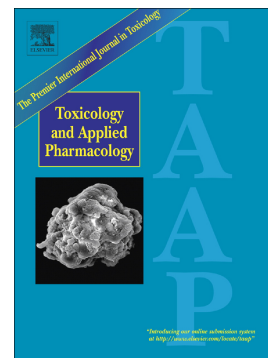


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Dendritic cells' death induced by contact sensitizers is controlled by Nrf2 and depends on glutathione levels

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ORIGINAL ARTICLE

DENDRITIC CELLS' DEATH INDUCED BY CONTACT SENSITIZERS IS CONTROLLED BY Nrf2 AND DEPENDS ON GLUTATHIONE LEVELS

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¹ ACD: allergic contact dermatitis, BMDC: bone marrow dendritic cells, CinA: cinnamaldehyde
CHS: contact hypersensitivity, CS: contact sensitizer, DNCB: 2,4-dinitrochlorobenzene,
GSH: glutathione, GPX1: Glutathione peroxidase 1, GSR: Glutathione-S-reductase, GST:
Glutathione-S-transferase, IL: interleukine, HO-1: heme oxygenase 1, Keap1: Kelch-like ECH-
associated protein 1, NQO1: NADPH- quinone oxidoreductase 1, MAPK: mitogen activated
protein kinase, MoDC: dendritic cells derived from monocytes, Nrf2: nuclear factor 2 related
factor 2, NOS2: nitric oxide synthase 2, RS: reactive species, TNCB: Trinitrochlorobenzene

ABSTRACT

Dendritic cells (DC) are known to play a major role during contact allergy induced by contact sensitizers (CS). Our previous studies showed that Nrf2 was induced in DC and controlled allergic skin inflammation in mice in response to chemicals. In this work, we raised the question of the role of Nrf2 in response to a stress provoked by chemical sensitizers in DC. We used two well-described chemical sensitizers, dinitrochlorobenzene (DNCB) and cinnamaldehyde (CinA), known to have different chemical reactivity and mechanism of action. First, we performed a RT-qPCR array showing that CinA was a higher inducer of immune and detoxification genes compared to DNCB. Interestingly, in the absence of Nrf2, gene expression was dramatically affected in response to DNCB but was slightly affected in response to CinA. These observations prompted us to study DC's cell death in response to both chemicals. DNCB and CinA increased apoptotic cells and decreased living cells in the absence of Nrf2. The characterization of DC apoptosis induced by both CS involved the mitochondrial-dependent caspase pathway and was regulated *via* Nrf2 in response to both chemicals. Oxidative stress induced by DNCB, and leading to cell death, was regulated by Nrf2. Unlike CinA, DNCB treatment provoked a significant reduction of intracellular GSH levels and up-regulated *bcl-2* gene expression, under the control of Nrf2. This work underlies that chemical reactivity may control Nrf2-dependent gene expression leading to different cytoprotective mechanisms in DC.

Keywords:

Allergy

dendritic cells

Nrf2

contact sensitizers

reactive species

GSH,

apoptosis

bcl-2

ho-1

INTRODUCTION

Allergic contact dermatitis (ACD) is a common skin disease known to be induced by contact sensitizers (CS) and involving dendritic cells (DC) (Martin et al., 2011). CS are low molecular weight compounds (< 500 Da) having electrophilic properties and able to bind covalently to nucleophilic residues from cutaneous proteins leading to the formation of a hapten-carrier complex with immunogenic properties (Christensen and Haase, 2012).

The hypothesis that CS can be perceived as a danger signal by DC has been also proposed based on signaling pathways (MAPK, NF κ B) identified upon CS treatments and known to be triggered by danger signals such as toll-like receptor agonists (Ade et al., 2007; Boisleve et al., 2004). Such chemicals can also alter the redox glutathione (GSH/GSSG) balance in human DC derived from monocytes (MoDC) (Mizuashi et al., 2005). DNCB and other CS like dinitrofluorobenzene (DNFB), trinitrochlorobenzene (TNCB) can lead to the production of reactive species (RS) in BMDC (Esser et al., 2012) or MoDC (Byamba et al., 2010).

The main pathway responsible for cell defense against oxidative stress and maintaining the cellular redox balance at physiological levels is the nuclear factor-erythroid 2-related-factor 2 (Nrf2) pathway (Stepkowski and Kruszewski, 2011). Under homeostatic conditions, Nrf2 is sequestered in the cytosol and binds to Kelch-like ECH-associated protein 1 (Keap1) (Lee et al., 2007) allowing its proteasomal degradation (Lo et al., 2006). In the presence of a chemical stress, oxidants or electrophiles, Keap1's conformation is modified leading to the release of Nrf2 and its nuclear translocation. In the nucleus, Nrf2 binds to the consensus sequence ARE (Antioxidant Response Element) allowing the transcription of many target genes [*heme*

oxygenase-1 (hmox1 or ho-1), NAD(P)H: quinone oxidoreductase 1 (nqo1), glutathione-S-transferase (gst)] (Watai et al., 2007). Many studies showed that the decreased levels of phase II detoxification enzymes and antioxidant proteins make *nrf2*^{-/-} mice highly sensitive to cytotoxic electrophiles compared to *nrf2*^{+/+} (wild-type) mice (Lee and Johnson, 2004; Ma et al., 2006; Talalay et al., 2003). Indeed, Murine Embryonic Fibroblasts (MEF) isolated from *nrf2*^{-/-} mice showed higher level of cell death in response to the redox-cycling RS generator menandione and the GSH-depleting anticancer agent cisplatin (Jung and Kwak, 2010). Thus, Nrf2-mediated antioxidant response represents a critically important cellular redox homeostasis and limits oxidative damage (Aw Yeang et al., 2012). We have previously demonstrated that a strong CS such as DNCB or a moderate CS such as CinA, both induced Nrf2 in DC (Ade et al., 2009; Migdal et al., 2013). Recently, we showed that contact hypersensitivity (CHS) induced with DNCB was exacerbated in *nrf2*^{-/-} mice compared to *nrf2*^{+/+} mice while in response to CinA, CHS was only observed in *nrf2*^{-/-} mice (El Ali et al., 2013).

A key antioxidant molecule in the regulation of the redox state, which also plays a role in cytoprotection, is glutathione (GSH). GSH is the most abundant intracellular low molecular weight thiol, and plays a major role in detoxification processes that maintain cellular redox homeostasis. Its protective action is based on oxidation of the thiol group of its cysteine residue, resulting in the formation of oxidized glutathione (GSSG); this in turn is catalytically reversed to GSH by GSH reductase (Franco and Cidlowski, 2012). GSH depletion is an early hallmark in the progression of cell death in response to different apoptotic stimuli (Franco and Cidlowski, 2006).

Since DNCB has been found to be a stronger sensitizer compared to CinA, we investigated the difference of response between DNCB and CinA to underlie differences of mechanisms in DC activation in response to these molecules. A RT-qPCR array was performed in *nrf2*^{+/+} and *nrf2*^{-/-} DC that allowed us to show that CinA was a stronger inducer of genes compared to DNCB. Then, we investigated Nrf2's role in DC survival upon treatment with DNCB and CinA by studying the mechanism triggering apoptosis. Our results showed that Nrf2 controlled apoptosis induced by DNCB through oxidative stress and *bcl-2* gene expression while in response to CinA, Nrf2 controlled apoptosis independently of GSH and probably through a higher expression of detoxification genes, particularly HO-1.

RESULTS

Expression of detoxification genes by Nrf2 is chemical-dependent.

Nrf2 is known as a multiorgan protector against xenobiotic stress (Lee et al., 2005) and has been shown to be a critical regulator of the innate immune response after LPS treatment (Thimmulappa et al., 2006). Its target genes have been found to be involved in several key survival pathways (Banerjee et al., 2012; Kawamoto et al., 2011; Radjendirane et al., 1998). We thus conducted a RT-qPCR array designed for 43 target genes in BMDC to address the question whether gene expression regulated by Nrf2 could be altered differently depending on the type of CS. For this purpose, *nrf2*^{+/+} and *nrf2*^{-/-} BMDC were treated with DNCB or CinA or DMSO (vehicle) at different time of stimulation (4 h, 8 h and 24 h) (Figure 1A).

Based on the ANOVA model, we showed a significant interaction between the three factors: groups of genes, CS and genotype (p-value = 0.02). We measured a higher induction of detoxification genes compared to immune genes in *nrf2*^{+/+} BMDC in the presence of both CS (Figure 1B).

Nrf2 deficiency markedly blocked the up-regulation of antioxidant genes after 4 h and 8 h of treatment with both CS (Figure 1A). At 24 h, genes expression was markedly delayed in *nrf2*^{-/-} leading to a significant up-regulation of genes in *nrf2*^{-/-} than in *nrf2*^{+/+} BMDC with both CS. In our hands, Nrf2 positively controlled antioxidant genes like *glutathione s reductase (gsr)*, *catalase*, *glutathione peroxydase (gpx)* and *nitric oxide synthase 2 (nos2)* in response to DNCB and CinA. These genes scavenge ROS production and play a role in contributing to a decrease in cell death (Bechtel and Bauer, 2009; Gouaze et al., 2002).

However, Nrf2 negatively regulated some genes in response to CS, such as *il-1 β* , *cxcl10*, *ccl3*, *cxcl1* and *il-12 α* (Figure 1A).

In addition, CinA was a better inducer of all genes compared to DNCB, and the induction was even stronger for detoxification genes in the absence of Nrf2. Relating to immune genes, they were more induced in *nrf2*^{-/-} than detoxification genes in the presence of CinA while in response to DNCB, they were less induced (Figure 1 A & B).

Nrf2 rescues DC from cell death in response to contact sensitizers.

Since detoxification genes were down-regulated by both chemicals in the absence of Nrf2, apoptosis provoked by DNCB or CinA in *nrf2*^{-/-} BMDC was measured as a possible consequence. For this purpose, apoptosis was measured using AnV/7-AAD. This assay is used to measure cells that have undergone apoptosis. Apoptosis will be detected by initially staining the cells with Annexin V and 7-AAD solution followed by flow cytometry analysis. It is based on the principle that normal cells are hydrophobic in nature as they express phosphatidyl serine (PS) in the inner membrane (side facing the cytoplasm) and when the cells undergo apoptosis, the inner membrane flips to become the outer membrane, thus exposing PS. The exposed PS is detected by Annexin V, and 7-AAD stains the necrotic cells, which have leaky DNA content that help to differentiate the apoptotic and necrotic cells.

Our results showed that *nrf2*^{-/-} BMDC treated with either DNCB or CinA showed an increase of dead cells compared to *nrf2*^{+/+} BMDC. Apoptosis was triggered 24 h after DNCB or CinA treatments in *nrf2*^{-/-} BMDC compared to *nrf2*^{+/+} BMDC (Figure 2A). The percentage of apoptotic cells (AnV⁺/7-AAD⁻ & AnV⁺/7-AAD⁺) was significantly increased in *nrf2*^{-/-} BMDC compared to

nrf2^{+/+} BMDC in the presence of DNCB (48 % to 80 %) or CinA (52 % to 75 %). The percentage of living cells represented by AnV⁻/7-AAD⁻ decreased significantly in the presence of DNCB (48 % to 14 %) or CinA (47 % to 22 %) in *nrf2*^{-/-} BMDC compared to *nrf2*^{+/+} BMDC (Figure 2A).

The protective role of Nrf2 was then also evaluated in human DC derived from monocytes (MoDC). Our results showed that Nrf2 was expressed after 2 h of treatment and this expression persisted at 4 h and 6 h in response to DNCB and CinA compared to DMSO (Figure 2B). To address the role of Nrf2 in human DC survival, MoDC were transfected with small interfering RNA (siRNA) to invalidate *nrf2* transcripts (supplementary data 1) and then treated with DNCB or CinA for 18 h. In the absence of chemical treatment, knocking down *nrf2* in MoDC (*nrf2*^{-/-} MoDC, siRNA *nrf2*) induced a significant increase showed no difference in the percentage of apoptotic cells (AnV⁺/7-AAD⁻ & AnV⁺/7-AAD⁺) compared to control cells (siRNA Rd) (Figure 2C). In *nrf2*^{-/-} MoDC, the percentage of apoptotic cells increased from 44 % to 64 % in the presence of DNCB and increased from 31 % to 52 % in response to CinA. In addition, a significant decrease of the percentage of living cells (AnV⁻/7-AAD⁻) was observed in response to DNCB (50 % to 28 %) or CinA (62 % to 46 %) in *nrf2*^{-/-} MoDC compared to *nrf2*^{+/+} MoDC (Figure 2C).

These results demonstrated the cytoprotective effect of Nrf2 in response to CS in both human and murine DC with DNCB being slightly more cytotoxic than CinA.

Both contact sensitizers induce apoptosis through mitochondrial and caspase-3/7 pathways while only DNCB induced *bcl-2* gene expression.

Since both chemicals induced higher apoptosis in the absence of Nrf2, we next characterize the mechanism of cell death induced by the two CS, DNCB and CinA. For this purpose,

mitochondrial membrane potential ($\Delta\Psi_m$) and caspase-3/7 activity were measured in DC. DiOC6(3) is a cell-permeant, green-fluorescent, lipophilic dye which accumulates in mitochondria due to their large negative membrane potential, it is used to monitor the mitochondrial membrane potential using flow cytometric detection. $\Delta\Psi_m$ is an important parameter of mitochondrial function and an indicator of cell health. Depletion of $\Delta\Psi_m$ suggests the loss of mitochondrial membrane integrity reflecting the initiation of the pro-apoptotic signal.

In the absence of Nrf2, a significant increase in the percentage of DiOC6(3)^{low} cells was observed at all tested times for each CS (Figure 3A).

To measure caspase-3/7 activity, we used the NucView™ 488 caspase-3/7 substrate. NucView™ 488 Caspase-3/7 substrate is a novel cell membrane-permeable fluorogenic caspase substrate designed for detecting caspase-3/7 activity within live cells in real time. This substrate detects caspase-3/7 activity within individual intact cells without inhibiting caspase activity. Our results showed that CinA did not induce any significant increase of caspase-3/7 activity in *nrf2*^{+/+} BMDC over time while a slight effect was observed with DNCB 24 h post treatment ($p < 0.1$). However, in *nrf2*^{-/-} BMDC treated with DNCB or CinA at 6 h and 24 h, caspase-3/7 activity was significantly increased compared to *nrf2*^{+/+} BMDC (Figure 3B). In summary, cell death provoked by these two molecules involved both an induction of caspase-3/7 activity and a rapid loss of $\Delta\Psi_m$ in *nrf2*^{-/-} BMDC treated with DNCB or CinA (Figures 3A & B).

Since an ARE sequence has been identified in the *bcl-2* promoter (Niture and Jaiswal, 2012), we investigated the regulation of *bcl-2*, which is known to be a key factor in the mitochondrial-dependent pathway of apoptosis, in response to CS in DC. For this purpose, *nrf2*^{+/+} and *nrf2*^{-/-}

BMDC were treated for 4 h and 8 h with DNCB or CinA (Figure 3C). Results revealed that DNCB ~~but not CinA~~ treatment increased *bcl-2* mRNA in *nrf2*^{+/+} BMDC 8 h post treatment (Figure 3C). Regarding CinA, our results show a tendency for an increase of *bcl-2* mRNA at 8 h but this increase was not significant ($p = 0.06$). However, in *nrf2*^{-/-} BMDC treated with DNCB or CinA, *bcl-2* mRNA levels were significantly lower compared to their respective control cells (*nrf2*^{+/+}) at 8 h (Figure 3C). Taken together, our data demonstrate that Nrf2 controls the intrinsic apoptotic pathway and regulates positively *bcl-2* gene expression in BMDC in response to DNCB and to a lesser extend in response to CinA.

Reactive species production and GSH levels are differently controlled by DNCB or CinA.

Since many antioxidant genes are down-regulated in response to both CS in BMDC in the absence of Nrf2 and since Nrf2 is known to regulate cytoprotective responses caused by RS and electrophiles, we addressed the role of RS production in apoptosis in response to both CS in DC. RS production was measured by flow cytometry using H2DCF-DA, a membrane permeable non-fluorescent dye producing a green fluorescence when the cleaved dye is oxidized by RS. *nrf2*^{+/+} and *nrf2*^{-/-} BMDC were treated with DNCB or CinA during 30 min, 1 h or 2 h. RS levels reached a peak 30 min after treatment with DNCB or CinA and then decreased gradually compared to the control (DMSO) in both *nrf2*^{+/+} and *nrf2*^{-/-} BMDC. RS production was higher in response to DNCB (25 %) compared to CinA (12 %). RS production was significantly higher in *nrf2*^{-/-} BMDC compared to *nrf2*^{+/+} BMDC for both DNCB and CinA (Figure 4A). Furthermore, basal levels of RS were also higher in *nrf2*^{-/-} BMDC.

GSH is one of the key antioxidant molecules responsible for maintenance of DC intracellular redox homeostasis. Cellular GSH levels are mainly determined by Nrf2 activity in regulating genes involved in GSH biosynthesis such as the glutamate-cysteine ligase catalytic (GCLC) subunit (MacLeod et al., 2009). We measured intracellular GSH levels at steady state and confirmed as previously described by Yeang *et al.*, (Yeang et al., 2012) that *nrf2*^{-/-} BMDC have lower basal GSH levels than *nrf2*^{+/+} BMDC (Figure 4B). Then, we measured GSH levels in *nrf2*^{+/+} and *nrf2*^{-/-} BMDC after 1 h of treatment with DNCB or CinA (Figure 4C). Our data showed that both DNCB and CinA depleted GSH in both genotypes. DNCB dramatically reduced GSH levels up to 67 % in *nrf2*^{+/+} BMDC and up to 76 % in *nrf2*^{-/-} BMDC compared to DMSO. However, for CinA, we observed a slight reduction of GSH levels up to 10 % in *nrf2*^{+/+} BMDC and up to 14 % in *nrf2*^{-/-} BMDC compared to DMSO. In control cells (DMSO), GSH levels were also lower in *nrf2*^{-/-} BMDC compared to *nrf2*^{+/+} BMDC (Figure 4C).

Nrf2 regulates oxidative stress induced by DNCB but not by CinA.

According to our results described above and the literature both CS were able to induce chemical/oxidative stress. To investigate whether apoptosis induced by DNCB or CinA in Nrf2 deficient BMDC was dependent on oxidative stress, an antioxidant scavenger, N-acetylcysteine (NAC), was used.

nrf2^{+/+} and *nrf2*^{-/-} BMDC were pre-treated with NAC, washed and then incubated for 24 h with DNCB or CinA. Pre-treatment of *nrf2*^{+/+} BMDC with NAC permitted DC survival in response to DNCB (34 % to 56.7 %). On the contrary, in response to CinA, no difference was observed concerning DC survival (48.8 % to 43.3 %) when adding NAC. In response to DNCB, cell survival

was restored in *nrf2*^{-/-} BMDC pre-treated with NAC (11.6 % to 57.5 %) with a decrease of total apoptotic cells % (78.8 % to 31.5 %). In response to CinA, a slight difference in cell survival was observed in *nrf2*^{-/-} BMDC pre-treated with NAC (19.8 % to 28.1) (Figure 5A). Statistical analyses have been done to demonstrate the differences between *nrf2*^{+/+} and *nrf2*^{-/-} BMDC in the presence or the absence of NAC for living cells (Figure 5B) and apoptotic cells (Figure 5C). These data suggested that RS production was mainly responsible for DNCB-induced cell death whereas this is probably not the case for CinA in *nrf2*^{-/-} BMDC suggesting an alternative mechanism (Figure 5).

DISCUSSION

Cells have developed an adaptive defense mechanism in response to oxidative and chemical stress that leads to a rapid and efficient expression of detoxifying enzymes (phase II enzymes) and antioxidant pathway (Kang et al., 2005) mainly mediated *via* the transcription factor Nrf2. We previously reported that Nrf2 was induced in the presence of CS (Migdal et al., 2013) but not in the presence of irritants in human DC (Ade et al., 2009). Several studies also described a major role for Nrf2 in cell survival against chemicals (Kaspar et al., 2009; Niture et al., 2010). It has been previously demonstrated that the stabilization of Nrf2 conferred a protection against oxidative stress-induced cell death in different cell types (Li et al., 2005; Miura et al., 2011; Reuland et al., 2013; Yang et al., 2012). Our published results evidenced striking differences between DNCB and CinA regarding Nrf2 expression (Migdal et al., 2013) prompting us to understand the implication of Nrf2 in DC survival when exposed to these CS.

We observed an induction of cell death in response to DNCB and CinA in DC, in accordance with Cruz *et al.*, showing that apoptotic death of skin DC occurred after exposure to DNFB (Cruz et al., 2003). However, our results are the first evidence for a role of Nrf2 in DC survival in response to CS and consistent with other studies revealing the protective role of Nrf2 against cytotoxic molecules in other cell types (Aleksunes et al., 2010; Chen and Shaikh, 2009; Shin et al., 2010). We also showed that mitochondrial membrane permeability and caspase-3/7 activity induced by DNCB or CinA were augmented in *nrf2*^{-/-} BMDC and demonstrated the role of Nrf2 in the intrinsic pathway of apoptosis also called 'mitochondrial pathway'. In the outer mitochondrial wall, Bcl-2 resides and inhibits cytochrome c release. Since an ARE sequence identified in the reverse strand of the *bcl-2* promoter has been identified as essential for the

up-regulation of Bcl-2 by chemicals and irradiations (Niture and Jaiswal, 2012), we addressed the status of Bcl-2 in DC in response to CS. Our results demonstrated that Nrf2 controlled *bcl-2* gene expression in response to DNCB suggesting that Nrf2 might prevent cell death through *bcl-2* gene expression.

The increase in RS production following CS exposure in the absence of Nrf2 (*nrf2*^{-/-} BMDC), was significantly higher upon DNCB or CinA treatments compared to *nrf2*^{+/+} BMDC. It is well known that only a small amount of intracellular GSH is sufficient to rescue cell viability under oxidative stress and that GSH is essential for cell survival upon oxidative stress (Hattem et al., 2014). In the absence of Nrf2, we showed that GSH levels were altered as previously described (Aw Yeang et al., 2012). Alteration in GSH levels leads to an increase in RS levels and less detoxification of DNCB and CinA electrophilic reactivity. Our hypothesis is that in cells treated with equivalent amounts of CS, there is less biologically available amount of CS in Nrf2 WT cells or in NAC treated cells vs Nrf2 KO cells, necessary to trigger apoptosis. Indeed, in our experiments, NAC treatment of BMDC leading to an increase of the cellular levels of GSH, prevented CS-induced RS production (supplementary data 2) and decreased DNCB-triggered apoptosis in *nrf2*^{-/-} BMDC. Besides its activities as a GSH precursor and RS scavenger, NAC is, *per se*, responsible for protective effects in the extracellular environment, mainly due to its nucleophilic and antioxidant properties, influencing the toxicokinetics of xenobiotics (De Flora et al., 2001). An alternative *scenario* with NAC buffering the electrophilicity of DNCB and consequently RS production cannot be excluded.

Relating to CinA, the mechanism of induced apoptosis seems to be different compared to DNCB. CinA is known to react with Cys residues of proteins *via* Michael's addition. In our hands, CinA slightly depletes GSH, and NAC had a weak effect on rescuing the *nrf2*^{-/-} BMDC from apoptosis. The slight GSH depletion and the low level of RS production induced by CinA indicated that Nrf2 was not solely involved in regulating the cytoprotective effect in DC. According to our ANOVA model of analysis, CinA was a better inducer of all genes. Among them, *ho-1* could be an interesting candidate and has been described to afford cytoprotection against cell death (Gozzelino et al., 2010).

Our work highlights the role of Nrf2 in the control of mechanism of DC's cell death induced by chemical sensitizers. Nrf2 controls cell death induced by DNCB or CinA in DC through two different mechanisms, leading to a rapid loss of $\Delta\Psi_m$ and activation of caspase-3/7. Depending on CS's reactivity and the specific genes profile induced by CS, a speculative model of the role of Nrf2 in DC functions is proposed (Figure 6). Nrf2 positively controlled antioxidant genes like *gsr*, *catalase*, *gpx*, *nos2* in response to DNCB and CinA. Expression of these genes participates to scavenge RS production playing a role in cell survival (Bechtel and Bauer, 2009; Gouaze et al., 2002). By regulating antioxidant genes to reduce RS production and immune genes to dampen inflammatory response, Nrf2, known to control skin inflammation in response to CS in mice (El Ali et al. 2013), would be a sensor of DC activation in response to CS.

Materials and methods

Generation of dendritic cells from human monocyte (MoDC) and bone marrow dendritic cells (BMDC)

The method for generating human MoDC was performed as previously described (Antonios et al., 2010). Briefly, Peripheral Blood Mononuclear Cells (PBMC) were purified from buffy coats obtained from Etablissement Français du Sang (EFS, Rungis, France) by density centrifugation with Ficoll gradient (PAA Laboratories GmbH, Pasing, Austria). Monocytes were isolated from the mononuclear fraction through magnetic positive selection using MiniMacs separation columns (Miltenyi Biotec, Bergish Glabash, Germany) and anti-CD14 antibodies coated on magnetic beads following provider's instructions (Direct CD14 isolation kit, Miltenyi Biotec, Bergish Glabash, Germany). Monocytes were cultured at 1×10^6 cells/ml in the presence of GM-CSF (550 U/ml) and IL-4 (550 U/ml) (both from Miltenyi Biotec, Bergish Glabash, Germany) in RPMI 1640 containing Glutamax I supplemented with 10 % heat inactivated fetal calf serum, 1 mM sodium pyruvate, 0.1 mg/ml streptomycin and 100 U/ml penicillin (RPMIc) (all from Gibco Invitrogen, Paisley, UK). Within 5 days, monocytes differentiate into DC were CD14^{low}, DC-SIGN^{high}, CD1a^{high}, CD83^{low} and CD86^{low} as analyzed by flow cytometry.

The method for generating murine BMDC was carried out according to previous publication (Lutz et al., 1999) with slight modifications. Briefly, cells derived from bone marrow collected from femurs and tibiae of 6 to 12 weeks old C57BL/6 *nrf2*^{+/+} and *nrf2*^{-/-} mice (Itoh et al., 1997a) seeded in bacteriological petri dishes in 10 ml in IMDM containing 10% heat inactivated fetal calf serum, streptomycin and penicillin (IMDMc) (all from Gibco Invitrogen), supplemented with 10 % GM-CSF culture supernatant from the cell line J558 (kindly provided by Sebastian

Amigorena, Institut Curie-France) and β -mercaptoethanol (50 μ M, Sigma®). At day 10, non-adherent cells and loosely adherent cells were collected by gentle pipeting and then centrifuged. The cells were then washed twice with IMDMc and incubated according to different protocols. BMDC phenotype was characterized and analyzed by flow cytometry by measuring the expression of cell surface markers CD11c^{high}, MHC II^{high} and CD86^{low}, CD80^{low}, CD103^{low}, CD207^{low}, Epcam^{low}.

Mice

nrf2^{-/-} mice were generated as described by (El Ali et al., 2013). *nrf2*^{-/-} mice were provided by the RIKEN BRC according to a MTA to Prof. S. Kerdine-Römer. Wild-type (*nrf2*^{+/+}) and *nrf2*^{-/-} mice, investigated in the present study, were generated from inbred C57BL/6J background *nrf2* heterozygous mice. The donating investigator reported that these mice were backcrossed to C57BL/6J for at least 10 generations. Mice were housed in a pathogen-free facility and handled in accordance with the principles and procedures outlined in Council Directive 86/609/EEC. Genotyping was performed by PCR using genomic DNA that was isolated from tail snips as described (Itoh et al., 1997b).

Chemical treatment

MoDC or BMDC were washed twice and then incubated at 1x10⁶ cells/ml. Cells were then treated with DNCB (10 μ M, Sigma®) or CinA (100 μ M, Sigma®) for different period of time according to the experiments. DNCB and CinA were dissolved in DMSO at 0.1% as a final concentration in complete medium.

NAC pre-treatment

At day 10 of differentiation, *nrf2*^{+/+} and *nrf2*^{-/-} BMDC were washed twice and then pre-treated with NAC (25 mM) for 2 h. The cells were then washed twice with complete medium and then re-suspended for further treatments.

Western blot analysis

Western blot was performed as previously described (Larange et al., 2012). Briefly, Cultured DC (10⁶ cells/ml) were washed in cold PBS before lysis in lysis buffer (20 mM Tris pH 7.4, 137 mM NaCl, 2 mM EDTA pH 7.4, 1 % Triton, 25 mM β -glycerophosphate, 1 mM Na₃VO₄, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM PMSF, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin and 5 μ g/ml pepstatin). The homogenates were centrifugated at 15,000 rpm for 20 min at 4°C. Equal amounts of denaturated protein were loaded onto 10 % SDS-PAGE gel and transferred on PVDF membrane (Amersham Biosciences, Les Ulis, France). Membranes were then incubated with Abs directed against Nrf2 (H-300, Santa Cruz biotechnology, Santa Cruz, USA). Immunoreactive bands were detected by chemiluminescence (ECL solution, Amersham Biosciences, Les Ulis, France). P38 MAPK was used as a loading control and revealed with Abs raised against p38 MAPK (Cell Signaling Technology, Ozyme, St-Quentin en Yvelines, France). Bands were measured using the ImageLab software (Bio-Rad, Marnes-La-Coquette, France).

Electroporation of MoDC with siRNA

Loading of siRNA in MoDC was performed as previously described (Larange et al., 2012). Briefly, at day 5 of differentiation, MoDC were washed once with serum-free medium and once with PBS. Cells were then resuspended in serum-free medium at 40×10^6 cells/ml. Equivalent amounts of non-silencing control siRNA (20 μ M, Rd siRNA from Qiagen, Courtaboeuf, France) or Nrf2 siRNA (20 μ M, ID s9492, Ambion Applied Biosystems, Foster City, CA, USA) were transferred into 4 mm cuvette (Biorad, Marnes-La-Coquette, France) and filled up to a final volume of 100 μ l with serum-free medium. 4×10^6 cells were added and pulsed in a GenePulser II (Biorad, Marnes-La-Coquette, France) (300V, 150 μ F). Cells were then resuspended in complete medium with GM-CSF (550 U/ml) and IL-4 (550 U/ml) at a concentration of 10^6 cells/ml for 18 h.

Measurement of apoptosis by Annexin V/7-AAD staining

After 24 h of treatment with chemicals (DNFB 10 μ M, CnA 100 μ M or DMSO), DC were then stained and incubated with PE Annexin V (AnV) and 7-AAD (BD Biosciences) according to the manufacturer's instructions. Stained cells were analyzed by flow cytometry using a FACSCalibur™ cell analyzer using the CellQuest™ software (Becton Dickinson). Apoptotic and dead cells were stained with both AnV and 7-AAD. Results were expressed as the percentage of living cell (AnV⁻/7-AAD⁻) and total apoptotic cells (AnV⁺/7-AAD⁻ & AnV⁺/7-AAD⁺).

Assessment of mitochondrial membrane potential

For assessment of mitochondrial membrane potential ($\Delta\Psi_m$), BMDC were stained with the cationic lipophilic fluorochrome 3,3'-dihexyloxacarbo-cyanide iodide [DiOC6(3)] (100 nM, Molecular Probes) for 30 min in the dark at 37°C. After different time of treatment with DNCB (10 μ M), CinA (100 μ M) or DMSO, stained cells were analyzed by flow cytometry. Results were expressed as the percentage of DiOC6(3) low cells.

Measurement of caspase-3/7 activity

Caspase-3/7 activity was determined by flow cytometry using the NucView™ (Biotium Inc., USA) assay. Briefly, after 24 h of treatment with DNCB (10 μ M), CinA (100 μ M) or DMSO, *nrf2*^{+/+} and *nrf2*^{-/-} BMDC were stained with NucView™ 488 caspase-3 substrate (5 μ M) for 30 min at RT in the dark. Results were expressed as the percentage of caspase-3/7 positive cells.

Reactive species (RS) production

Intracellular RS production was measured using the specific dye 2'-7'-dichlorodihydrofluorescein diacetate [(H2DCFDA), (FluoProbes, Interchim)], as described (Royall and Ischiropoulos, 1993). Briefly, cells were loaded for 30 min in the dark with H2DCFDA before treatment with DNCB (10 μ M), CinA (100 μ M) or DMSO. After different times of treatment with DNCB, CinA or DMSO, results were expressed according to the intensity of the green DCF fluorescence using flow cytometry.

GSH measurements

Intracellular GSH levels were quantified using the bioluminescent GSH-Glo glutathione assay (Promega), according to the manufacturer's instructions. Briefly, BMDC *nrf2*^{+/+} and *nrf2*^{-/-} were treated with DNCB (10 μ M), CinA (100 μ M) or vehicle (DMSO) for 1 h. Cells were then washed once with cold PBS and resuspended with 100 μ L of GSH-Glo lysis and reaction buffer (Promega). Cells were then dispatched in a 96-well plate. After further addition of 100 μ L of GSH-Glo Luciferin detection reagent (Promega) to each well, luminescence was measured using Berthold Tristar luminometer. Luminescence values were converted to GSH concentrations based on a standard curve created by serial dilution of a GSH standard according to the manufacturer's instructions.

Total RNA isolation

nrf2^{+/+} and *nrf2*^{-/-} BMDC (10⁶ cells/ml) were treated with DNCB (10 μ M) or CinA (100 μ M) for 4 h, 8 h and 24 h. Total RNA isolation was performed as previously described (Larange et al., 2012).

Briefly, total RNA was extracted after lysis of cells in TRIzol reagent (Invitrogen) by the guanidium thiocyanate method according to the manufacturer's instructions.

RT-qPCR array

nrf2^{+/+} and *nrf2*^{-/-} BMDC were treated with DNCB (10 μ M), CinA (100 μ M) or DMSO for 4 h, 8 h and 24 h.

For measurement of mRNA expression, single-stranded cDNA was reverse-transcribed from 1 μ g of total RNA, with random hexamers and oligo-dT priming using the iSCRIPT enzyme (Bio-

Rad), according to the manufacturer's instructions. Custom StellarArray™ qPCR plates (Lonza) were designed with two 48-well arrays per plate, including 43 target genes, 4 reference genes (*18S*, *β-actin*, *tata box binding protein (tbp)*, *peptidylpropyl isomerase B (ppib)*) and one negative control.

The screening was performed on a pool of 3 independent experiments of *nrf2*^{+/+} and *nrf2*^{-/-} BMDC, each individual sample being previously validated by measuring the expression of two known target genes of Nrf2 (*hmox1* and *nqo1*) and then pooled to be used in the qPCR array screening. Selected target genes comprised six functional classes, according to the GeneOntology annotation (<http://amigo.geneontology.org/cgi-bin/amigo/go.cgi>), which were grouped into two major categories including genes relative to defense against oxidative stress and genes involved in immune response. Results were expressed as mRNA fold change in compound-treated vs DMSO-treated cells, either up- (red), down- (green) or not (black) regulated. The relative amount of transcript expression in samples was determined using the $2^{-\Delta\Delta Cq}$ where $\Delta\Delta Cq = (Cq_{\text{target}} - Cq_{\text{reference}})_{\text{sample}} - (Cq_{\text{target}} - Cq_{\text{reference}})_{\text{calibrator}}$. Fold-changes values in the range 0.76-1.32 fell within the technical variability of the screening assay, and thus were considered stable expression. These data were obtained from the pool of three independent experiments. The genes significantly up- or down-regulated in *nrf2*^{+/+} and/or *nrf2*^{-/-} DC treated with DNCB or CinA compared to DMSO cells, were counted in each functional class considered. A Pearson correlation coefficient was used to measure the similarity of gene expression fold-changes between screening and repeated RT-qPCR measurements, and between different treatment conditions from the screening assay. A Fischer's exact test was performed to analyze contingency tables of up- and down- regulated genes in cells from both Nrf2 genotypes.

bcl-2 forward primer: 5'-CAA CCC AAT GCC CGC TGT GC-3'

bcl-2 reverse primer : 5'-GAG AAG TCA TCC CCA GCC CG-3'

Statistical analysis

The comparison of two data groups was analyzed by Mann-Whitney's U test ($p < 0.05$) or One way ANOVA followed by Bonferroni test. Data were analyzed with Statistica version 7.1 software. A Pearson correlation coefficient was used to measure the similarity of gene expression fold-changes between screening and repeated RT-qPCR measurements, and between different treatment conditions from the screening assay. A Fischer's exact test was performed to analyze contingency tables of up- and down- regulated genes in cells from both *Nrf2* genotypes.

We used a mixed-effect model approach (using SAS software, Version 9.3 of the SAS system for Unix) to take into account a hierarchical structure in the data: the genes were considered as random variables belonging to their category and linked by an unstructured covariance matrix. This ANOVA was used to estimate the effects of groups of genes (detoxification or immune response), chemical treatment (DNCB or CinA), time (4 h or 8 h) and *Nrf2* genotype (*nrf2*^{+/+} or *nrf2*^{-/-}) using the 'estimate' option of the MIXED procedure in the SAS software. The significance level of all tests was 0.05. Log transformation of fold induction (i.e., LogInduction) was applied in order to achieve the required normality assumption. To compare the differences of associations according to other factors, we performed interaction tests. Model assumptions were validated as the residuals were independantly and identically normally distributed. As a

sensitivity analysis, the exclusion of five outliers leading to high residual values did not alter the conclusions.

ACCEPTED MANUSCRIPT

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

- Ade, N., D. Antonios, S. Kerdine-Romer, F. Boisleve, F. Rousset, and M. Pallardy. 2007. NF-kappaB plays a major role in the maturation of human dendritic cells induced by NiSO(4) but not by DNCB. *Toxicol Sci* 99:488-501.
- Ade, N., F. Leon, M. Pallardy, J.L. Peiffer, S. Kerdine-Romer, M.H. Tissier, P.A. Bonnet, I. Fabre, and J.C. Ourlin. 2009. HMOX1 and NQO1 genes are upregulated in response to contact sensitizers in dendritic cells and THP-1 cell line: role of the Keap1/Nrf2 pathway. *Toxicol Sci* 107:451-460.
- Aleksunes, L.M., M.J. Goedken, C.E. Rockwell, J. Thomale, J.E. Manautou, and C.D. Klaassen. 2010. Transcriptional Regulation of Renal Cytoprotective Genes by Nrf2 and Its Potential Use as a Therapeutic Target to Mitigate Cisplatin-Induced Nephrotoxicity. *Journal of Pharmacology and Experimental Therapeutics* 335:2-12.
- Antonios, D., P. Rousseau, A. Larange, S. Kerdine-Romer, and M. Pallardy. 2010. Mechanisms of IL-12 synthesis by human dendritic cells treated with the chemical sensitizer NiSO4. *J Immunol* 185:89-98.
- Aw Yeang, H.X., J.M. Hamdam, L.M. Al-Huseini, S. Sethu, L. Djouhri, J. Walsh, N. Kitteringham, B.K. Park, C.E. Goldring, and J.G. Sathish. 2012. Loss of transcription factor nuclear factor-erythroid 2 (NFE2) p45-related factor-2 (Nrf2) leads to dysregulation of immune functions, redox homeostasis, and intracellular signaling in dendritic cells. *J Biol Chem* 287:10556-10564.
- Banerjee, P., A. Basu, B. Wegiel, L.E. Otterbein, K. Mizumura, M. Gasser, A.M. Waaga-Gasser, A.M. Choi, and S. Pal. 2012. Heme oxygenase-1 promotes survival of renal cancer cells through modulation of apoptosis- and autophagy-regulating molecules. *The Journal of biological chemistry* 287:32113-32123.
- Bechtel, W., and G. Bauer. 2009. Catalase protects tumor cells from apoptosis induction by intercellular ROS signaling. *Anticancer Res* 29:4541-4557.
- Boisleve, F., S. Kerdine-Romer, N. Rougier-Larzat, and M. Pallardy. 2004. Nickel and DNCB induce CCR7 expression on human dendritic cells through different signalling pathways: role of TNF-alpha and MAPK. *J Invest Dermatol* 123:494-502.
- Byamba, D., T.G. Kim, D.H. Kim, J.H. Je, and M.G. Lee. 2010. The Roles of Reactive Oxygen Species Produced by Contact Allergens and Irritants in Monocyte-derived Dendritic Cells. *Ann Dermatol* 22:269-278.
- Chen, J., and Z.A. Shaikh. 2009. Activation of Nrf2 by cadmium and its role in protection against cadmium-induced apoptosis in rat kidney cells. *Toxicology and Applied Pharmacology* 241:81-89.
- Christensen, A.D., and C. Haase. 2012. Immunological mechanisms of contact hypersensitivity in mice. *APMIS* 120:1-27.
- Cruz, M.T., C.B. Duarte, M. Goncalo, A. Figueiredo, A.P. Carvalho, and M.C. Lopes. 2003. The sensitizer 2,4-dinitrofluorobenzene activates caspase-3 and induces cell death in a skin dendritic cell line. *International journal of toxicology* 22:43-48.
- De Flora, S., A. Izzotti, F. D'Agostini, and R.M. Balansky. 2001. Mechanisms of N-acetylcysteine in the prevention of DNA damage and cancer, with special reference to smoking-related end-points. *Carcinogenesis* 22:999-1013.
- El Ali, Z., C. Gerbeix, P. Hemon, P.R. Esser, S.F. Martin, M. Pallardy, and S. Kerdine-Romer. 2013. Allergic skin inflammation induced by chemical sensitizers is controlled by the transcription factor Nrf2. *Toxicological sciences : an official journal of the Society of Toxicology* 134:39-48.

- Esser, P.R., U. Wolfle, C. Durr, F.D. von Loewenich, C.M. Schempp, M.A. Freudenberg, T. Jakob, and S.F. Martin. 2012. Contact sensitizers induce skin inflammation via ROS production and hyaluronic acid degradation. *PLoS One* 7:e41340.
- Franco, R., and J.A. Cidlowski. 2006. SLCO/OATP-like transport of glutathione in FasL-induced apoptosis: glutathione efflux is coupled to an organic anion exchange and is necessary for the progression of the execution phase of apoptosis. *J Biol Chem* 281:29542-29557.
- Franco, R., and J.A. Cidlowski. 2012. Glutathione efflux and cell death. *Antioxid Redox Signal* 17:1694-1713.
- Gouaze, V., N. Andrieu-Abadie, O. Cu villier, S. Malagarie-Cazenave, M.F. Frisach, M.E. Mirault, and T. Levade. 2002. Glutathione peroxidase-1 protects from CD95-induced apoptosis. *J Biol Chem* 277:42867-42874.
- Gozzelino, R., V. Jeney, and M.P. Soares. 2010. Mechanisms of cell protection by heme oxygenase-1. *Annu Rev Pharmacol Toxicol* 50:323-354.
- Hatem, E., V. Berthonaud, M. Dardalhon, G. Lagniel, P. Baudouin-Cornu, M.E. Huang, J. Labarre, and S. Chedin. 2014. Glutathione is essential to preserve nuclear function and cell survival under oxidative stress. *Free Radic Biol Med* 67:103-114.
- Itoh, K., T. Chiba, S. Takahashi, T. Ishii, K. Igarashi, Y. Katoh, T. Oyake, N. Hayashi, K. Satoh, I. Hatayama, M. Yamamoto, and Y. Nabeshima. 1997a. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochemical and biophysical research communications* 236:313-322.
- Itoh, K., T. Chiba, S. Takahashi, T. Ishii, K. Igarashi, Y. Katoh, T. Oyake, N. Hayashi, K. Satoh, I. Hatayama, M. Yamamoto, and Y. Nabeshima. 1997b. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* 236:313-322.
- Jung, K.A., and M.K. Kwak. 2010. The Nrf2 system as a potential target for the development of indirect antioxidants. *Molecules* 15:7266-7291.
- Kang, K.W., S.J. Lee, and S.G. Kim. 2005. Molecular mechanism of nrf2 activation by oxidative stress. *Antioxid Redox Signal* 7:1664-1673.
- Kaspar, J.W., S.K. Niture, and A.K. Jaiswal. 2009. Nrf2:INrf2 (Keap1) signaling in oxidative stress. *Free Radic Biol Med* 47:1304-1309.
- Kawamoto, S., J.P. Flynn, Q. Shi, S.W. Sakr, J. Luo, and M.D. Allen. 2011. Heme oxygenase-1 induction enhances cell survival and restores contractility to unvascularized three-dimensional adult cardiomyocyte grafts implanted in vivo. *Tissue engineering. Part A* 17:1605-1614.
- Larange, A., D. Antonios, M. Pallardy, and S. Kerdine-Romer. 2012. Glucocorticoids inhibit dendritic cell maturation induced by Toll-like receptor 7 and Toll-like receptor 8. *J Leukoc Biol* 91:105-117.
- Lee, J.M., and J.A. Johnson. 2004. An important role of Nrf2-ARE pathway in the cellular defense mechanism. *J Biochem Mol Biol* 37:139-143.
- Lee, J.M., J. Li, D.A. Johnson, T.D. Stein, A.D. Kraft, M.J. Calkins, R.J. Jakel, and J.A. Johnson. 2005. Nrf2, a multi-organ protector? *FASEB J* 19:1061-1066.
- Lee, O.H., A.K. Jain, V. Papusha, and A.K. Jaiswal. 2007. An auto-regulatory loop between stress sensors INrf2 and Nrf2 controls their cellular abundance. *J Biol Chem* 282:36412-36420.
- Li, J., D. Johnson, M. Calkins, L. Wright, C. Svendsen, and J. Johnson. 2005. Stabilization of Nrf2 by tBHQ confers protection against oxidative stress-induced cell death in human neural stem cells. *Toxicol Sci* 83:313-328.
- Lo, S.C., X. Li, M.T. Henzl, L.J. Beamer, and M. Hannink. 2006. Structure of the Keap1:Nrf2 interface provides mechanistic insight into Nrf2 signaling. *EMBO J* 25:3605-3617.

- Lutz, M.B., N. Kukutsch, A.L. Ogilvie, S. Rossner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 223:77-92.
- Ma, Q., L. Battelli, and A.F. Hubbs. 2006. Multiorgan autoimmune inflammation, enhanced lymphoproliferation, and impaired homeostasis of reactive oxygen species in mice lacking the antioxidant-activated transcription factor Nrf2. *Am J Pathol* 168:1960-1974.
- MacLeod, A.K., M. McMahon, S.M. Plummer, L.G. Higgins, T.M. Penning, K. Igarashi, and J.D. Hayes. 2009. Characterization of the cancer chemopreventive NRF2-dependent gene battery in human keratinocytes: demonstration that the KEAP1-NRF2 pathway, and not the BACH1-NRF2 pathway, controls cytoprotection against electrophiles as well as redox-cycling compounds. *Carcinogenesis* 30:1571-1580.
- Martin, S.F., P.R. Esser, F.C. Weber, T. Jakob, M.A. Freudenberg, M. Schmidt, and M. Goebeler. 2011. Mechanisms of chemical-induced innate immunity in allergic contact dermatitis. *Allergy* 66:1152-1163.
- Migdal, C., J. Botton, Z. El Ali, M.E. Azoury, J. Guldemann, E. Gimenez-Arnau, J.P. Lepoittevin, S. Kerdine-Romer, and M. Pallardy. 2013. Reactivity of chemical sensitizers toward amino acids in cellulose plays a role in the activation of the Nrf2-ARE pathway in human monocyte dendritic cells and the THP-1 cell line. *Toxicological sciences : an official journal of the Society of Toxicology* 133:259-274.
- Miura, T., Y. Shinkai, H.Y. Jiang, N. Iwamoto, D. Sumi, K. Taguchi, M. Yamamoto, H. Jinno, T. Tanaka-Kagawa, A.K. Cho, and Y. Kumagai. 2011. Initial response and cellular protection through the Keap1/Nrf2 system during the exposure of primary mouse hepatocytes to 1,2-naphthoquinone. *Chemical research in toxicology* 24:559-567.
- Mizuashi, M., T. Ohtani, S. Nakagawa, and S. Aiba. 2005. Redox imbalance induced by contact sensitizers triggers the maturation of dendritic cells. *J Invest Dermatol* 124:579-586.
- Niture, S.K., and A.K. Jaiswal. 2012. Nrf2 protein up-regulates antiapoptotic protein Bcl-2 and prevents cellular apoptosis. *J Biol Chem* 287:9873-9886.
- Niture, S.K., J.W. Kaspar, J. Shen, and A.K. Jaiswal. 2010. Nrf2 signaling and cell survival. *Toxicol Appl Pharmacol* 244:37-42.
- Radjendirane, V., P. Joseph, Y.H. Lee, S. Kimura, A.J. Klein-Szanto, F.J. Gonzalez, and A.K. Jaiswal. 1998. Disruption of the DT diaphorase (NQO1) gene in mice leads to increased menadione toxicity. *The Journal of biological chemistry* 273:7382-7389.
- Reuland, D.J., S. Khademi, C.J. Castle, D.C. Irwin, J.M. McCord, B.F. Miller, and K.L. Hamilton. 2013. Upregulation of phase II enzymes through phytochemical activation of Nrf2 protects cardiomyocytes against oxidant stress. *Free radical biology & medicine* 56:102-111.
- Royall, J.A., and H. Ischiropoulos. 1993. Evaluation of 2',7'-dichlorofluorescein and dihydrorhodamine 123 as fluorescent probes for intracellular H₂O₂ in cultured endothelial cells. *Arch Biochem Biophys* 302:348-355.
- Shin, D.H., H.M. Park, K.A. Jung, H.G. Choi, J.A. Kim, D.D. Kim, S.G. Kim, K.W. Kang, S.K. Ku, T.W. Kensler, and M.K. Kwak. 2010. The NRF2-heme oxygenase-1 system modulates cyclosporin A-induced epithelial-mesenchymal transition and renal fibrosis. *Free Radical Biology and Medicine* 48:1051-1063.
- Stepkowski, T.M., and M.K. Kruszewski. 2011. Molecular cross-talk between the NRF2/KEAP1 signaling pathway, autophagy, and apoptosis. *Free Radic Biol Med* 50:1186-1195.
- Talalay, P., A.T. Dinkova-Kostova, and W.D. Holtzclaw. 2003. Importance of phase 2 gene regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis. *Adv Enzyme Regul* 43:121-134.

- Thimmulappa, R.K., H. Lee, T. Rangasamy, S.P. Reddy, M. Yamamoto, T.W. Kensler, and S. Biswal. 2006. Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. *The Journal of clinical investigation* 116:984-995.
- Watai, Y., A. Kobayashi, H. Nagase, M. Mizukami, J. McEvoy, J.D. Singer, K. Itoh, and M. Yamamoto. 2007. Subcellular localization and cytoplasmic complex status of endogenous Keap1. *Genes Cells* 12:1163-1178.
- Yang, B., J. Fu, H. Zheng, P. Xue, K. Yarborough, C.G. Woods, Y. Hou, Q. Zhang, M.E. Andersen, and J. Pi. 2012. Deficiency in the nuclear factor E2-related factor 2 renders pancreatic beta-cells vulnerable to arsenic-induced cell damage. *Toxicol Appl Pharmacol* 264:315-323.
- Yeang, H.X., J.M. Hamdam, L.M. Al-Huseini, S. Sethu, L. Djouhri, J. Walsh, N. Kitteringham, B.K. Park, C.E. Goldring, and J.G. Sathish. 2012. Loss of transcription factor nuclear factor-erythroid 2 (NF-E2) p45-related factor-2 (Nrf2) leads to dysregulation of immune functions, redox homeostasis, and intracellular signaling in dendritic cells. *J Biol Chem* 287:10556-10564.

FIGURES LEGENDS:

Figure 1: *Antioxidant and immune-related genes are regulated by Nrf2 in DC in response to contact sensitizers.*

(A) Expression of 19 antioxidant genes and 23 immune-related genes in DC screened by qPCR array in response to contact sensitizers. BMDC from *nrf2*^{+/+} and *nrf2*^{-/-} mice were treated with DNCB (10 μ M), CinA (100 μ M) or DMSO for 4 h, 8 h and 24 h. Gene expression of the 43 target genes was measured using RT-qPCR arrays. Results were expressed as mRNA fold change in compound-treated vs DMSO-treated cells, either up- (red), down- (green) regulated or unchanged (black).

(B) Induction of detoxification and immune genes in response to DNCB or CinA in both Nrf2 genotypes.

Log transformation of fold induction [i.e., Log(Induction)] was applied in order to achieve the required normality assumption. Results are expressed as log(Induction) for detoxification and immune genes in response to DNCB or CinA. *: DNCB compared to CinA in *nrf2*^{+/+} or *nrf2*^{-/-}, * $p < 0.05$, ANOVA test.

Figure 2: *Nrf2 controls DC survival in response to contact sensitizers.*

(A) Nrf2 controls DC survival in response to contact sensitizers in BMDC. *nrf2*^{+/+} BMDC and *nrf2*^{-/-} BMDC treated with DNCB (10 μ M), CinA (100 μ M) or DMSO for 24 h were analyzed by flow cytometry using AnV/7-AAD staining. Results represent the mean of three independent experiments $\pm$ SEM. *: *nrf2*^{+/+} cells compared to *nrf2*^{-/-} cells, #: treated cells compared to DMSO in *nrf2*^{-/-}, # * $p < 0.05$, Mann Whitney U-test. **(B)** Nrf2 protein accumulation in MoDC in response

to contact sensitizers. MoDC were treated with DNCB or CinA for the indicated times. Nrf2 protein expression was measured by Western blot. P38 MAPK was used as a loading control. Data represent one representative experiment out of three. **(C)** Nrf2 controls DC survival in response to contact sensitizers in MoDC. MoDC transfected with siRNA were treated with DNCB, CinA or DMSO for 18 h and apoptosis was analyzed by flow cytometry using AnV/7-AAD staining. These data represent the mean of three independent experiments $\pm$ SEM. *: siRNA Rd compared to siRNA *nrf2*, #: treated cells compared to DMSO in siRNA *nrf2*, # * $p < 0.05$, Mann Whitney U-test.

Figure 3: *Nrf2 controls BMDC apoptosis through the mitochondrial intrinsic pathway and bcl-2 gene expression.*

(A & B) *nrf2*^{+/+} BMDC and *nrf2*^{-/-} BMDC were treated with DNCB (10 μ M), CinA (100 μ M) or DMSO for 4 h, 6 h and 24 h. Mitochondrial membrane potential ($\Delta\Psi$ m) using DiOC6(3) **(A)** and caspase-3/7 activity **(B)** using NucViewTM were analyzed by flow cytometry. For caspase-3/7 activity, results were expressed as the percentage of positive cells while for $\Delta\Psi$ m loss, results were expressed as the percentage of DiOC6(3) low cells. These data represent the mean $\pm$ SEM of 3 independent experiments. *: *nrf2*^{+/+} cells compared to *nrf2*^{-/-} cells, #: treated cells compared to DMSO in *nrf2*^{-/-} cells, ϕ \$: treated cells compared to DMSO in *nrf2*^{+/+} cells, * # \$ $p < 0.05$, ϕ $p < 0.1$, Mann Whitney U-test.

(C) *Nrf2 positively regulates bcl-2 gene expression in response to contact sensitizers. bcl-2* gene expression was measured by RT-qPCR using Bio-Rad CFX in *nrf2*^{+/+} BMDC and *nrf2*^{-/-} BMDC, treated with DNCB or CinA for 4 h and 8 h. Results were expressed as fold change. These data

represent the mean $\pm$ SEM of 3 independent experiments. *: *nrf2*^{+/+} cells compared to *nrf2*^{-/-} cells, * $p < 0.05$, & $p = 0.06$, Mann Whitney U-test

Figure 4: Contact sensitizers produce more RS and reduce intracellular GSH levels in the absence of Nrf2.

(A) BMDC from *nrf2*^{+/+} and *nrf2*^{-/-} mice were treated with DNCB (10 μ M), CinA (100 μ M) for 30 min, 1 h and 2 h. RS production was measured by flow cytometry using the H2DCF-DA probe. Results were expressed using the mean fluorescent intensity (MFI). These data represent the mean $\pm$ SEM of 3 independent experiments. *: *nrf2*^{+/+} cells compared to *nrf2*^{-/-} cells, #: treated cells compared to DMSO in *nrf2*^{-/-}, \$: treated cells compared to DMSO in *nrf2*^{+/+}, * # \$ $p < 0.05$, Mann Whitney U-test.

(B) BMDC from *nrf2*^{+/+} and *nrf2*^{-/-} mice were treated with DNCB or CinA for 1 h. Intracellular GSH levels were quantified using the bioluminescent GSH-Glo glutathione assay. Results were expressed as the concentration of intracellular GSH in μ M. These data represent the mean $\pm$ SEM of 3 independent experiments. *: *nrf2*^{+/+} cells compared to *nrf2*^{-/-} cells, #: treated cells compared to DMSO in *nrf2*^{-/-}, \$: treated cells compared to DMSO in *nrf2*^{+/+}, * # \$ $p < 0.05$, ***, \$\$\$, ### $p < 0.001$, One way ANOVA followed by Bonferroni test.

Figure 5: oxidative stress is involved in BMDC apoptosis in the absence of Nrf2.

nrf2^{+/+} BMDC and *nrf2*^{-/-} BMDC were pre-treated with NAC for 2 h, washed and then treated with DNCB (10 μ M), CinA (100 μ M) or DMSO for 24 h. Apoptotic (AnV⁺/7-AAD⁻) and necrotic

(AnV⁻/7-AAD⁺) cells were measured by flow cytometry as described in material and methods.

Results represent one experiment out of three.

(B & C) Statistical analyses to show the differences between *nrf2*^{+/+} BMDC and *nrf2*^{-/-} BMDC in the presence or the absence of NAC for living cells **(B)** and total apoptotic cells **(C)**. ***p < 0.001, One way ANOVA followed by Bonferroni test.

Figure 6: *The Nrf2 pathway controls DC apoptosis in response to contact sensitizers.*

In *nrf2*^{+/+} DC, DNCB induce a RS production, a significant reduction of intracellular GSH levels, a loss of mitochondrial membrane potential ($\Delta\Psi_m$) and an activation of caspase-3/7 leading to apoptosis.

For CinA, the same mechanism is activated but to a lesser extent. In response to both CS, we observed that Nrf2 activation controls many genes allowing less apoptosis. In the case of DNCB, *bcl-2* gene expression seems to inhibit apoptosis whereas in response to CinA, *ho-1* seems to play a cytoprotective effect.

In *nrf2*^{-/-} DC, Nrf2 deficiency leads to the abolition of *bcl-2* gene expression and detoxification genes in response to DNCB. Furthermore, a higher RS production, a significant reduction of intracellular GSH levels, a rapid loss of $\Delta\Psi_m$ and an activation of caspase-3/7 lead to apoptosis in response to both chemicals.

→ : induction ; —| : inhibition

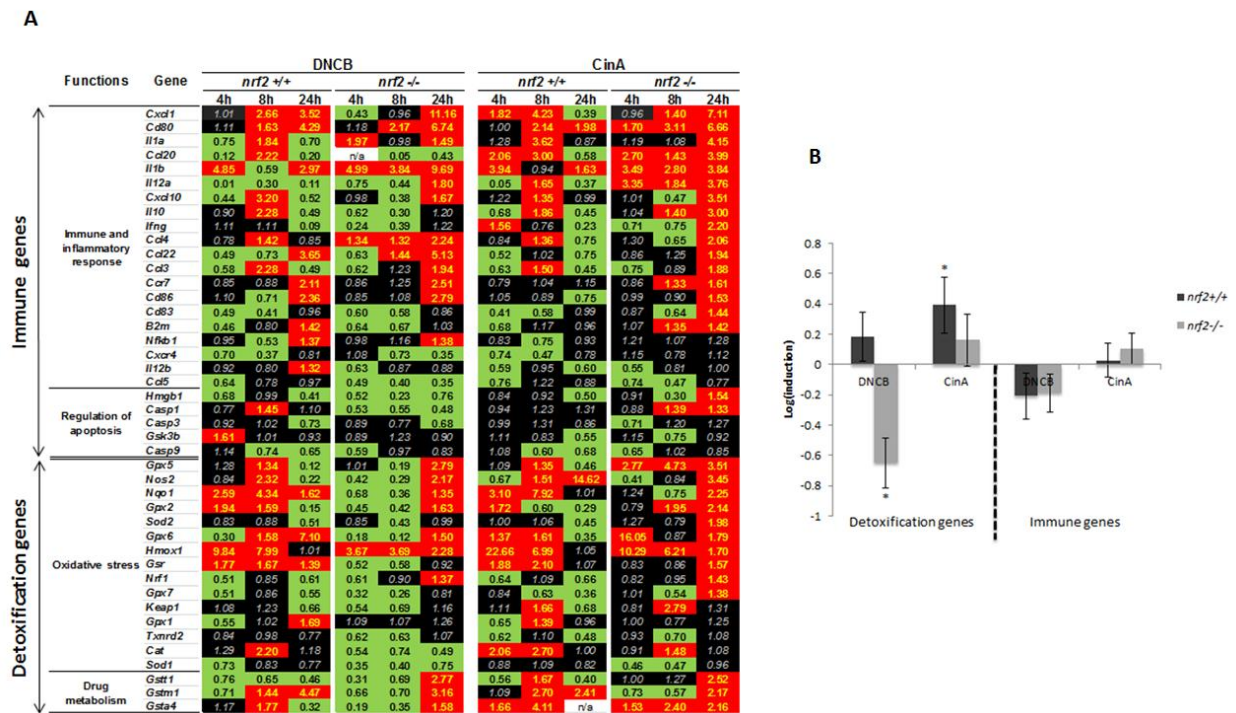
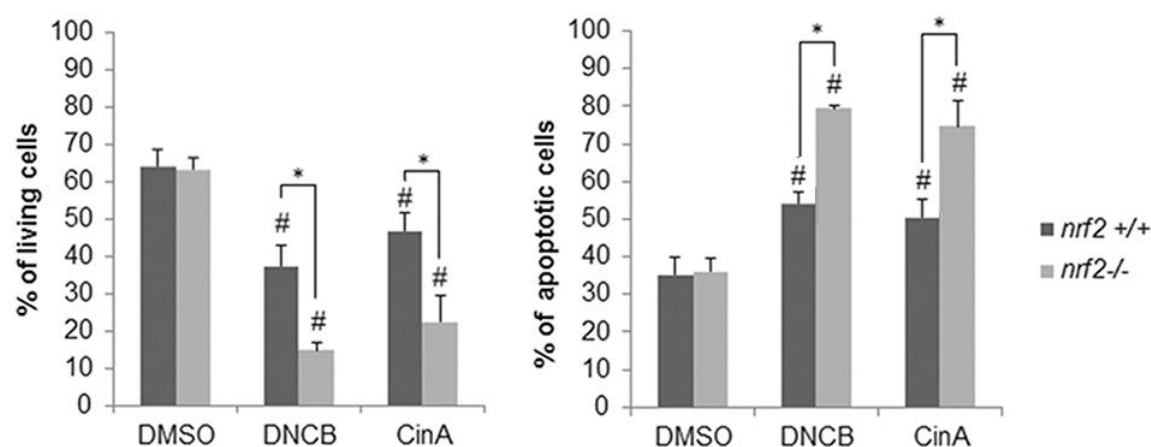


Figure 1

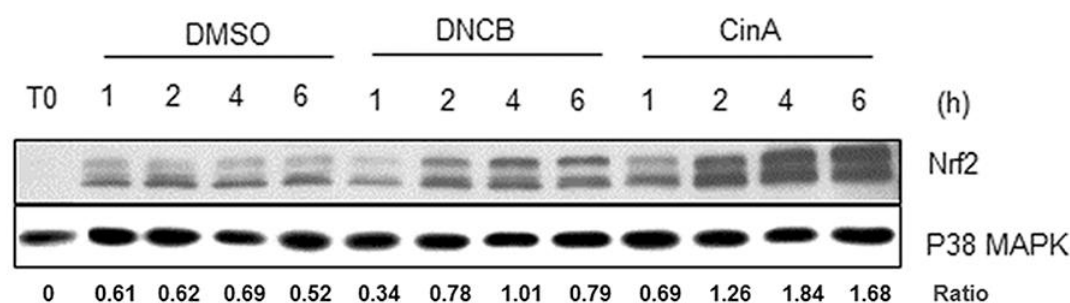
BMDC

A



MoDC

B



C

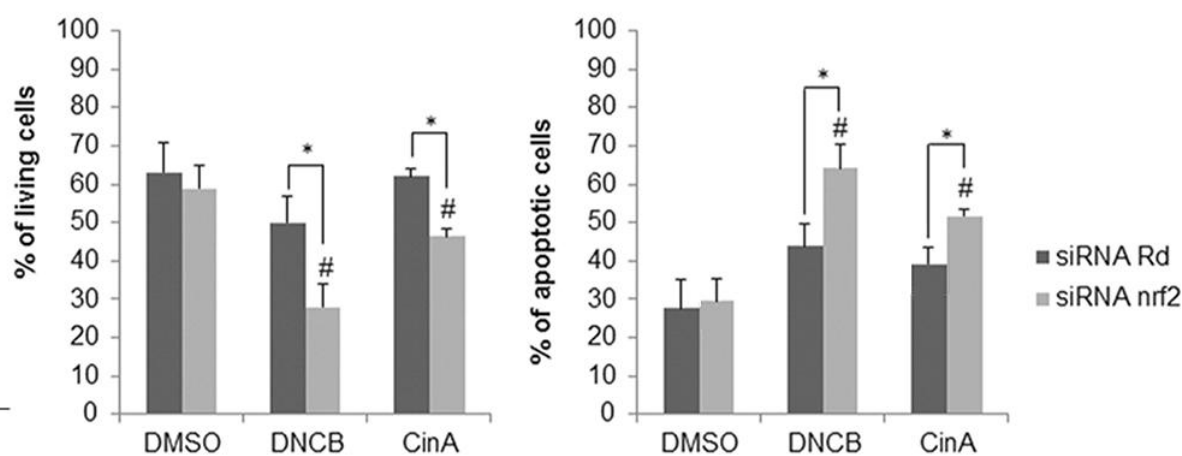


Figure 2

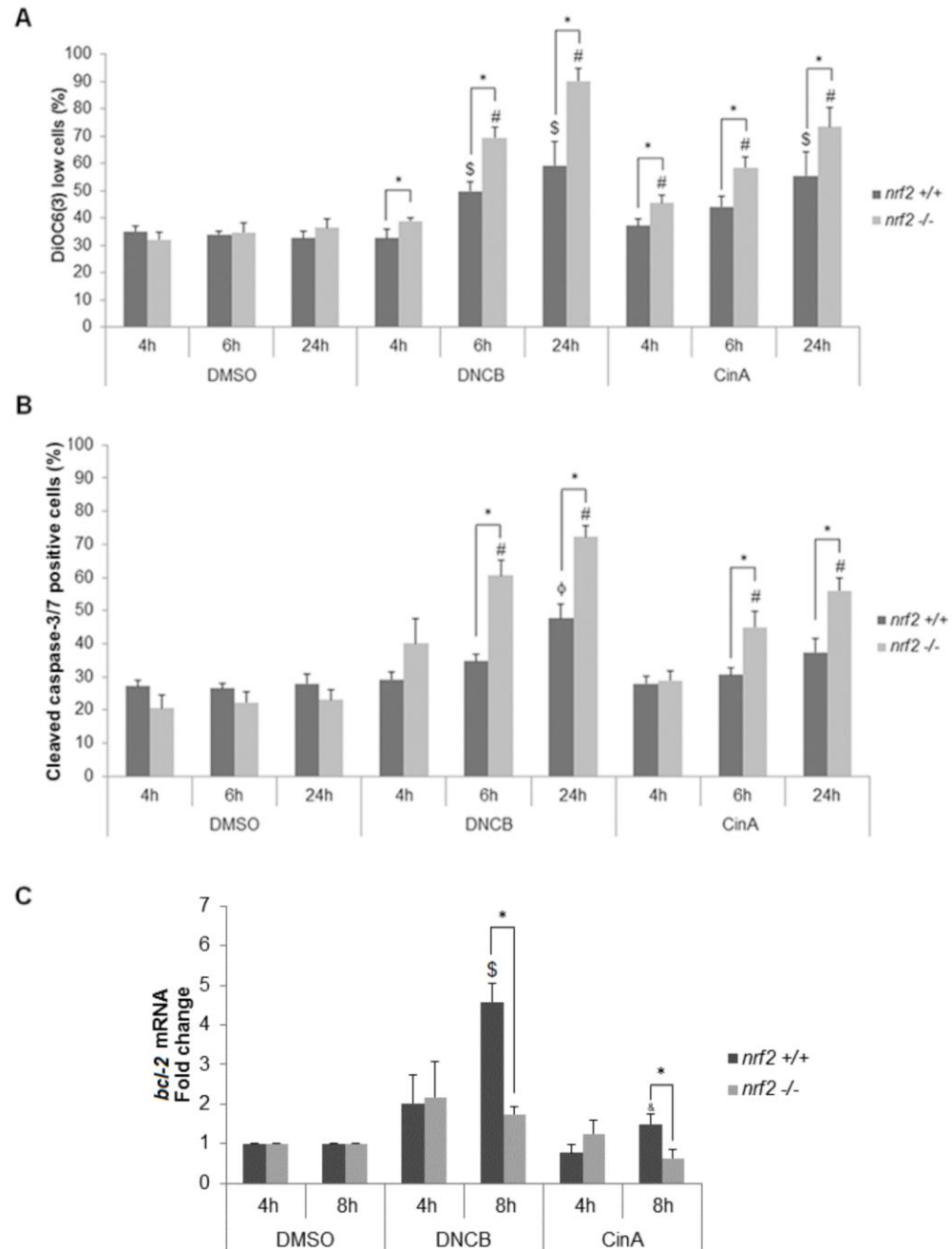
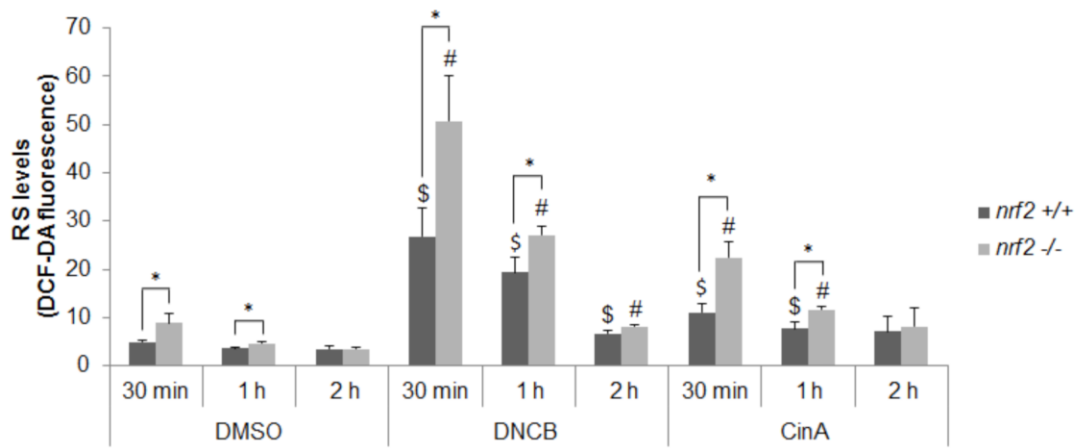
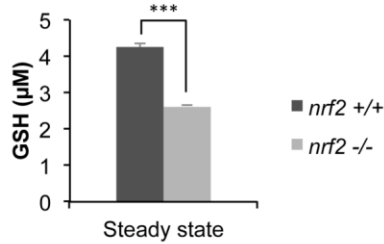


Figure 3

A



B



C

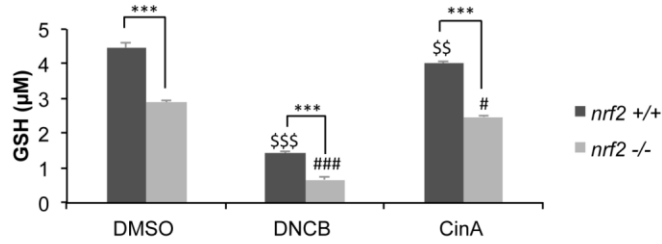


Figure 4

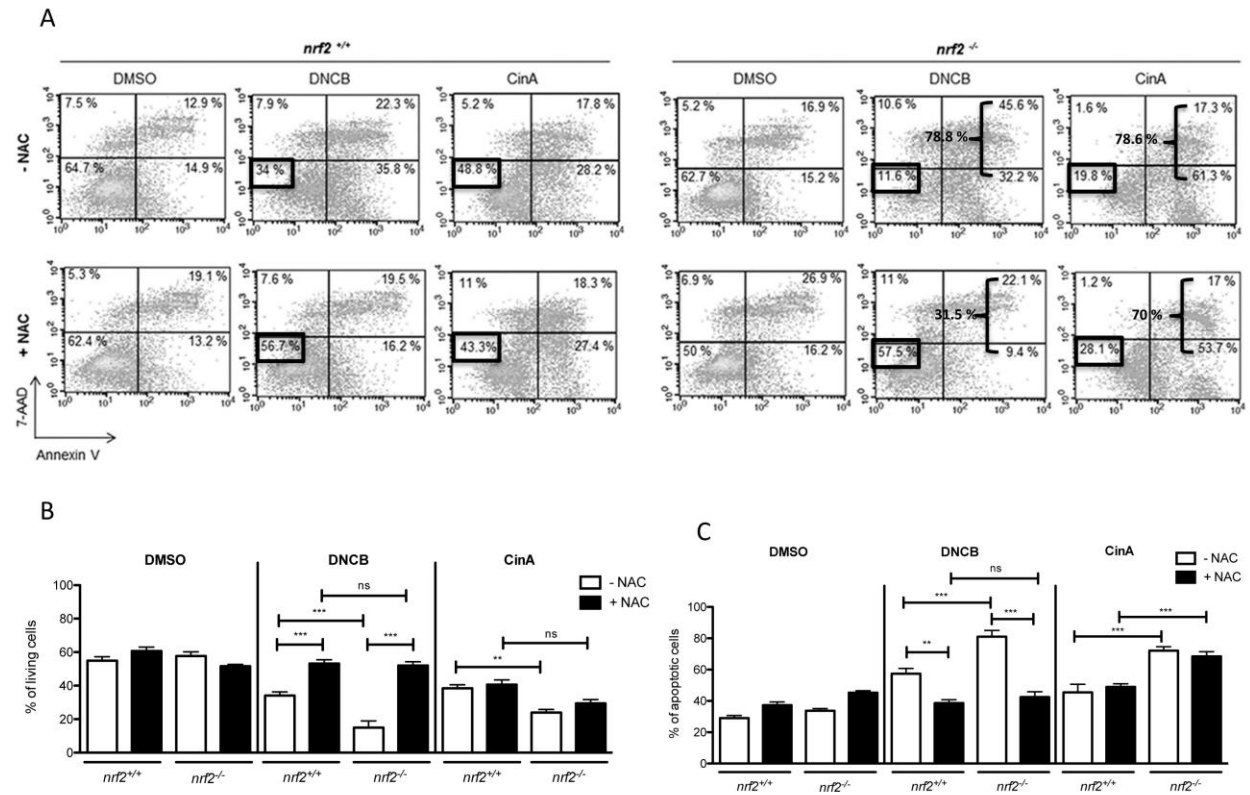


Figure 5

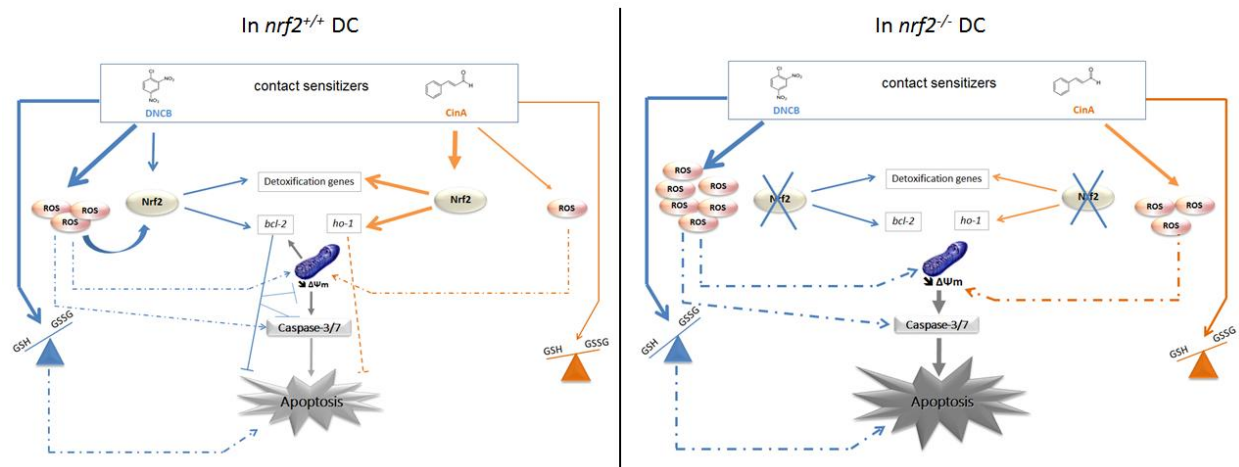
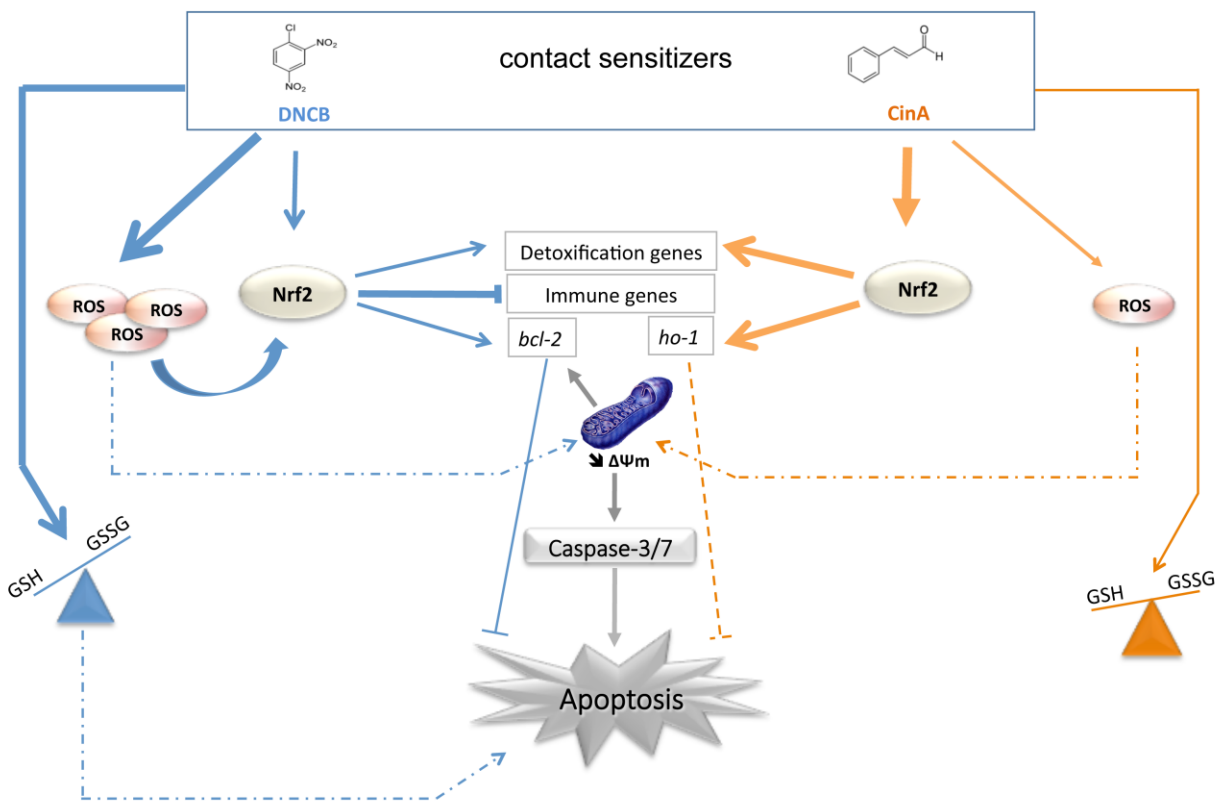


Figure 6



Graphical abstract

ORIGINAL ARTICLE

DENDRITIC CELLS' DEATH INDUCED BY CONTACT SENSITIZERS IS CONTROLLED BY NRF2 AND DEPENDS ON GLUTATHIONE LEVELS

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Short title: Nrf2 controls DC survival

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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ORIGINAL ARTICLE

HIGHLIGHTS

DENDRITIC CELLS' DEATH INDUCED BY CONTACT SENSITIZERS IS CONTROLLED BY NRF2 AND DEPENDS ON GLUTATHIONE LEVELS

- Nrf2 controls cell death induced by contact sensitizers in dendritic cells.
- DNCB reduced GSH levels and up-regulated *bcl-2* gene expression unlike CinA.
- Chemical reactivity controls Nrf2-dependent genes having protective effect in DC.