Irvine, Calif). MBB was purchased from Molecular Probes (Eugene, Ore). Antibodies for cellular staining of CD11c were obtained from BD PharMingen (San Diego, Calif). Primers for real-time PCR (see below) were purchased from E-Oligos (Hawthorne, NY). All organic solvents were of Fisher Optima grade, and the solid chemicals were of analytic reagent grade.

Single-cell preparation from spleens

The spleens were aseptically removed and gently grinded on a cell strainer in PBS. These single-cell suspensions were incubated with ammonium chloride to remove red blood cells. After washing with PBS, cells were resuspended in PBS.

Surface staining and flow cytometry

These procedures were previously described by us.[E1] Cell staining was performed with anti-CD11c phycoerythrin antibodies. Cells were incubated with antibodies for 30 minutes at 4°C in staining buffer in the dark. Samples were analyzed in the LSR flow cytometer (BD PharMingen) by using the excitation and emission settings of 488 nm and 575 nm (FL-2 channel), respectively. A minimum of 20,000 events were collected and analyzed with CellQuest software (Becton Dickinson, San Jose, Calif)

MBB staining to assess intracellular thiol content

Intracellular thiol levels in cells were measured by means of MBB loading.[E2] Working solutions of MBB (1 mmol/L) in PBS were made fresh from a 40 mmol/L MBB stock solution in dimethyl sulfoxide. Cells were resuspended in PBS at a concentration of 10^6 cells/mL, and MBB was added to a final concentration of 40 μmol/L for 10 minutes at room temperature. Where MBB fluorescence was combined with surface staining, this dye was added after surface staining, as described below. MBB fluorescence was excited by the UV laser tuned to 325 nm, and emission was measured at 510 nm (FL-4 channel) in the LSR flow cytometer.

Generation of BM-DCs

BM-DCs were generated as previously described by us with some modifications.[E1] Briefly, bone marrow cells were removed from the femurs of mice and cultured at a concentration of 2×10^6 cells per well in 6-well culture plates. Each well received 2 mL of RPMI-1640 supplemented with 10% FCS, 1% penicillin/streptomycin, 1% glutamine, 55 μmol/L 2-mercaptoethanol, GM-CSF (40 ng/mL), and IL-4 (100 pg/mL). The culture medium was refreshed every 3 days.

Magnetic bead separation of $CD11c^+$ cells

Magnetic cell sorting was performed as previously described.[E3] Briefly, splenocytes were prepared as described in the Online Repository and enriched with microbead-labeled anti-CD11c (Miltenyi Biotec). The labeled cells were separated by using the autoMACS (Miltenyi Biotec) system. The purity of the $CD11c^+$ population was confirmed by means of flow cytometry.

Hematoxylin and eosin staining

The left ear from each killed animal was excised and fixed in 10% buffered formalin phosphate. After processing and staining with hematoxylin and eosin, the sections were examined in a Fisher Digital Micromaster I (Fisher Scientific, Hampton, NH) at a magnification of ×20. At least 10 fields were examined for each tissue section.

RNA isolation

Total RNA was extracted from DCs or tissues by using the RNeasy Mini Kit (Qiagen, Inc, Valencia, Calif), according to the manufacturer's recommendations. Contaminating DNA was removed with a DNA-free kit (Ambion, Inc, Austin, Tex). Total RNA was spectrophotometrically quantitated. A 1-μg RNA sample was reverse transcribed by using the iScriptTMcDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, Calif). The cDNA templates were stored at −40°C.

Real-time RT-PCR

PCR was carried out with an iQTM SYBR Green Supermix (Bio-Rad Laboratories) by using an iCycler (Bio-Rad Laboratories), according to the manufacturer's instructions. The sequences for the primers for IFN-γ, IL-4, T-bet, heme oxygenase 1, *Nrf2*, NQO1, glutathione S-transferase (GST), γ-GCLR, GPx, and β-actin were recently described by us and are summarized in Table E1.[E2,E3,E4] The final PCR mixture contained 1 μL of cDNA template and 400 nM of the forward and reverse primers in a final volume of 25 μL. Samples were run concurrently with a standard curve prepared from the PCR products. Serial dilutions were performed to obtain appropriate template concentrations. β-Actin was used as a reference gene for the recovery of RNA, as well as reverse transcription efficiency. Melting curve analysis was used to confirm specific replicon formation.

Mice welfare

Mice were housed in the Division of Laboratory Animal Medicine vivarium at the University of California, Los Angeles. The UCLA Animal Research Committee approved all the experiments. Neither the CHS procedure nor SFN administration had any effects on the overall well-being or body weight of the animals.

DISCUSSION

When bound to its chaperone, Keap1, Nrf2 has a relatively short half-life (<20 minutes) and is continuously being degraded by an ubiquitin-26S proteosome pathway. Keap1 expresses 25 free cysteine residues, among which Cys-151 is critical for the binding and sequestration of Nrf2.[E5] One possibility is that aging could affect the oxidation status and function of this thiol group. Moreover, Nrf2 autoregulates its own gene expression, which means that a decrease in ARE activity could have detrimental effects on the expression of this transcription factor.[E6] This age-related decrease in ARE transcriptional activity also has to consider the influence of transcription factors that heterodimerize with Nrf2. Nrf2 binds to other bZip proteins, including members of the Jun/Fos family, Fra, small Maf, and activating transcription factor 4 proteins.[E7] Aging affects the transcriptional activity of a number of these binding partners.

Because GSH is such a potent antioxidant, small changes in GSH content could lead to sizeable biologic effects. Even a small decrease in GSH content can lead to a big decrease in the GSH/GSSG ratio. The GSH/GSSG ratio under conditions of redox equilibrium is typically in the range of 40:1 to 80:1. Assuming that most of the decrease in MBB fluorescence actually represents GSH conversion to GSSG, this would mean that converting 10% of GSH will result in a GSH/GSSG ratio of 9:1 to 2:1, whereas the corresponding ratio for a rate of 25% conversion will amount to 3:1. Thus a small change in GSH content can have a big effect on the ratio of the GSH/GSSG redox couple that initiates cellular responses. Moreover, this decrease could lead to even more significant consequences during aging.

There have been conflicting reports about the effect of thiol antioxidants on the CHS response.[E8-E12] There could be a number of reasons for these different outcomes, including differences in the experimental protocols, mouse strains, and contact-sensitizing chemicals used and different routes and times of antigen administration. Our finding is supported by the study by Särnstrand et al,[E8] who showed a dose-dependent increase of ear thickness by NAC treatment in OXA-treated BALB/c mice. They also showed that DiNAC, an oxidized disulfide form of NAC, could enhance the CHS response. However, the CHS response induced by NAC is inhibited by the contemporaneous administration of DiNAC and *vice versa*. One can explain this interference by the thiol groups competing for covalent binding. Covalent binding to endogenous proteins constitutes one of the mechanisms by which contact sensitizers induce immune activation.[E9,E10]

A study by Bruchhausen et al[E9] demonstrated that NAC blocked the binding of the contact sensitizer 2,4,6-trinitrochlorobenzene to cellular proteins and prevented tyrosine phosphorylation. This does not contradict our study because tyrosine phosphorylation could be indicative of oxidative stress that is reversed by an antioxidant. It is also important to note that our study differs from the Bruchhausen study in the time point at which NAC was administered. NAC administration at the time of TNCB sensitization (Brucchausen et al) creates the possibility of NAC binding to the chemical, thereby interfering in binding to cellular proteins. In our study DCs were pretreated with NAC, which was washed away before adding the contact sensitizer (Fig 5, *A*)

It is possible that different types of thiol-modulating compounds might affect the CHS response differently in different mouse strains. C57BL/6 mice are considered to have a T_H1 phenotype, and BALB/c mice are known to be more T_H2 prone.[E11] Särnstrand et al[E8] showed that DiNAC augmented the OXA response while decreasing the CHS response in BALB/c mice. Senaldy et al[E12] demonstrated that orally administered NAC (1.6 g/kg) reduced the TNCB response in BALB/c mice. Venkatraman et al[E13] reported that thiazolidinedione derivatives of the α-lipoic acid inhibited allergic contact dermatitis in OXA-treated NMRI mice.[E13] In contrast, we showed that NAC increases the CHS response to OXA and DNFB in C57/BL6 mice (Fig 5, *A*).[E3]

Our study is supported by the work of Furukawa et al,[E14] who demonstrated that dietary GSH supplementation in C57BL/6 mice reversed the age-associated decrease in the CHS response to DNFB. They confirmed an increase in the thiol content of splenocytes of old mice after GSH administration. Therefore it seems that thiol antioxidants could exert at least 2 effects on the CHS response, namely restoration of redox equilibrium and interference in the covalent binding of the CHS chemicals with endogenous proteins.

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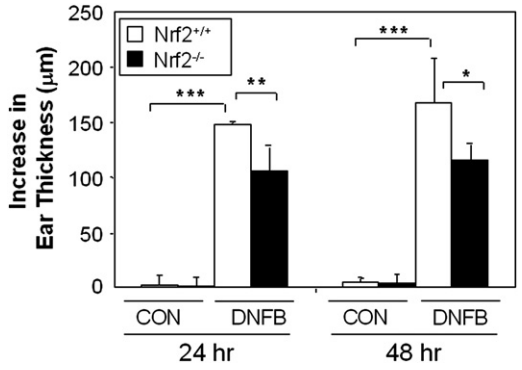

FIG E1. Nrf2 deficiency suppresses the CHS response induced by DNFB. The ear-swelling response was expressed as mean ± SD (n = 4). **P* < .05, ***P* < .01, and ****P* < .001. *CON*, Vehicle-treated control; *DFNB*, DNFB sensitized/challenged.

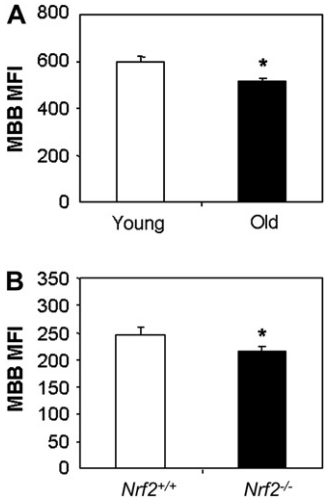

FIG E2. Thiol levels in the BM-DCs decreased by aging, Nrf2 deficiency, or both. Cultured BM-DCs were surface stained with phycoerythrin-labeled anti-CD11c, followed by MBB staining and flow cytometry. **A**, Mean fluorescence intensity for MBB staining in $CD11c^+$ splenocytes from young and old mice (means ± SDs). **B**, Mean fluorescence intensity for MBB staining in $CD11c^+$ splenocytes from old $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice (mean ± SD, n = 4). *$P < .05$. *MFI*, Mean fluorescence intensity.

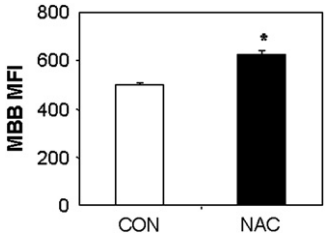

FIG E3. Increased thiol levels in the BM-DCs by NAC treatment. Cultured BM-DCs were incubated with or without NAC (20 mmol/L for 24 hours) and then washed. Cells were surface stained with phycoerythrin-labeled anti-CD11c, followed by MBB staining and flow cytometry. The graph shows mean fluorescence intensity for MBB staining in $CD11c^+$ BM-DCs (means ± SDs, n = 4). *$P < .05$. *CON*, Control group.

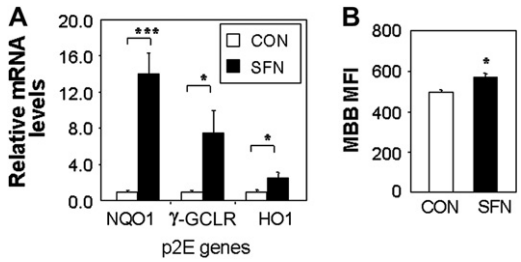

FIG E4. SFN treatment upregulates p2E message and thiol levels. Cultured BM-DCs were incubated with or without SFN (5 μmol/L for 24 hours) and then washed. **A**, Total RNA was extracted from $CD11c^+$ BM-DCs to perform real-time PCR for p2E. **B**, Cells were surface stained with phycoerythrin-labeled anti-CD11c, followed by MBB staining and flow cytometry. The graph shows mean fluorescence intensity for MBB staining in $CD11c^+$ BM-DCs (means ± SDs, n = 4). $*P < .05$, $***P < .001$. CON, Control group; HO1, heme oxygenase 1.

TABLE E1. The primer sequences for real-time PCR analysis

Primers	Forward (5′-3′)	Reverse (5′-3′)
IFN-γ	ACTGGCAAAAGGATGGTGAC	TGAGCTCATTGAATGCTTGG
IL-4	TCAACCCCCAGCTAGTTGTC	TGTTCTTCGTTGCTGTGAGG
T-bet	CAACAACCCCTTTGCCAAAG	TCCCCCAAGCAGTTGACAGT
HO-1	CACGCATATACCCGCTACCT	CCAGAGTGTTCATTCGAGCA
Nrf2	CTCGCTGGAAAAAGAAGTGG	CCGTCCAGGAGTTCAGAGAG
NQO1	TTCTCTGGCCGATTCAGAGT	GGCTGCTTGGAGCAAAATAG
GST	CGCCACCAAATATGACCTCT	CCTGTTGCCCACAAGGTAGT
γ-GCLR	TGGAGCAGCTGTATCAGTGG	AGAGCAGTTCTTTCGGGTCA
GPx	GTCCACCGTGTATGCCTTCT	TCTGCAGATCGTTCATCTCG
β-actin	AGCCATGTACGTAGCCATC	CTCTCAGCTGTGGTGGTGA

HO-1, Heme oxygenase 1; *GST*, glutathione S-transferase.