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## **Editor's Highlight: Fragrance Allergens Linalool and Limonene Allylic Hydroperoxides in Skin Allergy: Mechanisms of Action Focusing on Transcription Factor Nrf2**

Chloé Raffalli, Elodie Clouet, Salen Kuresepi, Marie-Hélène Damiens, Jean-Pierre Lepoittevin, Marc Pallardy, Pierre-Jacques Ferret, Elena Giménez-Arnau, Saadia Kerdine-Römer

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## Research Article

### Fragrance allergens linalool and limonene allylic hydroperoxides in skin allergy: mechanisms of action focusing on transcription factor Nrf2

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## Summary

Allergic contact dermatitis (ACD) is regarded as the most frequent expression of immunotoxicity in humans. Many odorant terpenes commonly used in fragrance compositions are considered as low skin sensitizers, whereas some of their autoxidation products, allylic hydroperoxides, are classified as strong sensitizers according to the Local Lymph Node Assay (LLNA). However, their mechanism of action on the immune system remains unclear.

Since dendritic cells play a key role in ACD, we studied their activation by the frequently used linalool (LINA) and limonene (LIMO), and their respective sensitizing allylic hydroperoxides (LINA-OOH, LIMO-OOH). As a concern of developing alternatives to animal experiments, the THP-1 cell line was used as it is currently employed in the validated h-CLAT *in vitro* test.

After 30 min of treatment, LINA-OOH and LIMO-OOH oxidized cell surface thiols inducing a ROS production and a decrease of GSH/GSSG ratio. This oxidative stress led to the cytoplasm accumulation of Nrf2, transcription factor for genes production such as *ho-1*, *nqo1* and *il-8*. Furthermore, MAPKs and NF- $\kappa$ B pathways were activated and cell-surface markers CD54 and CD86 were induced after 24 h of exposure. LINA and LIMO do not induce any phenotype maturation.

Results show that LINA and LIMO need to be oxidized to activate THP-1 cells. LINA-OOH seems to be a stronger activator of THP-1 cells than LIMO-OOH. The activation is first induced by the decrease of surface thiols, resulting in a cascade of events: glutathione depletion, Nrf2 and MAPK activation leading to a maturation of cells.

Keywords: allergic contact dermatitis, terpenes, allylic hydroperoxides, Nrf2, cell surface thiol

## Introduction

Allergic contact dermatitis (ACD) is a major cause of occupational skin disease. It results from an adverse reaction of the immune system to low-molecular weight chemicals called haptens. It is a T-cell mediated delayed type hypersensitivity reaction (Martin, 2015). Two phases are distinguished in this pathology, the sensitization phase and the elicitation phase. During the sensitization phase, the sensitizer penetrates into the epidermis through the stratum corneum barrier and binds to a carrier protein. Haptens must thus react with proteins in the skin to form an antigenic complex that will be recognized by the immune system. This immunogenic complex is captured and processed by dendritic cells (DCs). DCs are then activated and migrate to the draining lymph nodes where they present the haptened peptide to naïve T cells. These T lymphocytes will then become activated in order to induce a specific response to the haptened peptide (Martin, 2004; Vocanson et al., 2009). Furthermore, T cells proliferate and emigrate out of the lymph nodes to the blood where they recirculate between the lymphoid organs and the skin. Different types of haptens were described by Lepoittevin (2006). Real haptens are directly reactive towards proteins, prohaptens need to be converted into reactive compounds to become sensitizing *via* metabolization, and prehaptens *via* non-metabolic pathways such as UV exposure and air oxidation.

Terpenes are widespread fragrance compounds of natural origin, used in the production of fine perfumes, fragranced cosmetics, household and industrial products. Some of them behave as prehaptens as they autoxidize on air exposure, such as limonene (LIMO) and linalool (LINA). This oxidation leads to the formation of a cocktail of oxidized molecules such as epoxides, diols and allylic hydroperoxides (Fig. 1). Among them, allylic hydroperoxides have been identified as potent sensitizers in predictive animal tests. Allylic hydroperoxides derived from autoxidation of LIMO and LINA were tested with the local lymph node assay (LLNA) and classified as strong sensitizers, with EC<sub>3</sub> values between 1 and 10% (Karlberg et al., 2013). On the other hand, recent international multicenter studies showed frequent contact allergic reactions to oxidized linalool and oxidized R-limonene in consecutively tested dermatitis patients. On 2900 consecutive patients tested, 5.2% patients were positive to oxidized R-limonene 3% (containing 0.33% hydroperoxides) and 6.9% to oxidized linalool 6% (containing 1% hydroperoxides) (Bråred Christensson et al., 2012, 2013). Hydroperoxides derived from the autoxidation of LIMO and LINA are thus considered as strong sensitizers while the corresponding terpenes are classified as weak sensitizers. To be immunogenic, allylic hydroperoxides must react with proteins in the skin. It has been reported that these compounds form antigens most probably *via* mechanisms involving radical intermediates, starting with the homolytic cleavage of O-O/O-H bonds of weak dissociation energies (Kao et al., 2014). The cleavage is an easy process affording initially alkoxyl RO<sup>•</sup>/peroxyl ROO<sup>•</sup>

radicals, which can further lead via intramolecular cyclization, fragmentation or hydrogen abstraction to the formation of longer half-life reactive carbon-centred radicals  $R^\bullet$ . The presence of these oxygenated and carbon radical intermediates in the cells could lead to the activation of intracellular Nrf2, the nuclear factor-erythroid 2-related factor 2. The Keap1-Nrf2 cellular sensor pathway is implicated in the antioxidant response of the cell (Baird and Dinkova-Kostova, 2011). Moreover, in a recent study we have also shown that electrophilic allergens, depending on their chemical structure, may also activate the Nrf2 pathway in DCs (Migdal et al., 2013).

Under basal conditions, Nrf2 is sequestered by the Kelch-like EC-associated protein 1 (Keap1) in the cytoplasm and its expression is maintained to be low due to constant proteasomal degradation. In the presence of oxidative or electrophilic stress cysteine residues of Keap1 are oxidized. Nrf2 degradation is interrupted, leading to its rapid accumulation (Itoh et al., 1999). Nrf2 translocates to the nucleus, where it activates the Antioxidant Response Element (ARE)-dependent gene expression in association with small Maf proteins. It leads to the transcription of genes mostly involved in detoxification, such as heme oxygenase-1 (*ho-1*) or NADPH-quinone oxidoreductase 1 (*nqo1*). Nrf2 is activated in response to different haptens (Natsch and Emter, 2016).

The main objective of this work was to investigate the activation of immune cells by terpenes and their autoxidation products. We studied LINA and LIMO terpenes and their respective sensitizing allylic hydroperoxides. One allylic hydroperoxide resulting from the autoxidation of limonene was tested, 2-hydroperoxylimonene (LIMO-OOH). A mixture of two allylic hydroperoxides and one single compound were tested for LINA, 6 and 7-hydroperoxy-3,7-dimethylocta-1,7-dien-3-ol (LINA-OOH 6 + 7) and LINA-OOH 7. To be in accordance with the 3R concept, we tested these molecules on the THP-1 cell line, which is the model currently used in the h-CLAT *in vitro* test (Nukada et al., 2011).

The two terpenes behave differently as LIMO led to the activation of MAPK and NF- $\kappa$ B pathways while LINA did not. It appears that only the hydroperoxides were able to oxidize cell-surface thiols, deplete intracellular GSH and induce ROS production. These autoxidation products also led to the activation of the Nrf2 pathway and to the induction of the transcription of target genes *ho-1*, *nqo1* and *il-8*. The expression of CD54 and CD86 was also measured. According to our results, LINA-OOH seems to behave as a stronger activator of THP-1 cells than LIMO-OOH. No evidence of a significant difference between single LINA-OOH 7 and the LINA-OOH 6 + 7 isomers mixture was measured.

**Abbreviations:**

2-Hydroperoxylimonene: LIMO-OOH

6-Hydroperoxy-3,7-dimethylocta-1,7-dien-3-ol: LINA-OOH 6

7-aminoactinomycin : 7-AAD

7-Hydroperoxy-3,7-dimethylocta-1,7-dien-3-ol: LINA-OOH 7

Allergic contact dermatitis: ACD

Cinnamaldehyde: CinA

Dendritic cell: DC

Dichloro-dihydro-fluorescein diacetate: DCFH-DA

Dimethyl sulfoxide (DMSO)

Dithiothreitol: DTT

Ethylenediaminetetraacetic acid: EDTA

Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid: EGTA

Heme oxygenase-1: *ho-1*

Interleukine 8: *il-8*

Kelch-like EC-associated protein 1: Keap1

Linalool: LINA

Limonene: LIMO

Local lymph node assay: LLNA

NADPH-quinone oxidoreductase 1: *nqo1*

Nuclear factor-erythroid 2-related factor 2: Nrf2

Reactive oxygen species: ROS

Phenylmethylsulfonyl fluoride: PMSF

*tert*-butylhydroperoxide: tBHP

## **Materials and methods**

### **Chemicals**

Acetonitrile (CH<sub>3</sub>CN), dithiothreitol (DTT), (R)-(+)-limonene (LIMO), linalool (LINA) and *tert*-butylhydroperoxide (tBHP) were all purchased from Sigma-Aldrich (St Quentin-Fallavier, France).

LIMO-OOH and the mixture of isomers LINA-OOH 6 + 7 (6:4 ratio) were synthesized according to a procedure reported previously (Kao et al., 2011). These two compounds being extremely difficult to isolate from the mixture, isomer LINA-OOH 7 was kindly provided by Natsch, Givaudan (Switzerland).

### **Culture of THP-1 cell line**

The human monocytic cell line THP-1 (ATCC-TIB-202; LGC, Molsheim, France) was grown in RPMI 1640 medium with 25 mM HEPES GlutaMAX (Gibco, Invitrogen, Saint Aubin, France) supplemented with 10% heat-inactivated fetal bovine serum (Biowest, AbCys, Courtaboeuf, France), 1% sodium pyruvate (Gibco, Invitrogen), antibiotics [100 units/ml penicillin, 100 µg/ml streptomycin (Gibco, Invitrogen)] and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich) at 37°C in humidified air containing 5% CO<sub>2</sub>.

### **Chemical treatment**

THP-1 cells were cultured for at least 12 h at  $8 \times 10^5$  cells/ml in complete medium without 2-mercaptoethanol. On the next day, the cells were seeded in complete medium at  $1 \times 10^6$  cells/ml and treated with chemicals. CinA was diluted in DMSO and tBHP, terpenes and allylic hydroperoxides in CH<sub>3</sub>CN as they were water-insoluble. Final concentrations of solvent in culture media did not exceed 0.1%.

### **Measurement of cell viability**

THP-1 cells were seeded in 24-well plates ( $1 \times 10^6$  cells/ml, 1 ml/well) with various concentrations of each chemical ranged from 0.25 to 1.5 mM for 24 h (Tab. 1). Cells were washed twice with PBS and harvested cells were incubated with annexin-V-PE and 7-aminoactinomycin D (7-AAD) according to the manufacturer's protocol (BD Biosciences). Cell viability was measured using the FL-3 wavelength on a FACScalibur flow cytometer (Becton Dickinson, Le Pont de Claix, France). Data acquisition was performed using the CellQuest software (BD Biosciences). At least four independent experiments were performed for each chemical.

### **Analysis of cell-surface thiols by flow cytometry**

As previously described, the Alexa Fluor 488 C<sub>5</sub> maleimide (AFM) (Fisher Scientific, Illkirch, France) has been used to measure cell-surface thiols (Hirota et al., 2010). Briefly, THP-1 cells were seeded at  $1 \times 10^6$  cells/ml. After treatment with test chemicals at subtoxic concentration, THP-1 cells were recovered and washed with PBS twice, then incubated with 100  $\mu$ L of AFM (10  $\mu$ M) PBS solution for 30 min at 37°C. Then, the cells were washed and were analyzed by flow cytometry. A total of 10000 living cells were analyzed. RFI was calculated by using the following formula: RFI (% of control) = (MFI of chemical treated cells/MFI of vehicle control cells) x 100. The results are expressed as RFI - 100, meaning that values are negative when thiols are oxidized.

### **Quantification of intracellular GSH and GSSG**

After treatment with chemicals at subtoxic concentration for 1 h,  $1 \times 10^6$  cells were collected by pipetting and washed twice with cold PBS. 25  $\mu$ L of cells diluted in PBS were put in the 96-well plate, one set of cells for total glutathione measurement and one set for GSSG measurement. The GSH/GSSG-Glo™ Assay kit from Promega (Madison, USA) was used. The GSH/GSSG ratio was measured according to the protocol given by the manufacturer. To measure the total glutathione, a reducing agent converts all the glutathione, GSH and GSSG in a cell lysate to the reduced form GSH. To only measure the oxidized form GSSG, a reagent is added that blocks all the GSH while leaving the GSSG intact. The cells were lysed with 25  $\mu$ L of Total Glutathione Lysis Reagent or Oxidized Glutathione Lysis Reagent depending on the configuration. The plate was shaken for 5 min. 50  $\mu$ L of Luciferin Generation Reagent was added to all wells, and assays were mixed and incubated for 30 min. 100  $\mu$ L of Luciferin Detection Reagent was added to all wells, assays were mixed and after 15 min of incubation, luminescence was measured with a Tristar LB 941 from Berthold (Germany). The levels of total GSH and GSSG were calculated after interpolation of glutathione concentrations from standard curves. The content of GSH was obtained by subtracting the amount of GSSG from total GSH content.

### **ROS detection**

Intracellular production of ROS in THP-1 cells following the treatment was determined by measuring DCF-derived fluorescence by flow cytometry. THP-1 cells ( $2 \times 10^6$  cells/ml) were pretreated for 30 min with 2  $\mu$ M DCFH-DA (Sigma Aldrich) in serum-free RPMI at 37 °C. Cells were washed and resuspended in PBS ( $1 \times 10^6$  cells/ml). Following incubation at 37 °C with chemicals at subtoxic concentration (30 min and 1 h), fluorescence intensity of DCF was then measured in the FL1 channel. A total of 10000 living cells were analyzed. RFI was calculated by using the following formula: RFI (% of control) = (MFI of chemical-treated cells/MFI of vehicle control cells) x 100.



## Immunoblotting

Western blot was performed as previously described (Migdal *et al.*, 2013). THP-1 cells ( $10^6$  cells/ml, 2 ml/well) were exposed to test compounds at subtoxic concentration. Cells were washed twice in cold PBS before lysis in 40  $\mu$ L of lysis buffer (20 mM Tris pH 7.4, 137 mM NaCl, 2mM EDTA pH 7.4, 2mM sodium pyrophosphate, 1% Triton, 10% glycerol, 1mM PMSF, 1mM  $Va_3VO_4$ , 25 mM  $\beta$ -glycerophosphate, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin and 10  $\mu$ g/ml pepstatine) and left in ice for 20 min. Then, they were centrifuged at 17600 x g for 20 min at 4°C, before the supernatants were collected. Total protein concentration was evaluated using bicinchoninic acid assay, and equal amounts of denatured proteins (30  $\mu$ g) were loaded on 12% polyacrylamide gels (TGX Stain-Free FastCast; Bio-Rad Laboratories, Hercules, CA) for approximately 30 min at 300V in running buffer. Stain-free gels were activated by exposure to UV for 1 min. Proteins were transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA) using the Bio-Rad Trans-Blot Turbo Transfer System for 7 min. Total proteins on membranes were detected using the Stain-free method (Gilda and Gomes, 2013). For the Nrf2/Keap1 analysis pathway, membranes were incubated with antibodies raised against Nrf2 (H-300; Santa Cruz Biotechnology, Heidelberg, Germany). For the MAPKs and NF- $\kappa$ B analysis pathways, membranes were incubated with antibodies raised against the phosphorylated forms of p38 MAPK, JNK 1/2 or ERK 1/2 (all from Cell Signaling Technology, Ozyme, St-Quentin en Yvelines, France). Immunoreactive bands were detected by chemoluminescence (ECL solution, Amersham Biosciences).

## Immunofluorescent staining and flow cytometry analysis

After 24 h of incubation with chemicals at subtoxic concentration, THP-1 cells were harvested, washed once in PBS and  $3 \times 10^5$  cells (per antibody) were incubated in PBS containing 0.05% bovine serum albumin (BSA) for 30 min at 4°C with the monoclonal antibodies PE-conjugated anti-CD86 (555658, BD) and allophycocyanin (APC)-conjugated anti-CD54 (559771, BD). Appropriate antibody isotypes (IgG1, BD) were used at the same concentrations as the controls. After incubation, cells were washed twice in PBS + 0.05% BSA and once in PBS. THP-1 cell phenotype modifications were detected using a FACSCalibur flow cytometer. For each sample, 10000 cells were acquired from a gated homogenous cell population selected from a sideversus forward-scatter dot plot and analyzed using the CellQuest software. Results were expressed using relative fluorescence intensity (RFI) calculated with the following formula, where MFI is the mean fluorescence intensity: (MFI of chemical-treated cells labeled with antigen-specific antibody – MFI of chemical-treated isotype control cells)/(MFI of vehicle control cells labeled with antigen-specific antibody – MFI of vehicle isotype control cells).

## Total RNA isolation

THP-1 cells ( $10^6$  cells/ml, 2 ml/well) were exposed to test compounds at subtoxic concentration for 6 h. This time point was determined in preliminary experiments and was found to be optimal to study expression of Nrf2 target genes (data not shown). For the RNA extraction, the Macherey Nagel kit was used according to the manufacturer protocol. Treated cells were washed twice with cold PBS. 350  $\mu$ L of Lysis Buffer was added to the cell pellet and the tubes were vortexed vigorously. The viscosity was reduced and the lysate was cleared by filtration through a filter, the mixture was added to the filter and the tubes were centrifuged for 1 min at 11000 x g. The filter was discarded and 350  $\mu$ L of ethanol (70%) was added and mixed by pipetting up and down in order to adjust the RNA binding conditions. The lysate was loaded to a new column which binds to the RNA and the tubes were centrifuged 30 s at 11000 x g. The column was placed on a new tube and 350  $\mu$ L of Membrane Desalting Buffer was added. The tubes were centrifuged 1 min at 11000 x g to dry the membrane. Then the silica membrane was washed three times with different buffers and the RNA eluted in 40  $\mu$ L of RNase-free water. Total RNA yield was quantified by spectrophotometry using two dilutions (total RNA diluted at 1:100 and 1:200, two independent measurements).

## Reverse transcription

RNA concentrations were adjusted (80 ng/ $\mu$ L for THP-1 cells in 11  $\mu$ L final volume) and combined with 2  $\mu$ L of deoxynucleotide triphosphate mixture and 2  $\mu$ L of 50  $\mu$ M oligo(dT) primers. Mixes were denatured at 70°C for 10 min and then quickly cooled at 4°C in a Biometra thermocycler (Goettingen, Germany). Reverse transcription was carried out in AMV Reverse Transcriptase Reaction Buffer (Promega, Charbonnières-les-Bains, France) with final concentrations of 2 U/ $\mu$ L of RNase inhibitor (RNasine, Promega) and 0.2 U/ $\mu$ L of AMV Reverse Transcriptase (Promega), and RNase-free water to make up a final volume of 10  $\mu$ L. The reaction was then performed for 1 h 30 min at 42°C in the thermocycler. A control without reverse transcriptase was performed to confirm that no DNA contamination had occurred. The final product was stored at -20°C and used in the PCR step.

## Analysis of gene expression using real-time PCR

Real-time PCR analysis was performed using the SYBR Green technology on a Biorad CFX96 system. Each reaction consisted of cDNA (1:50 diluted for THP-1 cells in 4  $\mu$ L final volume of nuclease-free water), 0.5  $\mu$ M each of the forward and reverse primers (*ho-1* [5'-GGCCTGGCCTTCTTCACCTT-3' and 5'-GAGGGGCTCTGGTCCTTGGT-3'], *nqo1* [5'-GGGCAAGTCCATCCCAACTG-3' and 5'-GCAAGTCAGGGAAGCCTGGA-3'], *il-8* [5'-TCTCTTGGCAGCCTTCTGA-3' and 5'-TGGGGTGGAAAGTTTGG AG-3'], *gapdh* [5'-GGGCAAGTCCATCCCAACTG-3' and 5'-GCAAGTCAG- GGAAGCCTG GA-3'], or  $\beta$ -*actin* [5'-GGCATCCTCACCTGAAGTA-3' and 5'-GCACACGCAGCTCATTGTAG']), and Sso

Fast EvaGreen Supermix (Bio-rad, Marnes la Coquette, France) in a total reaction volume of 10  $\mu$ l. After 30 s at 95°C for Sso7d-fusion polymerase activation, amplification was allowed to proceed up to 44 cycles, each consisting of denaturation at 95°C for 5 s and annealing/extension at 62°C for 5 s. Six fold serial dilutions of mixed cDNA (from different samples) were analyzed for each target gene and allowed us to construct linear standard curves, from which the efficiency ( $E$ ) of each PCR reaction was evaluated and taken into account. The SYBR green fluorescence was detected at the end of each elongation cycle, after which a melting curve was assessed to confirm the specificity of the PCR products. The quantification was performed with the Biorad CFX Manager software, and data were analyzed using the delta-delta Ct method ( $\Delta\Delta Ct$ ). Ratio was calculated as the geometrical mean of  $(1 + E)^{-\Delta\Delta Ct}$  values, where  $E$  is the efficiency and  $\Delta\Delta Ct$  is the target gene expression of treated cells compared with normal levels in untreated cells and corrected using the expression of the reference genes *gapdh* and  *$\beta$ -actin*. Results were expressed as fold induction (i.e., ratio of  $(1 + E)^{-\Delta\Delta Ct}$  of treated cells/ $(1 + E)^{-\Delta\Delta Ct}$  of vehicle-treated cells).

### **Measurement of Nrf2 DNA binding**

DNA binding was analyzed using specific ELISA-based TransAM kits from Active Motif as described by (Macocho et al., 2015). After being treated THP-1 cells were lysed in a hypertonic buffer constituted of Non-diet P40 (NP40), 20% glycerol, 20 mM HEPES-KOH pH 7.9, 420 mM NaCl, 1 mM DTT, 1mM sodium orthovanadate, 1 mM pyrophosphate sodium, 125 mM okadaic acid, 62.5 mM EDTA, 40 mM EGTA, 0.5 mM PMSF, 1 g/ml aprotinin, 1 g/ml pepstatin and 1 g/ml leupeptine. After being left in ice for 20 min, THP-1 cells were centrifuged 20 min at 4°C at 17600 x g. Total protein concentration was evaluated using bicinchoninic acid assay and 20  $\mu$ g of protein were used for the assay. Total protein cell extracts (20 mg) were incubated for 1 h in a 96-well plate to which oligonucleotides, containing consensus binding sites for Nrf2, had been immobilized. After washing, the plate was incubated for 1 h with the appropriate primary Ab, which specifically detected an epitope accessible only when Nrf2 is activated and bound to its cognate oligonucleotide. The plate was then washed and incubated with horseradish peroxidase-conjugated secondary Ab (1:1000) for 1 h at room temperature. Colorimetric readout was quantified by spectrophotometry at 450 nm. Independent experiments were repeated at least three times.

## Results

### Cytotoxicity Measurement

For each chemical, several concentrations were first tested on THP-1 cells to assess cell viability after 24 h of treatment using 7-AAD staining (data not shown). According to these results, one concentration for each chemical was selected for further experiments. This concentration was the maximal concentration inducing no more than 30% cytotoxicity. For molecules without any cytotoxicity, the highest concentration (1.5 mM) was used (Tab. 1). All experiments were also realized at different concentrations to measure the relationship dose-response. For negative and positive controls DTT, CinA and tBHP, we used subtoxic concentrations which had previously been determined (Migdal et al., 2013).

### Measurement of cell-surface thiols, glutathione depletion and ROS production after terpenes and hydroperoxides exposure

A decrease of cell-surface thiols was described as a trigger of activation of intracellular signal transduction in hapten-treated THP-1 cells (Kagatani et al., 2010). We first measured this on THP-1 cells after treatment with the terpenes and the allylic hydroperoxides by flow cytometry. We used the Alexa fluor C5 maleimide, a specific impermeant thiol-reactive compound able to covalently bind to membrane surface cysteines. Thiol antioxidant DTT and oxidizing agent tBHP were also tested as negative and positive controls (Fig. 2B). Our results show that the terpenes and the hydroperoxides significantly induced a decrease of cell-surface thiols after 30 min of treatment. After 1 h of treatment this decrease was not anymore measured with LINA and LIMO, while it was still significant with LINA-OOH and LIMO-OOH. This effect was not anymore significant 2 h post exposure possibly explained by a recycling of the proteins at the membrane (Fig. 2A).

Since hydroperoxides treatment decreased cell surface thiols, GSH depletion was measured. The results showed a significant decrease of GSH/GSSG *ratio* in response to LINA-OOH (7 and 6 + 7) with a significant difference between LINA and LINA-OOH (Fig. 3A). No difference between LINA-OOH 7 and the mixture 6 + 7 was measured. LIMO-OOH induced a slightly decrease of the *ratio* in comparison with LIMO but the difference was not significant. Both LINA-OOH and LIMO-OOH induced a significant GSH depletion with a stronger effect for LINA-OOH. The depletion induced by the terpenes and/or their hydroperoxides is quite similar to positive controls (CinA or tBHP) (Fig. 3B).

Next, we measured ROS production at the same time since GSH is an important antioxidant, directly reacting with ROS and other reactive species. LINA-OOH 7 induced a significant production of ROS comparable to levels induced by tBHP, a positive control (Fig. 4B), whereas LINA, LIMO and LIMO-OOH did not (Fig. 4A).

### **Nrf2 is differently activated according to terpenes and hydroperoxides**

In response to an oxidative stress to the cells, the Nrf2/Keap1 pathway is activated in order to induce the synthesis of antioxidant enzymes, known to play a role in the generation of intracellular GSH (Dickinson et al., 2004). To investigate the activation of Nrf2 pathway after terpenes and hydroperoxides exposure, the accumulation of the Nrf2 protein was measured by western blot. Our results showed that in response to LINA-OOH 7 and 6 + 7, Nrf2 is accumulated. With LIMO and LIMO-OOH, a slight accumulation of Nrf2 was measured (Fig. 5A).

In order to activate the transcription of target genes, accumulated Nrf2 need to translocate to the nucleus and bind to ARE (Antioxydant Responsive Element) consensus sequence. Therefore, Nrf2 DNA binding activity was measured 6 h post stimulation. We showed that LINA-OOH and LIMO-OOH induced a significant increase of Nrf2 DNA binding while LINA and LIMO did not (Fig. 5B). The fold induction induced by both hydroperoxides was comparable to tBHP, our positive control (Fig. 5C). LINA-OOH 7 and 6 + 7 significantly induced the expression of *ho-1*, *nqo1* and *il-8*, target genes of Nrf2, while LINA did not. LIMO-OOH induced the expression of *ho-1* and *il-8* while LIMO did not (Fig. 6). However, induction of *ho-1* and *il-8* is lower with LIMO-OOH stimulation in comparison with LINA-OOH.

### **MAPKs and NF- $\kappa$ B pathways activation of THP-1 cells exposed to terpenes and hydroperoxides**

MAPK pathways have been described to be involved in the maturation of human DCs (Boislève et al., 2005). Furthermore, a link between their activation following oxidation of cell-surface thiols has been shown (Hirota et al., 2010). Therefore, the activation of MAPK and NF- $\kappa$ B pathways was measured in response to terpenes and hydroperoxides by western blot. The results showed that LINA-OOH 7 and 6 + 7 induced a significant activation of p38, ERK and p65 while LINA did not. LIMO induced the phosphorylation of JNK, ERK and p65 while LIMO-OOH induced the phosphorylation of p38 too. In conclusion, except for LINA, all chemicals induced MAPK and NF- $\kappa$ B pathways activation (Fig. 7).

### **Phenotypic characterization of THP-1 cells exposed to terpenes and hydroperoxides**

We analyzed whether changes of cell-surface thiols correlate with a maturation of THP-1 cells. The phenotype of THP-1 cells was analyzed after 24 h of treatment with terpenes and hydroperoxides. These modifications were expressed as RFI values. Vehicle-treated THP-1 cells had an immature phenotype as they expressed low levels of CD86 and CD54, which are the two activation markers used in the h-CLAT test. LINA and LINA-OOH 7 and 6 + 7 induced a significant expression of CD54. Concerning CD86, the expression was only significant with LINA-OOH 7 and 6 + 7 but not with LINA. A significant expression of CD54 was observed in response to LIMO-OOH, but not with LIMO. CD86 was significantly induced in response to

chemicals, LIMO and LIMO-OOH (Fig. 8A). The phenotype induced by hydroperoxydes is quite similar to the phenotype induced in response to positive control (Fig. 8B).

## Discussion

According to the European Commission, about 1 to 3% of the general population in Europe is affected by ACD to fragrance sensitizers (Consumer Safety Scientific Committee, 2011). 26 allergens need to be labelled on the packaging of cosmetic products and LIMO and LINA are part of them (European Commission, 1976). Some of their autoxidation products, such as allylic hydroperoxides, are indeed considered as moderate to strong sensitizers (Karlberg et al., 2013). Since these compounds could provoke an oxidative stress, we studied their mechanism of action on the THP-1 cell-line.

Kagatani et al. (2010) have reported that cells surface thiols on human monocytes THP-1 are oxidized by the treatment with some haptens. These modifications of thiols surface are playing a crucial role in the activation of MoDC and THP-1 cell lines. Once the proteins are oxidized, they need to be recycled to ensure the balance of the cell. GSH acts in conjunction with GSH peroxidases and glutathione S-transferases to detoxify ROS and to recycle oxidized protein thiols (Requejo et al., 2010). In our work, both terpenes and their derived hydroperoxides led to the oxidation of cell-surface thiols but the effect of the hydroperoxides was stronger. However, no significant ROS production was measured after exposure with LIMO-OOH, even though it induced a decrease of intracellular GSH.

The oxidation of cell surface thiols by exogenous GSSG can phosphorylate p38 MAPK (Filomeni et al., 2003). This kinase was also activated in response to DNCB, a strong sensitizer (Miyazawa et al., 2008). In our hands, LINA-OOH, LIMO and LIMO-OOH induced the phosphorylation of three MAPKs (JNK, ERK and p38) but only hydroperoxides led to the upregulation of both CD54 and CD86. According to the sensitizer, oxidation of thiols and activation of MAPKs pathway seems not to be necessary linked to the phenotype maturation of the cell. Our experiments realized with LINA-OOH 7 and 6 + 7 allowed us to figure out the impact of the mixture on activation of the cells. No significant differences between exposure with LINA-OOH 7 and 6 + 7 were measured so there is no evidence that the mixture of LINA-OOH is a stronger activator of THP-1 than LINA-OOH 7. The difference of results observed between LIMO-OOH and LINA-OOH could be explained by the suspected mechanisms for their reaction with skin proteins involving radical intermediates. Initially obtained alkoxyl RO•/peroxyl ROO• radicals can further lead via intramolecular cyclization, fragmentation or hydrogen abstraction to the formation of longer half-life reactive carbon-centred radicals R• through different radical rearrangements. It was proved by Kao *et al.* (2011) that some of these carbon-centred radicals R• derived from LINA-OOH and LIMO-OOH can react and form adducts with some amino acids such as cysteine, lysine and tryptophan. It was shown that the possibilities of radical rearrangements were more limited in the case of LIMO-OOH than in the case of LINA-OOH. Thus, one hypothesis could be that more rearrangements leading to a higher concentration of carbon-centred radicals R• intermediates induce a stronger

activation of THP-1 cells, explaining then why cells are more activated after treatment with LINA-OOH.

Our results allow us to propose that the mechanism of action of both terpenes should be different. LIMO could act as a pro-hapten and would be metabolized by THP-1. Once metabolized, the product would activate MAPKs pathways inducing an incomplete maturation of THP-1 cells. Bråred Christensson et al. (2016) mentioned that some terpenes could be activated to become sensitizers through both biotic and abiotic activation (geraniol and  $\alpha$ -terpinene for instance). Biotic oxidation can occur in the skin, through enzyme activity such as cytochrome P450 or peroxidase enzymes (Moss and Lepoittevin, 2016). Keratinocytes seem to play a role in the metabolic activation of prohaptens and conjugation of haptens (Hennen et al., 2011; Hennen and Blömeke, 2016). Meesters et al. (2007) studied the oxidative metabolism of LINA by cytochrome P450 by enzymatic assays. They identified different enzymatic products of allylic hydroxylation and epoxidation such as 8-hydroxylinalool and 6,7-epoxy-linalool. Epoxides can react as  $S_N2$  electrophiles with skin proteins (Roberts et al., 2016) and might also lead to the activation of intracellular pathways.

We also focused on the activation of Nrf2/Keap1 pathway, as it was described as a signaling pathway activated by chemical sensitizers (Ade et al., 2009). Once activated, Nrf2 binds to ARE sequences, leading to the transcription of antioxidant genes. Chemical sensitizers induced the transcription of Nrf2 target genes *ho-1* and *nqo1* (Migdal et al., 2013). Nrf2 is known to regulate oxidative stress response and its transcriptional activity is the biomarker of the KeratinoSens. This *in vitro* sensitization test quantifies luciferase gene induction as a measure of the activation of Nrf2 and was validated by the OECD in 2015 (OECD 442d). Our results show that allylic hydroperoxides led to the activation of Nrf2 while LINA or LIMO induced no accumulation of Nrf2 or a slight one respectively. Both terpenes act in a different way for Nrf2 activation. Some evidences indicated that phosphorylation of Nrf2 at several serine and threonine residues by MAPKs (Sun et al., 2009) is needed for its activation. It not excluded that the activation of MAPKs pathway by the hydroperoxides can be a part of the explanation of activation of Nrf2. With those measurements, we could propose a mechanism of action describing the allylic hydroperoxides leading to the maturation of the DCs. The first step would be the oxidation of thiols group of cysteine residues of protein at the membrane of the cell. It leads to the decrease of intracellular GSH and ROS production. Then, cysteine residues of Keap1 are oxidized and accumulated Nrf2 translocates to the nucleus. Nrf2 induces the transcription of *ho-1*, *nqo1* and *il-8*. Meanwhile, phosphorylation of MAPKs and NF- $\kappa$ B pathways proteins (p38, p65, JNK and ERK) occurs. The activation of these pathways also probably leads to an accumulation of Nrf2 and may participate to the acquirement of a mature phenotype with the expression of CD86 and CD54 at its surface (fig. 9).



These results made us to conclude that LIMO-OOH induces the oxidation of thiols and GSH depletion on THP-1 cells, leading to a slight activation of Nrf2 in a ROS independent manner. In comparison, LINA-OOH induces a strong ROS production associated with an activation of Nrf2 comparable with positive controls. Our study underlies that the tested allylic hydroperoxides seem not to be equal for DC activation in regard to ROS production and Nrf2 triggering.

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**Conflict of interest**

The authors declare that there are no conflicts of interest.

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