



Evaluation of an optimized protocol using human peripheral blood monocyte derived dendritic cells for the *in vitro* detection of sensitizers: Results of a ring study in five laboratories



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ABSTRACT

Allergic contact dermatitis is a delayed T-cell mediated allergic response associated with relevant social and economic impacts. Animal experiments (e.g. the local lymph node assay) are still supplying most of the data used to assess the sensitization potential of new chemicals. However, the 7th amendment to the EU Cosmetic Directive have introduced a testing ban for cosmetic ingredients after March 2013.

We have developed and optimized a stable and reproducible *in vitro* protocol based on human peripheral blood monocyte derived dendritic cells to assess the sensitization potential of chemicals. To evaluate the transferability and the predictivity of this PBMDs based test protocol, a ring study was organized with five laboratories using seven chemicals with a known sensitization potential (one none-sensitizer and six sensitizers, including one pro-hapten).

The results indicated that this optimized test protocol could be successfully transferred to all participating laboratories and allowed a correct assessment of the sensitization potential of the tested set of chemicals. This should allow a wider acceptance of PBMDs as a reliable test system for the detection of human skin sensitizers and the inclusion of this protocol in the toolbox of *in vitro* methods for the evaluation of the skin sensitization potential of chemicals.

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1. Introduction

Allergic contact dermatitis (ACD) is a type-IV allergy of the skin associated with significant social and economic impact. The evaluation of the skin sensitization potential of chemicals is thus a crucial parameter in toxicological evaluation of chemicals that is usually performed using animal models (e.g. the Local Lymph Node Assay (LLNA) (Baskett et al., 2002)). However, the 7th Amendment to the European Cosmetics Directive (Directive 76/768/EEC) severely restricts the use of animal tests.

Different *in vitro* test protocols have thus been developed and optimized for the detection of skin sensitizers (see for example

(Aebly et al., 2010; Mehling et al., 2012)). Most of them use cell lines and measure similar dendritic cell (DC) activation markers. For example, the h-CLAT protocol measures the expression of CD86 and CD54 on THP-1 cells (Sakaguchi et al., 2006); the MUSST assay evaluate the expression of CD86 on U937 cells (Aebly et al., 2010). However, due to the difficulties associated with cell lines such as altered genome, genomic instability, metabolic deficiencies or limited functional properties (See for example (Klein et al., 2006; Sabatier et al., 2005)), we decided to further evaluate a stable and reproducible *in vitro* protocol based on human peripheral blood monocyte derived dendritic cells (PBMDs) that may provide additional and complementary information to cell line based assays.

We have thus reinvestigated and optimized an *in vitro* approach based on PBMDs ((Aebly et al., 2004; Degwert et al., 1997; Reutter

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et al., 1997; Staquet et al., 2004) and established an improved and stable protocol resulting in reduced donor to donor variability, reproducible results and a significant correlation with the local lymph node assay (LLNA). This test method is based on human PBMDs and flow cytometric measurement of CD86 expression as an activation marker (Reuter et al., 2011). Moreover, a slightly modified version of this protocol has already been shown to be successful in identifying photosensitizers *in vitro* (Karschuk et al., 2010).

The intra-laboratory performance of this test protocol was considered as very promising. However its transferability and reliability had to be confirmed. Thus a comprehensive ring study was organized including five laboratories. A test set of seven chemicals was first defined according to the following criterion: weak to extreme sensitizers, including pre and pro-hapten and challenging chemicals such as sodium lauryl sulfate (considered as a false positive in the LLNA, see for example (Garcia et al., 2010)).

Each participating laboratory tested the same set of chemicals using a variety of laboratory equipments and different adaptations of the test protocols (fresh or fixed cells, see below).

In this paper, we describe the setup, the performance of this ring study and present and discuss the obtained results.

2. Material and methods

2.1. Participating laboratories

Lead laboratory:

Beiersdorf AG, Research and Development, Unnastrasse 48, D-20245 Hamburg, Germany (BDF).

Participating laboratories:

- BASF Product Safety – Experimental Toxicology and Ecology, BASF SE, GV/TB – Z570, D-67056 Ludwigshafen, Germany (BASF; Lab 1).
- Institut de Recherche Pierre Fabre, Centre Expérimental et Pharmacocinétique de Campans, Bel Air – Route de Lautrec, F-81106 Castres cedex (PF; Lab 4).
- Johnson & Johnson GmbH, Johnson & Johnson Platz 2, D-41470 Neuss, Germany (J&J; Lab 2).

- The Procter & Gamble Company, Mason Business Center, 8700 Mason Montgomery Road, Mason, Ohio 45040, USA (P&G; lab 3).

2.2. Chemicals

The test chemicals were dissolved either in dimethylsulfoxide (DMSO, Merck 1.029.50) or RPMI 1640 medium without phenol red (R7509, Sigma, Germany, or USA). The desired test concentrations were then obtained by diluting the solubilized chemicals in RPMI 1640. When DMSO was used, its final in-well concentration of DMSO was always 0.5%. LPS (L5014, Sigma, USA) was included in each test run as a functional control. Cytotoxicity was measured by the incorporation of 7-aminoactinomycin D (7-AAD, 559925, BD Biosciences, USA).

The chemicals included in the ring study test set are indicated in Table 1. They were ordered independently by each participating laboratory and were not blindly tested.

2.3. Antibodies

All antibodies were obtained from BD Biosciences, San Jose, CA 95131, USA.

PE Mouse Anti-Human CD86: Cat. Nr. 555658.

PE Mouse Anti-Human CD1a: Cat. Nr. 555807.

FITC Mouse Anti-Human CD14: Cat. Nr. 345784.

Non-specific staining was determined using the following relevant isotypic controls in parallel:

PE-Mouse IgG1: Cat. Nr. 349043.

FITC-Mouse IgG2a: Cat. Nr. 349051.

2.4. Culture medium

Cells were cultured in RPMI 1640 without phenol red (R7509, Sigma, Germany, or USA) supplemented with 10% fetal calf serum (FCS Gold, A15-151, PAA, Austria or FCS Gold USA origin, A15-251, PAA, Austria, respectively), previously heat inactivated for 30 min at 56 °C and 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin (P11-013, PAA, Austria; or G1146, Sigma, USA).

Table 1

List, characteristics and test concentrations of the chemicals included in the ring study test set.

Name	Abbreviation	LLNA classification	CAS number	Source	Purity	Test concentrations (µM)
Eugenol	EUG	Weak (pro-hapten)	97-53-0	Aldrich Cat. Nr. E51791 Lot BCBB3186	99%	50, 200, 350, 500, 650, 800, 950, 1100
Hydroxycitronellal	HCIT	Weak	107-75-5	BASF Cat. Nr. 50001174 Lot 874256PO	≥96%	350, 425, 500, 575, 650, 725 [§]
Alpha-Hexylcinnamaldehyde	HCA	Moderate	101-86-0	SAFC Cat. Nr. W256900 Lot 22297EJ010	95%	50, 60, 70, 80, 90, 100
Nickel sulfate	NiSO ₄	Moderate	10101-97-0	Sigma Cat. Nr. 039K0127 Lot 039K0127	99.9%	50, 100, 150, 200, 250, 300
2,4-Dinitrochlorobenzene	DNCB	Extreme	97-00-7	Sigma Cat. Nr. 237329 Lot 237329	≥99%	2.5, 5.0, 7.5, 10.0, 12.5, 15.0
Sodium lauryl sulfate	SDS	*	151-21-3	Merck Cat. Nr. 112533 Lot L58114533 004	≥99%	100, 200, 300, 400, 500, 600
4-Hydroxybenzoic Acid	HA	Non sensitizer	99-96-7	Aldrich Cat. Nr. 240141 Lot S79620-010	≥99%	5000, 6000, 7000, 8000, 9000, 10000

* Non sensitizer in human, considered false positive in the LLNA and many cell based *in vitro* assays.

[§] Labs 1, 3 and 4 used 700 µM instead of 725 µM.

2.5. Purification of human monocytes and differentiation into PBMDcs

PBMDcs were prepared as described (Reuter et al., 2011). Briefly, fresh buffy coats prepared by centrifugation were randomly and anonymously obtained as residual product from whole blood preservation production. Enriched mononuclear cells were obtained by gradient density centrifugation (Lymphocyte Separation Medium LSM 1077, J15-004, PAA, Austria or USA, 15 min at 800g, 20 °C) as previously described (Degwert et al., 1997). Alternatively, Leucosep separation tubes (Leucosep-Tubes, 227290 Greiner Bio One, Germany) can be used according to the manufacturer's instructions as an alternative to facilitate the separation. CD14+ monocytes were then isolated by a positive selection using anti-CD14-Ig coupled magnetic microbeads (MACS CD14 MicroBeads, human (130-050-201); Miltenyi Biotec, Germany or USA). The isolated monocytes were frozen ($1 - 1.5 \times 10^7$ cells/ml in RPMI cryo medium (80% RPMI-medium + 10% DMSO + 10% FCS heat inactivated) and stored at $-80\text{ }^{\circ}\text{C}$ for a maximum period of three months. Before differentiation to PBMDcs, cells were thawed, resuspended in RPMI medium with cytokines (10% FCS (heat inactivated 30 min at $56\text{ }^{\circ}\text{C}$), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 U/ml rh IL-4 (CS-C1064, Cellsystems, Germany) and 200 U/ml recombinant human GM-CSF (CS-C1038, Cellsystems, Germany)), and their CD14 phenotype was measured by flow cytometry.

The following acceptance criteria were defined: Only cell samples containing > 70% CD14+ cells were used to generate PBMDcs by incubating the cells (1×10^6 /ml) from each donor in the RPMI medium with cytokines (see above) for five days ($37\text{ }^{\circ}\text{C}$, 5%CO₂) in six well culture plates.

Table 2
Classification obtained in the five different laboratories: +: Sensitizer; -: Non sensitizer; +/-: Equivocal; The detailed results are presented in Figs. 1–7.

	SDS	HA	EUG	HCIT	HCA	NiSO4	DNCB
Lab BDF	+/-	-	+/-	+	+	+	+
Lab 1	+/-	-	+	+	+	+	+
Lab 2	-	-	+	+	+	+	+
Lab 3	+	-	+	+	-	+	+
Lab 4	-	-	+	+	Doubtful*	+	+

* Relevant increases in CD86 expression (>20%) were observed in 8 out of 9 donors.

3. Experimental procedure

3.1. Dose selection process

As a first step, the cytotoxicity of the chemicals included in the test set was evaluated by the lead laboratory within a large concentration range limited only by their solubility. Briefly, PBMDcs were exposed for 48 h to the test chemical at dose-range concentrations, and the cytotoxicity was measured against an untreated control: the concentration inducing 20% cytotoxicity or a 20% induction of CD86 was first determined by staining with 7-AAD (BD559925; BD Biosciences, Basel, Switzerland) incorporation to determine dead cells or CD86-FITC antibody respectively (Reuter et al., 2011; Schmid et al., 1992), and flow cytometry analysis was performed. The final test range (five concentrations) was then defined by choosing a minimum of four lower and one higher test concentrations and communicated to the participating laboratories (see Table 1).

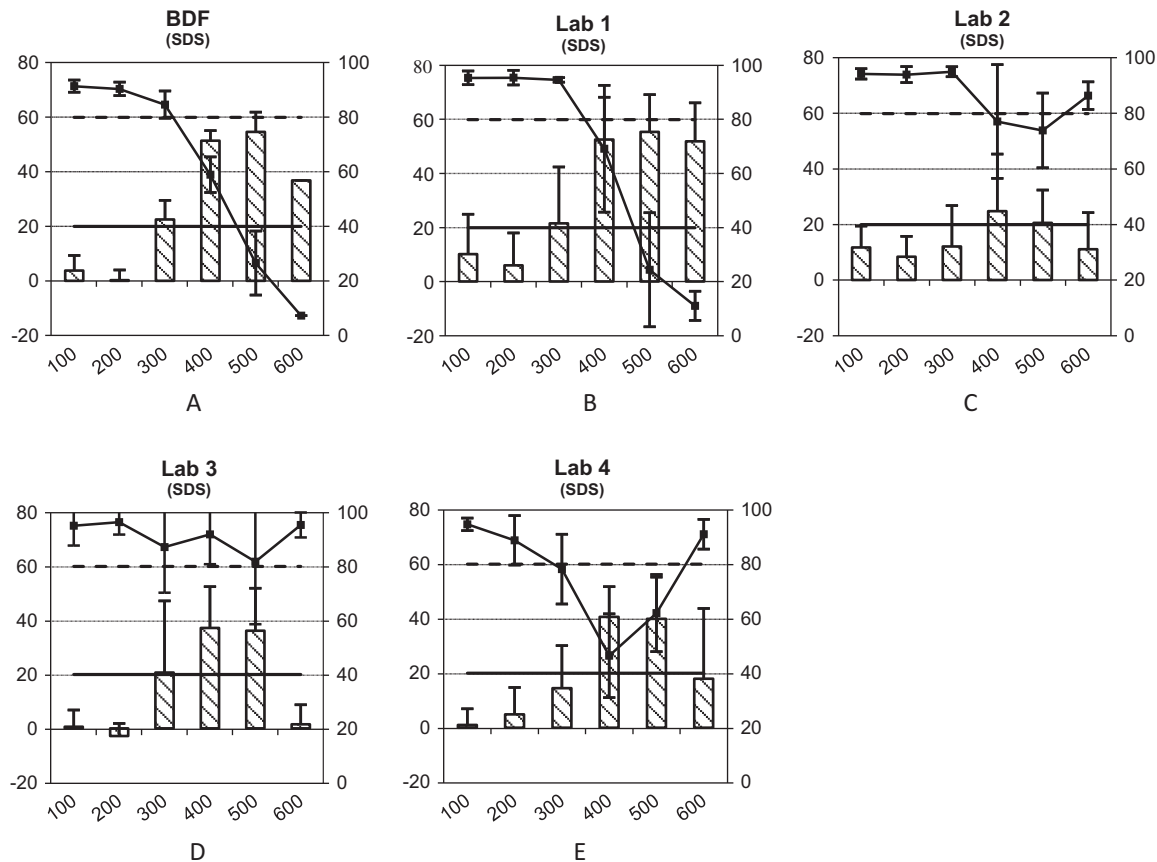


Fig. 1. Results obtained with PBMDc exposed for 48 h to the indicated SDS concentrations.

PBMDs were then exposed for 48 h to the chosen test chemical concentration range and then analyzed by flow cytometry for CD86 expression according to the protocol described below.

3.2. Chemical treatment of PBMDs

After thawing and five days in culture in six-well plates, the cells were harvested and seeded in 24-well plates (7.5×10^5 cells/ml; 0.4 ml per well). The PBMDs were then exposed for 48 h (37 °C; 5% CO₂) to the test chemicals (0.4 ml) at the 5 chosen concentrations and (in an additional well) to 100 ng/ml of LPS as a functional control (Reuter et al., 2011).

At the end of the exposure period, the cells were collected and prepared for flow cytometry analysis: 5×10^5 cells were transferred into fresh Eppendorf reaction tubes and centrifuged for 10 s at 1.3×10^3g . The supernatant was withdrawn and the cell pellet resuspended in 100 µl FACS-buffer (PBS (without Ca²⁺ and Mg²⁺) containing 0.05% sodium azide). 15 µl of the antibody solution (test antibody or isotype control) were added and cells were incubated for 25 min at 4 °C in the dark. The cells were then washed with 500 µl of FACS-buffer (centrifugation) and resuspended in 500 µl of FACS-buffer for analysis.

For viability measurement, the antibody stained cells were pelleted and resuspended in 100 µl FACS-buffer before addition of 2.5 µl (0.125 µg) 7-AAD. The cells were then incubated for 10 min at 4 °C and 400 µl FACS-buffer added before analysis.

Alternatively, to facilitate the work of laboratories without onsite flow cytometry facility, control cells (for monitoring the CD14 phenotype at day 0 or CD1a phenotype at day 5) were fixed in PBS with 1% para-formaldehyde and 0.05% sodium azide (freshly prepared or thawed) and analyzed later.

3.3. Flow cytometry analysis

The flow cytometry analysis of the cell viability (7-AAD incorporation) and CD86 surface expression was performed as already described (Reuter et al., 2011): Briefly, 10^4 cells were analyzed and gates for lymphocytes and debris exclusion were set based on forward and sideward scatter signals. Dead cells were assessed based on 7-AAD signals (Schmid et al., 1992) and excluded from the CD86 analysis. The isotype control was set to 1%. The percentage of cells positive for the analyzed marker (e.g. CD86) was defined as the % cells showing a signal above the 1% threshold set with the isotype control.

3.4. Data analysis

Each chemical was tested in at least five independent experiments corresponding to 5 different donors and for each test concentration the mean of the results obtained with the 5 donors was calculated. CD1a analysis was performed on the negative control only and CD86 and 7-AAD values were determined for all samples. For each experiment, the following acceptance criteria were set for the negative control: CD86 positive cells < 60%, CD1a positive cells > 60% and 7-AAD positive cells < 8.5%. Experiments not fulfilling these criteria were discarded.

The induction of CD86 expression after treatment was calculated as the difference to untreated controls ($\Delta\text{CD86} = \% \text{CD86}^+ \text{ cells in substance treated sample} - \% \text{CD86}^+ \text{ cells in untreated sample}$).

If a $\Delta\text{CD86} > 20\%$ was obtained for any concentration within the acceptable cytotoxicity range (cytotoxicity < 20%, see above), it was considered as a relevant increase and classified the substance as a sensitizer. However, if only one concentration reach the

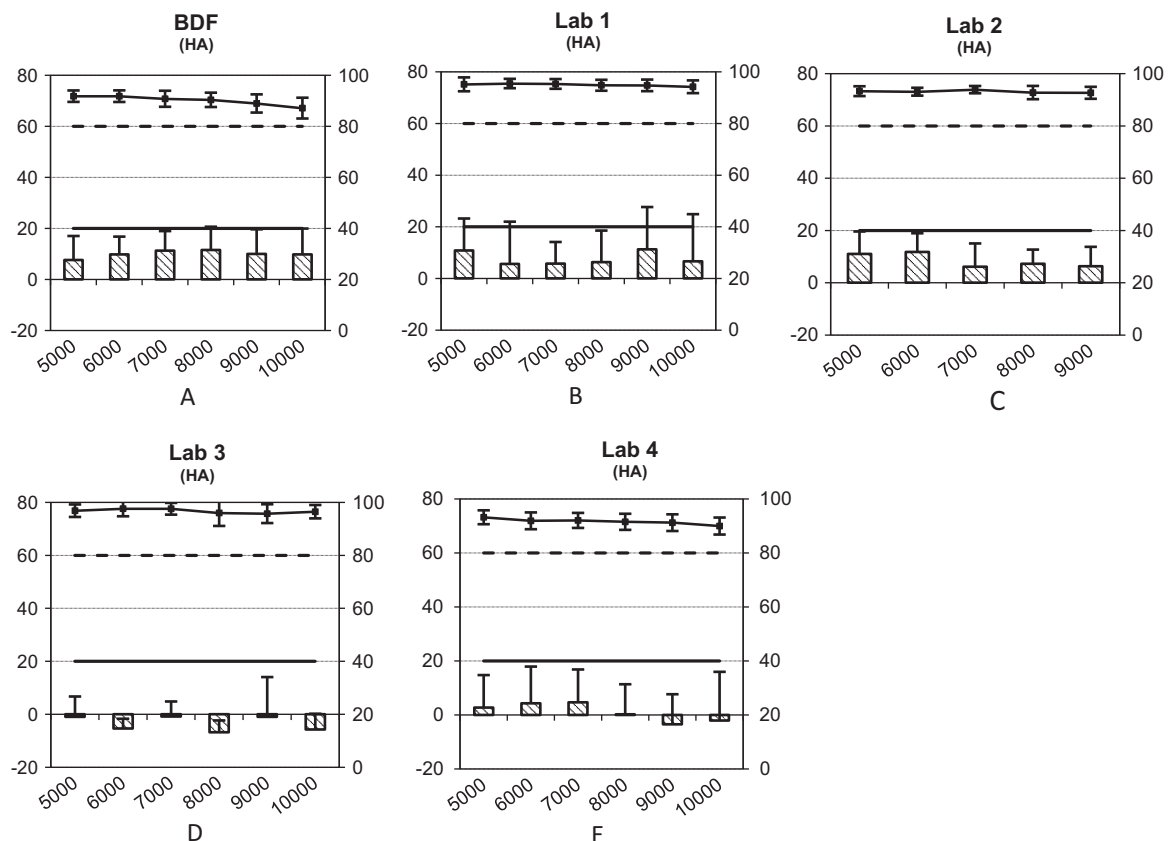


Fig. 2. Results obtained with PBMDs exposed for 48 h to the indicated HA concentrations.

$\Delta\text{CD86} > 20\%$ threshold before the cytotoxicity range, the result is considered as equivocal (+/–).

The extrapolated concentration inducing a $\Delta\text{CD86} \approx 20\%$ (intersection of the 20% threshold) was calculated through a linear regression between the first concentration inducing the $\Delta\text{CD86} > 20\%$ and the one just below (mean of 5 donors).

3.5. Statistical analysis

Descriptive and inductive statistics were performed on the inter-laboratory data according to the method two-way ANOVA with interaction using the SAS® statistical computing package for Windows V9.2 (www.sas.com).

The inductive statistic results presented in Table 3b has been obtained using the following parameters: Two sided hypothesis test and a 0.05 significance level. Moreover, for multiple comparisons, two types of significance were computed: “significant on multiple levels” (a multiple level of 0.05 was used) and “significant at the local level” (a local level of 0.05 was also used). Both types of significance are considered relevant to the claim support. Compliance with the multiple levels ensures a higher reproducibility of the significances obtained in this study.

An analysis of variance with laboratory and substance as the main effects and an interaction between laboratory and substance was calculated. The main effects describe the global differences between the laboratories. The interaction describes the differences between the laboratories per substance.

4. Results

4.1. The test protocol was successfully transferred to all participating laboratories

Each participating laboratory was first trained in the lead laboratory facility.

Transferability was first assessed by the lead laboratory through a functional test with LPS and the correct classification of HA (negative) and DNCB (positive) by all participating laboratories (Data not shown).

At the beginning the ring study, all laboratories experienced a similar high rate (up to 30%) of experiment rejection (see acceptance criteria under Data analysis). This was mainly due to the use of >24 h old buffy coats. This rejection rate was reduced >10% in subsequent experiments through the use of fresher (<24 h) buffy coats and additional hands-on experience with the DC isolation procedure.

4.2. Results obtained with the chemical test set

Each participating laboratory evaluated the chemical test set at the predetermined concentrations (Table 1) according to the shared protocol (see above). Experiments not fulfilling the described acceptance criteria (negative control values: $\text{CD86} < 60\%$, $\text{CD1a} > 60\%$ and $7\text{-AAD} < 8.5\%$) were rejected and not included in the final evaluation. The induction of CD86 expression after treatment was measured and the corresponding ΔCD86 calculated.

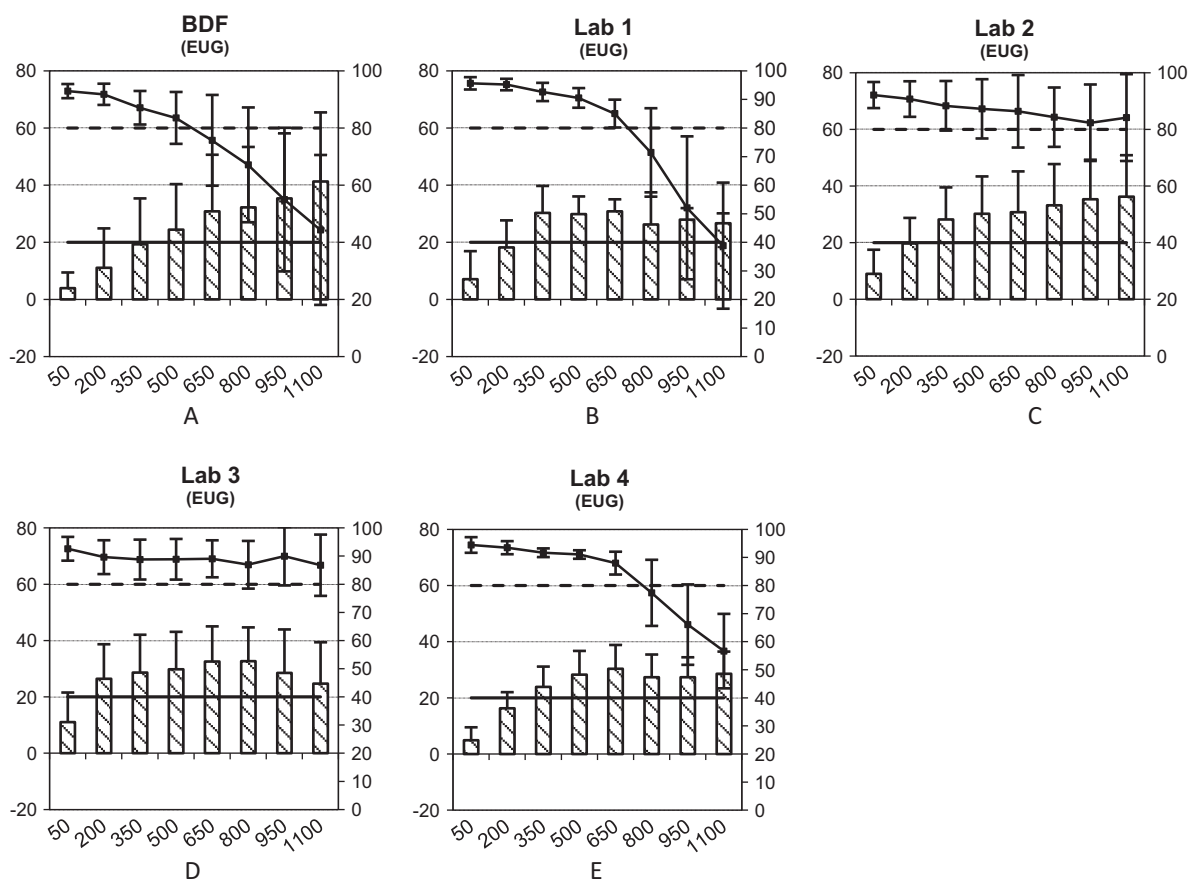


Fig. 3. Results obtained with PBMDc exposed for 48 h to the indicated EUG concentrations.

A test compound was categorized as sensitizer when the Δ CD86 was $>20\%$ within the acceptable cytotoxicity range. The overall results are summarized in Table 2.

SDS: (non-sensitizer, irritant; Fig. 1): In all laboratories SDS induced a $>20\%$ increase in CD86 expression at concentrations ranging from 289 μ M to 362 μ M with a median of 297 μ M (values obtained by linear regression intersection of the 20% threshold). However, in Labs 2 and 4, $>20\%$ increases in CD86 expression were only observed at cytotoxic concentrations ($>20\%$ cytotoxicity) and were thus considered as non-relevant, negative results. In the lead Lab and in Lab 1, the results were considered as equivocal since weak increases (EC20: 289 μ M and 290 μ M) were only observed at one concentration (300 μ M) before inducing cytotoxic effects.

HA: (non-sensitizer; Fig. 2): In all laboratories, HA did not induce any cytotoxicity (cytotoxicity $<20\%$) or increase in CD86 expression (Δ CD86 $<20\%$) up to the highest tested concentration (10,000 μ M). No dose effect was observed. Due to the absence of CD86 induction up to the highest tested concentrations, HA was classified as a non-sensitizer by all laboratories.

EUG: (Weak sensitizer, pro-hapten; Fig. 3): The lead lab and labs 1, 2 and 4 observed cytotoxic effects at test concentrations ranging from 650 to 1100 μ M (Linear regression intersection of the 20% threshold at 567 μ M to over 1100 μ M). In Lab 3, EUG did not induce any cytotoxicity (above the set thresholds) up to the highest tested concentration (1100 μ M). In all laboratories EUG induced increases in CD86 expression (Δ CD86 $>20\%$) at concentrations ranging from 137 μ M to 371 μ M with a median at 222 μ M (linear regression intersection of the 20% threshold). Due to the induction of increases of CD86 expression at non-cytotoxic concentrations, labs 1–4 classified EUG as a sensitizer. In the lead lab EUG

induced a Δ CD86 $>20\%$ at only one concentration before the cytotoxicity range and was thus classified as equivocal (+/–).

HCIT: (Weak sensitizer; Fig. 4): In all laboratories HCIT induced Δ CD86 $>20\%$ at concentrations ranging from 431 μ M to 643 μ M with a median of 505 μ M (values obtained by linear regression intersection of the 20% threshold). Due to the induction of these CD86 expression at non-cytotoxic concentrations, all laboratories classified HCIT as a sensitizer.

HCA: (Moderate sensitizer (LLNA); Fig. 5): All laboratories but one (Lab 3) observed cytotoxic effects at test concentrations >80 (lab 1) or at 100 μ M. In Lab 3, HCA did not induce any cytotoxicity $>20\%$ up to the highest tested concentration (100 μ M). All laboratories but one (Lab 3) observed Δ CD86 $>20\%$ at concentrations ranging from 51 μ M to 77 μ M with a median of 55 μ M (values obtained by linear regression intersection of the 20% threshold). However, Lab 4 also observed dose related increases in CD86 expression at concentrations up to 90 μ M but the mean increases did not reach the 20% threshold. Nevertheless, Δ CD86 $>20\%$ were observed in 8 out of 9 donors but the mean value obtained for the 9 donors did not reach the pre-defined 20% threshold. This result was thus considered as doubtful. In Lab3, increases in the expression of the CD86 marker were observed at the 60–100 μ M test concentrations. However, these increases did not reach the 20% threshold and Lab 3 classified HCA as a non-sensitizer.

NiSO₄: (Moderate sensitizer; Fig. 6): Within the chosen test range, a $>20\%$ (but weak) cytotoxic effect was only observed in laboratory 4, at the highest test concentration (300 μ M; linear regression intersection of the 20% threshold at 279 μ M). Nevertheless, in all laboratories NiSO₄ induced Δ CD86 $>20\%$ at concentrations ranging from 54 μ M to 196 μ M with a median of 75 μ M (values

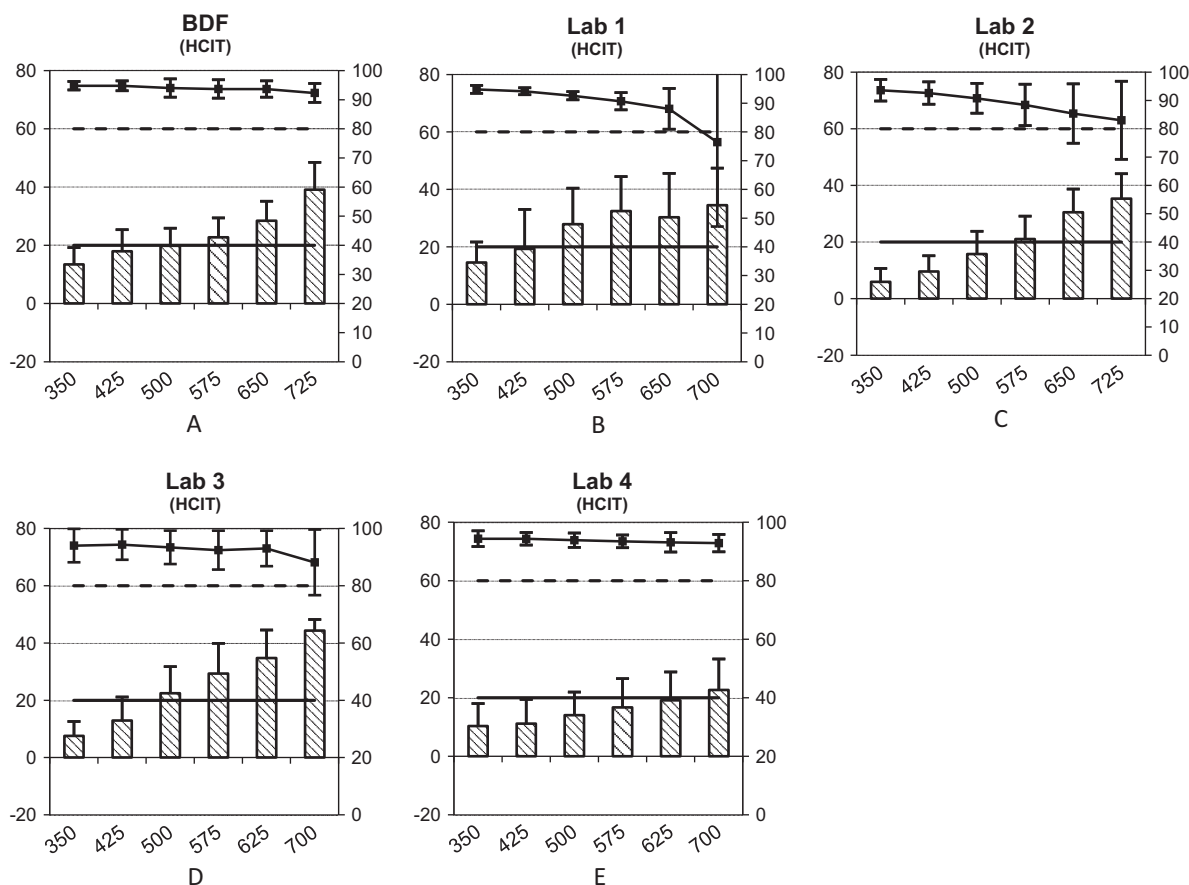


Fig. 4. Results obtained with PBMDc exposed for 48 h to the indicated HCIT concentrations.

obtained by linear regression intersection of the 20% threshold). Due to the induction of these increases of CD86 expression at non-cytotoxic concentrations, all laboratories classified NiSO₄ as a sensitizer.

DNCB: (Extreme sensitizer; Fig. 7): The selected test concentrations for this highly cytotoxic compound were comparatively low (2.5–15.0 μ M). According to the linear regression intersection of the 20% threshold, cytotoxicity was observed by the lead Lab and Labs 1 and 4 at concentrations higher than 11.1 μ M. Cytotoxicity values obtained in Lab 2 and 3 did not reach the 20% threshold. With DNCB, all laboratories observed increases in CD86 expression over the 20% threshold (linear regression intersection of the 20% threshold) at test concentrations ranging from 3.8 and 8.7 μ M (median 7 μ M). DNCB was thus classified as a sensitizer by all laboratories. These results also confirmed the successful transfer of the test protocol in all participating laboratories.

4.3. Statistical analysis

The descriptive statistical results are summarized in Fig. 8. The CV80 (test concentration inducing 20% cytotoxicity, e.g. viability < 80%) and EC20 (test concentration inducing a >20% increase in CD86 expression) values for the different substances in different study laboratories are summarized. Inductive statistical results are summarized in Table 3a. The computed values indicate that the hypothesis of relevant differences between substances is true. Moreover an inductive statistical comparison of the results between laboratories and substances (see Supplementary Table S1) indicates that there was no statistically significant difference between the results obtained by the laboratories with all

tested chemicals except for one: NiSO₄. However, this had no consequence since all labs classified NiSO₄ as a sensitizer! These results confirm the good inter-laboratory reproducibility of the evaluated protocol. Table 3b presents the results of the inductive comparison between laboratories. Again, no statistically relevant difference could be observed between the results obtained in each laboratory.

5. Discussion

We have recently published an optimized test protocol using PBMDs for the *in vitro* detection of contact allergens (Reuter et al., 2011). The choice of PBMDs as the test system instead of cell lines such as THP-1 or U-937 (Ade et al., 2006; Ashikaga et al., 2006; Bauch et al., 2012; Python et al., 2007; Sakaguchi et al., 2006) was based on the fact that PBMDs are primary human cells that are not degenerated through the aberrant expression of oncogenes or modified signaling pathways and are thus physiologically and metabolically very similar to functional DCs. Through a comprehensive review, evaluation and optimization of the already published PBMDs based protocols (Aeby et al., 2004; Degwert et al., 1997; Reuter et al., 2011; Reutter et al., 1997; Staquet et al., 2004) a simple and robust approach was developed. Preliminary evaluation of this optimized protocol by the lead laboratory, BDF (Reuter et al., 2011), using a limited test set of chemicals demonstrated its excellent performance: A correct classification (7 sensitizers/5 non-sensitizers) was obtained for all chemicals but one (benzalkonium chloride). However, a detailed and meaningful statistical analysis of the within- or between-laboratory reproducibility, robustness and specificity

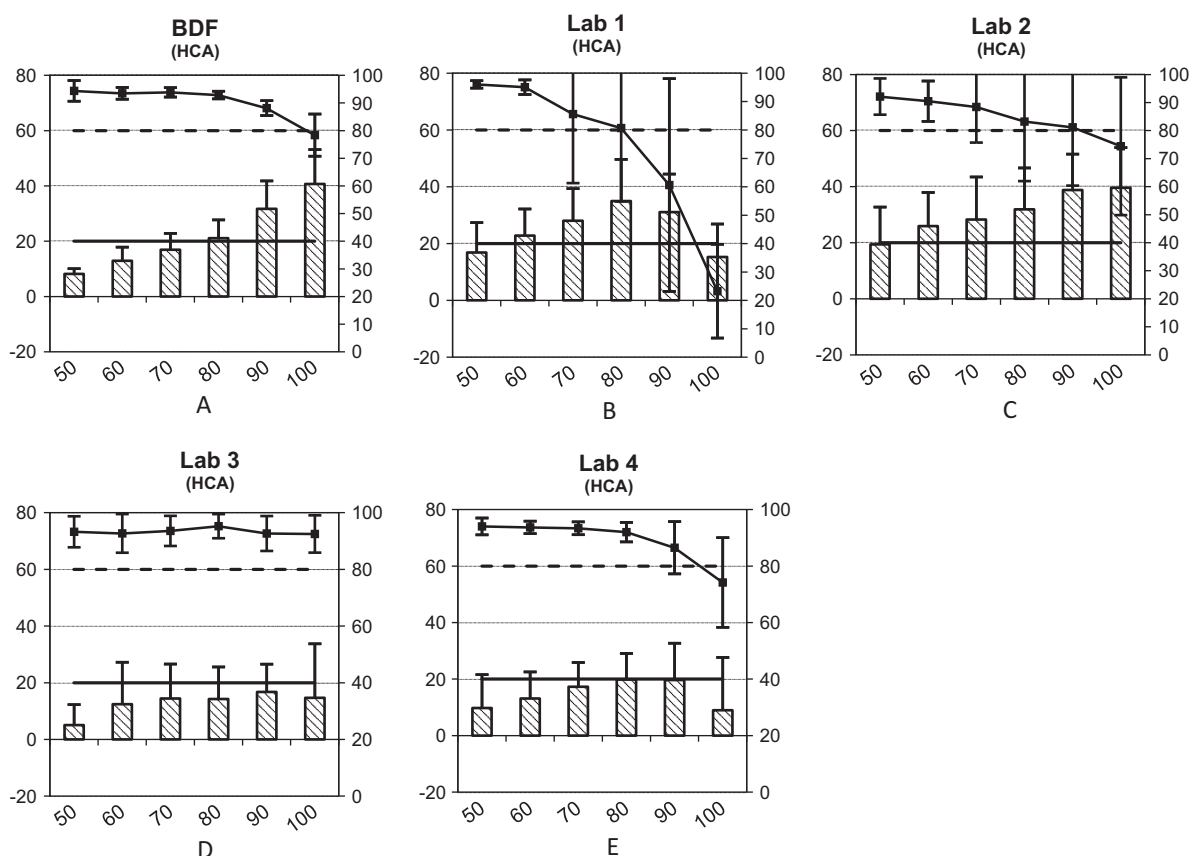


Fig. 5. Results obtained with PBMDs exposed for 48 h to the indicated HCA concentrations.

aspects needs a larger data set and a more comprehensive multi laboratory ring study.

In this paper, we present the results of such a ring study performed with five participating laboratories with quite diverse previous expertise concerning the isolation and culture of human monocytes and using a variety of equipments. As a starting point, a test set of seven chemicals was first defined according to the following criterion: Weak to extreme sensitizers, including pro-haptens and a metal and non-sensitizers (irritant and non-irritant). The test set (see Table 1) defined according to these criterion was: eugenol as a weak pro-hapten, hydroxycitronellal and alpha-hexylcinnamaldehyde as weak or moderate direct acting sensitizers, nickel sulfate as a moderate, metal sensitizer and 2,4-dinitrochlorobenzene as an extreme sensitizer (Gerberick et al., 2004; Kimber et al., 2003; Loveless et al., 1996). The selected non-sensitizers were sodium lauryl sulfate (irritant, non sensitizer in human but considered false positive in the LLNA (Basketter and Kimber, 2011) and many cell based *in vitro* assays) and 4-hydroxybenzoic acid. Each participating laboratory received and tested the same lot of each of the test chemicals.

The primary goal of this ring study was to evaluate the transferability and to assess the reliability (specificity) of the proposed test protocol in different laboratory environments. Indeed, this optimized protocol could be, after a short (3 days) learning phase organized within the lead laboratory (BDF), easily transferred to the participating laboratories that all had a different background regarding the technologies and procedures needed for the evaluation of the proposed protocol:

Lab 1: The personnel involved in this study had limited previous experience with flow cytometry and was never involved in PBMDs isolation procedures such as density gradient centrifugation, and bead-based cell isolation.

Lab 2: The personnel had a limited prior experience with *in vitro* cell culture but no previous practice with the isolation, culture and activation of PBMDs or flow cytometry analysis. This facility should thus be considered as a true naïve laboratory without previous experience with the evaluated PBMD activation protocol.

Lab 3: The personnel had an extensive expertise in PBMD isolation, culture, activation as well as flow cytometry analysis. They were already involved in the development of alternative *in vitro* methods.

Lab 4: The personnel had experience in cell culture procedures, however with only a limited previous knowledge of PBMD isolation and culture and nearly no experience of flow cytometry.

The successful transfer of the protocol to laboratories with such different experiences and equipment indicates that this is an easily deployable test protocol that was resilient to the inevitable laboratory adaptation. The fact that each participating laboratory could successfully isolate its own biological material (PBMD) further stress the robustness of the protocol. The preliminary and encouraging observations made during the transfer phase were confirmed by the ring study results discussed below:

HA was correctly classified by all laboratories as a non-sensitizer since it did not induce any $\Delta\text{CD86} > 20\%$ up to the highest test concentration (10,000 μM).

On the other hand, with SDS, different and sometimes borderline results were obtained: two laboratories (Lab 2 & 4) categorized

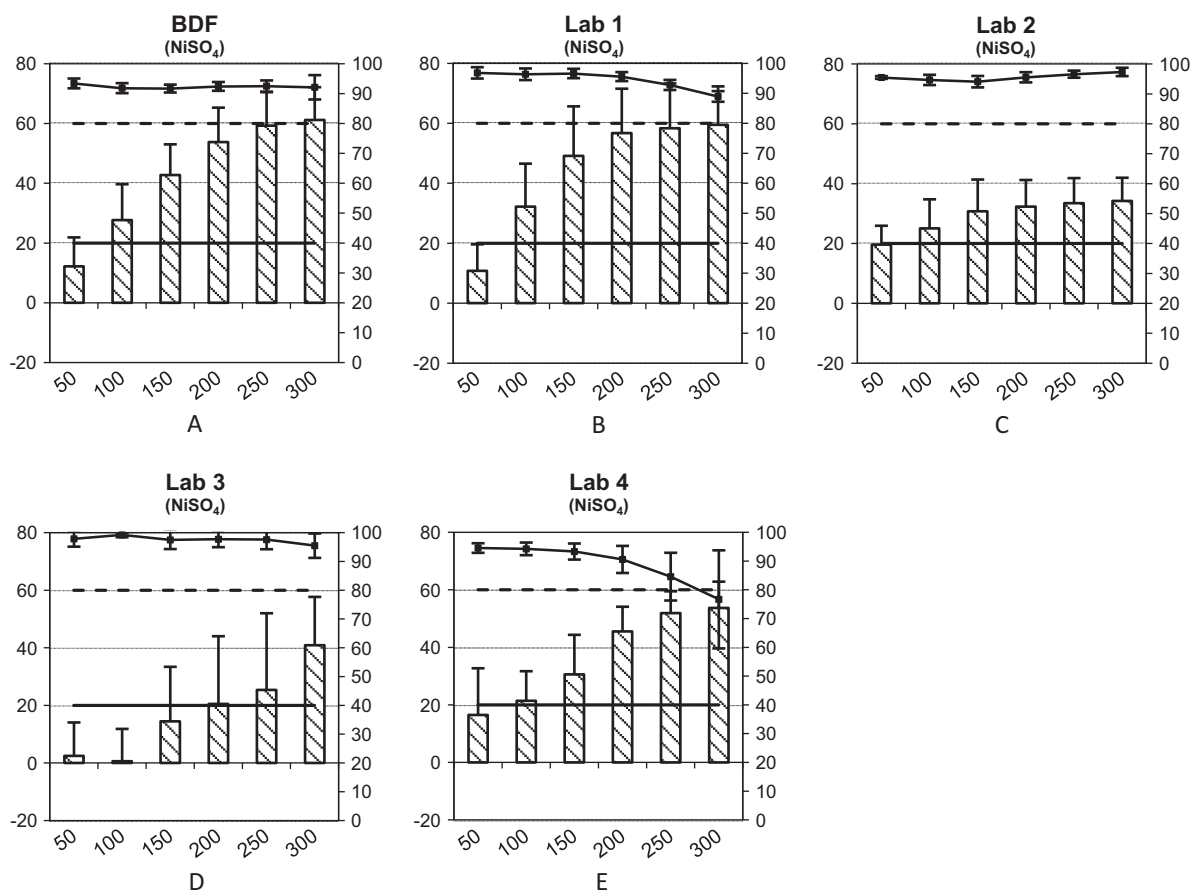


Fig. 6. Results obtained with PBMD exposed for 48 h to the indicated NiSO₄ concentrations.

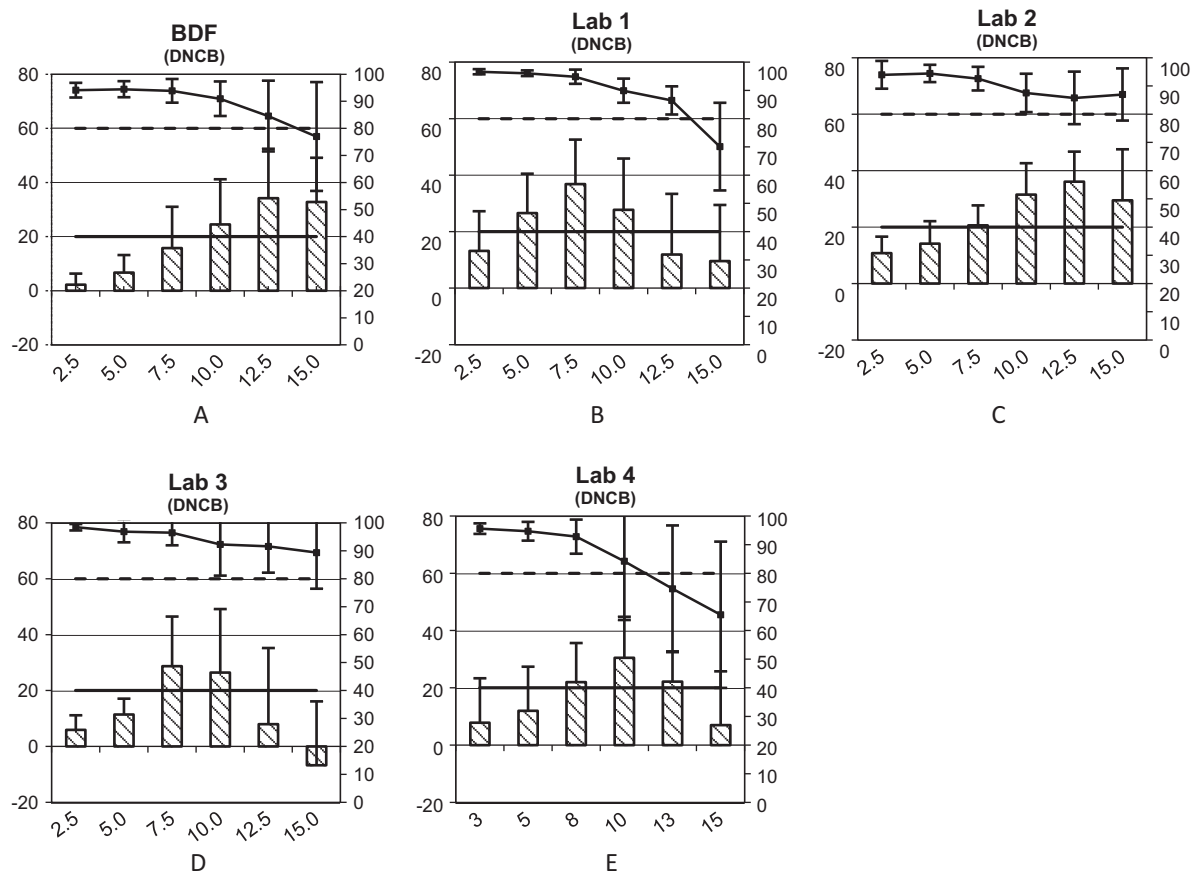


Fig. 7. Results obtained with PBMDc exposed for 48 h to the indicated DNCB concentrations.

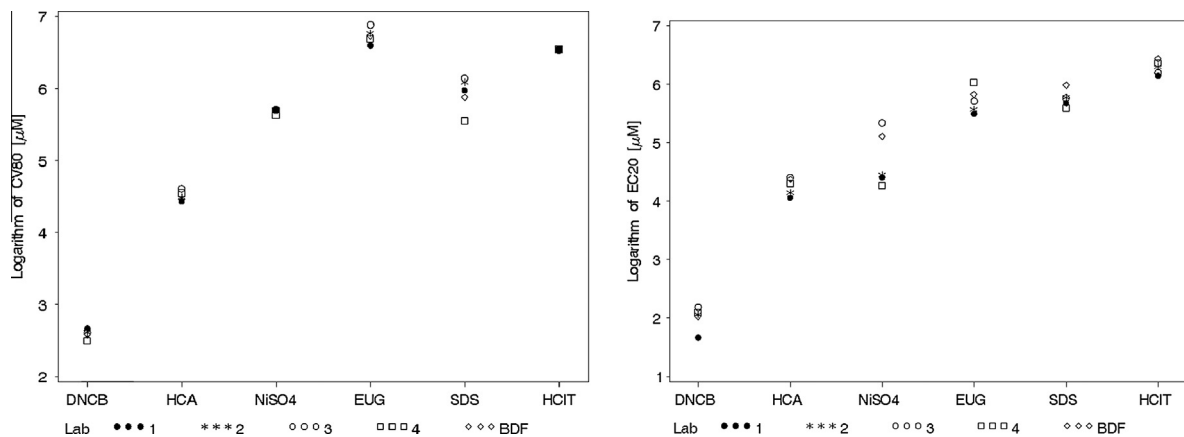


Fig. 8. CV80 (test concentration inducing 20% cytotoxicity, e.g. viability < 80%) and EC20 (test concentration inducing a >20% increase in CD86 expression) values obtained for the different substances in the indicated laboratories. X-axis: Test substance; Y-axis: Test substance concentration (in μM).

SDS as a non-sensitizer, one laboratory (Lab 3) as a sensitizer and the two others (Lead Lab and Lab 1) had equivocal results. All laboratories observed $\Delta\text{CD}86 > 20\%$ on cells exposed to SDS. However in two laboratories, these increases were only observed outside the acceptable cytotoxicity threshold (20%). SDS was classified as a non sensitizer in these laboratories. In Lab 3, SDS induced a $\Delta\text{CD}86 > 20\%$ at 400 and 500 μM within the acceptable cytotoxic range (<20% cytotoxicity) and was thus categorized a sensitizer.

In Lab 1 and in the lead Lab, a borderline situation was observed: SDS induced a weak increase in CD86 expression at only one concentration before inducing cytotoxic effects. These laboratories thus categorized SDS as an equivocal sensitizer. However, the measurement of the proportion of dead cells after exposure to surfactants such as SDS is quite challenging since lysed cells will not be detected by the flow cytometer and the cytotoxic effects are thus grossly under evaluated. Moreover, cell responses observed

in the cytotoxic range can be due to various artifacts (e.g. danger signals due to the release of cell components by dying or dead cells or to cytokines released by stressed cells (Reuter et al., 2011) and should thus not be considered for the categorization of a chemical. SDS is a known human irritant classified as a non-sensitizer in humans and as a false positive in the LLNA (Basketter and Kimber, 2011). It has already proven to be a difficult chemical when tested in various *in vitro* approaches (Hooyberghs et al., 2008; Python et al., 2007).

EUG is known as a weak sensitizer acting as a pro-hapten (e.g. it needs to be metabolically converted into a compound that is chemically reactive (Barratt and Basketter, 1992; Bertrand et al., 1997; Jäckh et al., 2012). Laboratories 1–4 correctly categorized this compound as a sensitizer since it induced $\Delta\text{CD86} > 20\%$ at non-cytotoxic concentrations. The lead lab indicated an equivocal result since EUG induced a relevant increase at only one concentration before the cytotoxicity range. This successful detection of a typical pro-hapten also supports our hypothesis that a test system based on primary human cells has the potential to detect chemicals classified as pro-haptens. This should be confirmed by testing more pro-haptens. Moreover, donor-to-donor-variations in detecting pro-haptens may arise from differences in metabolic capacity.

HCIT is known as a direct acting, weak sensitizer (Gerberick et al., 2004). As expected, it induced $\Delta\text{CD86} > 20\%$ at non-cytotoxic concentrations and all laboratories correctly categorized it as a sensitizer. Interestingly, within the tested range (350–725 μM) HCIT did not induce reproducible cytotoxic effects (only Lab 1 detected a relevant ($>20\%$) cytotoxic reaction at the highest tested concentration (700 μM)). A clear ΔCD86 dose response was nevertheless observed indicating that the increase in CD86 expression is not necessarily correlated with relevant cytotoxic effects.

HCA has been classified in the LLNA as moderate sensitizer (Gerberick et al., 2004). In this ring study, three laboratories observed $\Delta\text{CD86} > 20\%$ at non-cytotoxic concentrations and a fourth one obtain a doubtful result since it observed relevant increases in CD86 expression ($>20\%$) in 8 out of 9 donors but the mean value including this negative donor did not reach the pre-defined 20% threshold. HCA was thus classified as a sensitizer in three laboratories and as a non-sensitizer by Lab 3. However, the negative classification of Lab 3 could be due to the limited test concentration range indicated by the lead laboratory. Under standard experimental conditions, Lab 3 would have tested HCA at higher,

up to sub-cytotoxic, concentrations as defined in the test protocol and may have observed a $\Delta\text{CD86} > 20\%$. It is interesting to note that a large variability in the response to HCA has already been reported by other groups: In humans, only few positive HCA patch-test reactions were reported by (Heydorn et al., 2003) indicating that negative results may be expected when using a test system based on cells of human origin. Negative results were also observed by (Python et al., 2007) using the U937 cells that are also of human origin. HCA also gave equivocal results in the COLIPA h-CLAT ring trial (Sakaguchi et al., 2010).

On the other hand, NiSO₄ is a well-known moderate human sensitizer that generally tests negative in the LLNA. This discrepancy has recently been elucidated and is due to binding to the human TLR-4 receptor but not to the corresponding murine receptor (Mandervelt et al., 1997; Schmidt et al., 2010). In this ring study, it induced $\Delta\text{CD86} > 20\%$ at non-cytotoxic concentrations and was thus clearly categorized as a sensitizer by all laboratories. This result again emphasizes the advantage of using cells of human origin for the detection of human allergens and fully agrees with the current understanding of the species-specific Ni²⁺-induced TLR4 activation process.

DNCB has been categorized as an extreme sensitizer in the LLNA (Gerberick et al., 2004). It is nevertheless a challenging compound when tested in cell based *in vitro* assays due to its relatively high cytotoxicity and the reduced concentration range (narrow test window) where DC activation occurs before inducing unacceptable cytotoxic effects. DNCB was nevertheless correctly classified as a sensitizer in all the laboratories participating to the ring trial. It is interesting to note that all laboratories observed increases in CD86 expression over the 20% threshold at very low test concentrations (median 7 μM , calculated from a linear regression intersection of the 20% threshold), indicating the possibility of using the intersection of the 20% threshold as an indicator of the potency of test substance.

6. Conclusion

This ring study exercise has confirmed the transferability of this optimized PBMDc protocol in laboratories with a wide range of expertise regarding the isolation, culture and analysis of human monocytes. Furthermore, its robustness was also demonstrated by the concordant and correct results obtained using different versions of the protocol adapted to the available laboratory equipment (e.g. four different flow cytometry apparatus) and by the successful use of a biological source material (buffy coats) obtained from different populations originating from Germany, France and the United States (Fig. 8).

Quality information to characterize the sensitizing properties of a chemical will rely on the combination of multiple *in vitro* or *in silico* test methods. This test protocol using DC derived from fully functional human primary cells as the test system does not suffer from the inherent functional and metabolic limitations of cell line based assays. It can thus provide additional and/or confirmatory information to other assays (e.g. DPRA, h-CLAT, MUSST) and be included in a comprehensive test strategy (Goebel et al., 2012). A successful double blinded study with a larger chemical test set would support its validation and acceptability for inclusion in the toolbox of *in vitro* methods for the evaluation of the skin sensitization potential of chemicals, together with already validated assays such as the DPRA and the h-CLAT.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Table 3
Inductive statistical results on the ring study.

(a)		
EC20		
Effect	F-value	Pr > F
<i>Inductive statistics for the global model; F-value: test factor; Pr > F: p-value</i>		
Labor	2.0	0.0974
Substance	3.998E7	<.0001
(b)		
Comparison	Adjusted p-value	Result
<i>Inductive comparisons between laboratories across substances. n.s.: not significant</i>		
BASF ~ BDF	0.0593	n.s.
BASF ~ J&J	0.9198	n.s.
BASF ~ P&G	0.5496	n.s.
BASF ~ PF	0.4497	n.s.
BDF ~ J&J	0.3508	n.s.
BDF ~ P&G	0.7732	n.s.
BDF ~ PF	0.7852	n.s.
J&J ~ P&G	0.9597	n.s.
J&J ~ PF	0.9310	n.s.
P&G ~ PF	1.0000	n.s.

Transparency Document

The [Transparency document](#) associated with this article can be found in the online version.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tiv.2015.03.021>.

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Table S1: Inductive statistical results on the ring study. Comparison between laboratories and substances.

Comparison	Adjusted p-Value	Result
BASF, DNCB ~ BDF, DNCB	1.0000	n.s.
BASF, Eugenol ~ BDF, Eugenol	1.0000	n.s.
BASF, HCA < BDF, HCA	0.6928	n.s.
BASF, 4-Hydroxybenzoic Acid ~ BDF, 4-Hydroxybenzoic Acid	1.0000	n.s.
BASF, Hydroxycitronellal < BDF, Hydroxycitronellal	0.919	n.s.
BASF, NiSO ₄ < BDF, NiSO ₄	0.6035	n.s.
BASF, SDS ~ BDF, SDS	0.9998	n.s.
BASF, DNCB ~ J&J, DNCB	1.0000	n.s.
BASF, Eugenol ~ J&J, Eugenol	1.0000	n.s.
BASF, HCA ~ J&J, HCA	1.0000	n.s.
BASF, 4-Hydroxybenzoic Acid ~ J&J, 4-Hydroxybenzoic Acid	1.0000	n.s.
BASF, Hydroxycitronellal ~ J&J, Hydroxycitronellal	1.0000	n.s.
BASF, NiSO ₄ ~ J&J, NiSO ₄	1.0000	n.s.
BASF, SDS ~ J&J, SDS	1.0000	n.s.
BASF, DNCB ~ P&G, DNCB	0.9996	n.s.
BASF, Eugenol ~ P&G, Eugenol	1.0000	n.s.
BASF, HCA < P&G, HCA	0.6614	n.s.
BASF, 4-Hydroxybenzoic Acid ~ P&G, 4-Hydroxybenzoic Acid	1.0000	n.s.
BASF, Hydroxycitronellal ~ P&G, Hydroxycitronellal	1.0000	n.s.
BASF, NiSO ₄ < P&G, NiSO ₄	0.0252	m.s.
BASF, SDS ~ P&G, SDS	1.0000	n.s.
BASF, DNCB ~ PF, DNCB	1.0000	n.s.
BASF, Eugenol ~ PF, Eugenol	1.0000	n.s.
BASF, HCA < PF, HCA	0.9734	n.s.
BASF, 4-Hydroxybenzoic Acid ~ PF, 4-Hydroxybenzoic Acid	1.0000	n.s.
BASF, Hydroxycitronellal ~ PF, Hydroxycitronellal	0.9952	n.s.
BASF, NiSO ₄ ~ PF, NiSO ₄	1.0000	n.s.
BASF, SDS ~ PF, SDS	1.0000	n.s.
BDF, DNCB ~ J&J, DNCB	1.0000	n.s.
BDF, Eugenol ~ J&J, Eugenol	1.0000	n.s.
BDF, HCA < J&J, HCA	0.9894	n.s.
BDF, 4-Hydroxybenzoic Acid ~ J&J, 4-Hydroxybenzoic Acid	1.0000	n.s.
BDF, Hydroxycitronellal ~ J&J, Hydroxycitronellal	1.0000	n.s.
BDF, NiSO ₄ > J&J, NiSO ₄	0.7091	n.s.
BDF, SDS ~ J&J, SDS	1.0000	n.s.
BDF, DNCB ~ P&G, DNCB	1.0000	n.s.
BDF, Eugenol ~ P&G, Eugenol	1.0000	n.s.

BDF, HCA ~ P&G, HCA	1.0000	n.s.
BDF, 4-Hydroxybenzoic Acid ~ P&G, 4-Hydroxybenzoic Acid	1.0000	n.s.
BDF, Hydroxycitronellal ~ P&G, Hydroxycitronellal	0.9924	n.s.
BDF, NiSO4 ~ P&G, NiSO4	1.0000	n.s.
BDF, SDS ~ P&G, SDS	1.0000	n.s.
BDF, DNCB ~ PF, DNCB	1.0000	n.s.
BDF, Eugenol ~ PF, Eugenol	1.0000	n.s.
BDF, HCA ~ PF, HCA	1.0000	n.s.
BDF, 4-Hydroxybenzoic Acid ~ PF, 4-Hydroxybenzoic Acid	1.0000	n.s.
BDF, Hydroxycitronellal ~ PF, Hydroxycitronellal	1.0000	n.s.
BDF, NiSO4 > PF, NiSO4	0.2393	n.s.
BDF, SDS ~ PF, SDS	0.9931	n.s.
J&J, DNCB ~ P&G, DNCB	1.0000	n.s.
J&J, Eugenol ~ P&G, Eugenol	1.0000	n.s.
J&J, HCA < P&G, HCA	0.9819	n.s.
J&J, 4-Hydroxybenzoic Acid ~ P&G, 4-Hydroxybenzoic Acid	1.0000	n.s.
J&J, Hydroxycitronellal ~ P&G, Hydroxycitronellal	1.0000	n.s.
J&J, NiSO4 < P&G, NiSO4	0.039	m.s.
J&J, SDS ~ P&G, SDS	1.0000	n.s.
J&J, DNCB ~ PF, DNCB	1.0000	n.s.
J&J, Eugenol ~ PF, Eugenol	1.0000	n.s.
J&J, HCA ~ PF, HCA	1.0000	n.s.
J&J, 4-Hydroxybenzoic Acid ~ PF, 4-Hydroxybenzoic Acid	1.0000	n.s.
J&J, Hydroxycitronellal ~ PF, Hydroxycitronellal	1.0000	n.s.
J&J, NiSO4 ~ PF, NiSO4	1.0000	n.s.
J&J, SDS ~ PF, SDS	1.0000	n.s.
P&G, DNCB ~ PF, DNCB	1.0000	n.s.
P&G, Eugenol ~ PF, Eugenol	1.0000	n.s.
P&G, HCA ~ PF, HCA	1.0000	n.s.
P&G, 4-Hydroxybenzoic Acid ~ PF, 4-Hydroxybenzoic Acid	1.0000	n.s.
P&G, Hydroxycitronellal ~ PF, Hydroxycitronellal	1.0000	n.s.
P&G, NiSO4 > PF, NiSO4	0.0034	m.s.
P&G, SDS ~ PF, SDS	1.0000	n.s.

Table S1: Inductive comparisons between laboratories and substances. n.s.: not significant m.s.: significant on multiple level: adjusted p-value ≤ 0.05 .