

# Unsaturated compounds induce up-regulation of CD86 on dendritic cells in the in vitro sensitization assay LCSA

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**Abstract** Unsaturated compounds are known to cause false-positive reactions in the local lymph node assay (LLNA) but not in the guinea pig maximization test. We have tested a panel of substances (succinic acid, undecylenic acid, 1-octyn-3-ol, fumaric acid, maleic acid, linoleic acid, oleic acid, alpha-linolenic acid, squalene, and arachidonic acid) in the loose-fit coculture-based sensitization assay (LCSA) to evaluate whether unspecific activation of dendritic cells is a confounder for sensitization testing in vitro. Eight out of 10 tested substances caused significant up-regulation of CD86 on dendritic cells cocultured with keratinocytes and would have been classified as sensitizers; only succinic acid was tested negative, and squalene had to be excluded from data analysis due to poor solubility in cell culture medium. Based on human data, only undecylenic acid can be considered a true sensitizer. The true sensitizing potential of 1-octyn-3-ol is uncertain. Fumaric acid and its isomer maleic acid are not known as sensitizers, but their esters are contact allergens. A group of 18- to 20-carbon chain unsaturated fatty acids (linoleic acid, oleic acid, alpha-linolenic acid, and arachidonic acid) elicited the strongest reaction in vitro. This is possibly due to the formation of pro-inflammatory lipid mediators in the cell culture causing nonspecific activation of dendritic

cells. In conclusion, both the LLNA and the LCSA seem to provide false-positive results for unsaturated fatty acids. The inclusion of T cells in dendritic cell-based in vitro sensitization assays may help to eliminate false-positive results due to nonspecific dendritic cell activation. This would lead to more accurate prediction of sensitizers, which is paramount for consumer health protection and occupational safety.

**Keywords** Sensitization assay · Regulative toxicology · Dendritic cells · Keratinocytes · Coculture

## Introduction

The European Union legislation “Registration, Evaluation, Authorisation and Restriction of Chemicals” (REACH) requires testing of chemicals for their sensitizing potential. The local lymph node assay (LLNA) in mice is accepted as “first-choice method” by European regulatory bodies (Angers-Loustau et al. 2011). Although many alternative (non-animal) test assays are under evaluation by the European Union Reference Laboratory for alternatives to animal testing, none has yet been approved as a replacement for the LLNA [for an overview of alternative methods, see: Adler et al. (2011) and Mehling et al. (2012)].

The general consensus is that no single assay will be suitable to detect a chemical’s sensitizing potential but rather a combination of several assays (Veen et al. 2014). Even though the LLNA is validated as a stand-alone assay for sensitization testing, there are several drawbacks to it such as false positives, e.g., irritants, and false negatives, e.g., metal allergens (Ikarashi et al. 1992, 1993; Montelius et al. 1994; Mandervelt et al. 1997; Montelius et al. 1998; Vohr and Ahr 2005; Basketter et al. 2009; Ball et al. 2011). Interesting results were shown for an array of mostly

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endogenous unsaturated compounds where six out of nine tested substances would have been labeled as false positives in the LLNA (Kreiling et al. 2008). The development of alternative in vitro methods to the LLNA is not only an issue of animal welfare but also an opportunity to improve sensitivity and specificity of predictive testing for sensitizing potential.

For in vitro sensitization testing, we chose the same panel of lipid compounds (eight unsaturated and one saturated lipid compounds) which had been tested by Kreiling et al. (2008) in the LLNA and guinea pig maximization assay (GPMT). We used a combined in vitro assay for sensitizing and irritative potential which had been developed by our working group in the past: the loose-fit coculture-based sensitization assay [LCSA; Schreiner et al. (2007)]. This assay is based on a monolayer of human primary keratinocytes on which human monocytes (purified or as part of peripheral blood mononuclear cells) are seeded and generated into a class of dendritic cells we termed dendritic cell-related cells. Application of sensitizers to this coculture leads to up-regulation of CD86 on dendritic cells. Earlier studies have shown that the LCSA already proved the capacity to discriminate between sensitizers and irritants (Wanner et al. 2010), the latter often tested false positive in the LLNA. Metal allergens, often unrecognized using standard LLNA protocols, are also detected by our in vitro assay (Schreiner et al. 2008). In this publication, in vitro sensitization data from the above-mentioned lipid compounds are compared to the in vivo data published by Kreiling et al. (2008).

## Materials and methods

### Chemicals

Compounds for testing were chosen in accordance with Kreiling et al. (2008). When identical preparations were not available, substances were obtained from different suppliers. Additionally, arachidonic acid was tested. Eight out of 10 chemicals are endogenous substances abundant in the human body, food, and consumer products. Two chemicals (1-octyn-3-ol and undecylenic acid) are widely used in cosmetics. Table 1 lists all the reagents used with their respective chemical formulae, manufacturers, purity, and significant features.

### Cryopreservation of human keratinocytes (KCs)

Human skin from anonymous donors was received as residual material from plastic surgery. After 16–18 h of incubation in phosphate-buffered saline (PBS, without calcium/

magnesium) containing 2  $\mu$ /ml of dispase I (Roche, Mannheim, Germany) at 4 °C, epidermal sheets were stripped off the dermal layer. Epidermis was then dissociated in PBS with 40 % TrypLE Express (Invitrogen, Carlsbad, USA) containing 0.1 ng/ml DNase (Roche) for 1 h at 37 °C. Single-cell suspension was obtained by passaging through a 40- $\mu$ m cell strainer (BD Biosciences, Heidelberg, Germany). Cells were washed with PBS and resuspended in serum-free keratinocyte growth medium-2 (KGM-2, PromoCell, Heidelberg, Germany) supplemented with complete SupplementPack without  $\text{CaCl}_2$  (PromoCell), 100  $\mu$ /ml penicillin, and 100  $\mu$ g/ml streptomycin (both Biochrom, Berlin, Germany). Keratinocytes (KCs) were then seeded on Costar Cell Culture Flasks (Corning, Shipl- Rijk, the Netherlands) at a density of  $2\text{--}5 \times 10^5$  cells/cm<sup>2</sup>. Cells were cultured until confluence with medium changes on alternate days. KCs were harvested by trypsinization and cryopreserved in fetal calf serum (FCS, Biochrom) with 10 % dimethyl sulfoxide (DMSO Hybrimax, Sigma-Aldrich) at a density of  $1.3\text{--}2 \times 10^6$  cells/ml.

### Cryopreservation of human peripheral blood mononuclear cells (PBMCs)

In this study, PBMCs were used as source of monocytes. PBMCs were enriched from fresh buffy coat preparations (Deutsches Rotes Kreuz, Berlin, Germany) by density centrifugation on Ficoll-Paque (Biochrom). PBMCs were cryopreserved as described above for KCs at a density of  $3.3\text{--}4 \times 10^7$  cells/ml.

### Sensitization assay (LCSA)

KCs were thawed at 37 °C, washed with PBS, and resuspended in KGM-2. KCs were seeded in 12-well plates (Costar Cell Culture Cluster, Corning) at a density of  $2\text{--}6 \times 10^4$  cells/cm<sup>2</sup>. When cells were adherent, wells were rinsed with PBS to remove dead cells. PBMCs were thawed and resuspended in KGM-2, and then seeded onto KCs at a density of  $1\text{--}3 \times 10^6$  cells/cm<sup>2</sup>. Interleukin-4 (IL-4, ImmunoTools Friesoythe, Germany), granulocyte macrophage colony-stimulating factor (GM-CSF, ImmunoTools), and transforming growth factor- $\beta$  (TGF- $\beta$ , R&D Systems, Minneapolis, USA) were added to the coculture at final concentrations of 100, 100, and 10 ng/ml, respectively. After 48 h of incubation, test substances were added. Increasing concentrations of test substances were tested until significant cytotoxicity occurred as measured by 7-aminoactinomycin D (7-AAD, Cell Viability Solution, BD Biosciences) staining. Each substance was tested with 2–4 different PBMC/KC donor pairs in duplicates. The strong contact allergen 2,4,6-trinitrobenzenesulfonic acid (TNBS, Sigma-Aldrich,

**Table 1** Substances used in the present study with their respective properties

| Substance (purity)                       | CAS no.  | Formula           | Manufacturer                       | Cat. no.    | Biological and/or industrial significance                                                | Immunomodulatory properties                                                                                                      |
|------------------------------------------|----------|-------------------|------------------------------------|-------------|------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------|
| Arachidonic acid ( $\geq 99\%$ )         | 506-32-1 | $C_{20}H_{32}O_2$ | Sigma-Aldrich (Steinheim, Germany) | A3555-10MG  | Component of cell membranes                                                              | Precursor of multiple pro- and anti-inflammatory lipid mediators (e.g., prostaglandins, leukotrienes)                            |
| Linoleic acid ( $\geq 99\%$ )            | 60-33-3  | $C_{18}H_{32}O_2$ | Sigma-Aldrich                      | L1012       | Essential fatty acid in humans                                                           | Arachidonic acid precursor                                                                                                       |
| $\alpha$ -Linolenic acid ( $\geq 98\%$ ) | 463-40-1 | $C_{18}H_{30}O_2$ | Cayman chemical (Ann Arbor, USA)   | 90,210      | Essential fatty acid in humans                                                           | Precursor of docosahexaenoic acid <sup>a</sup> , which can be converted to mostly anti-inflammatory lipid mediators <sup>b</sup> |
| Oleic acid ( $\geq 99\%$ )               | 112-80-1 | $C_{18}H_{34}O_2$ | Sigma-Aldrich                      | O1383       | Abundant in the human body (e.g., adipose tissue)                                        | Controversial, possibly anti-inflammatory <sup>c</sup>                                                                           |
| Squalene ( $\geq 98\%$ )                 | 111-02-4 | $C_{30}H_{50}$    | Sigma-Aldrich                      | S3626       | Precursor of steroids; moisturizer in cosmetics; vaccine adjuvant (MF59)                 | Differentiation of monocytes to dendritic cell phenotypes <sup>d</sup>                                                           |
| Undecylenic acid (98 %)                  | 112-38-9 | $C_{11}H_{20}O_2$ | Sigma-Aldrich                      | 124,672     | Topical fungicide; cosmetic ingredient                                                   | Two case reports of allergic contact dermatitis <sup>e,f</sup>                                                                   |
| 1-Octyn-3-ol ( $\geq 98\%$ )             | 818-72-4 | $C_8H_{14}O$      | TCI (Eschborn, Germany)            | O0196       | Flavor and fragrance ingredient                                                          | No data                                                                                                                          |
| Succinic acid ( $\geq 99\%$ )            | 110-15-6 | $C_4H_6O_4$       | Sigma-Aldrich                      | S9512       | Part of citric acid cycle                                                                | Pro-inflammatory mediator <sup>g</sup> ; immediate-type hypersensitivity reactions known <sup>h,i</sup>                          |
| Fumaric acid ( $\geq 99\%$ )             | 110-17-8 | $C_4H_4O_4$       | Sigma-Aldrich                      | 240,745-5G  | Part of citric acid cycle; esters used for treatment in psoriasis and multiple sclerosis | Immunomodulatory action not fully understood <sup>j</sup> ; allergic contact dermatitis to esters was observed <sup>k,l</sup>    |
| Maleic acid ( $\geq 99\%$ )              | 110-16-7 | $C_4H_4O_4$       | Sigma-Aldrich                      | 63,180-100G | Isomer of fumaric acid                                                                   | One case report of allergic contact dermatitis to maleic acid ester <sup>m</sup>                                                 |

<sup>a</sup> Barceló-Coblijn and Murphy (2009), <sup>b</sup> Serhan (2007), <sup>c</sup> Carrillo et al. (2012), <sup>d</sup> Seubert et al. (2008), <sup>e</sup> Gelbfarb and Leider (1960), <sup>f</sup> Anguita et al. (2002), <sup>g</sup> Tamahill et al. (2013), <sup>h</sup> Caimmi et al. (2008), <sup>i</sup> Walker et al. (2011), <sup>j</sup> Gold et al. (2012), <sup>k</sup> Lammintausta et al. (2010a), <sup>l</sup> Lammintausta et al. (2010b), <sup>m</sup> Hansson and Thörneby-Andersson (2003)

Steinheim, Germany) at a concentration of 100  $\mu\text{mol/l}$  served as positive control. As all test substances were dissolved in DMSO, the highest total concentration of DMSO added to an individual well during each set of experiments served as negative control (final concentrations in cell culture medium did not exceed 0,1 %). After 48 h of incubation with the test substances, floating cells were pipetted off the coculture and prepared for fluorescence-activated cell sorting (FACS) analysis.

### FACS analysis

For FACS analysis, cells were stained with PE-conjugated anti-CD86 (clone: 2331 (FUN-1), mouse IgG1, BD Biosciences), PE-conjugated isotype control (clone: MOPC-21, mouse IgG1, BD Biosciences), and 7-AAD. Analysis was performed on a FACSCalibur flow cytometer (BD Biosciences). Cells were gated to a distinct population of dendritic cells according to their scatter properties [for details, see: Schreiner et al. (2007)]. The experiments were assumed to be reliable, when the addition of TNBS led to an at least 1.5-fold increase in CD86 expression compared to negative controls. Relative up-regulation of CD86 expression was calculated by the following equation: mean fluorescence intensity (MFI) of CD86 on treated cells/MFI of CD86 of vehicle control. 7-AAD staining was used to determine and exclude dead cells from the calculation. Dose–effect relationships for relative CD86 expression and viability were determined using Flowing Software 2.4.0 (Cell Imaging Core, Turku Centre for Biotechnology, Turku, Finland).

Strength of sensitizing potential in the LCSA is measured by the estimated concentration of a test substance that leads to half-maximal increase in CD86 expression ( $\text{EC}_{50_{\text{sens}}}$ ). We defined the following categories for sensitizers: “extreme” ( $<12.5 \mu\text{mol/l}$ ), “strong” ( $<50 \mu\text{mol/l}$ ), “moderate” ( $<100 \mu\text{mol/l}$ ), and “weak” ( $>100 \mu\text{mol/l}$ ). Similarly, categorization of irritative potential is based on the concentration that results in 50 % cell death ( $\text{EC}_{50_{\text{vit}}}$ ) of dendritic cells as compared to vehicle controls. Categories for irritants are: “irritative” ( $<50 \mu\text{mol/l}$ ), “weakly irritative” ( $<1000 \mu\text{mol/l}$ ), “not irritative” ( $>1000 \mu\text{mol/l}$ ). EC values were calculated based on concentration curves of test substances [for details, see Wanner et al. (2010)].

### Results

Squalene did not lead to a significant increase in CD86 on dendritic cells at concentrations up to 200  $\mu\text{mol/l}$  (not shown). However, we decided to exclude squalene from data analysis as we observed precipitations in the

aqueous cell culture medium with concentrations as low as 2  $\mu\text{mol/l}$ .

According to the concentrations needed to up-regulate CD86 on dendritic cells and decrease the viability of dendritic cells, we classified the substances into three categories: late (or no) up-regulation and low cytotoxicity (Fig. 1a), an intermediate group (Fig. 1b), and early up-regulation and high cytotoxicity (Fig. 2). Except for succinic acid, all substances elicited a significant up-regulation of CD86 and would have been classified as sensitizers in the LCSA.

Oleic acid,  $\alpha$ -linolenic acid, and linoleic acid would even have been classified as extreme sensitizers according to an  $\text{EC}_{50_{\text{sens}}}$  value  $<12.5 \mu\text{mol/l}$  (Fig. 3). As  $\alpha$ -linolenic acid and linoleic acid are precursors of eicosanoids, which are the source of a plethora of pro-inflammatory mediators, we also tested the eicosanoid precursor arachidonic acid in the LCSA: Up-regulation of CD86 was similarly strong compared to the other three fatty acids (Fig. 2).

Strength of irritative potential correlated with strength of sensitizing potential for all substances (Fig. 3). Oleic acid,  $\alpha$ -linolenic acid, linoleic acid, and arachidonic acid yielded  $\text{EC}_{50_{\text{vit}}}$  values  $<50 \mu\text{mol/l}$  and were thus classified as “irritative.” Undecylenic acid was classified as “weakly irritative” with an  $\text{EC}_{50_{\text{vit}}}$  value of 851  $\mu\text{mol/l}$ . All other substances were rated “not irritative” according to their  $\text{EC}_{50_{\text{vit}}}$  values  $>1000 \mu\text{mol/l}$ .

### Discussion

Similar to the in vivo data collected by Kreiling et al. (2008), we found an unexpected high number of lipids which would have been classified as sensitizers in the in vitro sensitization assay LCSA. A comparison of in vivo and in vitro results is shown in Table 2.

#### Undecylenic acid is most likely a weak sensitizer based on human data, LCSA, LLNA, and GPMT

The topical fungicide and cosmetic ingredient undecylenic acid is the only substance which was tested positive as a sensitizer in all three assays. Moreover, two anecdotal reports exist on contact allergies to undecylenic acid in humans (Gelbfarb and Leider 1960; Anguita et al. 2002). So, this substance is most likely properly defined as a sensitizing substance.

#### Data for 1-octyn-3-ol are inconclusive

1-Octyn-3-ol is widely used in cosmetics. In contrast to its widespread use, no reports on contact allergies in humans

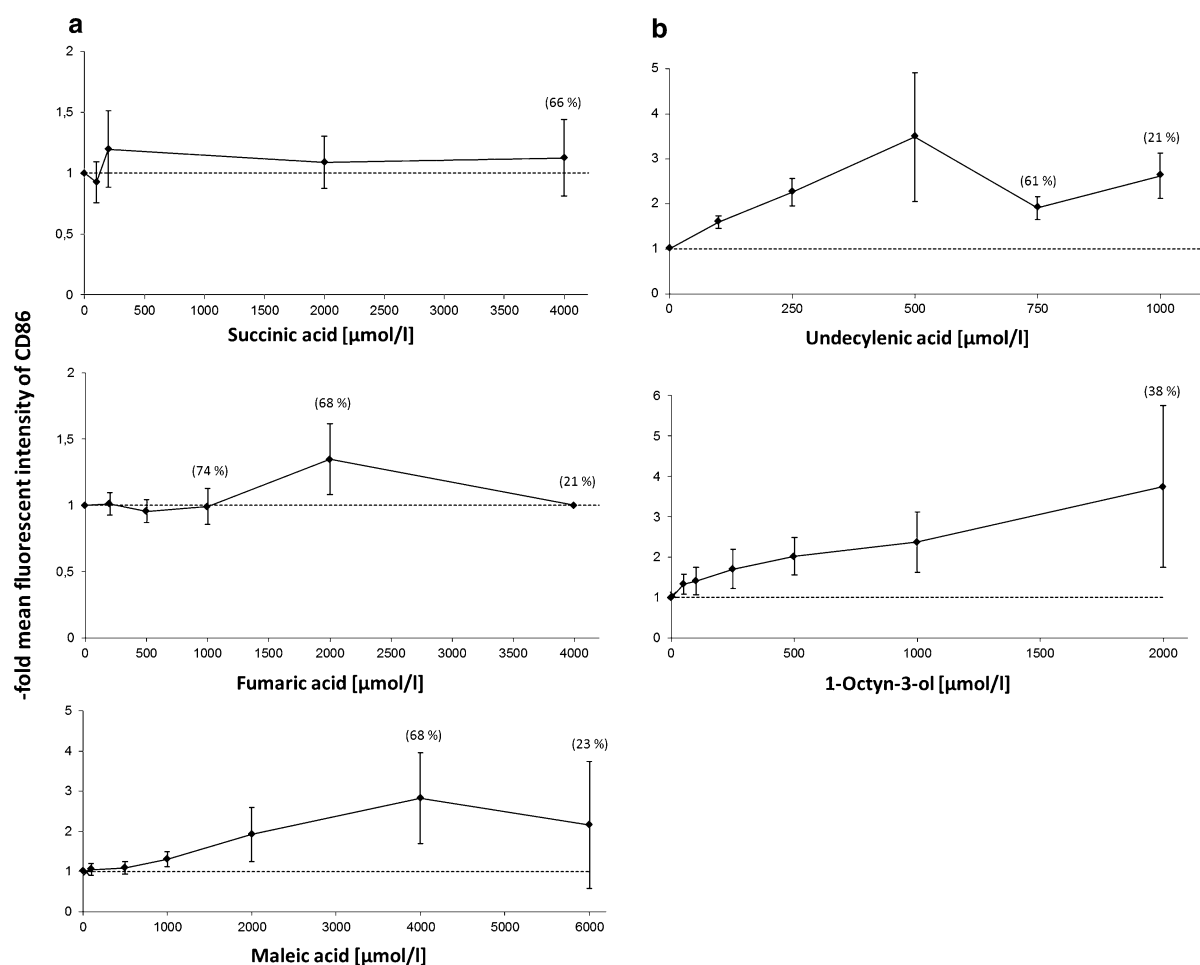


**Fig. 1** Relative up-regulation of CD86 on dendritic cells in the coculture after application of test substances is shown (mean values  $\pm$  SD of 2–4 independent experiments in duplicates). Substances showed no (succinic acid) or relatively low up-regulation of CD86

only at high concentrations **a**. Two substances elicited a more marked response **b**. When general cytotoxicity became noticeable (i.e., dendritic cell viability <75 %), the mean viability of dendritic cells is noted in parentheses over data points

were found in the literature. Therefore, it seems unlikely for 1-octyn-3-ol to have a strong sensitizing potency. Its classification based on  $EC_{50_{sens}}$  values in the LCSA and  $EC_3$  values in the LLNA would be weak and moderate, respectively. No sensitizing properties were detected in the GPMT. In this case, data are inconclusive and do not allow for any classification.

#### Succinic acid was labeled non-sensitizing by LCSA, LLNA, and GPMT

The data for the remaining substances must be interpreted carefully as they are endogenous substances abundant in mammalian organisms. However, allergic reactions to endogenous substances are not unknown. Succinic acid is known to cause immediate-type hypersensitivity reactions after intravenous administration as corticosteroid ester for medical treatment (Caimmi et al. 2008; Walker et al. 2011).

No reports for delayed-type hypersensitivity (i.e., allergic contact dermatitis) were found. Appropriately, succinic acid has been labeled non-sensitizing by all three assays.

#### Poor penetration might cause negative reactions to fumaric acid in vivo and a positive reaction in the LCSA

Fumaric acid showed no sensitizing potential in vivo but elicited a weak increase in CD86 in vitro. Allergic contact dermatitis to fumaric acid is unknown but its ester dimethyl fumarate caused an outbreak of “furniture-related dermatitis” in Europe (Lammintausta et al. 2010a, b). Presumably, fumaric acid esters penetrate better through the skin than fumaric acid due to higher lipophilicity. This may account for the negative reactions to fumaric acid in vivo and positive reaction in the LCSA, which does not simulate penetration.

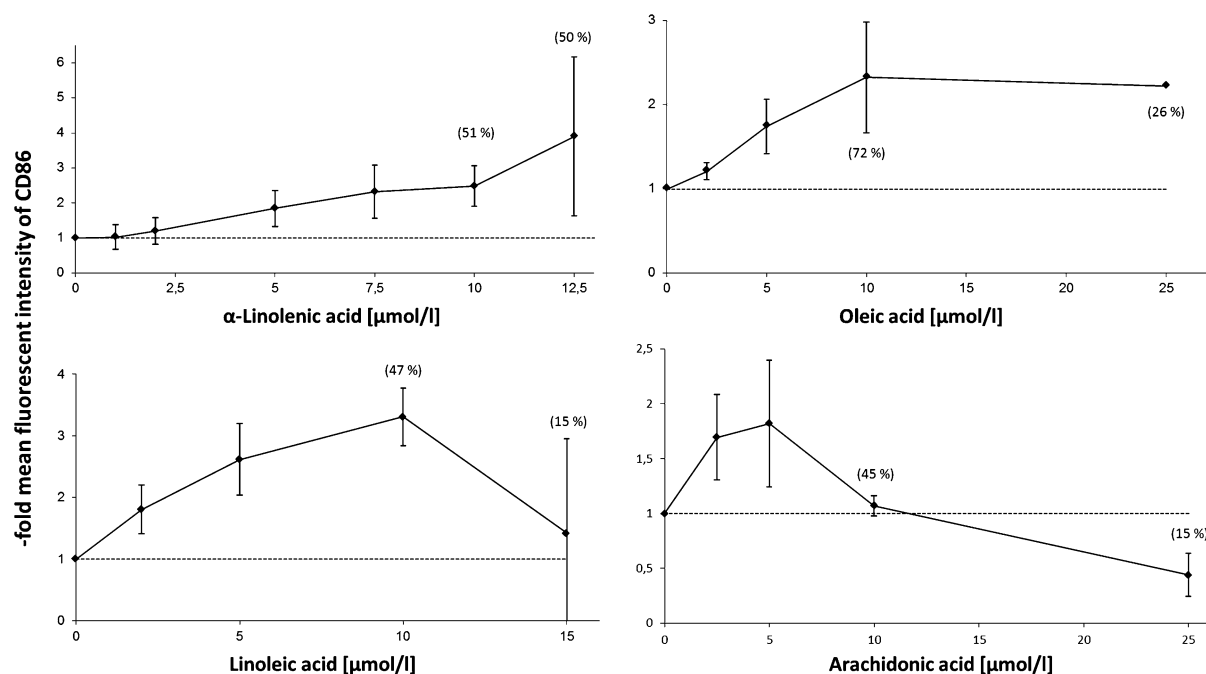


**Fig. 2** Relative up-regulation of CD86 on dendritic cells in the coculture after application of test substances is shown (mean values  $\pm$  SD of 2–4 independent experiments in duplicates). Substances showed a pronounced reaction at low concentrations. When general

cytotoxicity became noticeable (i.e., dendritic cell viability <75 %), the mean viability of dendritic cells is noted in parentheses next to data points

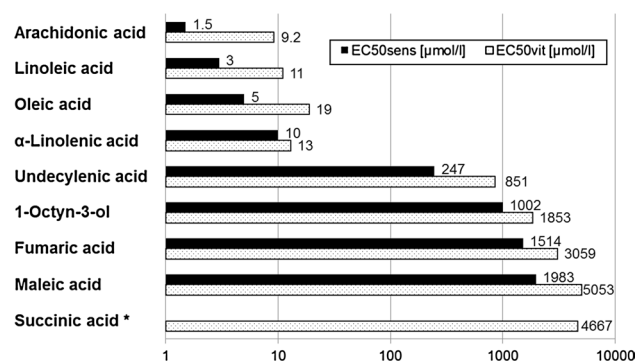


**Fig. 3** Estimated concentrations for half-maximal CD86 up-regulation and 50 % loss of viability of dendritic cells in coculture are shown as EC50<sub>sens</sub> and EC50<sub>vit</sub> values, respectively. Calculation of EC50<sub>sens</sub> and EC50<sub>vit</sub> is based on mean values of 2–4 independent experiments in duplicates. \* Succinic acid elicited no significant up-regulation of CD86; therefore, no EC50<sub>sens</sub> value can be determined

### Sensitizing potential for maleic acid is more accurately measured by the LCSA than by the LLNA

Maleic acid would have been classified as weak, moderate, or no sensitizer in the LCSA, LLNA, and GPMT, respectively. It is isomeric with fumaric acid and may be converted by enzymatic or non-enzymatic reactions to its cis-isomer. It has even been speculated that, from a stereochemical point of view, maleic acid and fumaric acid

give rise to the same complete allergen after haptization of a protein, only in different quantities (Hansson and Thörneby-Andersson 2003). This may explain the similar shape of the dose–response curves observed after testing these substances in the LCSA, with the curve of maleic acid being shifted to the right as compared to fumaric acid (Fig. 1a). While maleic acid had the tendency to induce a weaker reaction than fumaric acid in vitro, it was markedly stronger than fumaric acid in the LLNA (where it would have been classified as “moderate” contact allergen). Clinically, only one case report of allergic contact dermatitis to maleic acid esters was found—with negative skin reaction to maleic acid itself (Hansson and Thörneby-Andersson 2003). Considering the widespread use of maleate salts in pharmaceutical formulations, the sensitizing potency for maleic acid, if any, should be very low. Therefore, we think the sensitizing potential of this substance is more accurately measured by the LCSA than by the LLNA.

### 18-Carbon chain unsaturated fatty acids are tested false positive in the LCSA and LLNA

The LCSA revealed unexpected results for linoleic acid, oleic acid, and α-linolenic acid. Low concentrations ranging from 3 to 10 μmol/l were sufficient for half-maximal up-regulation of CD86 on dendritic cells in the LCSA,

**Table 2** Comparison of relative sensitizing potency which would have been assigned to the test compounds noted above (EC50<sub>sens</sub>: estimated concentration to elicit half-maximal CD86 increase on

dendritic cells in the LCSA; EC3: estimated concentration to elicit a stimulation index of three in the LLNA; n/d: not determinable)

|                  | LCSA (EC50 <sub>sens</sub><br>in µmol/l) | Sensitizing<br>potency (LCSA) | LLNA<br>(EC3 in %) | Sensitizing<br>potency (LLNA) | GPMT (animals<br>w/reproducible<br>positive reactions) | Sensitizing<br>potency (GPMT) |
|------------------|------------------------------------------|-------------------------------|--------------------|-------------------------------|--------------------------------------------------------|-------------------------------|
| Arachidonic acid | 1.5                                      | Extreme                       | (No data)          | (No data)                     | (No data)                                              | (No data)                     |
| Linoleic acid    | 3                                        | Extreme                       | 14.1               | Weak                          | Data inconclusive                                      |                               |
| Oleic acid       | 5                                        | Extreme                       | 10.5               | Weak                          | 2/10                                                   | None                          |
| α-Linolenic acid | 10                                       | Extreme                       | 9.9                | Moderate                      | (No data)                                              | (No data)                     |
| Undecylenic acid | 247                                      | Weak                          | 19.4               | Weak                          | 4/10                                                   | Yes                           |
| 1-Octyn-3-ol     | 1002                                     | Weak                          | 4.8                | Moderate                      | 0/10                                                   | None                          |
| Fumaric acid     | 1514                                     | Weak                          | n/d                | None                          | 0/10                                                   | None                          |
| Maleic acid      | 1983                                     | Weak                          | 7.5                | Moderate                      | 0/10                                                   | None                          |
| Succinic acid    | n/d                                      | None                          | n/d                | None                          | 0/10                                                   | None                          |
| Squalene         | (No data)                                | (No data)                     | 7.9                | Moderate                      | 2/10                                                   | None                          |

Calculation of EC50<sub>sens</sub> is based on mean values of 2–4 independent experiments in duplicates. LLNA and GPMT data were taken from Kreiling et al. (2008). EC3 values were calculated from these data by linear interpolation or log-linear interpolation as suggested by Ryan et al. (2007)

which led to a classification as “extreme” sensitizers (Table 2). In the LLNA, these substances were also classified as sensitizers, albeit with lesser potency. Given the widespread occurrence in food, their abundance in the human body, and lack of clinical evidence for contact sensitization, these fatty acids were most likely false positives. In comparison with the other substances in this study, they bear structural similarities as 18-carbon chain unsaturated fatty acids. Linoleic acid and α-linolenic acid are essential fatty acids and eicosanoid precursors, which can be converted to a magnitude of pro- and anti-inflammatory lipid mediators. Linoleic acid is a direct precursor of the eicosanoid arachidonic acid, which gives rise to several pro-inflammatory mediators, e.g., prostaglandin E2 (Khanapure et al. 2007). Therefore, we decided to also test arachidonic acid in the LCSA and found a similar dose–response curve compared to linoleic acid, only shifted to the left (Fig. 2). In fact, arachidonic acid elicited the strongest reaction of all substances in this study with half-maximal CD86 up-regulation at 1.5 µmol/l.

#### False-positive reactions to 18-carbon chain unsaturated fatty acids might be due to the formation of pro-inflammatory lipid mediators and/or TLR-4 signaling

We hypothesize that up-regulation of CD86 on dendritic cells in the LCSA after application of 18-carbon chain unsaturated fatty acids may be due to the formation of lipid mediators in the dendritic cell/keratinocyte coculture. The conversion of linoleic acid to arachidonic acid is plausible but needs yet to be confirmed by analytical methods. In

this respect, the reaction of α-linolenic acid seems surprising considering that omega-3 fatty acids (unlike omega-6, such as linoleic acid or arachidonic acid) rather generate the anti-inflammatory spectrum of lipid mediators (Calder 2006; Calviello et al. 2013). However, a recent study with cultured endothelial cells suggests that peroxidation of unsaturated fatty acids (also omega-3 fatty acids) may exert pro-inflammatory effects by Toll-like receptor 4 (TLR-4) activation (Mutoh and Ueda 2013). Interestingly, oleic acid was also among the panel of fatty acids causing TLR-4 signaling. In an earlier study, we could prove CD86 up-regulation on dendritic cells in the LCSA coculture after application of the TLR-4 ligand lipopolysaccharide (Schreiner et al. 2007). The idea that TLR-4 activation is involved in the up-regulation of CD86 on dendritic cells after application of unsaturated fatty acids to the LCSA coculture is tempting and needs further investigation.

Considering the possible function of 18-carbon chain unsaturated fatty acids (or their metabolites) as pro-inflammatory danger signals, it seems surprising that sensitization to these substances does not seem to occur in humans. Generation of immunological memory needs a foreign antigen and a pro-inflammatory environment, and many strong allergens are known to act both as antigen and danger signal promoting this condition, e.g., nickel is a TLR-4 ligand (Schmidt et al. 2010). The 18-carbon chain unsaturated fatty acids used in this study are common food ingredients and are thus taken up via the oral route—a route which is known to cause immunological tolerance rather than sensitization (Pabst and Mowat 2012), which may account for the lack of contact allergies to these substances in humans.

### Test strategies combining several in vitro assays which represent different mechanistic steps of sensitization are necessary for replacement of the LLNA

In summary, both the in vitro sensitization assay LCSA and the in vivo sensitization assay LLNA gave false-positive results after application of long-chain unsaturated fatty acids; sensitizing potential was even rated higher in vitro than in vivo. The most likely explanation is unspecific activation of dendritic cells by inflammatory mediators. The hallmark of allergy is the generation of a specific immunologic memory during the induction phase and production of clinical signs in the effector phase. In the GPMT, which simulates both phases, oleic acid was tested negative and linoleic acid elicited only nonspecific skin lesions (Table 2). The divergent behavior between GPMT on one side and LLNA and LCSA on the other side illustrates a challenge for sensitization assay development. In contrast to the GPMT, alternative assays only reproduce certain mechanistic steps rather than the whole process of sensitization. According to Adler et al. (2011), the LCSA represents the “mechanistic step 4” (dendritic cell activation). Possibly, mechanistic step 3 (epidermal inflammation) is also indirectly represented in the LCSA coculture by establishing an environment that facilitates the generation of allergen-sensing dendritic cells from monocytes. Integrated test strategies combining several mechanistic steps are needed to predict the sensitizing potential of substances: For example, a simple “2 out of 3 model” using two positive results out of three different in vitro sensitization assays for the prediction of sensitizers showed a slightly better accuracy than the LLNA (Urbisch et al. 2014).

### The LCSA has the potential to include a T cell readout, which might give it an advantage over other in vitro assays

Unlike other in vitro sensitization assays, the LCSA makes use of primary human cells as opposed to continuous cell lines. While this approach is more time-consuming, it has the advantage that T cells are already present in the coculture as part of PBMC. Modification of culture methods could make use of this advantage by facilitating T cell proliferation and establishing new read-out parameters representing T cell responses. This might contribute to future test strategies as there is still a lack of assays representing “mechanistic step 6” of sensitization, i.e., T cell proliferation. Introducing a T cell assay into integrated test strategies might improve the prediction of sensitizers.

However, it must be noted that—unlike continuous cell line assays—a consistent ratio of cell types in coculture cannot be granted. Fractions of cell types in PBMC vary from donor to donor, and so does proliferation of keratinocytes during assay culture. Exact PBMC/keratinocyte ratios are of lesser importance as long as the positive control TNBS shows an adequate response. For standardization, we determined an at least 1.5-fold increase in CD86 after application of 100 µmol/l TNBS as a prerequisite for utilization of results from an individual experiment.

### The LCSA shows a good correlation to the LLNA, but might be prone to the same false-positive reactions to unsaturated fatty acids

Sensitizing potential determined by the LCSA showed a good correlation to LLNA results and human data

**Table 3** Comparison of relative sensitizing potency of some known sensitizers and non-sensitizers as measured by LCSA, LLNA, and human data (sensitizing category: 1: “highest intrinsic sensitizing

potency”; 2: “strong intrinsic potency”; 3: “substantial degree of exposure necessary for sensitization”; 5: “very low intrinsic potency”; 6: “non-sensitizers”); n/d: not determinable

|                                                       | LCSA (EC50 <sub>sens</sub><br>in µmol/l) | Sensitizing potency<br>(LCSA) | LLNA<br>(EC3 in %) | Sensitizing potency<br>(LLNA) | Sensitizing category<br>in humans |
|-------------------------------------------------------|------------------------------------------|-------------------------------|--------------------|-------------------------------|-----------------------------------|
| Methylchloroisothiazolinone/<br>methylisothiazolinone | 0.8 <sup>a</sup>                         | Extreme                       | 0.05               | Extreme                       | 1                                 |
| 2,4-Dinitrochlorobenzene                              | 3 <sup>b</sup>                           | Extreme                       | 0.08               | Extreme                       | 1                                 |
| <i>p</i> -Phenylenediamine                            | 16 <sup>b</sup>                          | Strong                        | 0.11               | Strong                        | 1                                 |
| Cinnamal                                              | 60 <sup>b</sup>                          | Moderate                      | 2                  | Moderate                      | 2                                 |
| Isoeugenol                                            | 140 <sup>b</sup>                         | Weak                          | 1.2                | Moderate                      | 2                                 |
| Coumarin                                              | 777 <sup>c</sup>                         | Weak                          | 30                 | Weak                          | 3                                 |
| Hexylcinnamal                                         | 3 <sup>b</sup>                           | Extreme                       | 8                  | Moderate                      | 5                                 |
| Salicylic acid                                        | n/d <sup>b</sup>                         | None                          | n/d                | None                          | 6                                 |
| Phenol                                                | n/d <sup>b</sup>                         | None                          | n/d                | None                          | 6                                 |
| Sodium lauryl sulfate                                 | n/d <sup>b</sup>                         | None                          | 14                 | Weak                          | 6                                 |

Human sensitizing categories and LLNA data were taken from Basketter et al. (2014). LCSA data were taken from <sup>a</sup> Sonnenburg et al. (2014),

<sup>b</sup> Wanner et al. (2010), and <sup>c</sup> unpublished data

(Table 3). Notable deviations (e.g., hexylcinnamal—a weak-to-moderate sensitizer in humans and the LLNA but “extreme” in the LCSA) can occur in substances which penetrate poorly through the epidermis: Their sensitizing potential is overestimated in the LCSA as it does not simulate skin penetration (Wanner et al. 2010). The present study also shows that the LCSA might be prone to the same false-positive reactions toward unsaturated fatty acids as the LLNA.

Determination of the sensitizing potential of substances is mandatory for risk assessment. From the view of a consumer or employee with allergies, sensitization assays should possess a high sensitivity. However, high numbers of false-positive results are disadvantageous for both consumers and manufacturers as substances with beneficial properties are excluded from products due to unnecessary concerns. Our study may help to develop more accurate sensitization assays in the future.

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