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ORIGINAL PAPER

Effect of lipopolysaccharide, skin sensitizers and irritants on thioredoxin-1 expression in dendritic cells: relevance of different signalling pathways

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Abstract Thioredoxin-1 is a ubiquitous protein involved in phenotypical and functional changes in dendritic cells (DC). We investigated the effect of lipopolysaccharide (LPS), skin sensitizers, and irritants on thioredoxin-1 by Western blot and immunofluorescence and on mRNA by real-time PCR. As DC models, we used a skin DC line and DC derived from human blood monocytes. We observed that all tested chemicals increased thioredoxin-1 expression, which is only transient for irritants, being the strongest effect observed for LPS ($63 \pm 15\%$). To address the involvement of thioredoxin-1 in DC maturation, we analysed the effect of an activator of thioredoxin-1 expression, hydrogen peroxide, on CD86 expression, a marker of DC maturation. We found that hydrogen peroxide increases thioredoxin-1 and CD86 expression reinforcing thioredoxin-1 involvement in DC maturation. Because mitogen-activated protein kinases and PI3K are activated upon DC maturation, we also analysed their involvement in thioredoxin-1 modulation. We verified that LPS-induced upregulation of thioredoxin-1 expression was dependent on PI3K pathway.

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Introduction

Allergic contact dermatitis (ACD), a common occupational and environmental health problem, is a delayed-type hypersensitivity reaction induced by small reactive chemicals following skin contact [19, 35]. A critical step in the induction of ACD is the activation and subsequent migration of immature dendritic cells (DC) of the skin, namely the Langerhans cells (LC). LC typify the sentinel role of immature DC, translating changes in their local microenvironment into specific immune responses. Following an encounter with skin sensitizers, LC become activated, migrate to lymph nodes and mature into immunostimulatory DC capable of presenting antigen to naïve T cells [10, 50]. During maturation, LC experiment morphological, functional and phenotypical changes, such as alteration of surface markers [2, 20, 58], production of cytokines and chemokines [3, 16, 20] and the induction of signal-transduction pathways [20, 46]. Lipopolysaccharide (LPS) and skin sensitizers, in contrast to irritants, have been described to stimulate DC maturation [3, 9, 13, 16, 28, 57, 60, 64]. However, the exact cellular and molecular mechanisms of DC activation are not yet fully understood, being this knowledge crucial to elucidate the mechanisms involved in the acquisition of skin sensitization.

Thioredoxin (Trx)-1, a ubiquitous protein with a conserved dithiol motif in the active site, is involved in the maintenance of proteins in their reduced state. Trx-1 has an important role in defence against cellular oxidative stress [8, 65], being its expression regulated by nuclear factor

erythroid-derived 2-related factor (Nrf2)/antioxidant responsive element (ARE) pathway [29, 67]. Recently, Nrf2/ARE regulatory pathway has been proposed for the screening of the sensitization potential of novel chemicals [1, 42]. Trx-1 functions [8] also include control of growth, regulation of the activation of several transcription factors (Nrf2, nuclear factor [NF]-κB, activator protein [AP]-1 and p53), modulation of apoptosis by direct inhibition of apoptosis signal-regulating kinase 1 (ASK1) [36, 51] and also co-cytokine and chemokine activity [11]. Hence, beyond its intrinsic antioxidant activity against oxidative stress induced by oxidants and electrophiles such as skin sensitizers, Trx-1 has also a signalling intermediate role that could be responsible for phenotypical and functional changes in DC following exposure to chemical sensitizer. Accordingly, it was previously demonstrated that Trx genes were upregulated in DC after treatment with the DC maturation inducer agent LPS [45, 55] and the strong allergen 2,4-dinitrobenzene sulphonic acid [23, 48].

Previous studies, performed by us and other authors, reported the involvement of intracellular signalling pathways, namely mitogen-activated protein kinases (MAPK), phosphoinositide-3-OH kinase (PI3K)-Akt and the transcription factor NF-κB, in the survival and maturation of DC [7, 9, 14–17, 37, 38, 43, 46, 64]. Accordingly, it was previously demonstrated by us and other authors that LPS and skin sensitizers activate MAPK signalling pathways in DC [4, 31, 37, 40, 57]. In addition, MAPK are involved in the induction of Nrf2/ARE-dependent gene expression [66]. Therefore, analysis of intracellular signalling pathways, particularly their involvement in Trx-1 protein expression, could provide a promising tool for the elucidation of the DC maturation process.

To understand the skin sensitization process and the intracellular mechanisms involved in DC maturation, we investigated: (1) the effect of LPS, skin sensitizers with different chemical structures and with different potencies, namely 2,4-dinitrofluorobenzene (DNFB), 1,4-phenylenediamide (PPD) and nickel sulphate ($NiSO_4$), and irritants with different structures, in particular sodium dodecyl sulphate (SDS) and benzalkonium chloride (BC) in Trx-1 protein expression in DC; (2) the correlation between thioredoxin-1 expression and DC maturation, by addressing the role of hydrogen peroxide, an activator of thioredoxin-1[49], on the conventional marker of DC maturation CD86 [20], and recently demonstrated by us to be upregulated in a mouse skin DC line by LPS [44]; (3) finally, we investigated which intracellular signalling pathways were responsible for Trx-1 protein increase induced by LPS. As experimental DC cell models, we used the fetal skin-derived dendritic cell line (FSDC) and DC derived from human peripheral blood monocyte (MoDC). FSDC is a skin DC precursor with morphological, phenotypical and functional characteristics of LC [24] and, in contrast to other DC cell lines, did not require exogenous growth factors for their continued proliferation, when cultured in serum-containing medium. As major findings, we observed that (1) all tested chemicals increased thioredoxin-1 expression, which is only transient for irritants, being the strongest effect observed for LPS; (2) there is a correlation between thioredoxin-1 expression and DC maturation; (3) the upregulation of thioredoxin-1 expression induced by LPS was dependent on the PI3K pathway.

Materials and methods

Materials

Iscove's modified Dulbecco's medium (IMDM), RPMI-1640, DNFB, SDS, PPD, dexamethasone, cyclosporin A and Tween-20 were obtained from Sigma-Aldrich Química (Madrid, Spain). $NiSO_4$ and BC were purchased from Sigma Chemical Co. (Madrid, Spain). LPS from *Escherichia coli* (serotype 026:B6) was from Sigma Chemical Co. (St Louis, MO, USA). LY294002 and SP600125 were from Calbiochem (San Diego, CA, USA). SB203580 was a kind gift of Dr J.L. Adams from SmithKline Pharmaceuticals (King of Prussia, PA, USA) and PD098059 was obtained from RBI (Natick, MA, USA). Fetal calf serum and trypsin were purchased from Gibco (Paisley, UK) and human GM-CSF and IL-4 were from PeproTech (London, UK). The protease and phosphatase inhibitor cocktails were obtained from Roche (Amadora, Portugal). Lymphoprep was from Axis-Shield (Oslo, Norway) and the MACS colloidal supermagnetic microbeads conjugated with anti-human CD14 monoclonal antibody (CD14 microbeads) were purchased from Miltenyi Biotec Inc. (Bergisch Gladbach, Germany). Acrylamide was obtained from Promega (Madison, WI, USA) and the polyvinylidene difluoride (PVDF) membranes were from Millipore Corporation (Bedford, MA, USA). The rabbit polyclonal antibody against CD86 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit polyclonal antibody against Trx-1 was purchased from Abcam (Cambridge, UK) and the mouse monoclonal anti-β-tubulin I antibody was from Sigma-Aldrich Química (Madrid, Spain). The alkaline phosphatase-linked secondary antibodies and the enhanced chemifluorescence (ECF) reagent were obtained from GE Healthcare (Carnaxide, Portugal). The Vectashield mounting medium was purchased from Vector, Inc. (Burlingame, CA, USA) and Alexa 488-conjugated goat anti-rabbit antibody was from Molecular Probes (Eugene, OR, USA). TRIzol reagent was purchased from Invitrogen (Barcelona, Spain). iScript kit and SYBR Green were obtained from Bio-Rad (Amadora, Portugal). Primers were from MWG

Biotech (Ebersberg, Germany). All other reagents were from Sigma Chemical Co. (Madrid, Spain) or from Merck (Darmstadt, Germany).

Cell culture

The FSDC cell line, kindly supplied by Dr G. Girolomoni (Laboratory of Immunology, Istituto Dermopatico dell'Immacolata, IRCCS, Rome, Italy), has a surface phenotype consistent with an LC progenitor (H-$2^{d.b+}$, I-$A^{d.b+}$, $CD54^+$, $CD11b^+$, $CD11c^+$, $MHCII^+$, $MHCI^+$, $B7.2^+$, $B7.1^-$, $CD44^+$, $B220^-$, $CD3^-$) [24], a phenotype confirmed in our laboratory for the most relevant surface markers [44]. FSDC was cultured in IMDM, supplemented with 10% (v/v) heat-inactivated fetal calf serum, 35.9 mM sodium bicarbonate, 1% (w/v) glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

MoDC were obtained from $CD14^+$ monocytes that were generated from peripheral blood mononuclear cells, as described previously [43]. Blood was collected from healthy human donors after their informed consent. The $CD14^+$ monocytes were cultured in modified RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 800 U/ml IL-4 and 1,000 U/ml GM-CSF. After 7 days of culture, the cell phenotype is consistent with an immature DC phenotype: HLA-DR^+, $CD1a^+$, $CD83^-$, $CD80^{low}$ and $CD86^{low}$ [26].

Chemical treatment

For determination of Trx-1 protein levels by Western blot analysis, FSDC was plated at 0.5×10^6 cells/ml in 12-well microplates or at 0.33×10^6 cells/ml in 24-well microplates and then incubated with the chemicals for 24 and 48 h, respectively. MoDC were plated at 0.3×10^6 cells/ml in 24-well plates. Cells were incubated with 5 μg/ml LPS, or with the skin sensitizers DNFB (1 μg/ml), PPD (50 μg/ml) and $NiSO_4$ (50 μg/ml), or with the irritants SDS (50 μg/ml) and BC (1 μg/ml), or with the Trx activator H_2O_2 (5 or 20 μM), for 24 and 48 h. SDS, BC, PPD and $NiSO_4$ were dissolved in PBS, while DNFB and DCNB were first solubilised in dimethyl sulphoxide and subsequent dilutions were performed in PBS. The final concentration of dimethyl sulphoxide never exceeded 0.01% (v/v), and was without effect on cell viability and on Trx-1 expression (data not shown). To investigate the signalling pathways involved in Trx-1 protein expression induced by LPS, cells were incubated with 5 μg/ml LPS alone or in the presence of the different signalling pathways inhibitors for 48 h. LY294002 (2.5 μM) was used as PI3K inhibitor, SB203580 (20 μM) as p38 MAPK inhibitor, PD098059 (20 μM) as extracellular signal-regulated protein kinase (ERK)1/2 inhibitor and SP600125 (1 μM) as c-Jun N-terminal kinase (JNK) inhibitor. In addition, the effect of the immunomodulators such as dexamethasone (5 μM) and cyclosporin A (1 μM) was also evaluated on Trx-1 protein expression induced by LPS. Dexamethasone and cyclosporin A are immunosuppressors that inhibit the transcription factor NF-κB in FSDC, as previously demonstrated by us [17, 63].

For immunocytochemistry analysis, FSDC was plated at 0.1×10^6 cells/well on glass coverslips. To analyse the Trx-1 immunoreactivity, cells were then incubated with 5 μg/ml LPS, 1 μg/ml DNFB or 1 μg/ml BC for 48 h. To investigate the surface levels of CD86, cells were incubated with 5 μg/ml LPS or 20 μM H_2O_2 for 24 h.

For determination of the Trx-1 mRNA levels by RT-PCR, FSDC was plated at 0.5×10^6 cell/ml in six-well microplates and then incubated with 5 μg/ml LPS, 1 μg/ml DNFB, 50 μg/ml $NiSO_4$ or 50 μg/ml SDS for 24 h.

Concentrations of the chemicals used to perform the experiments (optimal concentrations) were those that induced until 30% of cytotoxicity, evaluated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide reduction assay (data not shown), since it has been described that some degree of cytotoxicity may play an important role in DC activation [27, 52]. Control experiments were performed with non-treated cells.

Western blot analysis

To prepare total cell lysates for Western blot analysis, cells were harvested in ice-cold lysis buffer containing 0.25 M sucrose, 10 mM Tris–HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, protease inhibitor cocktail and phosphatase inhibitor cocktail. Cell lysates were incubated 30 min on ice, and then sonicated to disrupt the cells. Protein concentration of the cell lysates was determined by the bicinchoninic acid protein assay. Cell lysates were then denatured and either used immediately for SDS-PAGE electrophoresis, or frozen at −20°C until use.

Briefly, equivalent amounts of protein (5–20 μg) were separated by electrophoresis on a 12% (v/v) SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were saturated with 5% (w/v) fat-free dry milk in Tris-buffered saline, containing 0.1% (v/v) Tween-20 (TBS-T) for 1 h at room temperature. The levels of Trx-1 and CD86 protein were detected using a rabbit anti-thioredoxin antibody (1:2,000) and a rabbit anti-CD86 antibody (1:200), respectively, for 1 h at room temperature, followed by incubation with an alkaline phosphatase-conjugated anti-rabbit antibody (1:20,000). The immune complexes were detected using the ECF system and the membranes were scanned for blue excited fluorescence on the Storm 860 (GE Healthcare). The generated signals were quantified by scanning the membranes with a fluorescence scanner

and analysed using the software ImageQuant TL®. To demonstrate equivalent protein loading, membranes were stripped and reprobed with a monoclonal anti-β-tubulin I antibody (1:20,000), followed by incubation with an alkaline phosphatase-conjugated anti-mouse antibody (1:20,000). All the antibodies were prepared in 1% (w/v) fat-free dry milk in TBS-T.

Immunofluorescence assay

After FSDC incubation with chemicals, cells were fixed and permeabilized with cold methanol:acetone (1:1) for 10 min. For immunocytochemistry detection of CD86 on the surface level, the cells were only fixed with paraformaldehyde 4% (w/v) in PBS. Non-specific binding was blocked by incubating the cells with PBS supplemented with 1% (w/v) BSA for 45 min at room temperature. Cells were then incubated with a rabbit polyclonal anti-Trx-1 antibody (1:200) for 2 h at room temperature or with a rabbit anti-CD86 antibody (1:25) overnight at 4°C, followed by incubation with Alexa 488-conjugated goat anti-rabbit antibody (1:500) for 45 min. Antibodies were diluted in PBS supplemented with 0.5% (w/v) BSA. After a washing step, a DAPI solution (0.1 μg/ml) was added to cells for 1 min to stain the nucleus. The coverslips were mounted with Vectashield mounting medium. Negative control experiments were performed as described above, except for the omission of the primary antibody, and resulted in weak non-specific staining. Fluorescence labelling was visualised in a Zeiss Axiovert 200 microscope, and images acquired with a coupled AxioCamHR camera and analysed with AxioVision v.4.5 software. In each experiment, the optimal acquisition parameters were defined for the condition that had the highest fluorescence and then maintained for all the other conditions, within the same experiment.

RNA isolation, cDNA synthesis and real-time PCR

Total RNA was isolated by TRIzol method. Briefly, after chemical stimulation, cells were scrapped in TRIzol. Chloroform was added, and the samples were centrifuged at 12,000*g*, for 15 min at 4°C to separate the phases. The aqueous phase was transferred to a fresh tube and the RNA precipitated by mixing with isopropyl alcohol. After centrifugation at 12,000*g*, for 10 min at 4°C, the supernatant was removed and the RNA pellet washed with 75% ethanol. The RNA was then resuspended in RNase-free water. The amount and purity of the RNA samples was evaluated by

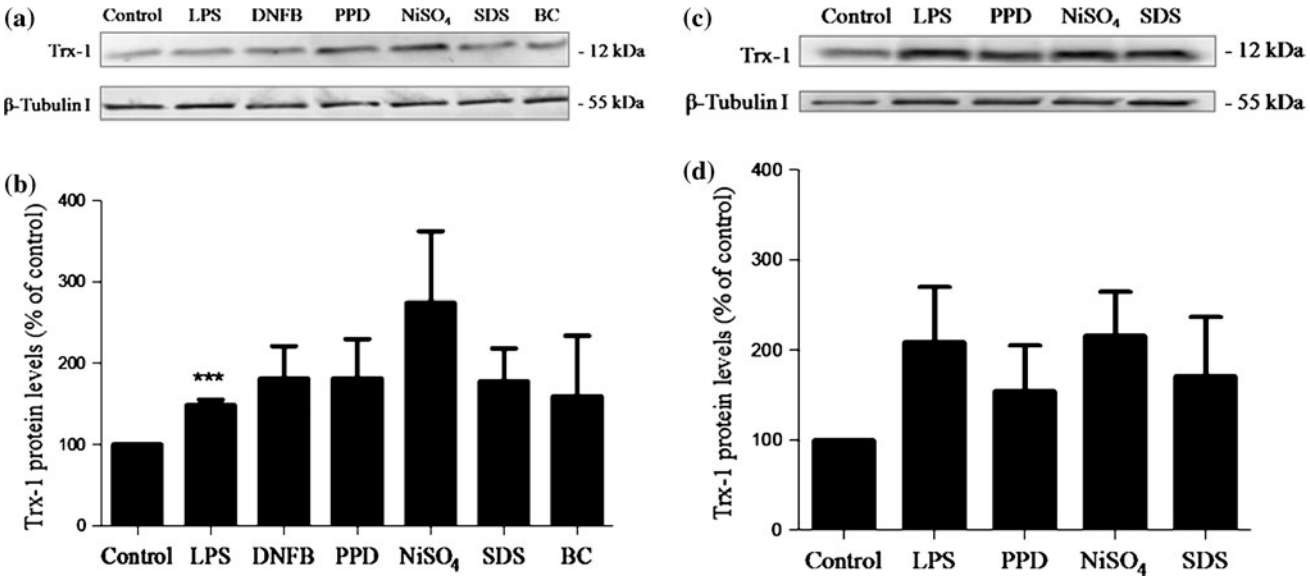

Fig. 1 Modulation of Trx-1 expression by LPS, skin sensitizers and irritants, after 24 h of DC stimulation. FSDC (**a**) was incubated, in culture medium, in the absence (control), or in the presence of 5 μg/ml LPS, 1 μg/ml DNFB, 50 μg/ml PPD, 50 μg/ml $NiSO_4$, 50 μg/ml SDS or 1 μg/ml BC, for 24 h. Equal amounts of protein, obtained from total cell extracts, were electrophoresed through SDS-PAGE, transferred to PVDF membranes and subjected to Western blot analysis using an anti-Trx-1 antibody. To ensure that there were similar amounts of protein in each sample, the membranes were stripped, and reprobed with a monoclonal anti-β-tubulin I (1:20,000) followed by incubation with an alkaline phosphatase-conjugated anti-mouse antibody (1:20,000). **b** The blots were quantified and the results were expressed as % of Trx-1 protein levels relatively to the control and each value represents the mean ± SEM of three to four independent experiments. **c**, **d** The results of Western blot analysis obtained in FSDC was confirmed in MoDC. MoDC, obtained by culturing human monocytes in GM-CSF and IL-4-supplemented culture medium for 7 days, were incubated, in culture medium, in the absence (control), or in the presence of 5 μg/ml LPS, 50 μg/ml PPD, 50 μg/ml $NiSO_4$ or 50 μg/ml SDS, for 24 h. For MoDC (**d**), each value represents the mean ± SEM of two independent experiments. The results were statistically compared using Student's *t* test (***$P < 0.001$ as compared to the control)

spectrophotometry, using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc.). The RNA was stored at −80°C. Reverse transcription of RNA was performed using iScript™ select cDNA synthesis kit. In all reactions, cDNA was synthesised in 20 µl using 1 µg of total RNA and 2 µl of random primers. The resulting cDNA was stored at −20°C until assayed by real-time PCR using SYBR Green fluorescence dye on a iCycler iQ real-time PCR detection system. Fifty nanograms of cDNA was used in the real-time PCR reaction mixture that also includes forward and reverse primers (250 nM) and the SYBR Green Supermix (2×). cDNAs of the two housekeeping genes HPRT1 and β-actin, used as endogenous controls, and Trx-1 were amplified by PCR using specific oligonucleotide primers selected within the coding regions of the genes. The primers were designed using Beacon Designer 7 software (Premier Biosoft International). Trx-1 primers used were 5′-GAAGTGGATGTGGATGAC-3′ (sense) and 5′-AGGCATATTCAGTAATAGAGG-3′ (antisense), designed to produce a 151-bp product; HPRT1 primers used were 5′-GTTGAAGATATAATTGACACTG-3′ (sense) and 5′-GGCATATCCAACAACAAAC-3′ (antisense), designed to produce a 180-bp product; and β-actin primers used were 5′-GTGCGTGACATCAAAGAG-3′ (sense) and 5′-GCCACAGGATTCCATACC-3′ (antisense), designed to produce a 195-bp product. Real-time PCR was performed in My Cycler iQ5 (Bio-Rad) for 40 cycles, after an initial incubation for 3 min at 95°C. Each cycle consisted of a denaturation step during 10 s at 95°C, an annealing step during 30 s at 55°C and an elongation step during 30 s at 72°C. The SYBR Green fluorescence was detected at the end of each annealing step and the specificity of the amplification products was checked by the melting curve analysis. The quantification data were then analysed by Vandesompele method [59].

Statistics

The results are expressed as mean ± SEM of the indicated number of experiments. The statistical analysis was performed using Student's t test or using one-way ANOVA with Dunnett's post test. The significance level was $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Results

Modulation of Trx-1 protein levels by LPS, skin sensitizers and irritants in DC

The effect of LPS, skin sensitizers and irritants on Trx-1 protein levels was evaluated by Western blot analysis, after FSDC and MoDC exposure to the chemicals for 24 h. In FSDC (Fig. 1a), LPS increased the Trx-1 protein levels by 49 ± 7% ($^{***}P < 0.001$). All the skin sensitizers, namely DNFB, PPD and $NiSO_4$, and the irritants SDS and BC increased the Trx-1 protein levels, although without statistical significance (Fig. 1b). Therefore, the Trx-1 protein levels were not differentially modulated, in FSDC, by skin sensitizers or irritants after 24 h of cell stimulation. As shown in Fig. 1c, d, the exposure of MoDC, for 24 h, to LPS, PPD, $NiSO_4$ and SDS also increased the Trx-1 protein levels confirming the pattern of Trx-1 expression observed in FSDC.

Next, we investigated Trx-1 protein levels, in FSDC, after exposure to the same chemicals for 48h, by Western blot analysis (Fig. 2). LPS increased Trx-1 protein levels by 63 ± 15% ($^{**}P < 0.01$). Trx-1 protein levels were also increased after stimulation with DNFB, PPD and $NiSO_4$, although without statistical significance. In contrast, SDS and BC did not modify Trx-1 protein levels when compared with control.

To confirm the Western blot results, we also analysed the effect of LPS, the skin sensitizer DNFB and the irritant BC on the cellular distribution of Trx-1 protein, using immunofluorescence (Fig. 3). According to the results obtained by Western blot analysis, LPS and DNFB upregulate Trx-1

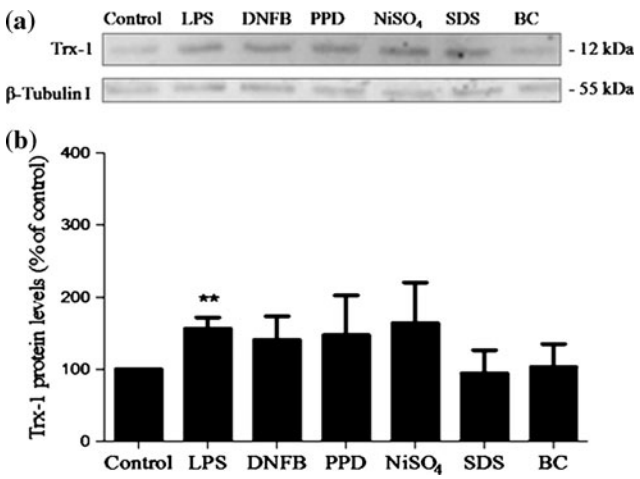

Fig. 2 Modulation of Trx-1 protein levels induced by LPS, skin sensitizers and irritants after 48 h of FSDC stimulation. **a** FSDC was incubated in culture medium in the absence (control), or in the presence of 5 µg/ml LPS, 1 µg/ml DNFB, 50 µg/ml PPD, 50 µg/ml $NiSO_4$, 50 µg/ml SDS or 1 µg/ml BC, for 48 h. Total cell extracts were electrophoresed through SDS-PAGE, transferred to PVDF membranes and subjected to Western blot analysis using an anti-Trx-1 antibody. To ensure that there were similar amounts of protein in each sample, the membranes were stripped, and reprobed with a monoclonal anti-β-tubulin I antibody (1:20,000) followed by incubation with an alkaline phosphatase-conjugated anti-mouse antibody (1:20,000). **b** The blots were quantified and the results were expressed as % of Trx-1 protein levels relatively to the control. Each value represents the mean ± SEM of three to eight independent experiments and the results were statistically compared using Student's t test ($^{**}P < 0.01$ as compared to the control)

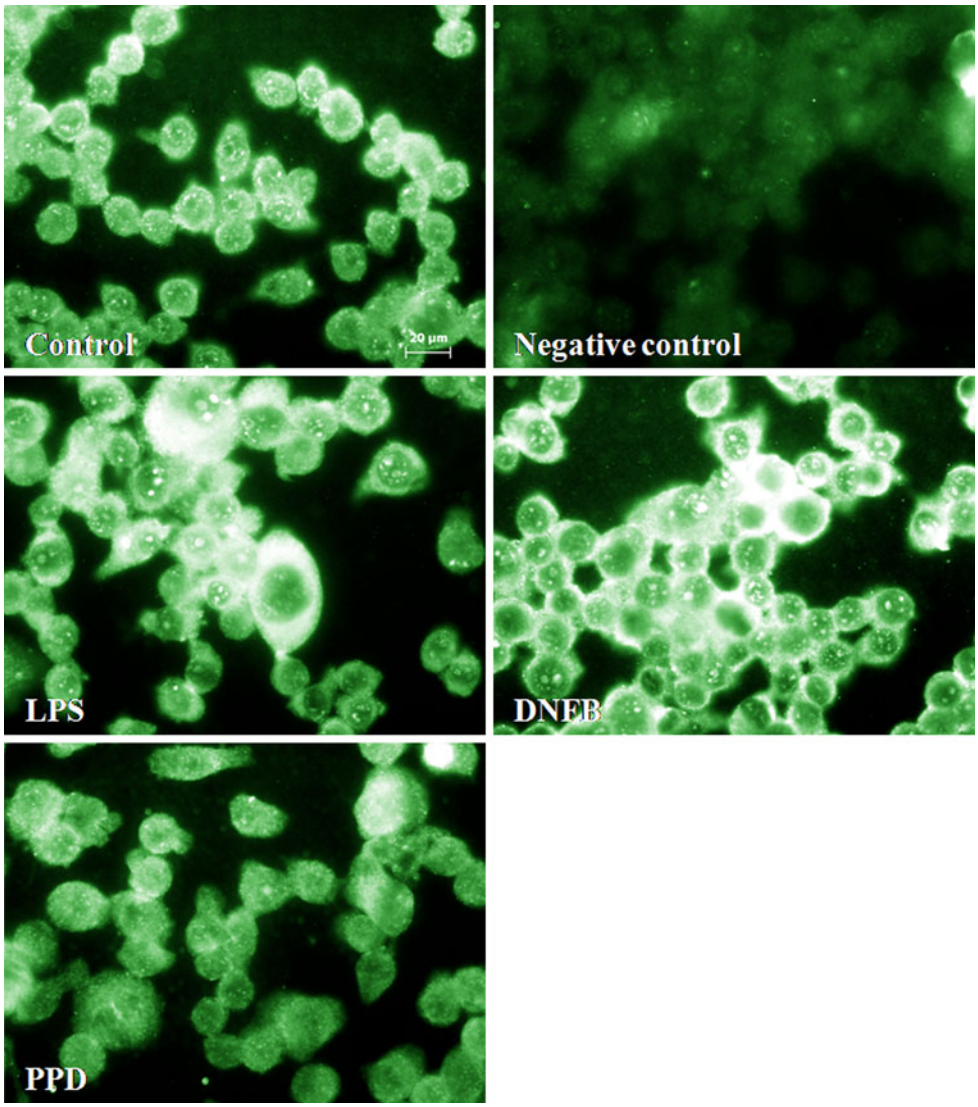

Fig. 3 Immunofluorescence analysis of the effect of LPS, skin sensitizers and irritants on Trx-1 protein levels. FSDC was subjected to immunostaining using an anti-Trx-1 antibody. The images shown are representative of two individual experiments yielding similar results (magnification ×630)

immunoreactivity, in contrast to BC which has no effect, yielding similar results to the control assay. The increases in Trx-1 immunoreactivity, induced by LPS and DNFB, were predominantly observed in the cytoplasm. Negative control experiment resulted in a weak non-specific staining (data not shown). Therefore, LPS and skin sensitizers increased Trx-1 protein levels after 24 h, being this effect still present at 48 h, whereas the increase in Trx-1 protein levels induced by irritants seem to be transient in time. These results suggest that LPS, skin sensitizers and irritants differentially modulates Trx-1 protein levels in FSDC.

Modulation of Trx-1 mRNA expression induced by LPS, skin sensitizers and irritants

Because protein levels are regulated both by protein degradation and by synthesis of new protein, we investigated the effect of LPS, skin sensitizers (DNFB and $NiSO_4$) and irritant SDS on the synthesis of new protein, through evaluation of the Trx-1 mRNA levels by real-time PCR analysis (Fig. 4). LPS and $NiSO_4$ increased the Trx-1 mRNA expression by 1.73 ± 0.21 ($^*P < 0.05$) and 1.91 ± 0.33 ($^*P < 0.05$) fold, respectively, after 24 h of FSDC stimulation. Both DNFB (1.16 ± 0.29 fold) and SDS (1.12 ± 0.21 fold) had no effect on Trx-1 mRNA expression. To determine housekeeping genes, a panel of six possible genes was analysed. Using the GenEx software (Multi D; Analyser. AB), HPRT1 and β-actin were selected as the two most stable for the treatment condition used (data not shown).

Effect of hydrogen peroxide on Trx-1 protein expression and on DC maturation

The effect of LPS and different concentrations of H_2O_2 on Trx-1 and CD86 protein levels were evaluated by Western blot analysis after FSDC exposure to the chemicals for 24

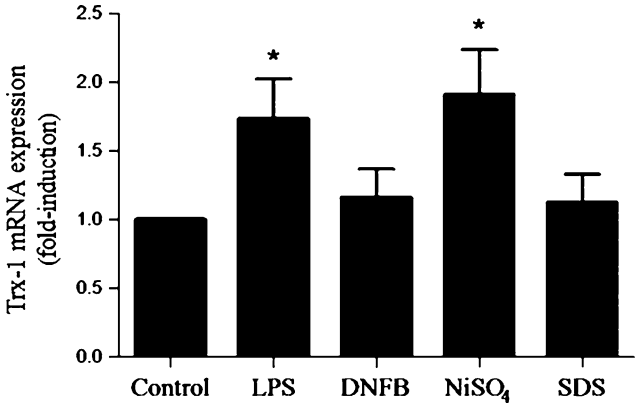

Fig. 4 Modulation of Trx-1 mRNA expression induced by LPS, skin sensitizers and irritants, after 24 h of FSDC stimulation. FSDC was incubated in culture medium in the absence (control) or in the presence of 5 μg/ml LPS, 1 μg/ml DNFB, 50 μg/ml $NiSO_4$ or 50 μg/ml SDS, for 24 h, and then analysed for Trx-1 mRNA expression. The results were expressed as fold induction of Trx-1 mRNA expression relatively to control. Each value represents the mean ± SEM of four independent experiments and the results were statistically compared using Student's *t* test (*$P < 0.05$ as compared to the control)

and 48 h (Fig. 5a, b). Our results demonstrated that the two H_2O_2 concentrations used increased both Trx-1 and CD86 expression. Because CD86 is a DC co-stimulatory molecule, we also verified its upregulation at the surface by performing immunocytochemistry in non-permeabilized cells (Fig. 5c). Our results demonstrated that both LPS and the Trx activator, H_2O_2, increased the surface levels of CD86 after 24 h of DC stimulation.

Intracellular signalling pathways involved in the increase of Trx-1 protein levels induced by LPS

The previous results demonstrated that LPS increased significantly Trx-1 protein levels in FSDC, after 24 and 48 h of stimulation and Trx-1 mRNA expression also increased after 24 h of exposure to LPS. We have recent data demonstrating that LPS induced an early activation of Akt, NF-κB and MAPKs signalling pathways in FSDC [44]. Therefore, we investigated the involvement of these signalling pathways on the modulation of Trx-1 protein levels induced by LPS, after 48 h of cell stimulation, by Western blot. For this purpose, cells were incubated with LPS alone or in the presence of different signalling pathway inhibitors (Fig. 6).

Lipopolysaccharide induced an increase in Trx-1 protein levels by 63 ± 15% (**$P < 0.01$), which was not inhibited by the intracellular signalling pathways inhibitors SB203580, PD098059 and SP600125. On the other hand, the PI3K inhibitor LY294002 induced an increase in the Trx-1 protein levels by 98.1% (*$P < 0.05$), when compared with LPS alone. Neither dexamethasone nor cyclosporin A, immunosuppressors that inhibit the transcription factor NF-κB in FSDC, as previously demonstrated by us [17, 63], had an effect on Trx-1 protein levels in LPS-stimulated cells.

These results indicated that the PI3K-Akt signalling pathway and not the MAPKs signalling pathways or NF-κB is involved in the modulation, in particular the inhibition, of Trx-1 protein levels induced by LPS in FSDC.

Discussion

Because DC have a crucial role in ACD, it is important to elucidate the mechanisms involved in DC maturation, which occurs during the sensitization process. In this work, we evaluated the effect of LPS, skin sensitizers and irritants on Trx-1 protein expression using the cell line FSDC as an experimental model of skin DC. We also evaluated whether H_2O_2, an activator of Trx-1, also increased CD86 protein expression. In addition, we studied the role of different signalling pathways on Trx-1 protein modulation induced by LPS, a well known inducer of DC maturation.

Analysing the effect of LPS, the skin sensitizers DNFB, PPD and $NiSO_4$, and the irritants SDS and BC on Trx-1 protein levels we demonstrated that LPS and all the chemicals used increased the Trx-1 protein levels, after 24 h of FSDC stimulation (Fig. 1). However, after 48 h of FSDC stimulation, only LPS and skin sensitizers increased Trx-1 protein levels, mainly in cytoplasm, whereas the irritants had no effect (Figs. 2, 3). The similar pattern of Trx-1 protein levels obtained in FSDC and MoDC (Fig. 1) indicates that the FSDC cell line could be a good model for the study of DC maturation mechanisms. Accordingly, we have previously demonstrated that FSDC cell line and DC derived from human peripheral blood monocytes respond in a similar way to contact sensitizers by upregulating the proteins CXCR4 and CD40 [43]. In addition, we recently published that, in FSDC, LPS induces a maturation programme similar to that described in other DC models, namely activation of NF-κB, MAPK and Akt, upregulation of co-stimulatory molecules and also production of the cytokines IL-6, CCL5, G-CSF, CCL2 and CXCL2 [44].

The increase in cytoplasmatic Trx-1 protein levels induced by LPS and skin sensitizers, after 48 h of FSDC stimulation, indicate that Trx-1, due to its functions in defence against oxidative stress, activation of transcription factors (NF-κB, Nrf2, AP-1 and p53), and also direct inhibition of ASK1 [8, 21, 36, 51, 65], could be partially responsible for the phenotypical and functional changes occurring during DC maturation and, consequently, in skin sensitization. Reinforcing the role of Trx-1 in skin sensitization, it was previously reported that upon DC cross talk with T cells, LPS and TNF-α induce DC Trx synthesis and its release providing a reducing microenvironment that facilitates immune response [6].

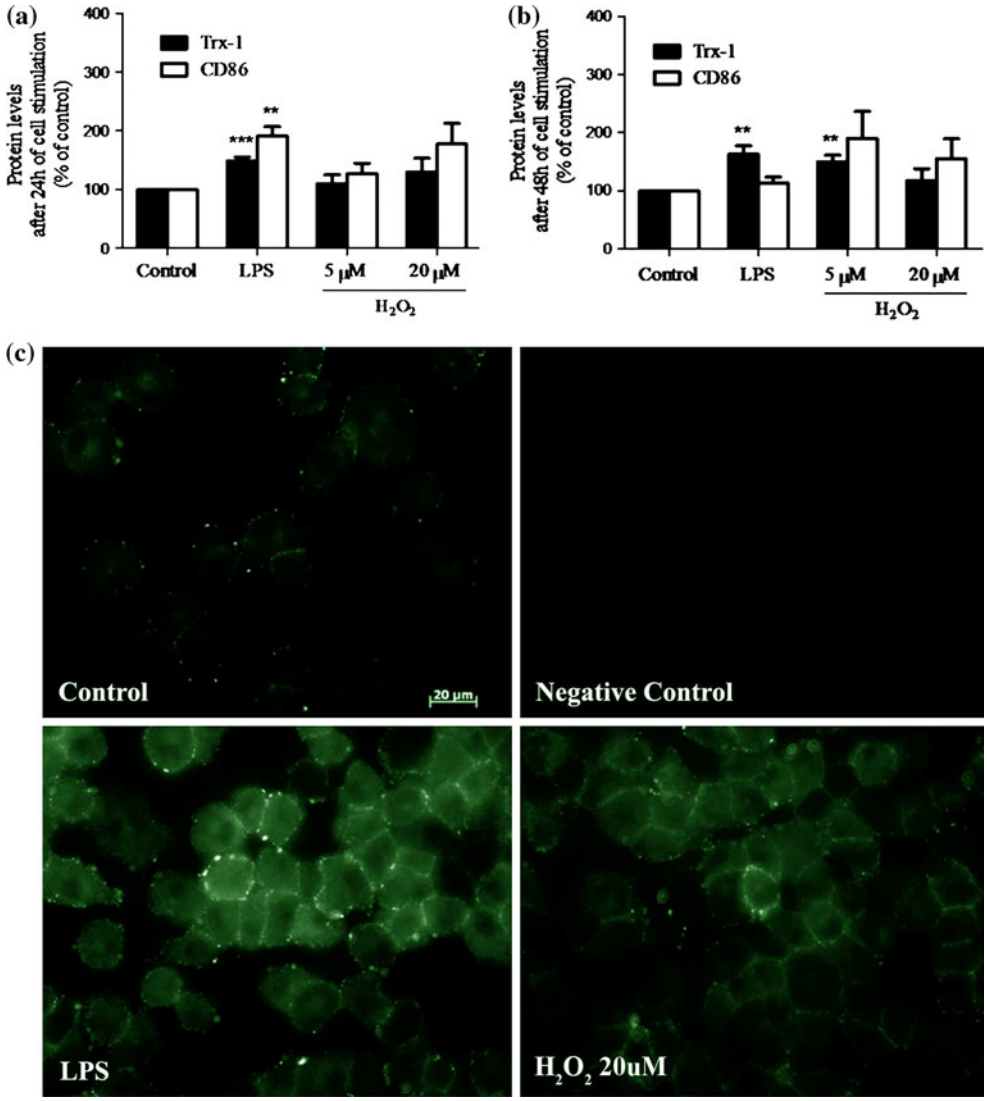

Fig. 5 Modulation of Trx-1 and CD86 protein levels by LPS and the activator of Trx-1 protein expression, H_2O_2. FSDC was incubated in culture medium in the absence (control), or in the presence of 5 μg/ml LPS, or H_2O_2 (5 or 20 μM), for 24 h (**a**) or 48 h (**b**). Total cell extracts were electrophoresed through SDS-PAGE, transferred to PVDF membranes and subjected to Western blot analysis using an anti-Trx-1 antibody or an anti-CD86 antibody. The blots were quantified and the results were expressed as % of protein levels relatively to the control. Each value represents the mean ± SEM of three to nine independent experiments and the results were statistically compared using Student's *t* test (**$P < 0.01$, ***$P < 0.001$, as compared to the control situation). **c** FSDC was incubated in culture medium in the absence (control), or in the presence of 5 μg/ml LPS, or 20 μM H_2O_2, for 24 h, and then subjected to immunostaining, without permeabilization, using an anti-CD86 antibody. The images shown are representative of two individual experiments yielding similar results (magnification ×630)

To investigate whether Trx-1 protein levels increase induced by LPS and skin sensitizers was due to an increase in Trx-1 mRNA expression, we performed real-time PCR analysis after 24 h of cell stimulation (Fig. 4). In accordance with other studies performed in other DC models [45, 55], LPS increased the Trx-1 mRNA expression in FSDC. $NiSO_4$ also increased the Trx-1 mRNA expression in our DC model. This increase in Trx-1 mRNA expression could explain the effect of LPS and $NiSO_4$ on Trx-1 protein levels, which was upregulated after 24 and 48 h of cell stimulation (Figs. 1, 2). DNFB and SDS had no effect on Trx-1 mRNA expression. Based on these results, we can conclude that the increase in Trx-1 protein levels induced by LPS and $NiSO_4$ is, at least in part, due to an increase in Trx-1 mRNA expression.

As described above, Trx-1 has an important role in defence against cellular oxidative stress, which could be induced by skin sensitizers due to its electrophilic properties [12, 22]. It was also demonstrated that the redox imbalance induced by skin sensitizers is important in the regulation of DC functions [5, 38, 39, 41, 47, 61]. Therefore, the increase in Trx-1 mRNA expression observed in

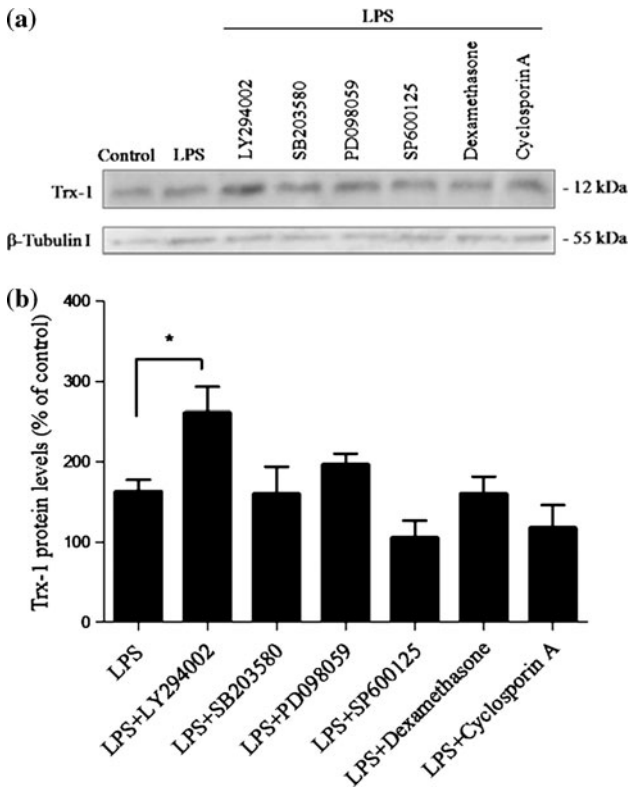

Fig. 6 Intracellular signalling pathways involved in Trx-1 protein levels modulation induced by LPS in FSDC. **a** FSDC was incubated in the presence of 5 μg/ml LPS alone or with PI3K inhibitor LY294002 (2.5 μM), p38 MAPK inhibitor SB203580 (20 μM), ERK1/2 activation inhibitor PD098059 (20 μM), JNK inhibitor SP600125 (1 μM) or with the immunomodulators dexamethasone (5 μM), or cyclosporin A (1 μM), for 48 h. Total cell extracts were electrophoresed through SDS-PAGE, transferred to PVDF membranes and subjected to Western blot analysis using an anti-Trx-1 antibody. To ensure that there were similar amounts of protein in each sample, the membranes were stripped, and reprobed with a monoclonal anti-β-tubulin I antibody (1:20,000) followed by incubation with an alkaline phosphatase-conjugated anti-mouse antibody (1:20,000). **b** The blots were quantified and the results were expressed as % of Trx-1 protein levels relatively to the control. Each value represents the mean ± SEM of three to four independent experiments. The results were statistically compared using one-way ANOVA test, with Dunnett's post test (*$P < 0.05$)

FSDC is probably a cellular response to redox imbalance. The expression of Trx genes is regulated by the transcription factor Nrf2/ARE regulatory pathway, one of the most important cellular defence mechanism against oxidative stress or electrophiles [29, 67]. Hence, our results support the utilisation of Nrf2/ARE regulatory pathway to screen the sensitization potential of novel chemicals, as recently proposed [1, 42]. Accordingly, it was previously demonstrated that the skin sensitizer Ni (II) could activate the Nrf2 signalling pathway in human monocytic cells [33].

To establish a correlation between thioredoxin-1 expression and DC maturation, we used the activator of thioredoxin-1, hydrogen peroxide [49], and addressed its role on CD86 expression, a conventional marker of DC maturation [20], and recently demonstrated by us to be upregulated in a mouse skin DC line by LPS [44]. We observed that the H_2O_2 concentration that increased Trx-1 protein expression also augmented CD86 on the surface level, reinforcing the involvement of Trx-1 in DC maturation.

Lipopolysaccharide has been shown to fully activate DC both in vitro and in vivo [13, 18, 60]. We have recently demonstrated that LPS activates Akt, NF-κB and MAPK, namely JNK1/2, ERK1/2 and p38 MAPK, in FSDC [44]. Therefore, stimulation with LPS could be used as a model to elucidate the intracellular signalling pathways involved in DC maturation, a crucial step for skin sensitization. Using specific inhibitors of PI3K-Akt (LY294002), p38 MAPK (SB203580), ERK1/2 (PD098059) and JNK1/2 (SP600125), we further investigated the intracellular signalling pathways involved in the increase of Trx-1 protein levels induced by LPS. We observed that the PI3K-Akt signalling pathway, and not the MAPK signalling pathways, is involved in the modulation, particularly the inhibition, of Trx-1 protein levels induced by LPS in FSDC (Fig. 5). In agreement, it was previously demonstrated that the activation of different signalling pathways and the induction of gene expression in LPS-treated monocytes was negatively regulated by the PI3K-Akt signalling pathway [25]. The immunosuppressors and inhibitors of the transcription factor NF-κB, dexamethasone and cyclosporin A, did not modify Trx-1 protein levels induced by LPS.

Our results indicate that LPS increased Trx-1 protein levels, partially due to an increase in Trx-1 mRNA expression, being the Nrf2/ARE regulatory pathway probably involved. In addition, the PI3K-Akt signalling pathway has an inhibitory role in this cell response to LPS. Based on our results and previous studies, we propose a possible cellular mechanism, by which LPS modulates the Trx-1 protein levels in FSDC. LPS is recognised by a LPS-activation cluster [56] that could activate two different pathways. Upon stimulation with LPS, ROS production occurs [39, 62], which appears to break the inactive complex reduced Trx-1/ASK1, thus allowing the subsequent recruitment of TRAF6 to the ASK1 signalosome [21, 54]. The activated ASK1 signalosome induces Nrf2/ARE-mediated expression [66], which is involved in Trx-1 protein expression [29, 67]. On the other hand, LPS activates PI3K-Akt signalling pathway [7, 44] that have an inhibitory role in ASK1/Nrf2/ARE-mediated Trx-1 expression, which is due to ASK1 phosphorylation that downregulates its activity [30, 53]. Therefore, LPS modulates Trx-1 protein expression by two opposite mechanisms: increases Trx-1 protein expression by activating the ASK1/Nrf2/ARE pathway, and inhibits Trx-1 protein expression by activating the PI3K-Akt pathway. Interestingly, the PI3K-Akt signalling pathway could be redox

regulated through the interaction between Trx-1 and the phosphatase PTEN [21, 32, 34].

Similarly to LPS, skin sensitizers can induce redox imbalance [38, 41] that activates the cellular mechanism proposed above and responsible for the increase in Trx-1 protein levels. These data suggest that the ASK1/Nrf2/ARE regulatory pathway could be activated by LPS and skin sensitizers in a similar way, being involved in DC maturation.

In conclusion, using FSDC as a cell model, our results suggest that LPS, skin sensitizers and irritants modulate Trx-1 protein levels by different pathways in DC. Based on the results obtained for the maturation agent LPS, we discussed the possible mechanism by which LPS, skin sensitizers and irritants differentially modulate Trx-1 protein levels and, therefore, induce different phenotypical and functional changes in DC. It can be stated that the analysis of Trx-1 redox state or protein expression and the signalling pathways involved in Trx-1 expression could be helpful in elucidating DC maturation process and, therefore, in understanding mechanisms involved in the acquisition of ACD. However, further research is needed to identify the early intracellular mechanisms involved in chemical-induced DC maturation, as well as the cross talk between different signalling pathways, to clarify the molecular mechanisms associated with the induction of skin sensitization.

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Conflict of interest statement None of the authors have any conflict of interest.

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