

A novel method to generate monocyte-derived dendritic cells during coculture with HaCaT facilitates detection of weak contact allergens in cosmetics

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Abstract The in vitro sensitization assay LCSA (Loose-fit Coculture-based Sensitization Assay) has proved reliable for the detection of contact sensitizers in the past. However, the coculture of human monocyte-derived dendritic cells (DCs) with primary human keratinocytes (KCs) in serum-free medium is relatively complex compared to other sensitization assays which use continuous cell lines. To facilitate high-throughput screening of chemicals, we replaced KCs with the HaCaT cell line under various culture conditions. Coculture of HaCaT with peripheral blood mononuclear cells in serum-supplemented medium leads to generation of CD1a⁺/CD1c⁺ DCs after addition of GM-CSF, IL-4, and TGF-β1 (as opposed to CD1a⁺/CD1c⁺ DCs which arise in the “classic” LCSA coculture). These cells resemble monocyte-derived DCs generated in monoculture, but, unlike those, they show a marked upregulation CD86 after treatment with contact allergens. All of the nine sensitizers

in this study were correctly identified by CD1a⁺/CD1c⁺ DCs in coculture with HaCaT. Among the substances were weak contact allergens such as propylparaben (which is false negative in the local lymph node assay in mice) and resorcinol (which was not detected by CD1a⁺/CD1c⁺ DCs in the “classic” LCSA). The level of CD86 upregulation on CD1a⁺/CD1c⁺ DCs was higher for most allergens compared to CD1a⁺/CD1c⁺ DCs, thus improving the assay’s discriminatory power. Three out of four non-sensitizers were also correctly assessed by the coculture assay. A false-positive reaction to caprylic (octanoic) acid confirms earlier results that some fatty acids are able to induce CD86 on DC in vitro. In conclusion, change of the LCSA protocol led to reduction of time and cost while even increasing the assay’s sensitivity and discriminatory power.

Keywords Dendritic cells · Keratinocytes · HaCaT · Sensitization assay · Coculture · Cosmetics

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Introduction

The sensitizing potency of a substance is an important factor for risk assessment, and its determination is mandatory under the European Union legislation “Registration, Evaluation, Authorisation and Restriction of Chemicals” (REACH). Concerning the massive amount of animals needed to comply with these regulations, alternatives for in vivo assays are sought after.

Several in vitro assays for contact sensitization have been developed (for overview: Mehling et al. 2012). One important group of in vitro sensitization assays focuses on dendritic cells (DCs), often represented by cell lines such as THP-1 in the human Cell Line Activation Test (h-CLAT; Ashikaga et al. 2006). We have chosen a different approach

using primary human keratinocytes (KCs) and human monocytes, which are differentiated to a KC/DC-related cell coculture: the Loose-fit Coculture-based Sensitization Assay (LCSA; Schreiner et al. 2008). Application of a coculture is favorable, as the presence of KCs increases sensitivity of DCs toward sensitizers—not only in monocyte-derived DCs (Schreiner et al. 2007) but also in THP-1 cells (Hennen et al. 2011). However, the isolation of primary human KCs adds more time and cost to an assay, both of which are undesired for high-throughput screening needed in the context of REACH. Therefore, it was our aim to replace primary human KCs with the keratinocyte cell line HaCaT in the LCSA.

Primary cells and continuous cell lines both have advantages and disadvantages for toxicity assay development. Primary cells are often criticized for potential inter-donor variability of results. Continuous cell lines are more readily available and do not require time-consuming isolation from tissues. They lack potential inter-donor variability, but other influences exist on the reproducibility of results. Continuous cell lines have a disposition to genetic instability and “evolve” in cell culture, leading to the existence of genetically diverse subtypes of a certain cell line in different laboratories (Hiorns et al. 2004). Genetic drift may lead to phenotypic drift: In some cell lines, experiments yielded divergent results with higher passage numbers (Briske-Anderson et al. 1997; Esquenet et al. 1997).

We aimed to use the advantages of both primary cells and cell lines while minimizing potential problems. Primary human monocytes were to be kept in the assay because blood as a human tissue is relatively easy to obtain, and we could show that monocyte-derived DCs express the assay’s readout parameter for sensitization (i.e., CD86) with low donor-to-donor variations (Sonnenburg et al. 2012). Primary human KCs were replaced with HaCaT to lower time and cost of the LCSA. We chose this cell line because genetic evolution of HaCaT cells has been studied extensively: They remain non-tumorigenic up to >300 passages, and despite growing numbers of chromosomal aberrations in later passages, no evidence for gross chromosomal imbalances was found (Boukamp et al. 1997).

In this study, 13 substances often used in cosmetics with known sensitizing potency (4 non-sensitizers and 9 sensitizers) were tested in different experimental setups using primary human KCs or HaCaT cells in the LCSA coculture. Since the “classic” setup of the LCSA cultures cells in serum-free medium, HaCaT cells were cocultured with DCs in both serum-free and serum-containing medium to exclude differences solely based on culture conditions.

Materials and methods

Reagents

The following test substances were used: 2,4-dinitrochlorobenzene (DNCB), p-phenylenediamine (PPD), resorcinol, methylparaben, ethylparaben, propylparaben (all: Sigma-Aldrich, Steinheim, Germany), isopropylparaben (TCI Deutschland GmbH, Eschborn, Germany), 2-mercaptobenzothiazole (MBT), monochlorobenzene (both: Fluka, Taufkirchen, Germany), coumarin (alternative name: 1-benzopyran-2-one), caprylic acid (alternative name: octanoic acid; both: Roth, Karlsruhe, Germany), glycerol (Amresco, Solon, Ohio), and salicylic acid (AppliChem, Darmstadt, Germany). Trinitrobenzenesulfonic acid (TNBS; Sigma-Aldrich) was used as a positive control.

Dimethylsulfoxide (DMSO; Sigma-Aldrich) was used as a vehicle for DNCB, MBT, and caprylic acid. Ethanol (Sigma-Aldrich) was used as a vehicle for coumarin, monochlorobenzene, and salicylic acid. Acetone (Fisher Scientific, Schwerte, Germany) was used as a vehicle for PPD. For the remaining substances, phosphate-buffered saline (PBS; Biochrom, Berlin, Germany) was used as a vehicle. Final concentrations of vehicles in cell culture medium did not exceed 0.1 %.

Interleukin-4 (IL-4), granulocyte macrophage colony-stimulating factor (GM-CSF), and transforming growth factor beta 1 (TGF- β 1) were purchased from eBioscience (San Diego, California). For flow cytometric analysis, cells were stained with PE-conjugated anti-CD86 (clone: 2331 (FUN-1), mouse IgG1, BD Biosciences, Heidelberg, Germany) or PE-conjugated isotype control (clone: MOPC-21, mouse IgG1, BD Biosciences), and 7-aminoactinomycin D (7-AAD; Cell Viability Solution, BD Biosciences). For some experiments, cells were stained with PerCP-conjugated anti-CD14 (clone: M ϕ P9), FITC-conjugated anti-HLA-DR (clone: G46-6), PE-conjugated anti-CD54 (clone: HA58; all: BD Biosciences), FITC-conjugated anti-CD1a (clone: HI149, Immunostep, Salamanca, Spain), FITC-conjugated anti-CD1c (clone: AD5-8E7, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and FITC-conjugated anti-CD11c (clone: BU15, AbD Serotech, Kidlington, UK). The respective isotypes were: FITC-conjugated mouse IgG1 (clone: X40), FITC-conjugated mouse IgG2a (clone: G155-178; both: BD Biosciences), and PerCP-conjugated IgG2b (clone: 133303, R&D Systems, Wiesbaden-Nordenstadt, Germany).

Isolation of human peripheral blood mononuclear cells (PBMCs)

PBMCs were enriched from fresh buffy coat preparations (Deutsches Rotes Kreuz, Berlin, Germany) by density centrifugation on Ficoll-Paque (Biochrom). PBMCs were cryopreserved in fetal calf serum (FCS; Lonza, Basel, Switzerland) with 10 % DMSO at a density of 4×10^7 cells/ml.

Isolation of primary human keratinocytes (KCs)

Human skin from anonymous donors was received as residual material from plastic surgery according to Declaration of Helsinki principles and after obtaining written informed consent. After 18 h of incubation in PBS containing 2 U/ml of dispase I (Roche, Mannheim, Germany) at 4 °C, epidermal sheets were stripped off the dermal layer and dissociated in PBS containing 0.25 % trypsin (Biochrom) and 0.01 % DNase (Roche) for 15 min at 37 °C. Single-cell suspension was obtained by passing through a 40- μ m cell strainer (BD Biosciences). KCs were washed with PBS and resuspended in serum-free keratinocyte growth medium-2 (KGM-2) supplemented with complete SupplementPack without CaCl_2 (Lonza), 100 U/ml penicillin, and 100 μ g/ml streptomycin (both: Biochrom). KCs were then seeded on culture flasks (Techno Plastic Products, Trasadingen, Switzerland) at a density of 5×10^5 cells/cm². KCs were cultured until confluence with medium changes on alternate days in serum-free KGM-2 at 37 °C under 5 % CO₂ and were passaged three times. KCs were harvested by trypsinization and cryopreserved as described above for PBMCs at a density of 2×10^6 cells/ml.

Cultivation of HaCaT

HaCaT were cultured in either serum-free KGM-2 or Dulbecco's modified Eagle's medium (DMEM; Biochrom) supplemented with 10 % FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C under 5 % CO₂.

HaCaT (passage number 30–40) were seeded on culture flasks (Techno Plastic Products) at a density of 5×10^5 cells/cm², cultured until confluence with medium changes on alternate days, and cryopreserved as described above for PBMCs at a density of 2×10^6 cells/ml.

Loose-fit coculture-based sensitization assay (LCSA)

The LCSA has been developed in our work group as an assay for the measurement of a substance's sensitizing and irritative potency. For details on the protocol, see earlier publications (Schreiner et al. 2008; Wanner et al. 2010). In this study, the LCSA was performed in three different setups: A) primary human KCs cocultured with PBMCs in

serum-free KGM-2; B) HaCaT cocultured with PBMCs in serum-free KGM-2; C) HaCaT cocultured with PBMCs in DMEM supplemented with 10 % FCS.

KCs or HaCaT were thawed at 37 °C, washed with PBS, and resuspended in either KGM-2 or DMEM. Cells were then seeded on 12-well plates (Techno Plastic Products) at a density of 6×10^4 cells/cm². When cells were adherent, wells were rinsed with PBS to remove dead cells. PBMCs were thawed and resuspended in either KGM-2 or DMEM, then seeded onto KCs at a density of 3×10^6 cells/cm². IL-4, GM-CSF, and TGF- β 1 were added to the coculture at final concentrations of 100, 100, and 10 ng/ml, respectively, to induce differentiation of monocytes to DC-related cells (DCrc). After 48 h, test substances were added. Increasing concentrations of test substances were tested until significant cytotoxicity occurred as measured by 7-AAD staining, or until precipitations were noted in cell culture medium.

Parabens were tested in two independent experiments in doubles with PBMCs from two different donors. Other substances were tested in three independent experiments in doubles with PBMCs from three different donors. TNBS at a concentration of 100 μ mol/l served as positive control. After another 48 h, floating cells were pipetted off the coculture and prepared for fluorescence-activated cell sorting (FACS) analysis.

FACS analysis

FACS analysis was performed on a FACS Calibur flow cytometer (BD Biosciences). Raw data were analyzed with Flowing Software 2.5.1 (Cell Imaging Core, Turku Centre for Biotechnology, Turku, Finland). Cells were gated to a distinct population of DCrc according to their scatter properties (for details, see Schreiner et al. (2008) and supplementary online material: Fig. S1). 7-AAD staining was used to exclude dead cells from calculations by gating to unstained cells only. Relative upregulation of CD86 was calculated by the following equation: geometric mean fluorescence intensity (MFI) of CD86 on substance-treated cells/MFI of CD86 on vehicle control-treated cells. Diagrams of dose–response relationships for relative CD86 expression and viability were prepared with GraphPad Prism software (GraphPad Software, San Diego, California). Results were assumed to be reliable when 100 μ mol/l TNBS caused at least a 1.5-fold increase in CD86 expression, otherwise experiments were excluded from data analysis. Hypothetical concentrations required to cause half-maximal CD86 expression (EC50_{sens}) allowed quantitative evaluation of substances' sensitizing potency. Similarly, the irritative potency of substances was assessed by 7-AAD staining (for details, see Wanner et al. 2010). The hypothetical concentration

which caused death of 50 % of the DCrc population as compared to vehicle controls was termed $EC_{50_{vit}}$. Both $EC_{50_{sens}}$ and $EC_{50_{vit}}$ were computed by linear interpolation between the two data points lying immediately below and above half-maximal CD86 expression or 50 % cell death of DCrc, respectively. If no peak or plateau could be detected in dose–response curves of relative CD86 expression, minimal $EC_{50_{sens}}$ values were computed using the highest measured CD86 expression as maximal CD86 expression. If significant cell death of DCrc occurred but did not reach 50 % as compared to vehicle controls, $EC_{50_{vit}}$ was computed using linear interpolation based on the two data points nearest to 50 %.

As defined in our earlier publications, the following categories for sensitizers were used: “extreme” ($EC_{50_{sens}} < 12.5 \mu\text{mol/l}$), “strong” ($EC_{50_{sens}} < 50 \mu\text{mol/l}$), “moderate” ($EC_{50_{sens}} < 100 \mu\text{mol/l}$), and “weak” ($EC_{50_{sens}} > 100 \mu\text{mol/l}$). Substances failing to elicit significant rise of CD86 expression at the highest concentration tested were deemed non-sensitizers. The following categories were defined for irritants: “irritative” ($EC_{50_{vit}} < 50 \mu\text{mol/l}$), “weakly irritative” ($EC_{50_{vit}} < 1000 \mu\text{mol/l}$), “not irritative” ($EC_{50_{vit}} > 1000 \mu\text{mol/l}$ or no significant reduction of vitality up to the highest concentration tested). For comparison with human data, sensitizing potency in humans was adopted from Basketter et al. (2014). The “Basketter classes” range from 1 (highest intrinsic skin sensitizing potency) to 6 (no evidence of contact allergy in humans). Monochlorobenzene is not covered in this publication but

does not yield any sensitizing potency in the local lymph node assay in mice (Ashby et al. 1995).

Results

Substances without evidence of contact allergy in humans (Basketter class 6)

Glycerol, monochlorobenzene, and salicylic acid were tested in ascending concentrations up to $1000 \mu\text{mol/l}$ in LCSA setup A (KC/PBMC coculture in serum-free KGM-2) and LCSA setup B (HaCaT/PBMC coculture in serum-free KGM-2). Neither significant upregulation of CD86 on DCrc nor significant reduction of DCrc viability was observed (Fig. 1). We then decided to test only the highest concentration ($1000 \mu\text{mol/l}$) of these three substances in LCSA setup C (HaCaT/PBMC in DMEM with 10 % FCS). As expected, no change in CD86 expression or DCrc viability was observed (Fig. 1).

In contrast, caprylic acid elicited linear upregulation of CD86 in all three LCSA setups up to 1.7-, 2.2-, and 1.4-fold in LCSA setups A, B, and C, respectively. Viability was not affected (Fig. 2a). Since no peak or plateau of CD86 upregulation could be shown in dose–response curves, only minimal $EC_{50_{sens}}$ values were computed using the highest measured CD86 upregulation instead of maximal CD86 upregulation. Thus, $EC_{50_{sens}}$ was $>252 \mu\text{mol/l}$ (setup A), $>669 \mu\text{mol/l}$ (setup B), and $>219 \mu\text{mol/l}$ (setup C). Sensitizing potency in vitro was determined as “weak” in all setups.

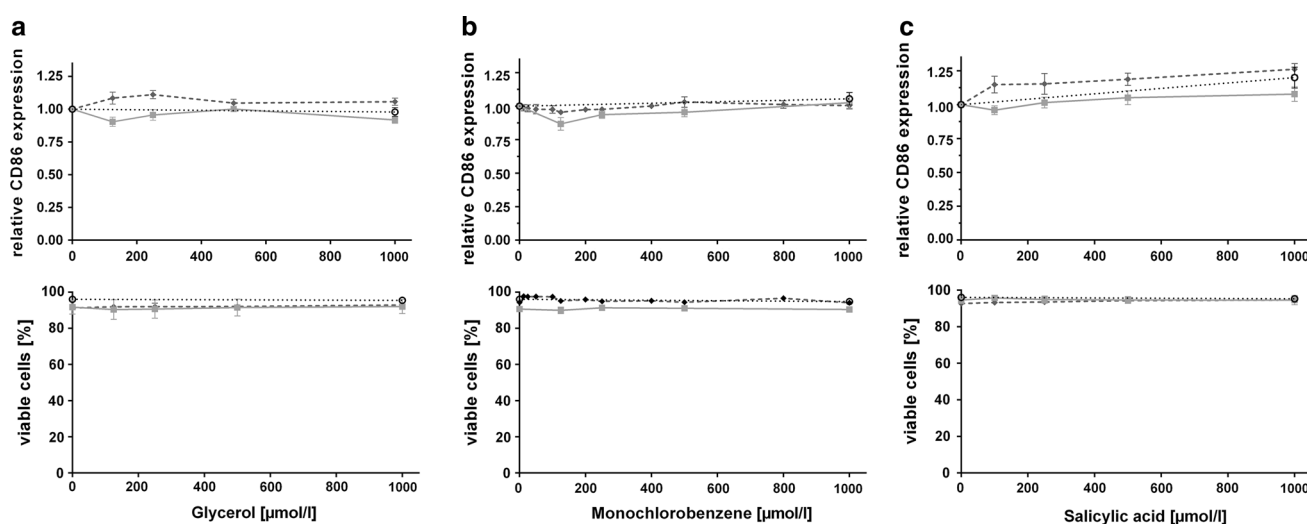


Fig. 1 Relative CD86 expression and viability (as determined by 7-AAD staining) of DCs after addition of increasing concentrations of glycerol (left), monochlorobenzene (middle), and salicylic acid (right) to cell culture medium. Solid line: coculture of $CD1a^+/CD1c^+$ DCs with primary human KCs in serum-free KGM-2 (“LCSA

setup A”); dashed line: coculture of $CD1a^+/CD1c^+$ DCs with HaCaT in serum-free KGM-2 (“LCSA setup B”); dotted line: coculture of $CD1a^+/CD1c^+$ DCs with HaCaT in DMEM supplemented with 10 % FCS (“LCSA setup C”). Each data point represents $n = 3 \pm \text{SEM}$

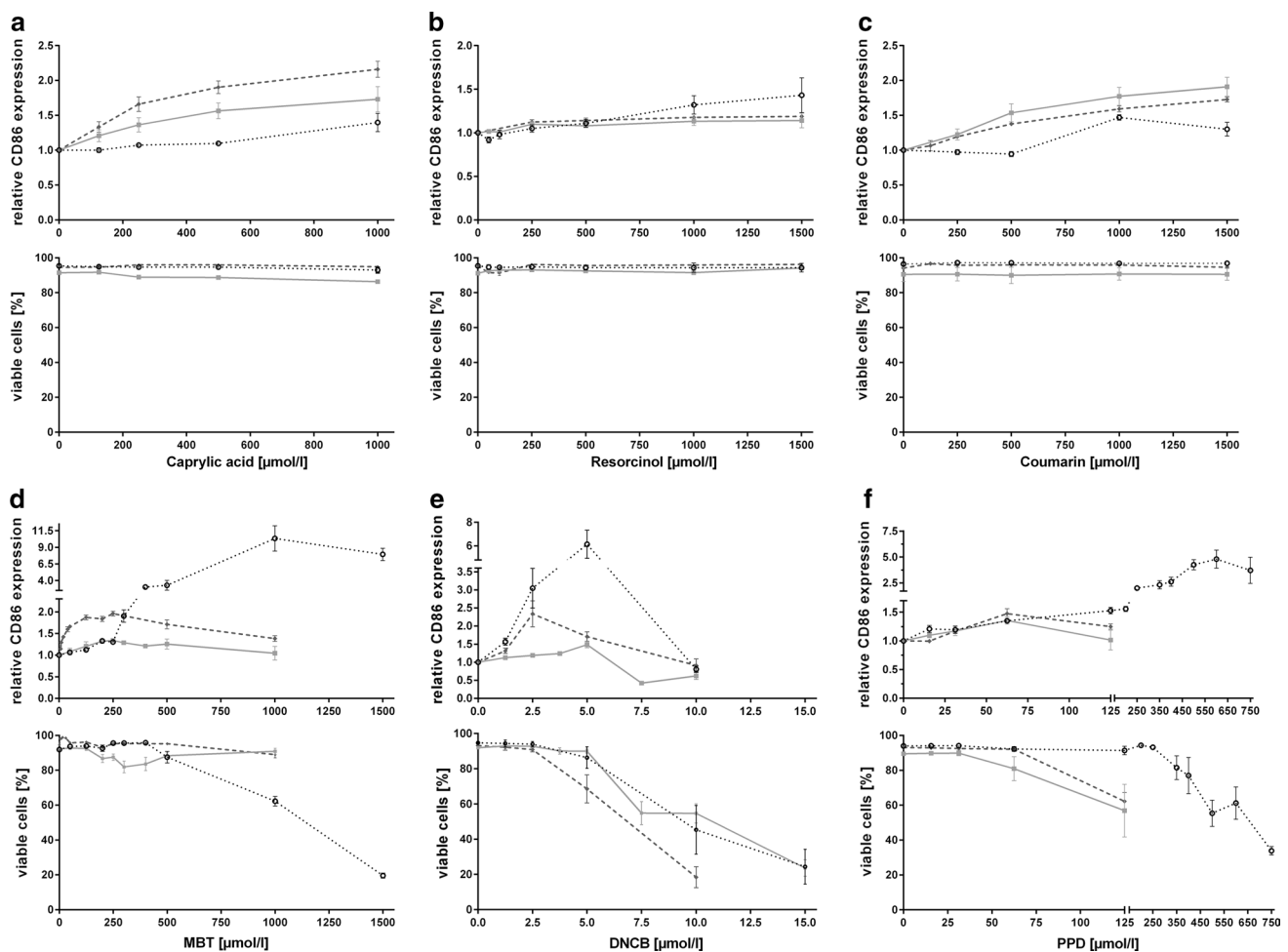


Fig. 2 Relative CD86 expression and viability (as determined by 7-AAD staining) of DCs after addition of increasing concentrations of caprylic acid (a), resorcinol (b), coumarin (c), MBT (d), DNCB (e), and PPD (f) to cell culture medium. *Solid line*: coculture of CD1a⁻/CD1c⁻ DCs with primary human KCs in serum-free KGM-2

(“LCSA setup A”); *dashed line*: coculture of CD1a⁻/CD1c⁻ DCs with HaCaT in serum-free KGM-2 (“LCSA setup B”); *dotted line*: coculture of CD1a⁺/CD1c⁺ DCs with HaCaT in DMEM supplemented with 10 % FCS (“LCSA setup C”). Each data point represents $n = 3 \pm \text{SEM}$

Substances needing a substantial degree of exposure to cause contact allergy in humans (Basketter classes 3 and 4)

Resorcinol (class 4) caused no significant upregulation of CD86 on DCrc in LCSA setups A and B (Fig. 2b). In LCSA setup C, relative upregulation of CD86 reached 1.4-fold after application of 1500 $\mu\text{mol/l}$ resorcinol. As no peak or plateau of CD86 upregulation could be shown in dose-response curves, $\text{EC}_{50\text{sens}}$ was computed as $>753 \mu\text{mol/l}$, which corresponds to a “weak” sensitizing potency. Viability of DCrc was not affected in all setups up to the highest concentration.

Coumarin (class 3) showed similar dose-response curves in all three LCSA setups (Fig. 2c). CD86 levels increased linearly without reaching a peak up to 1.9-fold (setup A) or 1.7-fold (setup B) after application of 1500 $\mu\text{mol/l}$

coumarin. $\text{EC}_{50\text{sens}}$ was computed as $>434 \mu\text{mol/l}$ (setup A) or $>482 \mu\text{mol/l}$ (setup B). In LCSA setup C, a peak of 1.5-fold CD86 upregulation was reached at 1000 $\mu\text{mol/l}$ and $\text{EC}_{50\text{sens}}$ was 777 $\mu\text{mol/l}$. This leads to classification of coumarin as a “weak” sensitizer in all three LCSA setups. Viability of DCrc was not affected in all setups up to the highest concentration.

Application of MBT (class 3) led to similar dose-response curves in LCSA setups A and B (Fig. 2d); maximal CD86 upregulation occurred at 250 $\mu\text{mol/l}$ and was 1.3-fold (setup A) or twofold (setup B) with $\text{EC}_{50\text{sens}}$ values of 103 $\mu\text{mol/l}$ (setup A) or 24.5 $\mu\text{mol/l}$ (setup B). Viability of DCrc was not affected in both setups. LCSA setup C yielded different results: Maximal CD86 expression occurred at 1000 $\mu\text{mol/l}$ and was markedly higher with a 10.3-fold upregulation compared to vehicle controls. Accordingly, $\text{EC}_{50\text{sens}}$ was higher with a concentration of

669 $\mu\text{mol/l}$. Different $\text{EC}_{50_{\text{sens}}}$ values led to deviations in sensitizing potency classification: In LCSA setups A and C, MBT would be classified as a “weak” sensitizer; in LCSA setup B, sensitizing potency would be “strong.” Viability of DCrc was not affected in LCSA setups A and B with concentrations tested up to 1000 $\mu\text{mol/l}$. However, in LCSA setup C significant cell death occurred at 1000 $\mu\text{mol/l}$ and vitality dropped below 50 % at 1500 $\mu\text{mol/l}$. $\text{EC}_{50_{\text{vit}}}$ was 1191 $\mu\text{mol/l}$, which would classify MBT as “not irritative.”

Substances with the highest intrinsic skin sensitizing potency in humans (Basketter class 1)

DNCB elicited CD86 upregulation on DCrc in all three setups (Fig. 2e). Maximal levels of relative upregulation were 1.5-, 2.3-fold, and sixfold in LCSA setups A, B, and C, respectively. Peak levels of CD86 expression were reached after application of 2.5 $\mu\text{mol/l}$ DNCB (setup B) or 5 $\mu\text{mol/l}$ (setup A and C). $\text{EC}_{50_{\text{sens}}}$ values were similar with 2.8, 1.7, and 2.6 $\mu\text{mol/l}$ for LCSA setups A, B, and C, respectively, all of which would lead to classification of DNCB as an “extreme” sensitizer. Dose–response relationships for DCrc viability were also similar in all three LCSA setups with $\text{EC}_{50_{\text{vit}}}$ values of 11.8, 7.2, and 9.8 $\mu\text{mol/l}$ in setups A, B, and C, respectively. Thus, DNCB would be labeled “irritative” in all setups.

PPD also elicited CD86 upregulation on DCrc in all three setups (Fig. 2f). Dose–response curves were similar in LCSA setups A and B with maximal relative upregulation of 1.4- and 1.5-fold, respectively, both occurring at 62.5 $\mu\text{mol/l}$. $\text{EC}_{50_{\text{sens}}}$ values were 44.1 $\mu\text{mol/l}$ (setup A) and 38.1 $\mu\text{mol/l}$ (setup B), both leading to classification of PPD as a “strong” allergen. DCrc viability was also similar in LCSA setups A and B with $\text{EC}_{50_{\text{vit}}}$ values of 157 and 158 $\mu\text{mol/l}$, respectively. In contrast, DCrc in LCSA setup C tolerated markedly higher concentrations of PPD, leading to an $\text{EC}_{50_{\text{vit}}}$ of 680 $\mu\text{mol/l}$. In all setups, PPD would be labeled “weakly irritative.” Maximal relative upregulation of CD86 was higher in setup C with 4.8-fold at 600 $\mu\text{mol/l}$. However, due to an $\text{EC}_{50_{\text{sens}}}$ value of 418 $\mu\text{mol/l}$, PPD would be classified as a “weak” allergen in setup C.

Characterization of DCs in LCSA setups B and C

The population of DCrc in setup C (coculture with HaCaT in DMEM with 10 % FCS) showed a higher granularity in scatter dot plots as compared to those in setups A and B (coculture with KCs or HaCaT in serum-free KGM-2). For further characterization of cells, several surface markers were stained: DCrc in setup C were CD11c^+ , HLA-DR^+ , CD14^- , CD54^- , and partially CD1a^+ and CD1c^+ (Fig. 3b). DCrc in setup B were CD11c^+ , HLA-DR^+ , CD14^- , CD54^- , CD1a^- , and CD1c^- (Fig. 3a). DCrc in

setup A have already been characterized in previous studies as CD11c^+ , HLA-DR^+ , CD14^- , CD1a^- , and CD1c^- (Schreiner et al. 2008). Thus, the presence of serum in cell culture medium in setup C gives rise to a different population of DCs, part of which express CD1a and CD1c. Both $\text{CD1a}^-/\text{CD1c}^-$ and $\text{CD1a}^+/\text{CD1c}^+$ cells in the “DCrc gate” are hereinafter referred to as “dendritic cells” (DCs).

Testing the sensitivity of $\text{CD1a}^+/\text{CD1c}^+$ DCs: substances with very low intrinsic ability to cause contact allergy in humans (Basketter class 5)

Parabens belong to the weakest known contact allergens in humans (Basketter et al. 2014). In a recent study, we have tested several parabens in the “classic” setup of the LCSA with $\text{CD1a}^-/\text{CD1c}^-$ DCs (i.e., setup A) and found a relationship between side chain length and sensitizing potency (Sonnenburg et al. 2015). We chose the four parabens with the weakest sensitizing potency (methyl-, ethyl-, propyl-, and isopropylparaben) to test the sensitivity of $\text{CD1a}^+/\text{CD1c}^+$ DCs which arise in serum-supplemented medium during coculture with HaCaT (i.e., setup C). For methyl- and ethylparaben, results were generally similar, leading to classification as “weak” contact allergens in both setups (supplementary online material: Fig. S2a-b). $\text{EC}_{50_{\text{sens}}}$ was 325 $\mu\text{mol/l}$ (setup A) or 765 $\mu\text{mol/l}$ (setup C) for methylparaben and 295 $\mu\text{mol/l}$ (setup A) or 300 $\mu\text{mol/l}$ (setup C) for ethylparaben. Propyl- and isopropylparaben elicited a stronger reaction in $\text{CD1a}^+/\text{CD1c}^+$ DCs (supplementary online material: Fig. S2c-d), which is reflected by classification as “moderate” in setup C and “weak” in setup A for both allergens. $\text{EC}_{50_{\text{sens}}}$ was 138 $\mu\text{mol/l}$ (setup A) or 67 $\mu\text{mol/l}$ (setup C) for propylparaben and 158 $\mu\text{mol/l}$ (setup A) or 72 $\mu\text{mol/l}$ (setup C) for isopropylparaben. Viability of $\text{CD1a}^+/\text{CD1c}^+$ DC was lower after treatment with high concentrations of parabens compared to $\text{CD1a}^-/\text{CD1c}^-$ DC, which leads to a lower $\text{EC}_{50_{\text{vit}}}$ for all four substances in setup C (Table 1). In one case, classification of irritative potency was affected: Ethylparaben would be labeled “not irritative” in setup A but “weakly irritative” in setup C.

Levels of CD86 upregulation are higher in $\text{CD1a}^+/\text{CD1c}^+$ DCs than in $\text{CD1a}^-/\text{CD1c}^-$ DCs for most allergens

While the hypothetical concentration needed to reach half-maximal relative CD86 upregulation is used for determination of sensitizing potency, the maximum of relative CD86 upregulation is important for determination whether a substance is a sensitizer at all. Only sensitizers which are able to elicit significant upregulation of CD86 on DCs can be recognized correctly. Therefore, we compared maximal

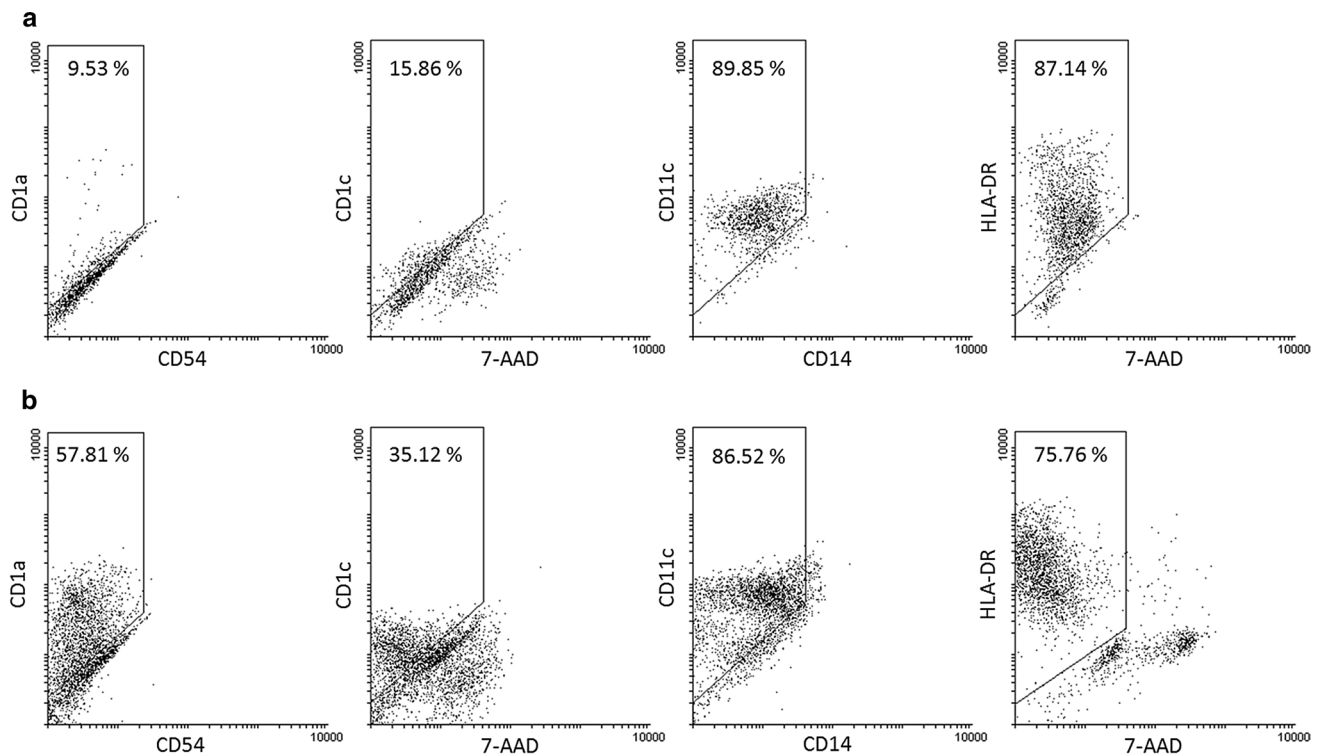


Fig. 3 Flow cytometric analysis of monocyte-derived DCs generated during coculture with HaCaT in serum-free KGM-2 (**a**) or DMEM supplemented with 10 % FCS (**b**). DCs were gated according to scatter properties as demonstrated in supplementary online material (Fig. S1). Dot plots of CD1a, CD1c, CD11c, HLA-DR, CD54, 7-AAD,

and CD14-staining are shown while gated to DCs. Percentages of positive DCs in each region are given. One representative experiment of two independent experiments with two different monocyte donors is shown

relative CD86 upregulation of CD1a⁺/CD1c⁺ DCs with CD1a⁺/CD1c⁺ DCs after treatment with sensitizers (Fig. 4). In 6 out of 9 contact allergens, maximal relative CD86 upregulation was higher on CD1a⁺/CD1c⁺ DCs. For some substances, maximal CD86 expression was even drastically enhanced on CD1a⁺/CD1c⁺ DCs (MBT 10.3-versus 1.3-fold, DNCB 6.1- versus 1.5-fold, and PPD 4.8-versus 1.4-fold).

Discussion

Presence of serum in culture medium leads to generation of CD1a⁺/CD1c⁺ DCs with higher sensitivity toward contact allergens

Monocyte-derived DCs generated during coculture in serum-free and serum-containing medium express different surface markers (Fig. 3) and show differences in CD86 expression after treatment with allergens (Fig. 4). DCs generated in serum-free medium were CD1a⁺/CD1c⁺ regardless if cocultured with primary human KCs or HaCaT.

In contrast, when DCs were cocultured with HaCaT in serum-containing medium, a heterogeneous population of cells developed which was partially CD1a⁺/CD1c⁺. This confirms that serum supplements are important in directing differentiation of monocytes to different DC subtypes. In particular, the presence of FCS is crucial for expression of CD1a (Duperrier et al. 2000). This DC population in coculture resembles “classic” monocyte-derived DCs (moDCs), which are differentiated during 6 days of monoculture and also consist of a heterogeneous population of cells which are partially CD1a⁺/CD1c⁺ (Grassi et al. 1998; Peiser et al. 2004). It is remarkable that “moDC-like” cells generated during coculture with HaCaT are much more sensitive toward contact allergens than their counterparts in monoculture: A concentration of 850 μmol/l TNBS was needed to induce approximately 1.5-fold upregulation of CD86 on moDCs in monoculture (Aeby et al. 2004); during coculture with HaCaT, only 100 μmol/l TNBS sufficed for a threefold upregulation of CD86 on CD1a⁺/CD1c⁺ DC (supplementary online material: Fig. S1). This increased sensitivity facilitated identification of the weak sensitizer resorcinol (see below).

Table 1 Comparison of sensitizing and irritative potency as determined by human data, the LLNA in mice, and the LCSA in three different setups (A = coculture of DCs with primary human KCs in serum-free KGM-2; B = coculture of DCs with HaCaT in serum-free KGM-2; C = coculture of DCs with HaCaT in DMEM supplemented with 10 % FCS); n/a = not available; n/d = not determinable

Substance	EC50 _{sens} (μmol/l) sensitizing potency			EC50 _{vit} (μmol/l) irritative potency			LLNA EC3 sensitizing potency ^b	Human sens. class ^b
	A	B	C	A	B	C		
DNCB	2.8 Extreme	1.7 Extreme	2.6 Extreme	11.8 Irritative	7.2 Irritative	9.8 Irritative	0.08 Extreme	1
PPD	44.1 Strong	38.1 Strong	418 Weak	157 Weak irr.	158 Weak irr.	680 Weak irr.	0.11 Strong	1
MBT	103 Weak	24.5 Strong	669 Weak	>1000 Not irrit.	>1000 Not irrit.	1191 Not irrit.	1.7 Moderate	3
Coumarin	>434 Weak	>482 Weak	777 Weak	>1000 Not irritative			30 Weak	3
Resorcinol	N/d Not sens.	N/d Not sens.	>753 Weak	>1000 Not irritative			6.3 Moderate	4
Methylparaben	325 ^a Weak	n/a	765 Weak	>2000 Not irrit.	n/a	1344 Not irrit.	n/d ^c Not sens.	n/a
Ethylparaben	295 ^a Weak	n/a	300 Weak	1601 Not irrit.	n/a	935 Weak irr.	n/a	n/a
Propylparaben	138 ^a Weak	n/a	67 Moder.	648 Weak irr.	n/a	342 Weak irr.	n/d Not sens.	5
Isopropylparaben	158 ^a Weak	n/a	72 Moder.	609 Weak irr.	n/a	351 Weak irr.	n/a	n/a
Glycerol	n/d Not sens.			>1000 Not irritative			n/d Not sens.	6
Monochlorobenzene	n/d Not sens.			>1000 Not irritative			n/d ^d Not sens.	n/a
Salicylic acid	n/d Not sens.			>1000 Not irritative			n/d Not sens.	6
Caprylic acid	>252 Weak	>669 Weak	>219 Weak	>1000 Not irritative			n/d Not sens.	6

^a Sonnenburg et al. (2015)

^b Unless otherwise noted, classification of sensitizing potency in humans and EC3 values were adopted from Basketter et al. (2014). Human sensitization classes range from 1 (highest intrinsic skin sensitizing potency) to 6 (no evidence of contact allergy in humans)

^c Gerberick et al. (2005)

^d Ashby et al. (1995)

For the majority of test substances, sensitizing potency as determined by different LCSA setups or the LLNA is in accordance with human data

The non-sensitizers glycerol, monochlorobenzene, and salicylic acid showed no sensitizing potency in the LCSA regardless of culture conditions. This coincides with historical data from the local lymph node assay (LLNA) in mice and human data (Table 1). The sensitizer DNCB which belongs to the strongest known contact allergens in humans yielded similar results in all three LCSA setups. Both LCSA and LLNA correctly identify the substance as “extreme” sensitizer. Coumarin is a contact allergen requiring “higher exposure for sensitization” in humans (Basketter et al. 2014). Accordingly, it was classified as “weak” in

all three LCSA setups and the LLNA. Minor inconsistencies were shown for MBT whose sensitizing potency in humans is comparable to coumarin. Sensitizing potency in humans is probably overestimated in LCSA setup B (“strong”) and in the LLNA (“moderate”), while EC50_{sens} values in LCSA setups A and C are in the same range as for coumarin so that MBT is likewise classified as a “weak” allergen.

Parabens are rare causes for contact allergy in humans (Thyssen et al. 2010), and their sensitizing potency in humans is presumably very weak (Basketter et al. 2014). LLNA data exist only for methyl- and propylparaben, and both were tested false negative. In contrast, the LCSA was able to detect all parabens in this study correctly. However, sensitizing potency might be overestimated in LCSA setup C for propyl- and isopropylparaben.

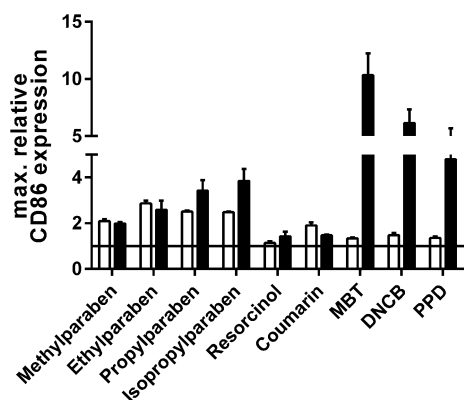


Fig. 4 Maximal relative CD86 expression was obtained from dose–response curves of each tested allergen for CD1a[−]/CD1c[−] DCs in LCSA setup A (open bars) and CD1a⁺/CD1c⁺ DCs in LCSA setup C (closed bars). Each column represents $n = 2 \pm \text{SEM}$ (for parabens) or $n = 3 \pm \text{SEM}$ (other substances)

The weak sensitizing potency of resorcinol in humans is inconsistently detected by in vivo and in vitro assays

Contact allergy to resorcinol is rare but well documented: In a multicenter study with eczema patients, 3 out of 2939 individuals (0.1 %) had positive patch test results (Søsted et al. 2013). For comparison, prevalence of PPD allergy was 45 times higher in the same study. Considering that both substances are equally common ingredients of current hair dyes (Hamann et al. 2014), this ratio might be a good approximation of the relation of these two substances' true sensitizing potency. Regarding the respective EC₃ values of PPD and resorcinol (0.11 and 6.3), the LLNA seems to reproduce this relationship well (Table 1). However, it must be noted that two out of three LLNA studies reported in the literature did not show a positive reaction to resorcinol, which was attributed to the vehicle used or a faulty negative control (Basketter et al. 2007). In our experiments with the LCSA, only setup C was able to detect the weak sensitizing potency of resorcinol. Other in vitro sensitization assays also showed inconsistent results. An integrated test strategy using three in vitro assays failed to detect resorcinol as a contact allergen (Urbisch et al. 2015). This was attributed to limited metabolic capacity in vitro as resorcinol is a putative pro-hapten which has to be metabolized in cell culture to form an immunogenic molecule. In the present study, replacement of KCs with HaCaT under the same culture conditions did not alter results significantly. In contrast, supplementation of cell culture medium with serum and formation of CD1a⁺/CD1c⁺ DCs led to significant upregulation of CD86 after application of resorcinol. If viewed together with the inconsistent LLNA results, limited metabolic capacity is presumably not the reason for failed detection of resorcinol in vitro—its sensitizing

potency might simply be too weak to be consistently detected by sensitization assays. Addition of serum to cell culture medium is crucial for detection of resorcinol. One explanation might be the presence of additional proteins which promotes formation of a complete allergen by chemical binding of the hapten. Considering the generally higher levels of CD86 upregulation on CD1a⁺/CD1c⁺ DC after treatment with sensitizers (Fig. 4), it seems more likely that serum supplementation leads to the development of more sensitive DCs with the ability to detect very weak contact allergens.

Differences in metabolic capacity of cocultures influence assessment of PPD

Peak CD86 expression and cytotoxic effects on DCs in LCSA setup C (coculture with HaCaT in serum-containing medium) were reached at approximately 10 times higher concentrations of PPD, as compared to the other two setups where primary KCs or HaCaT was cocultured in serum-free medium (Fig. 2f). This delayed reaction is most likely caused by a higher metabolic capacity of HaCaT cells. PPD is detoxified by N-acetyltransferase 1 (NAT1), which shows a fourfold higher activity in HaCaT than in primary human KCs (Bonifas et al. 2010a; Götz et al. 2012). The obvious difference in dose–response curves between cocultures with HaCaT in serum-free medium (i.e., setup B) and HaCaT in serum-containing medium (i.e., setup C) seems to contradict this hypothesis. However, it must be noted that NAT1 activity in the cited references was measured during culture of HaCaT in DMEM with FCS, which corresponds to the experimental conditions in setup C. We noticed slower proliferation of HaCaT in serum-free medium. As higher NAT1 activity in HaCaT is associated with increased cell proliferation (Bonifas et al. 2010b), we assume that NAT1 activity in setup C is higher than in the other two setups, which leads to underestimation of PPD's sensitizing potency.

The false-positive reaction to caprylic acid in the LCSA and other dendritic cell-based sensitization assays might be due to pro-inflammatory effects of medium-chain fatty acids

Caprylic acid showed a weak sensitizing potency in all LCSA setups. It was not possible to elicit contact sensitization to caprylic acid in a study with human volunteers (Opdyke 1981), and no case reports on contact allergy to this substance exist, so it has to be regarded as false positive in the LCSA. Interestingly, caprylic acid has been tested false positive in two other DC-based sensitization assays: the h-CLAT and the U937 test [supplemental material in: Urbisch et al. (2015)]. This leads to the assumption

that activation of DCs in the LCSA by caprylic acid is not a random effect.

Caprylic acid is able to elicit irritant contact dermatitis in human volunteers (Robinson et al. 1999), while the LCSA did not detect any irritative potency (Table 1). For the purpose of in vitro testing, it is often assumed that irritants have cytotoxic effects on skin cells, hence triggering release of pro-inflammatory cytokines (Gibbs 2009). Cell viability is the most important parameter for current in vitro irritation assays and the sole parameter for the only OECD adopted in vitro irritation test guideline (OECD 2013). The LCSA itself makes use of DC viability to measure a substance's irritative potency. We hypothesize that irritant contact dermatitis caused by caprylic acid is due to direct activation of DCs rather than cytotoxic effects on skin cells. This has implications for both sensitization and irritation assays in vitro: Irritation assays without a DC endpoint are prone to false-negative results when testing “dendritic cell activators”; sensitization assays based on DCs are prone to false-positive results when testing these substances. In a previous study, we have already found numerous fatty acids which were falsely recognized as sensitizers in the LCSA (Frohwein et al. 2015). Special care should be taken when interpreting results from fatty acids in DC-based sensitization assays because substances in this class seem to be frequent activators of DCs in vitro.

These findings underline the need for T cell-based sensitization assays as part of integrated test strategies because a T cell-specific response toward putative sensitizers might eliminate false-positive results, e.g., to fatty acids. Furthermore, irritant and allergic contact dermatitis seems to be more intertwined than previously thought. The separation between irritation and sensitization assays might prove disadvantageous in the future. The LCSA is already able to measure irritative and sensitizing potency simultaneously. Further implementation of a T cell response is needed to improve accuracy of this assay. The fact that T cells—as a fraction of PBMCs—are already present in LCSA cocultures facilitates addition of a T cell readout parameter and might be an advantage over other in vitro assays (a study about an extended LCSA with T cell readout is currently being undertaken).

Primary human KCs in the LCSA coculture can be replaced by HaCaT without compromising assay performance, which decreases costs and amount of work

In summary, the primary aim of this study was accomplished by showing that human KCs in the LCSA coculture can be replaced by HaCaT cells. For the first time, a coculture of HaCaT and monocyte-derived DC was used for in vitro sensitization testing. Moreover, it could be

shown that the presence or absence of serum in cell culture medium makes a crucial difference by directing differentiation of monocytes to one of two different populations of DCs in coculture. Both populations are able to detect contact allergens when cocultured with HaCaT cells. However, increase of CD86 expression after application of allergens is markedly higher in the partially CD1a⁺/CD1c⁺ population that is formed in the presence of serum, which improves the discriminatory power of the assay. Accordingly, the weak sensitizing potency of resorcinol could only be detected in experimental setup C.

For screening of chemicals, HaCaT cocultures in serum-containing medium (i.e., setup C) should be used because they possess a better discriminatory power and a higher sensitivity for contact allergens than the other two setups. However, the case of PPD exemplifies that differences in metabolic capacity between HaCaT and primary human KCs can influence results. The coculture with primary human KCs (i.e., setup A) is comparatively complex, but its metabolic capacity is presumably closest to human skin in vivo. It remains useful for fundamental research on contact allergy.

We encourage screening for “dendritic cell activators” and their avoidance in cosmetic products together with putative contact allergens

The medium-chain fatty acid caprylic acid (octanoic acid) is a common ingredient of cosmetic products. Its capacity to induce the costimulatory molecule CD86 on DCs might facilitate sensitization to weak allergens that would otherwise pose little risk for consumers. In a mouse model, the related fatty acid nonanoic acid facilitated the development of contact sensitization, which led to the hypothesis that certain fatty acids might act as “allergy enhancers” in vivo without being haptens themselves (Yamashita et al. 2013).

Therefore, we encourage screening of cosmetic ingredients—especially fatty acids—in DC-based sensitization assays even if a sensitizing potency has already been ruled out by in vivo assays or tests with human volunteers. By this means, “dendritic cell activators” can be detected and avoided in cosmetic formulations containing possible contact allergens.

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Compliance with ethical standards

Conflict of interest The LCSA is patented in Germany with the application number DE102007006736B4 (patent holder: Mrs. Dagmar Briechele). The authors have no financial relationship with the patent holder and declare no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

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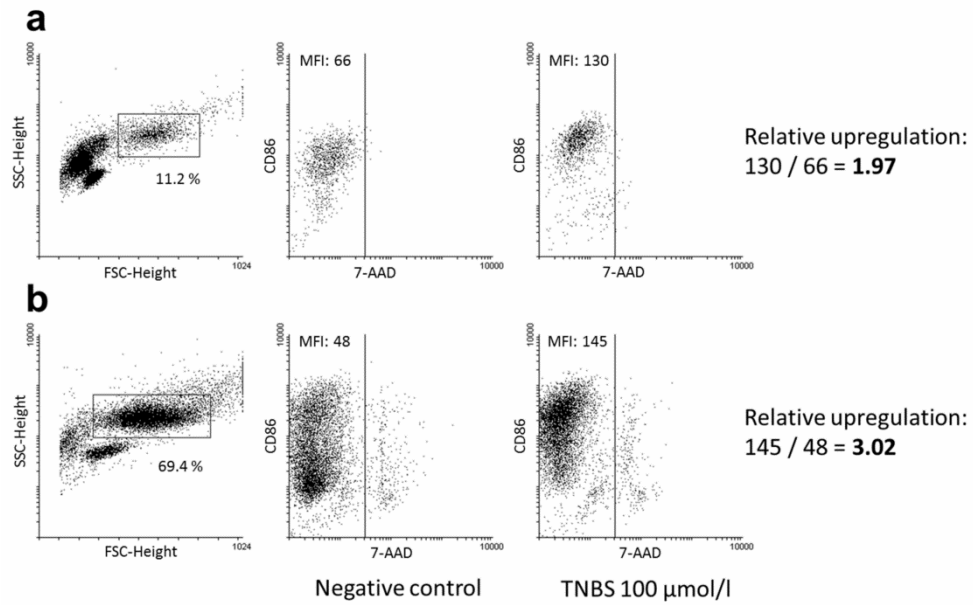


Fig. S1 Left: gating strategies according to scatter properties are shown for CD1a⁻/CD1c⁻ DCs (a) and CD1a⁺/CD1c⁺ DCs (b). Middle and right: an example for calculation of relative CD86 upregulation is demonstrated for the positive control TNBS. Note that only MFIs of 7-AAD negative DCs (i.e. viable DCs) in the left half of dot plots are used for calculation of relative CD86 upregulation

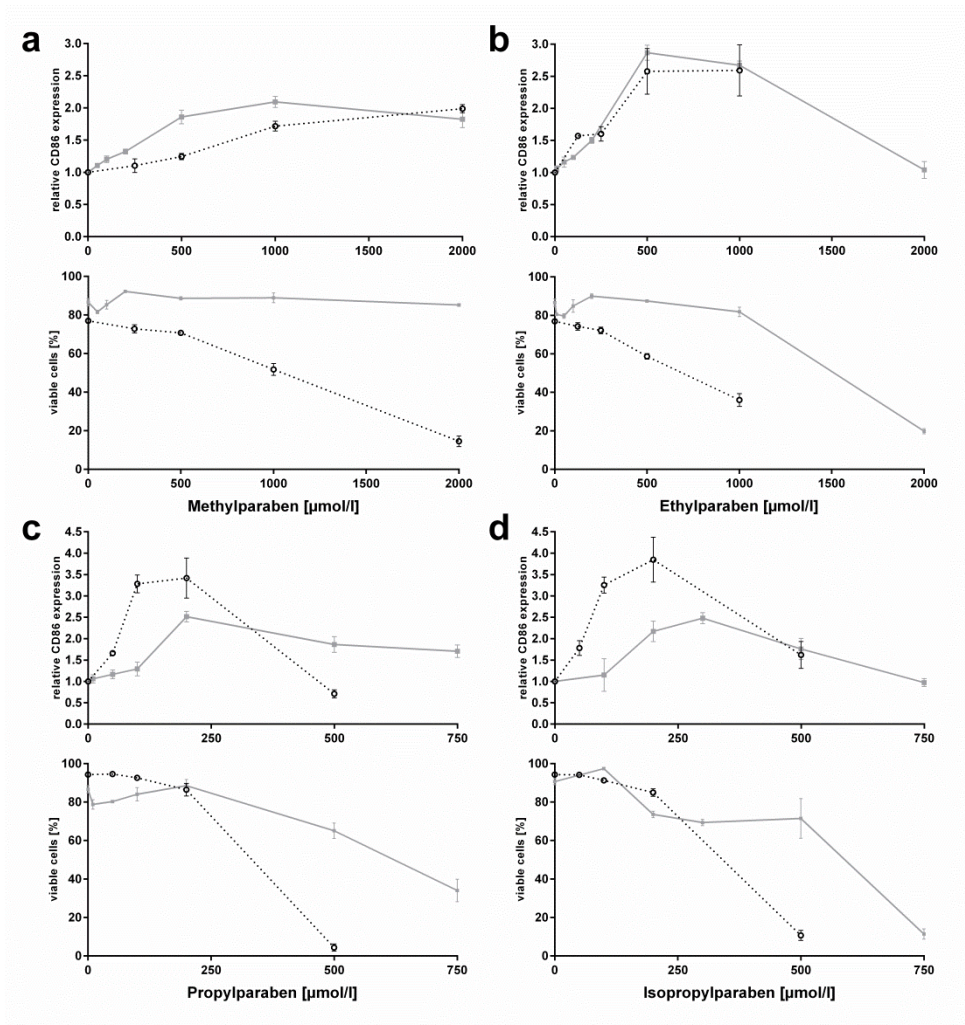


Fig. S2 Relative CD86 expression and viability (as determined by 7-AAD staining) of DCs after addition of increasing concentrations of methylparaben (a), ethylparaben (b), propylparaben (c), and isopropylparaben (d) to cell culture medium. Solid line: coculture of CD1a⁻/CD1c⁻ DCs with primary human KCs in serum-free KGM-2 ("LCSA-setup A"); dotted line: coculture of CD1a⁺/CD1c⁺ DCs with HaCaT in DMEM supplemented with 10 % FCS ("LCSA-setup C"). Each data point represents $n = 2 \pm \text{SEM}$