



The THP-1 cell toolbox: a new concept integrating the key events of skin sensitization

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Abstract

According to the current scientific consensus, one in vitro test is insufficient to cover the key events (KE) defined by the adverse outcome pathway (AOP) for skin sensitization. To address this issue we combined different end points in the same cell line to cover all KEs defined by the skin sensitization AOP. Since dendritic cells (DC) play a key role in the sensitization phase leading to the development of allergic contact dermatitis (ACD), we used THP-1 cells as a surrogate for DC. We measured ROS production and GSH depletion for KE1 (binding to proteins), Nrf2 activation pathway and gene expressions for KE2 (keratinocyte response), phenotype modifications using cell-surface markers and cytokine production for KE3 (DC activation), and T-cell proliferation for KE4 (T-cell activation). These measurements were performed using the THP-1 cell line and an original THP-1/T-cell co-culture system following exposure to a variety of chemicals, including irritant, non-sensitizers, and chemicals sensitizers (pro/prehaptens). Results showed that treatment with sensitizers such as cinnamaldehyde (100 μ M) or methylisothiazolinone (150 μ M) was able to trigger the three main key events (KE1, KE2, and KE3) of the sensitization phase of ACD in THP-1 cells. In addition, all sensitizers were able to induce T lymphocyte proliferation (KE4), while non-sensitizers and irritants did not. Our study shows for the first time that addressing the four main KE of skin sensitization AOP in a single cell line is an achievable task.

Keywords Allergic contact dermatitis · Adverse outcome pathway · In vitro sensitization assay · Co-culture · THP-1 · Lymphocyte

Abbreviations

ACD	Allergic contact dermatitis
AOP	Adverse outcome pathway
ARE	Antioxidant response element
BZK	Benzalkonium chloride
CinA	Cinnamaldehyde

CS	Contact sensitizer
DC	Dendritic cell
DNCB	Dinitrochlorobenzene
DPRA	Direct peptide reactivity assay
EUG	Eugenol
FAR	Farnesol
GER	Geraniol
GSH	Glutathione
GSSG	Glutathione disulfide
h-CLAT	human cell line activation test
ITS	Integrated testing strategy
KE	Key event
Keap1	Kelch-like ECH-associated protein 1
MIT	Methylisothiazolinone
Nrf2	Nuclear factor erythroid 2-related factor 2
RESO	Resorcinol
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
t-BHQ	tert-butylhydroquinone

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Introduction

ACD is a dermatological disease that occurs after a chemical contact with the skin (Kimber et al. 2011). ACD is a two-step process: (1) sensitization phase or skin sensitization is induced when dermal exposure occurs at sufficient levels leading to T-cell activation and proliferation and (2) elicitation phase occurs after subsequent dermal exposures that trigger the clinical symptoms. Skin sensitization is one of the key events to be addressed for human hazard characterization and risk assessment of chemicals and cosmetic ingredients. The skin sensitization potential of substances has been usually assessed using the murine local lymph node assay (LLNA) (OECD 2002).

In the context of the 2013 ban on animal testing imposed by EU Cosmetics Regulation, the ability to identify and classify skin sensitization potential of chemicals without animal testing is of high importance. Moreover, OECD has summarized the various steps of the biological mechanism of skin sensitization (Vandebriel and Loveren 2010) into an AOP (OECD 2012).

The four key events (KE) of skin sensitization AOP are covalent binding to skin proteins (KE1), keratinocyte responses (KE2), activation of DC (KE3), and T-cell proliferation (KE4) leading to the adverse outcome of ACD. Various non-animal test methods addressing one of these three KEs, have been developed and validated (OECD TG 442C, D, E). Five testing methods have achieved this process and have been validated: (1) the direct peptide reactivity assay (DPRA), an *in chemico* test method addressing KE1 by evaluating the reactivity of chemicals using model peptides containing cysteine or lysine residues (OECD 2015a), (2) the KeratinoSens™, an *in vitro* assay based on the activation of the Nrf2-Keap1 pathway in response to oxidative and electrophilic stress response addressing KE2 in HaCaT-derived human keratinocytes (Emter et al. 2010; OECD 2015b), and (3) three *in vitro* assays that address DC activation: the human cell line activation test (h-CLAT) measuring biomarkers of DC activation in THP-1 cells, the U937 cell line activation test (U-SENS™) or the IL-8 Luc assay. These last three methods address KE3 (OECD 2016a, 2017). It has been accepted that one single non-animal test method was not sufficient to cover the all AOP and to obtain a 100% accuracy compared with the murine LLNA. Thus, several integrated testing strategies (ITS) using multiple tests have been developed to evaluate the sensitizing potential of chemicals (OECD 2016b). Under REACH, combining *a minima* of three non-animal test methods can now be used to fulfill the regulatory information requirements (European Chemicals and Agency 2016).

The main objective of this work was to combine different tests measuring relevant endpoints within the same

cell line to propose a new model covering all KEs of the skin sensitization AOP. Since DC plays a key role in ACD, THP-1, a human promonocytic leukemia cell line that is commonly used as a DC surrogate, has been selected (Ashikaga et al. 2010; Sakaguchi et al. 2006). The reactivity of chemicals measured using reactive oxygen species (ROS) and glutathione (GSH) depletion measurements was selected to evaluate KE1; Nrf2-antioxidant response element (ARE) activation pathway assessment and genes expression mimicked the epithelial stress response KE2 (Emter et al. 2010); upregulation of surface markers CD86, CD54 and identification of cytokines addressed KE3 and T-cell proliferation using human CD4⁺/THP-1 co-culture addressed KE4.

Our results demonstrated for the first time that all KEs of the skin sensitization AOP can be evaluated within the THP-1 cell line for contact sensitizers (CS).

Materials and methods

Chemicals

Eleven different molecules (including controls as PBS and DMSO), non-sensitizers (BZK and t-BHQ), irritant (SDS), RESO and EUG as weak, FAR, GER and CinA as moderate, and one strong CS, MIT, were selected. They were all purchased from Sigma-Aldrich (St Quentin-Fallavier, France). Sensitizers were selected based on human evidence and to reflect various potency classes (Supplementary Table 1).

Culture of THP-1 cell and chemical treatment: modified h-CLAT

The human monocytic cell line THP-1 (ATCC-TIB-202; LGC, Molsheim, France) was grown 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) as described by Migdal et al. (2013). Briefly, THP-1 were washed twice and maintained at 1×10^6 cells/ml. Cells were then treated with subtoxic chemicals for different periods of time according to the experiments. Chemicals were diluted in their respective solvent and final concentrations of solvent in culture media did not exceed 0.1%. Cell viability after treatment was never less than 75% as assessed by trypan blue exclusion, propidium iodide and 7-aminoactinomycin D (7-AAD) (Supplementary Table 1).

Flow cytometry analysis of THP-1

After 24 h of incubation with chemicals, THP-1 cells were washed once in cold phosphate-buffered saline (PBS) and 3×10^5 cells 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 (Ab) isotypes (IgG1, BD) were used at the same concentrations as controls. THP-1 cell phenotype modifications were detected using a FACS Calibur flow cytometer. For each sample, 10,000 cells were acquired and analyzed using the CellQuest software (Becton Dickinson). 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 Ab-MFI of chemical-treated isotype control cells)/(MFI of vehicle control cells labeled with antigen-specific Ab-MFI of vehicle isotype control cells).

Quantitative determination of GSH/GSSG

Intracellular GSH levels were quantified using the bioluminescent GSH-Glo glutathione assay (Promega), according to the manufacturer's instructions and as described by El Ali et al. (2017). Briefly, THP-1 were treated with chemicals or vehicle for 1 h. Cells were then washed once with cold PBS and resuspended with 100 μ L of glutathione lysis and reaction buffer (Promega). Cells were then dispatched in a 96-well plate. After further addition of 100 μ L of luciferin detection reagent 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.

ROS detection

Intracellular ROS were measured using the specific dye 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) (Sigma-Aldrich), as described by El Ali et al. (2017) by flow cytometry. The non-fluorescent dye (H2DCF-DA) is a chemically reduced form of fluorescein and cell-permeable. Intracellular esterases cleave off the diacetate (DA) moiety which renders the molecule (H2DCF) sensitive to oxidation by ROS. In its oxidized form dichlorofluorescein (DCF) is highly fluorescent and easily detectable by flow cytometry. Briefly, cells were loaded for 30 min in the dark with H2DCF-DA (2 μ M) in serum-free RPMI at 37 °C before treatments. After different times of treatment, all results were compared together and to their respective vehicle at 30 min and finally expressed in fold increase according to

the intensity of the green DCF fluorescence (MFI of chemical-treated cells/MFI of vehicle control cells—30 min).

Immunoblotting

Western blot was performed as previously described by Migdal et al. (2013). Briefly, THP-1 cells (10^6 cells/well) were exposed during 6 h to chemicals. Cells were washed twice in cold PBS before addition of 40 μ L of lysis buffer (20 mM Tris pH 7.4, 137 mM NaCl, 2 mM EDTA pH 7.4, 2 mM sodium pyrophosphate, 1% Triton, 10% glycerol, 1 mM PMSF, 1 mM Na_3VO_4 , 25 mM β -glycerophosphate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and 10 μ g/ml pepstatin) and left in ice for 20 min. The homogenates were centrifuged at 15,000 rpm for 20 min at 4 °C. Equal amounts of denatured protein were loaded onto 12% polyacrylamide gel and transferred on PVDF membrane (Bio-Rad Laboratories, Hercules, CA). 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 (Cell Signaling Technology, Ozyme, St-Quentin en Yvelines, France). Bands were measured using the ImageLab software (Bio-Rad, Marnes-La-Coquette, France).

Measurement of Nrf2 transcription factor activity (DNA binding)

DNA binding was analyzed using specific ELISA-based TransAM kits from Active Motif as described by Raffalli et al. (2017). After being treated, THP-1 cells were lysed in a hypotonic buffer constituted of Non-diet P40 (NP40), 20% glycerol, 20 mM HEPES-KOH pH 7.9, 420 mM NaCl, 1 mM DTT, 1 mM Na_3VO_4 , 1 mM sodium pyrophosphate, 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 leupeptin. 20 μ g of protein cell extracts was incubated for 1 h in a 96-well plate-containing consensus binding sites for Nrf2. After washing, the plate was incubated for 1 h with the appropriate primary Ab, washed and then incubated with horseradish peroxidase-conjugated secondary Ab (1:1000) for 1 h at room temperature. Colorimetric readout was quantified by spectrophotometry at 450 nm.

Genes expression according to the SENS-IS method and adapted to THP-1 cells

THP-1 cells (2×10^6 cells/well) were exposed to chemicals for 6 h, collected and centrifuged at 3500 rpm for 5 min and were placed in a tube for snap freezing in liquid nitrogen. Then, the cell pellets were treated according to the SENS-IS protocol as described in Cottrez et al. (2015, 2016).

Cytokine array

Cytokine secretion was assessed using the Proteome Profiler Array: Human Cytokine Array Panel A, ARY005B (R & D Systems, Minneapolis, USA) as described by the manufacturer's instructions. Briefly, membranes were incubated with blocking buffer at room temperature for 1 h. Cell-treated supernatants (0.5 ml of THP-1 (1×10^6 cells/ml)) were collected and mixed with a biotinylated Ab cocktail, and then each was incubated on a membrane overnight at 4 °C. Cytokine arrays were detected by chemiluminescence (ChemiDoc XRS + System, Bio-Rad, Marnes-La-Coquette, France) using an ECL solution (Amersham Biosciences).

Cytokine quantification in THP-1 cell supernatants

CXCL8, IL-16, CCL5, and CCL4 levels in the culture supernatants were measured using DuoSet ELISA kits, respectively (R&D Systems, Abingdon, UK), according to the manufacturer's guidelines. 1×10^6 THP-1 cells were seeded in 24-well plates for 24 h. ELISA assays were performed on culture supernatants. The lower limit of detection was approximately 30 pg/ml for CXCL8, 15 pg/ml for IL-16, 15 pg/ml for CCL4/MIP-1 β and 15 pg/ml for CCL5. Colorimetric readout was quantified by spectrophotometry at 450 and 550 nm.

Co-culture of CD4⁺ T-lymphocytes and THP-1 cells

The capacity of THP-1 to activate CD4⁺ T cells was evaluated by the use of allogeneic CD4⁺ T cells labeled with 0.5 μ M carboxy fluorescein succinimidyl ester (CFSE) as described by de Bourayne et al. (2017). CD4⁺ T cells were isolated from PBMCs by positive selection with the MACS CD4 isolation kit II (Miltenyi Biotec). These T cells were confirmed to have purity greater than 95%, based on CD4 (555349; BD Biosciences) expression evaluated by flow cytometry (FACS Calibur, BD Biosciences).

THP-1 were stimulated with chemicals and positive controls such as NiSO₄ (500 μ M) and LPS (100 ng/mL) for 24 h in complete medium. Treated THP-1 were washed and then cultured with allogeneic total CD4⁺ T cells at 1:10 DC/THP-1 ratio for 5 d in complete RPMI 1640. On day 5, CD4⁺ T-cell proliferation was analyzed by flow cytometry and expressed as the percentage of CFSE^{low} cells. Cell division was next determined by analyzing CFSE dilution with a FACS Calibur flow cytometer and CellQuest software (BD Biosciences).

Statistical analysis

All results are recorded as means $\pm$ standard errors of the means (SEM) unless otherwise stated. Statistical testing

was performed using Prism 5.02 software (GraphPad Software Inc.). Unpaired *t* test, Mann–Whitney test, or two-way ANOVA with Bonferroni post hoc analysis were used to analyze differences between groups, as appropriate. Effects were designated significant at *p* < 0.05.

Results

Oxidative stress induced by sensitizers as KE1

Since CS can lead to an oxidative stress, we investigated ROS production and GSH depletion following THP-1 exposure to the selected chemicals at a maximal concentration inducing no more than 30% cytotoxicity (Supplementary Table 1). RESO, MIT and CinA induced a significant production of ROS after 30 min and 1 h exposure of THP-1 cells, compared to vehicle control (Fig. 1a). All non-sensitizers (SDS, BZK, t-BHQ) did not induce ROS production. For glutathione, we measured a significant decrease in GSH/GSSG ratio after 1 h of THP-1 exposure to MIT, CinA, RESO and BZK (Fig. 1b).

Nrf2 pathway and genes expression activated by sensitizers as KE2

Since the Nrf2/Keap1 pathway is activated to induce the synthesis of antioxidant enzymes (Dickinson et al. 2004) and to counter regulate oxidative stress, we measured its activation after exposure to chemicals. The level of Nrf2 accumulation measured by western blot was significantly higher for sensitizers such as CinA and MIT and also for t-BHQ a positive control for the Nrf2 pathway (Fig. 2a). These observations were associated with an increase of Nrf2 DNA-binding activity to an ARE consensus sequence, 6 h poststimulation (Fig. 2b) for CinA and MIT.

Since KE2 is also associated with gene expressions, we measured gene expression using an adapted version of the SENS-IS method. These experiments were performed within the THP-1 cell line in place of the 3D skin model used in the SENS-IS method. Among these genes, 11 genes for irritancy (IRR), 6 genes for SENS-IS, and 9 genes for ARE were retained (Supplementary Table 2). Intermediate and strong CS such as CinA, RESO, and MIT augmented the expression of inflammation-related genes such as *il-8*, *il-1 α* , *dusp1*, *cxcl1*, and *cxcl14*, included in IRR's and SENS-IS genes. They also induced ARE genes such as *nqo1*, *akr1b10*, and *hmox1* (Supplementary Table 2). A positive result was taken into consideration if genes expression was at least augmented by a fold of two compared to the respective controls.

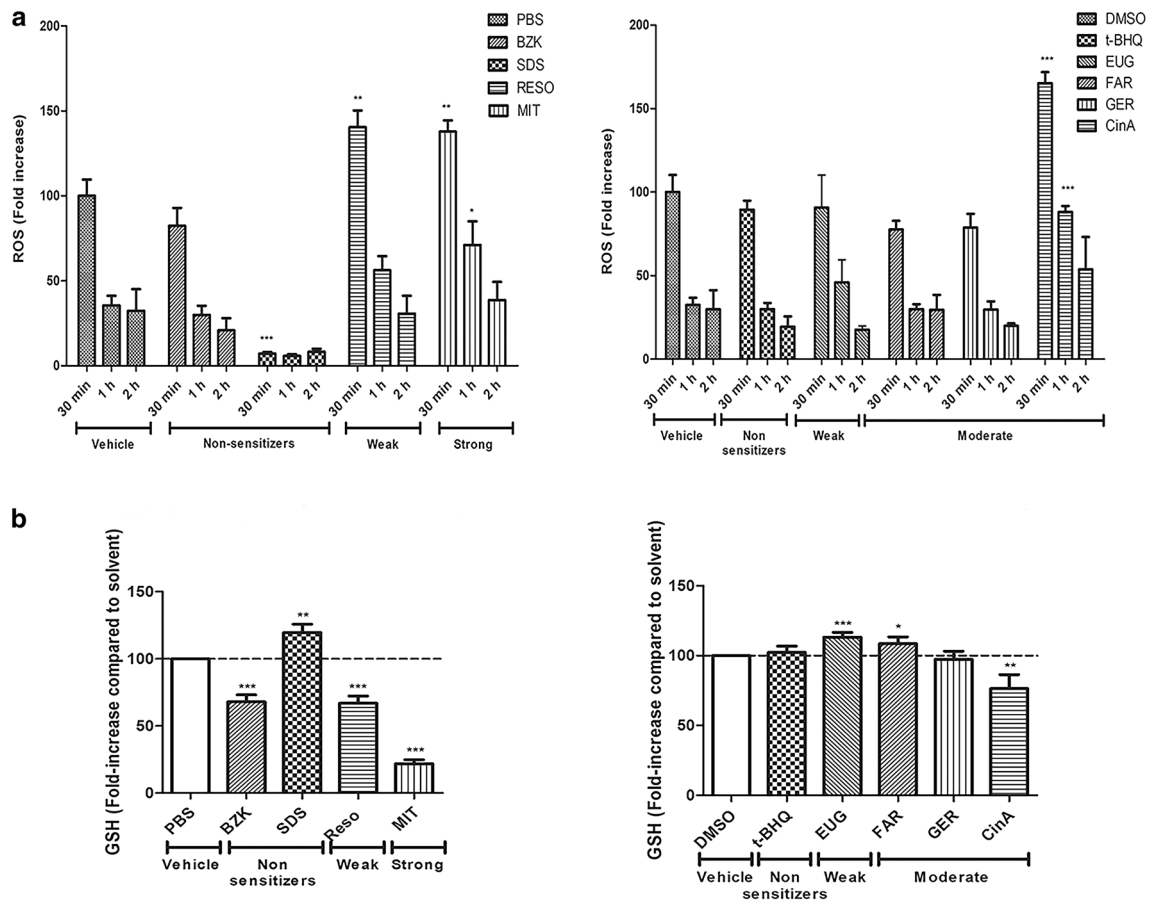


Fig. 1 ROS production and intracellular levels of GSH in THP-1 cells treated with chemical sensitizers. THP-1 (0.5×10^6) were exposed at subtoxic dose for 30 min, 1 h, 2 h for ROS production and 1 h for GSH depletion. Results were expressed as fold induction compared with vehicle-treated cells (PBS or DMSO). **a** ROS production was analyzed by flow cytometry using DCFDA. Data represent

the mean $\pm$ SEM ($n=4$). *** $p<0.001$, ** $p<0.01$, * $p<0.05$ (two-way ANOVA statistical analysis with Bonferroni post hoc analysis). **b** Depletion of GSH was analyzed by luminescence. Data represent the mean of GSH/GSSG ratio $\pm$ SEM ($n=8$) (except for Eug, Far, Ger: $n=5$). *** $p<0.001$ versus control cells, ** $p<0.01$, * $p<0.05$ (Mann–Whitney U tests)

Cellular responses induced by sensitizers as KE3

Next, phenotypes of THP-1 cells were analyzed by flow cytometry after 24 h of exposure according to the modified h-CLAT protocol (OECD 2016a). RESO, EUG, CinA, and MIT induced a significant increase in the expression of CD54. For CD86, its expression was significantly augmented with EUG, GER, CinA, and MIT (Fig. 3a).

In a second step, the extracellular level of cytokines produced by THP-1 cells was screened using a protein array (Fig. 3b). The main cytokines identified in the CS exposure group were IL-8, IL-16, CCL4, and CCL5. We confirmed by ELISA the enhanced production of CCL4 that was significantly increased for moderate and strong CS such as EUG, FAR, GER, CinA, and MIT (Fig. 3c). For CCL5, a significant increase in its production was measured after treatment with the antioxidant t-BHQ and the strong CS, MIT (Fig. 3d). In addition, we measured a significant

augmentation in the production of IL-8 for all CSs but not for irritants (Fig. 3e). A significant increase in the production of IL-16 was also observed for EUG, FAR, GER, CinA, and MIT treatment (Fig. 3f).

T-cell proliferation in response to THP-1 primed by contact sensitizers as KE4

To study the functional capacity of human THP-1 to activate $CD4^+$ T cells, a key step for initiating a full immune response, CS-stimulated THP-1, were co-incubated with allogeneic $CD4^+$ T-lymphocytes loaded with CFSE. Proliferation was measured on day 5 as a % of CFSE^{low} $CD4^+$ T cells, LPS and nickel were used as positive controls. In the absence of THP-1 cells, 18% of $CD4^+$ T cells were CFSE^{low}. Representative results for all chemicals are shown (Fig. 4a, b). Beside nickel and LPS, a significant increase in $CD4^+$ T-cell proliferation was measured for MIT-treated THP-1

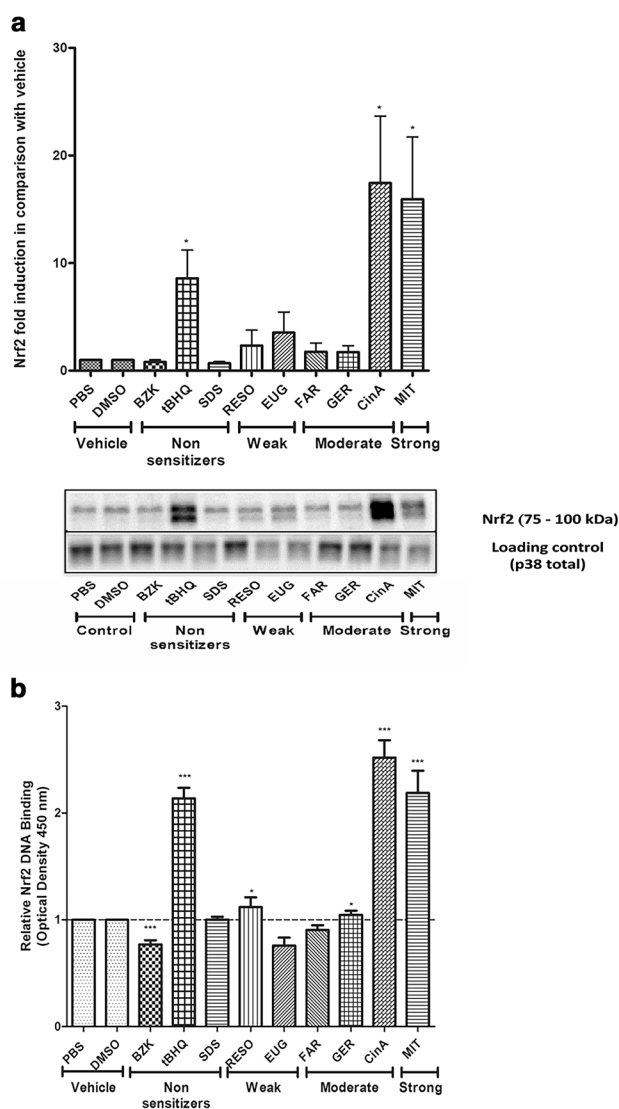


Fig. 2 Nrf2 activation following treatment with chemical sensitizers. THP-1 (3×10^6) were exposed at subtoxic dose for 6 h and Nrf2 was detected by chemiluminescence. Results were expressed as fold increase ratio of Nrf2 proteins to the loading control and were compared to the vehicle control (PBS or DMSO). Data represent the mean $\pm$ SEM ($n=4$). * $p<0.05$ (Mann–Whitney U tests). **a** A representative western blot after a 6-h exposure at subtoxic concentrations. **b** Measurement of DNA-binding activity for activated Nrf2 by ELISA. THP-1 cells (5×10^6) were exposed at subtoxic concentrations for 6 h and analyzed by spectrophotometry (450 nm). Data represent the mean $\pm$ SEM ($n=3$). *** $p<0.001$, * $p<0.05$ (Mann–Whitney U tests)

(Fig. 4c). Although, we did not detect any other significant increase in CD4⁺ T-cell proliferation, we noticed a trend for CD4⁺ T-cell proliferation after co-culture with all CS-treated THP-1 cells compared to their respective control. Interestingly, we did not observe any significant increase in CD4 T-cell proliferation after irritant treatment (Fig. 4a). Simultaneously, pretreated THP-1 led to an increase in CD4⁺ T cell

count in each cell generation, particularly in generations 3 and 4 with FAR, GER, and CinA and in 4 and 5 with RESO and MIT. These overall findings revealed that THP-1 stimulated by strong CS can induce an increase of CD4⁺ T-cell proliferation (Fig. 4d).

Discussion

To evaluate the sensitization potential of chemicals, numerous in vitro methods are available. However, to address hazard identification, *a minima* of three independent assays are required covering KE1 to KE3 of the AOP for chemical sensitization. In the present work, we are proposing a single cell line to combine all KEs using different assays and endpoints. Since DC plays a crucial role and is essential to promote T cell activation, the THP-1 cell line described as a surrogate of DC have been selected to combine all the KEs of AOP, ROS and glutathione for KE1, Nrf2 activation for KE2, CD86 and/or CD54 expression for KE3 and T-lymphocytes activation for KE4.

Full activation of DC is essential for the establishment of sensitization to CS. Electrophilic and oxidative stress are involved in this process (Ade et al. 2009). Indeed, ROS was found to be produced after CS treatment such as 2,4-dinitrochlorobenzene (DNCB) in DC (Migdal et al. 2010; Byamba et al. 2010). In our model, ROS production in THP-1 cells was significantly higher following stimulation with MIT or CinA, accompanied with decreased GSH levels (Franco and Cidowski 2012). In our study, ROS production and GSH depletion may act as a surrogate for protein binding, allowing us to address the KE1 in THP-1 cells.

The main pathway responsible for cell defense against oxidative stress and maintaining the cellular redox balance at physiological levels is the Nrf2 pathway (Aw Yeang et al. 2012). 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). Furthermore, the expression of Nrf2 protects DC against chemical stress and ROS production thus playing a role in their survival (El Ali et al. 2017). In addition, the KE2 is measured by the activation of the Keap1-Nrf2-ARE pathway in keratinocytes (Emter et al. 2010; OECD 2015b). In our study, CinA and MIT led to the accumulation of Nrf2 compared to a slight accumulation for RESO and GER known as weak and moderate sensitizers. Moreover, CinA and MIT led to an active Nrf2 since increase in Nrf2-binding activity to ARE sequence was measured in response to both CS. Results obtained in response to CinA and MIT are quite similar to those obtained with the KeratinoSens assay (KE2) based on Nrf2 transcriptional activity (Emter et al. 2010). However, pre/pro-haptens such as EUG, and FAR did not induce Nrf2 activation as observed in our study unlike what has been

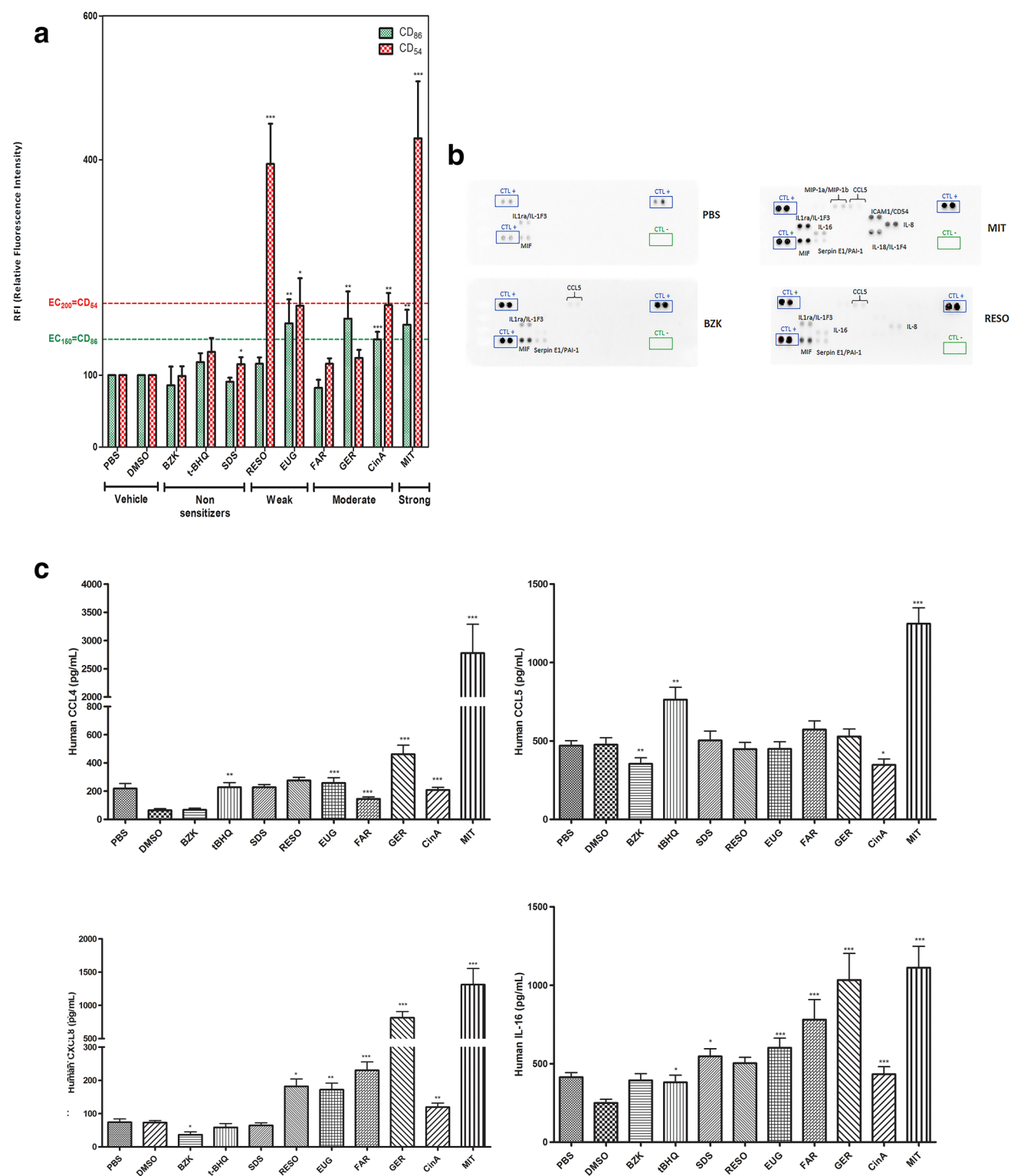


Fig. 3 Chemical sensitizers alter THP-1 phenotype. **a** Chemical sensitizers induce CD54 and C86 expression. After a 24-h exposure at subtoxic concentration, RFI was determined on THP-1 cells (1×10^6) by flow cytometry. Data represent the mean RFI $\pm$ SEM versus control cells ($n=7$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ (Mann–Whitney U tests). **b** Cytokines secretion after exposure to chemical sensitizers. THP-1 cells (1×10^6) were treated at subtoxic concentrations for 24 h

and supernatants were collected and analyzed using a cytokine array. Four representative membranes are shown. **c** Cytokines secreted in response to CS. THP-1 cells (1×10^6) were treated at subtoxic concentrations for 24 h and CCL4, CCL5, CXCL8, and IL-16 concentrations were measured by ELISA. Data represent the mean $\pm$ SEM ($n=8$). * $p < 0.05$ versus control cells or DMSO (Mann–Whitney U tests)

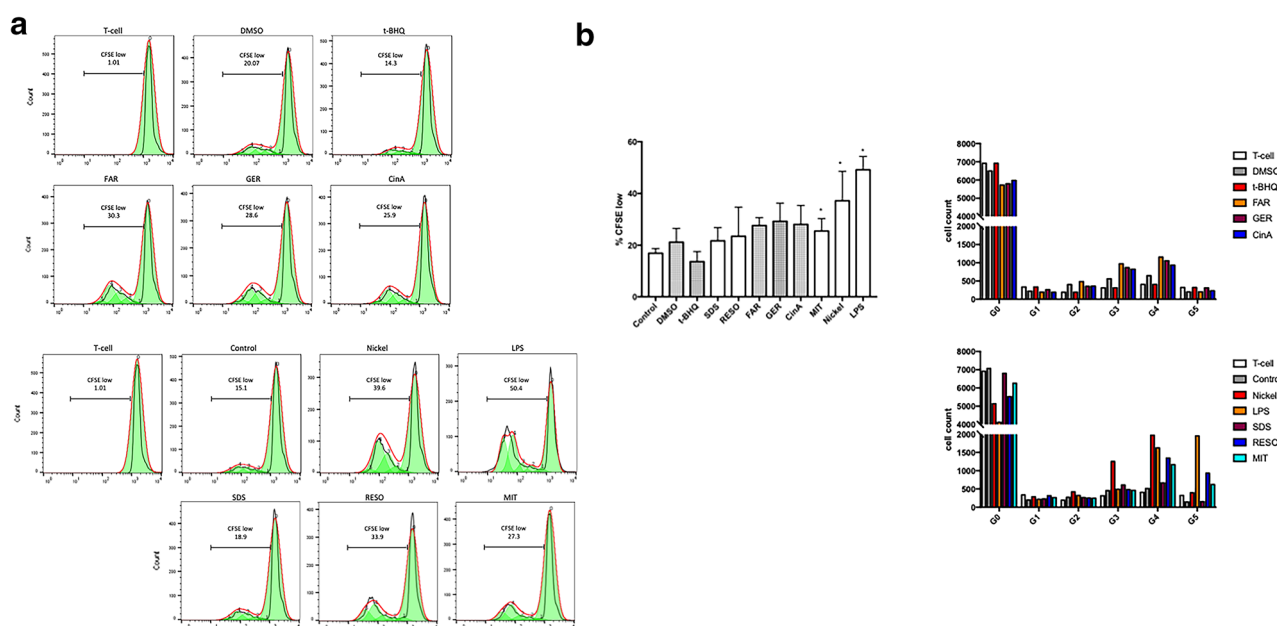


Fig. 4 THP-1 cells activated by CS induced CD4⁺ T-lymphocyte proliferation. CFSE-loaded CD4⁺ T-lymphocytes were cultured with THP-1 cells pretreated with chemicals sensitizers or NiSO₄ (500 mM) or LPS (100 ng/mL) for 24 h and then co-cultured (10⁵ cells/ml) for 5 days at a T cell/THP-1 cell ratio of 1/10. On day 6 the percentage of CFSE^{low} cells was determined by flow cytometry. Representa-

tive results are shown for chemicals solubilized in PBS (**a**) or DMSO (**b**). Data represent the percentage of CFSE^{low} cells, corresponding to T-cell proliferation (**c**) or as cell counts per cell division (**d**). Data represent the mean $\pm$ SEM ($n=3-4$). * $p<0.05$ versus control cells or DMSO (Mann-Whitney)

described in the literature. This discrepancy could be due to their lipophilic properties or to less metabolic capacities of THP-1 cells (Migdal et al. 2010; Saito et al. 2017). To further address KE2, we performed the SENS-IS test adapted to THP-1 cells using similar genes. This test measures expression of a large panel of genes with one gene set for irritancy (IRR) and two gene set (SENS-IS and ARE) for sensitization. In response to RESO, EUG, CinA, and MIT, a common panel of genes was induced but with different levels of magnitude. The genes identified, *gclc*, *txnr1*, *gclm*, *hmox1*, *nqo1* or *cxcl14*, *il-8*, *il-1a*, *ccl20*, *icam-1*, *dusp1*, were part of one of the IRR, SENS-IS or ARE categories.

Concerning the KE3, related to the modulation of CD86 and CD54 cell-surface markers on THP-1 cells, our results were similar as described in the literature for most of the CS used except for FAR.

Additionally, alteration of DC phenotype can be followed through cytokine production. Our work revealed that CS induced the production of many cytokines and chemokines that play a crucial role in skin inflammation. Among these cytokines and chemokines, CCL4, CCL5, CXCL8, and IL-16 were induced in response to intermediate or strong CS in THP-1 cells. CCL4 is a powerful chemoattractant for a wide variety of inflammatory cells, including T cells, NK cells, monocytes, and macrophages (Taub et al. 1995; Ugucioni et al. 1995). As described by Lim et al.,

(2008), we showed that CCL4 seemed to be a good candidate as a biomarker for CS. In response to MIT, CCL5 was secreted by THP-1 cells, as described for DNCB and CinA in MUTZ-3 cells (Williams et al. 2010). CCL5 plays a role in initiating migration of irritant-exposed human Langerhans cells out of the epidermis. CCL5 could be identified as biomarker for strong CS in THP-1 cells. For CXCL8, it has been tested under various conditions as a marker for sensitization (Ouweland et al. 2010; Python et al. 2009, 2007). As described by Ouweland et al. (2010) in MUTZ-3 cells, CXCL8 secretion in THP-1 cells was increased after exposure to almost all sensitizers, but not to non-sensitizers. For IL-16, this cytokine can act as an immunomodulatory cytokine that contributes to the regulatory process of T cell recruitment and activation at sites of inflammation (Cruikshank et al. 1987). Interestingly, a promoter polymorphism in the *il-16* gene has been suspected to be associated with susceptibility to ACD (Reich et al. 2003). In MoDCs, based on IL-16 secretion CS such as CinA, EUG have been identified as chemical sensitizers (Hitzler et al. 2013). Our study showed that most of all CS have induced IL-16 production thus identifying this cytokine as potential new biomarker of CS.

Concerning the KE4, no standardized in vitro assays are available; thus, KE4 cannot yet be measured in vitro. Only non-animal test methods addressing KE1, KE2 or KE3, are

Table 1 Hit-map showing results obtained for each KE.

		Key 1		Key 2					Key 3						Key 4	
		Chemical reactivity		Proteins activity	DNA-binding ELISA	Genes expression on THP-1 (SENS-IS)			Surface markers		Modulation by inflammatory cytokines	Cytokines quantification				CD4 ⁺ T cell proliferation
		ROS	GSH depletion	Western blot	Nrf2 TransAM	IRR	SENS-IS	ARE	CD ₅₄	CD ₈₆	Cytokine array	CCL4	IL-8	IL-16	IL-18	
NON SENSITIZERS	PBS					7	1	2			IL1-ra MIF					
	DMSO					6	2	2			IL1-ra MIF IL-16-/+ CCL5					
	BZK					14	4	7			IL-16++ CCL5					
	t-BHQ					11	1	11			IL-16++ CCL5+					
IRR	SDS					13	7	5			IL-16++ CCL5					
WEAK	RESO					16	4	12			CXCL8+ IL-16+ CCL5+					
	EUG					12	2	10			CXCL 8 IL-16-/+ CCL5					
MODERATE	FAR					9	8	7			CXCL8+ IL-16++ IL-18 CCL5 +					
	GER					7	4	4			CCXL8++ IL-16++ CCL5 +					
	CinA					18	6	13			IL-16 CXCL8+ IL-18+					
STRONG	MIT					17	7	11			IL-16+ CXCL8+++ IL-18++					

Red boxes correspond to a positive result and green boxes to a negative result. ROS production, glutathione depletion, NRF2 accumulation, DNA binding, cytokines quantifications and surface markers are considered positive as soon as the results are statistically different of the control. For the adapted SENS-IS, the number of genes induced is indicated. A result is positive for the IRR group if ≥ 12 genes are induced and for SENS-IS/ARE groups if ≥ 7 genes are modified

currently accepted as OECD test guidelines. Activated DC can present antigens to T cells and express co-stimulatory molecules, resulting in activation and proliferation of T cells (KE4). We have addressed the property of THP-1 cells pretreated with various chemicals to provoke CD4⁺ T cell proliferation using an original THP-1/T cells co-culture system. In this case, we are addressing the capacity of CS to modify THP-1 phenotype thus participating to the activation and proliferation of T cells. Our results showed that T cell proliferation in response to THP-1 cells activated by CS was readily quantified. An increase in T cell proliferation for all CS compared to their vehicle control and no significant proliferation after irritant treatment was measured. Interestingly,

the percentage of T-cell proliferation in response to nickel-treated THP-1 was very similar to the one found with nickel-treated monocyte-derived DC (Bechara et al. 2017) supporting the usefulness of THP-1 cells as a surrogate for DCs.

In summary, in THP-1 cells, the oxidative stress may lead to activation of signal transduction pathways such as the Nrf2 pathway leading to the transcription of cytokines and chemokines. These inflammatory proteins drive the maturation and activation of DCs and in this case THP-1 cells, allowing T cell proliferation.

Some chemicals with cysteine/lysine (CIN) and high cysteine (mainly MIT) reactivity were true activators of Nrf2 pathway in our model, whereas other chemicals such

as the lysine-reactive RESO showed an Nrf2-independent activation in vitro. Moreover, for pre- and pro-haptens such as EUG, FAR, and GER, a less metabolic capacity of THP-1 cells or another activation pathway than Nrf2 can be suspected. The combination of all the results using a hit-map allowed us to discriminate moderate and strong CS from the other molecules since for CinA and MIT, all the KE boxes were “positive” (red). For the other CSs, the KE4 box was “positive” and always associated with at least one other “positive” KE box (Table 1). This analysis using reference molecules clearly showed that the new test measuring KE4 is decisive for identifying a CS. Beyond any doubt, this new test needs to be improved with more chemicals and according a dose response curve to propose a relation between response and potency.

Conclusion

Experimental tests suggested that CS themselves could provide a danger signal, allowing the maturation of DCs (Ade et al. 2007; Casati et al. 2005). In the same way, Nrf2, which can be activated by chemical sensitizers (Ade et al. 2009) is one of the key players in contact allergy, integrating chemical sensitizer’s protein reactivity and translating this signal into a biological response in DCs (Kim et al. 2009). Likewise, THP-1 cells is known to be activated by a larger panel of CS confirming that this cell line is a useful tool for in vitro sensitization screening (Nukada et al. 2011). Considering these different parameters and since THP-1 cells can be used as surrogate cells for DC, our study aims to propose a strategy to categorize CS by combining multiple activation pathways within a same cell line, the THP-1.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest and also no sponsorship.

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