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Dendritic cell migration assay: A potential prediction model for identification of contact allergens

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ABSTRACT

This manuscript describes methodology and a prediction model for the MUTZ-LC migration assay. The assay represents the physiological change in Langerhans cell (LC) behavior after exposure to a sensitizing chemical, resulting in LC migration from the epidermis to the dermis. MUTZ-LC are derived from the commercially available MUTZ-3 cell line. Upon exposure to a sensitizer MUTZ-LC migrate preferentially towards CXCL12 whereas upon exposure to a non-sensitizer MUTZ-LC migrate towards CCL5. A CXCL12/CCL5 ratio >1.10 in 2/3 independent experiments is indicative of a sensitizer, whereas a CXCL12/CCL5 ratio ≤1.10 is indicative of a non-sensitizer. At non cytotoxic chemical concentrations 9 sensitizers (2,4-dinitrochlorobenzene, paraphenyldiamine, cinnamaldehyde, isoeugenol, nickel-sulfate, tetramethylthiuram disulfide, eugenol, cinnamic-alcohol, ammonium-hexachloroplatinate) were distinguished from 4 non sensitizers (sodium lauryl sulfate, salicylic acid, phenol, octanoic acid). Critical points in assay performance are (i) MUTZ-3 passage number after thawing (p6–p40); (ii) cell viability (>80%); (iii) standard curve to optimize correlation of fluorescence with cell number; and (iv) optimization of the concentration of rhCXCL12 and rhCCL5 in transwell. The protocol has been tested in three European laboratories and results suggest that it may provide working conditions for performing the DC migration assay which is aimed at distinguishing sensitizers from non sensitizers.

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1. Introduction

The aim of the Sens-it-iv EU funded project, (#018681) was to develop and optimize a battery of *in vitro* assays which when used together in a tiered strategy would be capable of predicting the potential of a chemical to induce sensitization in a human. Once validated, these assays may replace the Local Lymph Node Assay for risk assessment of potentially sensitizing substances (Kimber et al., 1990, 1991). During the development of the assays the following mechanistic steps were taken into account: (i) In order for a potential sensitizer to cause an allergic reaction it must first penetrate or damage the stratum corneum to exert its effect on the viable epidermal and dermal layers below. (ii) Once a chemical has penetrated the stratum corneum, it will trigger keratinocytes to release alarm signals in the form of cytokines and chemokines. In parallel, the chemical pro-hapten, needs metabolic activation

in order to become protein reactive. (iii) The Langerhans Cells (LCs) within the epidermis will then become activated by the hapten-protein complex and undergo maturation and differentiate from antigen-capture and processing cells into potent immunostimulatory dendritic cells (DCs) which are able to present antigen effectively to effector T-cells. (iv) The LCs migrate from the epidermis, to the dermis and then onto the paracortical area of the regional lymph nodes, where they present the allergenic epitope to naïve T-cells (Aiba et al., 1997; Lanzavecchia and Sallusto, 2001; Nosbaum et al., 2009; Reid et al., 2000; Saint-Mezard et al., 2004). (v) This results in expansion and differentiation of allergen reactive T cells, thereby forming specific effector and memory T cells, which migrate via the efferent lymphatics into the bloodstream and recirculate through the body (Sallusto et al., 1999). The individual is then sensitized to the chemical and will develop an allergic contact dermatitis when exposed again to the same chemical.

Five assays were identified which reflect the different steps involved in sensitization: (i) An epidermal equivalent potency assay for determining sensitizer potency (dos Santos et al., 2011; Spiekstra et al., 2009); (ii) A keratinocyte cell line (NCTC) based assay in which increased production of intracellular IL-18 may distinguish

Abbreviations: MUTZ-LC, MUTZ-3 derived Langerhans Cells; CMC, cytokine maturation cocktail.

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skin sensitizers from respiratory sensitizers and non sensitizers (Corsini et al., 2009; Galbiati et al., 2011); (iii) A genomic biosignature using the human MUTZ-3 cell line (GARD) which may identify sensitizers and determine sensitizer potency (Johansson et al., 2011); (iv) A MUTZ-3 derived Langerhans Cell (MUTZ-LC) migration assay which may distinguish sensitizers from non sensitizers based on differential chemokine receptor and migratory properties of MUTZ-LC after sensitizer and non sensitizer exposure (Rees et al., 2011; Ouwehand et al., 2010b); and (v) T cell assays based on T cell proliferation using radioactive labeling or dilution fluorochromes such as carboxyfluorescein succinimidyl ester (CFSE) and also T cell activation markers, such as CD137, CD154 (CD40L), and IFN- γ to identify antigen-activated T cells (Martin et al., 2010). This manuscript describes in detail the current methodology a prediction model for the MUTZ-LC migration assay. This assay represents the functional migration of LC from the epidermis to the dermis after exposure to a sensitizing chemical.

Migration of LC is associated with an increase in CXCR4 and a decrease in CCR1/CCR2/CCR5 receptors on the maturing LC's combined with an increase in the secretion of the chemokine CXCL12 (ligand for CXCR4) from fibroblasts in the dermis (Ouwehand et al., 2008) (Fig. 1). The increase in CXCL12 secretion by fibroblasts is a general stress signal since it is induced by TNF- α (Ouwehand et al., 2008). The mature LC's eventually travel in a CXCR4/CCR7 dependent manner to the lymph nodes where they may prime T cells resulting in sensitization (Villablanca and Mora, 2008). In contrast to sensitizer mediated LC migration via the CXCR4/CXCL12 axis, non sensitizer (irritant) mediated LC migration is mediated by maintained CCR1/CCR2/CCR5 expression and low CXCR4 expression on immature LC together with upregulated CCL5 secretion by dermal fibroblasts. Increased levels of CCL5 result in drawing CCR1/CCR2/CCR5 expressing LC from the epidermis into the dermis (Ouwehand et al., 2010a). The DC migration assay is based on the

differential chemokine receptor expression on LC after exposure to sensitizers (CXCR4) or non-sensitizers (CCR1, CCR2 and/or CCR5) and their ability therefore to migrate preferentially to CXCL12 or CCL5 respectively. This assay uses MUTZ-LC derived from the MUTZ-3 cell line (CD34+ human acute myeloid leukaemia cell line). MUTZ-3 have been shown to differentiate in a cytokine dependent manner into LC-like cells, expressing Langerin and Birbeck granules, which very closely represent the native counterpart (Masterson et al., 2002; Santegoets et al., 2008). Furthermore, mature MUTZ-LC have been shown to migrate preferentially towards CXCL12 whereas immature MUTZ-LC migrate preferentially towards CCL5 in a similar manner to native LC (Ouwehand et al., 2008, 2010a, 2010b). In the migration assay, in parallel wells of a transwell plate either human recombinant CXCL12 or CCL5 are placed in the lower chamber. MUTZ-LC, pre-exposed to non-cytotoxic concentrations of chemicals, are placed in the upper chamber of both CXCL12 and CCL5 containing wells. MUTZ-LC exposed to sensitizers (including pro-haptens) in the upper chamber of a transwell migrate preferentially towards CXCL12 in the lower transwell chamber, whereas MUTZ-LC, pre-exposed to non sensitizers in the upper chamber of a transwell migrate preferentially towards CCL5 in the lower transwell chamber (Ouwehand et al., 2010b; Rees et al., 2011). The read-out of the assay is the relative number of fluorescent MUTZ-LC entering the lower chamber expressed as a ratio of CXCL12: CCL5. A ratio >1.1 in 2/3 independent experiments indicates that the chemical is a sensitizer whereas a ratio $\leq$ 1.1 indicates that the chemical is a non sensitizer. This assay has been tested in a ring-study, whereby three different European laboratories (VU University Medical Centre, Amsterdam, The Netherlands; Università degli Studi di Milano, Italy; and University of West England, Bristol, UK) have shown that the assay is transferable. Intra-laboratory and inter-laboratory variation with regards to MUTZ-3 progenitor cell culture, differentiation into MUTZ-LC, maturation and assay

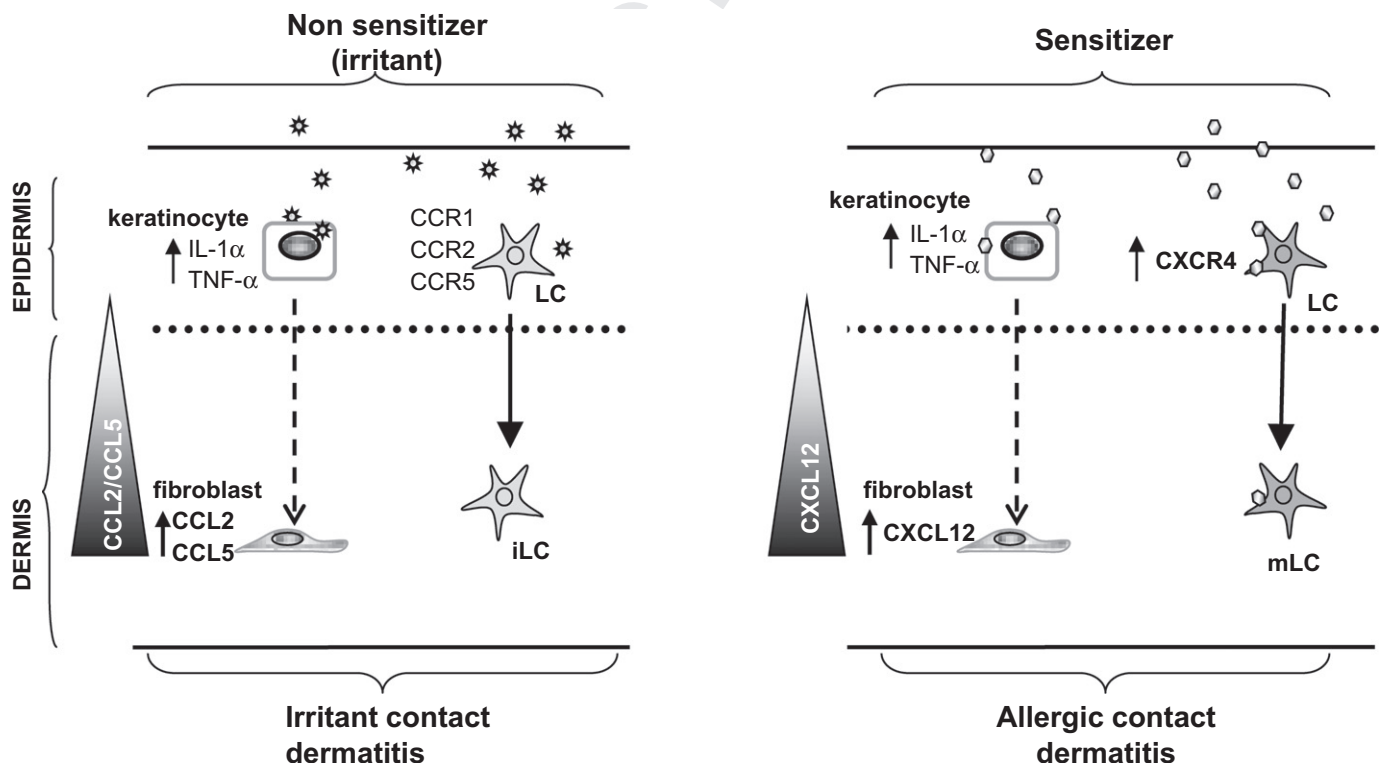


Fig. 1. Schematic overview of mechanism of LC migration in irritant and allergic contact dermatitis. The mechanism of LC migration is depicted. Large arrows pointing down indicate direction of LC migration; dotted arrows indicate cytokine conditioning; small upward arrows indicate upregulation of indicated molecules; shaded pyramids indicate chemokine gradients.

implementation showed that results were consistent between the laboratories, and the dose response data were reproducible in the three laboratories (Rees et al., 2011). Here we describe a prediction model for the assay which incorporates all results obtained to date as well as describing the detailed procedure and critical points for performing the assay.

2. Materials and methods

The entire procedure described in this manuscript is summarized in Fig. 2. In short it involves maintenance of the MUTZ-3 progenitor cell, differentiation into immature LC, labeling with carboxyfluorescein diacetate succinimidyl ester (CFSE), exposure to chemicals, and then migration towards either human recombinant CCL5 or CXCL12. These steps are described in detail below.

2.1. Maintenance of renal cell carcinoma 5637 cell line (5637 cells) and preparation of culture supernatant

The supernatant obtained from the renal cell carcinoma 5637 cell line (ACC 35; DSMZ, Braunschweig, Germany) is used in the maintenance medium of MUTZ-3 progenitor cells. The culture supernatant is prepared as previously described (Quentmeier et al., 1997). The culture medium for these adherent cells is described in Table 1 and the cells are passaged by standard culture and trypsinization methods. For generation of the conditioned culture supernatant, 5637 cells are seeded at a concentration of 5×10^5 /ml in 30 ml of medium in 175 cm² tissue culture flasks. Cells are grown to confluency (usually an overnight culture is sufficient). The culture medium is then discarded and 30 ml of fresh culture medium is added. The 5637 cells are then cultured for 48 h before harvesting culture supernatant. The conditioned culture supernatant is prepared by removing cell debris (by centrifugation) and filter sterilizing with a 0.2 µm filter. Conditioned supernatant is stored at -20°C .

2.2. Maintenance of MUTZ-3 progenitor cells

MUTZ-3 progenitor cells (ACC 295; DSMZ, Braunschweig, Germany) are maintained in MUTZ-3 medium (Table 1) at a concentration of $2\text{--}5 \times 10^5$ cells/ml in a single well within a 12 well tissue culture plate (Corning Costar, NY). Medium is refreshed every 3 to 4 days as follows: cells are gently aspirated in order to achieve a single cell suspension, cells are then transferred to a 50 ml tube and centrifuged at 300 g for 5 min. Cells are resuspended in

warmed culture medium. MUTZ-3 are re-seeded at 2×10^5 cells/ml and cultured in 2 ml of MUTZ-3 medium at 37°C , 5% CO₂, 95% humidity. It is not advisable to culture the cells in larger culture flasks. In addition, MUTZ-3 progenitor cells should be used between passage 6 and 40 after thawing to obtain optimal results in the migration assay.

2.3. Differentiation of MUTZ-3 into immature MUTZ-LC and phenotypic analysis (flow cytometry)

MUTZ-3 are differentiated into MUTZ-LC by culturing at 2×10^5 cells/ml in MUTZ-LC medium (Table 1) in a 12 well plate, with 2 ml cell suspension per well. Differentiation is achieved after 10 days of culture at 37°C , 5% CO₂, 95% humidity. Every 3 days fresh cytokines are added to the medium already in the well at a concentration of 50 ng/ml rhGM-CSF, 5 ng/ml TGF- and 1.25 ng/ml TNF- (in PBS, 0.1% BSA). This is an additional 50% cytokines to that already present in the culture medium.

The degree of MUTZ-3 differentiation is determined by flow cytometry for each independent experiment. Cells within a well are divided into FACS tubes (0.4×10^5 cells/tube) and washed with 3 ml of PBS containing 0.1% BSA and 0.1% sodium azide. After centrifugation, cells are resuspended in the same buffer. Cells are then incubated for 30 min with directly conjugated antibodies or isotype controls (to identify non specific binding), washed with PBS containing 0.1% BSA and 0.1% sodium azide and resuspended using the same buffer. Antibodies used were: CD14-FITC clone TÜK4 1:10 dilution (Miltenyi Biotec GmbH, Germany, cat. No. 130-080-701); CD34-PE clone 581 1:25 dilution (BD Pharmingen, cat. No. 555822); CD1a-FITC clone HI149 1:25 dilution (BD Systems, San Diego, CA, cat. No. 555806); CD207 (Langerin)-PE clone DCGM4 1:25 dilution (Immunotech, France, cat. No. pn im 3577); IgG1-FITC clone MOPC-21 1:25 dilution (BD Pharmingen, cat. No. 555748); IgG1-PE clone MOPC-21 1:25 dilution (BD Pharmingen, cat. No. 555749); IgG2a-FITC clone G155-178 1:25 dilution (BD Pharmingen, cat. No. 555573) and FcR blocker clone unknown 1:25 dilution (Miltenyi Biotec GmbH, cat. No. 130-059-901). During the analysis of the flow cytometry data, cells are gated to exclude any debris present (Fig. 4). Mean Fluorescence and % positive cells is determined.

MUTZ-3 progenitor cells should be Langerin negative, CD1a negative, CD34+ (>5%), CD14+ (>5%) (Fig. 4). The percentage of positive CD34 and CD14 cells may vary between passages. Viability by PI staining should be >80%. MUTZ-3 cells are suitable for the assay from passage 6 to passage 40 after thawing. MUTZ-LC should

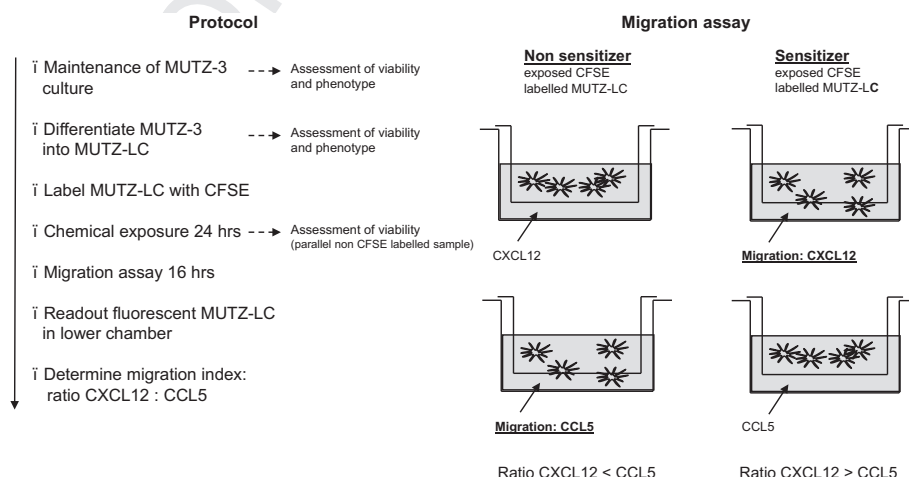


Fig. 2. Protocol for DC migration assay. The number of MUTZ-LC migrating towards either rhCXCL12 or rhCCL5 in the transwell assay system is expressed as a CXCL12/CCL5 ratio. A CXCL12/CCL5 ratio >1.1 is indicative of a sensitizer, whereas a CXCL12/CCL5 ratio ≤ 1.1 is indicative of a non-sensitizer.

Table 1
Culture media.

Component	5637 renal carcinoma cell line	MUTZ-3	MUTZ-LC	Migration assay upper chamber	Migration assay lower chamber
α -MEM ^a	–	YES	YES	YES	YES
RPMI 1640 ^b	YES	–	–	–	–
FBS (heat inactivated) ^c	10%	20%	20%	20%	0%
2-mercaptoethanol ^d	50 μ M	50 μ M	50 μ M	50 μ M	50 μ M
Penicillin–Streptomycin ^e	100 iE/ml,–100 μ g/ml	100 iE/ml,–100 μ g/ml	100 iE/ml,–100 μ g/ml	100 iE/ml,–100 μ g/ml	100 iE/ml,–100 μ g/ml
L-glutamine ^c	–	2 mM	2 mM	2 mM	2 mM
Conditioned 5637 supernatant	–	10%	–	–	–
GM-CSF	–	–	100 ng/ml	–	–
TGF- β	–	–	10 ng/ml	–	–
TNF- α	–	–	ng/ml	–	–

– = absent.

^a Alpha minimal essential medium including ribonucleosides, deoxyribonucleosides (Invitrogen, UK).^b Lonza, Belgium.^c Hyclone, Perbio Thermo scientific, UK.^d Sigma Aldrich, UK.^e Invitrogen, UK.

be Langerin+ (>30%), CD1a+ (>35%), CD34+ (<25%), CD14+ (<75%) (Fig. 4).

2.4. Cell viability

Cell viability is determined for MUTZ-3 and MUTZ-LC. MUTZ-LC viability is also determined on a cell sample directly after chemical exposure, before parallel CFSE labeled and chemical exposed cells are added to the upper transwell migration assay. Propidium iodide (PI) viability test (Invitrogen, Paisley, UK) is based on the PI binding to the DNA within the cell. When cells

are less viable, the cells become porous making PI able to leak into the cell. Cells (0.4×10^5 cells/tube) are transferred to FACS tubes (Beckton Dickinson) and washed with 3 ml of PBS containing 0.1% BSA and 0.1% sodium azide. After centrifugation, cells are resuspended in the same buffer. PI is added in a final concentration of 1 μ g/ml. Subsequently a 30 min incubation period will allow the PI to bind to the DNA in damaged cells. After the incubation, cells are washed again, centrifuged, and taken up into a small volume of FACS buffer. PI-positive stained cells can be visualized by FACS analysis at 488 nm. The percentage of positive PI cells in the MUTZ-LC population indicates the

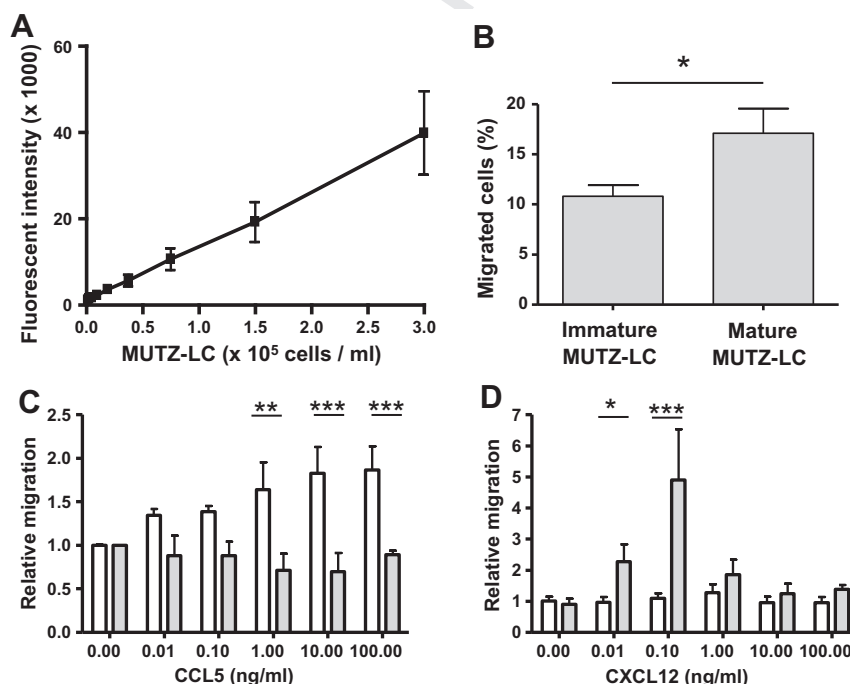


Fig. 3. Optimization of assay conditions. (A) standard curve of serial dilutions of 3×10^5 CFSE labeled MUTZ-LC for correlation of fluorescence with cell number ($n = 4$ in duplicate; Wilcoxon t -test); (B) percentage of unexposed and CMC exposed MUTZ-LC migrating from the upper chamber towards CXCL12 (0.01 ng/ml) in the lower chamber. An excess amount of Flow-Count Fluorospheres was added to 3×10^5 MUTZ-LC as described by the supplier (Beckman Coulter, Fullerton, CA, USA) and the percentage of migrating cells was determined with a FACSscan flowcytometer ($n = 4$, Mann–Whitney); (C) titration of rhCXCL5 and (D) rhCXCL12 in order to identify optimal chemokine concentrations ($n = 6$ in duplicate, two-way ANOVA test followed by a Bonferroni posttest). White bar indicates immature MUTZ-LC, gray bar indicates maturing MUTZ-LC exposed to CMC. Mean $\pm$ SEM is shown for totally independent biological experiments (n) with each experiment being from a different passage number of MUTZ-LC and also containing an intra-experiment duplicate. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$.

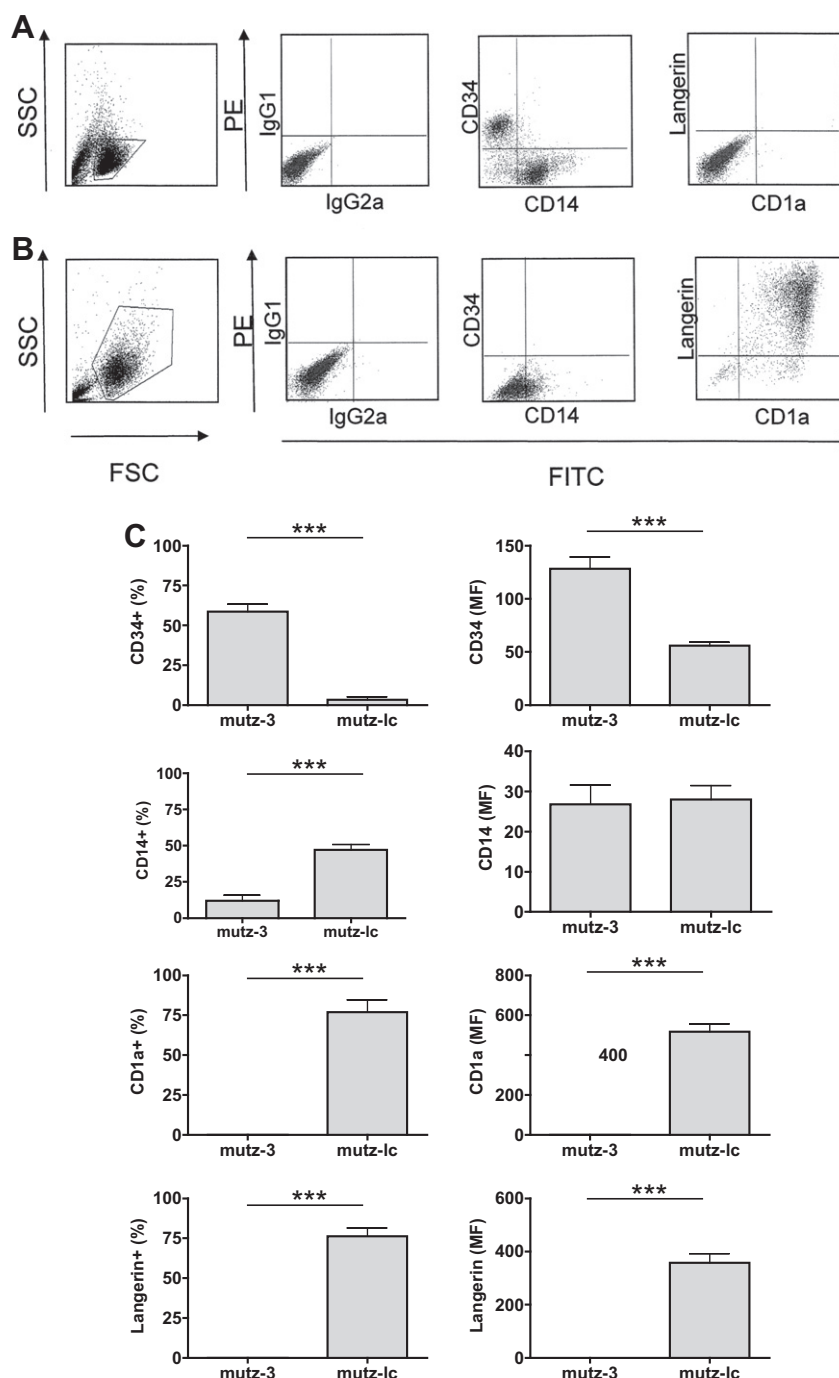


Fig. 4. Phenotypic analysis of MUTZ-3 and MUTZ-LC. (A) representative MUTZ-3 dot plot; (B) representative MUTZ-LC dot plot; (C) percentage positive cells and mean fluorescence (MF) after incubating with antibodies for CD34, CD14, CD1a and Langerin. Mean $\pm$ SEM is shown for at least seven totally independent biological experiments for the NL lab. (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$ by Wilcoxon t -test).

amount of non viable cells. The absolute viability of MUTZ-3 progenitor cells and MUTZ-LC should be $>80\%$ before chemical exposure. The highest chemical concentration used in the dose response studies should result in no more than 25% cytotoxicity relative to unexposed cultures (75% cell viability) (see below).

2.5. labeling MUTZ-LC with CFSE and correlation of fluorescence with cell number

MUTZ-LCs are resuspended at a final concentration of 1×10^6 cells/ml in PBS containing 0.1% BSA. 10 μ M CFSE (Cell-Trace™ CFSE Cell Proliferation Kit, Invitrogen) is added to 1 ml cell

suspension in a tube followed by incubation for 10 min. at 37 °C. The staining is quenched by addition of five volumes of ice cold MUTZ-LC medium followed by 5 min incubation on ice. A standard curve correlating the degree of fluorescence to the number of MUTZ-LC is then performed starting from 3×10^5 labeled cells placed in a 96 well flat bottomed plate (Greiner) (Fig. 3A). The fluorescence can be read at 485 nm excitation and 535 emission using e.g.: Victor² Wallac 1420 Multilabel counter; or a Fluorstar Optima (BMG Labtech) or a Thermo Scientific Ascent Fluoroskan.

The linear part of the graph shows the optimal correlation between cell number and fluorescence. Taking into account that in the DC migration assay only 5–30% of the total number of

Table 2
Chemicals used in migration assays.

Chemical	Abbreviation	CAS Number	LLNA Classification	Human Classification
2,4 dinitrochlorobenzene	DNCB	97-007	Extreme ^a	Extreme ^c
Paraphenyldiamine	PPD	106-50-3	Strong ^b	Strong ^c
Cinnamaldehyde	CIN	14371-10-9	Moderate ^b	Moderate ^d
Isoeugenol	IE	97-54-1	Moderate ^b	Moderate ^d
Nickel sulfate	NiSO ₄	7786-81-4	Moderate ^c	Moderate ^c
Tetramethylthiuram disulfide	TMTD	137-26-8	Moderate ^b	na
Eugenol	EU	97-53-0	Weak ^b	Weak ^d
Cinnamic alcohol	CA	104-54-1	Weak ^b	Weak ^d
Ammonium hexachloroplatinate	HCPT	16919-58-7	Respiratory sensitizer	na
Sodium Lauryl sulfate	SDS	151-21-3	Weak/irritant ^c	Non-sensitizer ^e
Salicylic acid	SA	69-72-7	Non-sensitizer ^c	Non-sensitizer ^c
Phenol	–	108-95-2	Non-sensitizer ^c	Non-sensitizer ^c
Octanoic acid	–	124-07-2	Non-sensitizer ^c	Non-sensitizer ^c

The chemicals were selected from a set of reference compounds chosen by the Sens-it-iv consortium. The criteria by which they were selected were that they were either well characterized sensitizers or chemicals that showed a lack of sensitizing activity according to mouse LLNA data and Human data (chemical dose per skin area leading to a sensitization incidence of 5% (DSA₀₅) or maximal no observed effect level (NOEL) (g/cm²) of chemicals). Of note, Sodium Lauryl sulfate is a well recognized false positive in LLNA. The chemicals had to be available commercially. Na = information not available. Vehicles were dimethylsulfoxide (DMSO) (Sigma Aldrich, UK) at a final concentration of 0.1% DMSO or endotoxin free water) (Sigma Aldrich, UK). NiSO₄ was purchased from Merk, Whitehouse Station, NY and all other chemicals were purchased from Sigma (St Louis, MO, USA) at the highest purity available.

^a Kimber et al. (2003).

^b Gerberick et al. (2005).

^c Schneider and Akkan (2004).

^d Basketter et al. (2005).

^e Basketter and Kimber (2011).

cells placed in the upper compartment migrate to the lower compartment (Fig. 3B) it can be concluded that approximately $1.5\text{--}3 \times 10^5$ cells/ml should be placed in the upper transwell compartment. This number is estimated taking into account variation in labeling efficiency and sensitivity of the multi-plate reader used. These optimal cell numbers are then used for further migration experiments. It is recommended that each laboratory performs this optimization step three times in order to obtain a mean value as the actual window of chemotaxis in response to CCL5 and CXCL12 is quite small and may vary slightly in different laboratories.

2.6. Maturation and chemical exposure of MUTZ-LC

All exposure experiments are performed in 24-wells plates, with each well containing approximately $2.5\text{--}3.0 \times 10^5$ CFSE labeled MUTZ-LC in 1 ml of culture medium.

Maturation: MUTZ-LC are exposed to a cytokine maturation cocktail (CMC) in MUTZ-LC medium for 24 h to obtain mature MUTZ-LC which are used as the positive control in the migration assay. The cytokine cocktail consists of 100 ng/ml IL-6, 25 ng/ml IL-1 β (Miltenyi Biotech, Germany), 50 ng/ml TNF- α , 1 μ g/ml Prostaglandin E₂ (Sigma Aldrich, UK).

Chemical exposure: MUTZ-LCs are exposed to chemicals (not blinded) in a dose dependent manner for 24 h. The chemicals are selected by ECVAM based on human clinical experience and on validated data collected by the LLNA assay for European research purposes on *in vitro* assay development (Table 2) (Casati et al., 2009). The highest chemical concentration used in the dose response studies correlates to no more than 25% cytotoxicity, 75% cell viability as determined by propidium iodide (PI) staining (Invitrogen, Paisley, UK) (see above). Then 50% stepwise decreases in chemical concentration are used in the dose range and at least three chemical concentrations were included in the dose response.

2.7. Transwell migration assay

Optimization of human recombinant CXCL12 (rhCXCL12) and CCL5 (rhCCL5) concentrations: each laboratory should identify the optimal concentration of rhCXCL12 and rhCCL5 for incorporation into the lower chamber in the transwell migration assay since the actual window of chemotaxis in response to CCL5 and CXCL12 is

quite small and may vary slightly in different laboratories particularly if different lot numbers of recombinant chemokines are used. Therefore a dose response for rhCCL5 (0, 0.01, 0.1, 1, 10 and 100 ng/ml) with immature unexposed MUTZ-LC and a dose response for rhCXCL12 (0, 0.01, 0.1, 1, 10 and 100 ng/ml) with CMC exposed maturing MUTZ-LC should first be performed (Fig. 3C and D). The optimal CXCL12 (e.g. 0.1 ng/ml) and CCL5 (e.g. 100 ng/ml) concentrations are then further used in the assay. CFSE labeled MUTZ-LC, after chemical or CMC exposure (see above) are harvested, centrifuged and re-suspended in 50 μ l of 20% FBS α -MEM with penicillin/streptomycin and glutamine (upper chamber migration assay medium: Table 1). The cell suspension, 3×10^5 cells/well in 50 μ l, is added to a 5 μ m poresize insert of a 24 transwell plate, (Corning Costar, NY). Either rhCCL5 or rhCXCL12 is added to the lower well in 500 μ l lower chamber migration assay medium (Table 1). The dilutions of chemokines are prepared in α -MEM with penicillin/streptomycin and glutamine only. The transwell plate is then incubated for 16 h at 37 $^{\circ}$ C and 5% CO₂. Hereafter, the cells from each lower chamber are harvested and transferred to a new well in a 96 well flat bottomed plate (Greiner). The fluorescence is read as described above. Data are presented as the fluorescence intensity of cells migrating towards CXCL12 divided by the fluorescence intensity of cells migrating towards CCL5.

2.8. Positive and negative controls in DC migration assay

CXCL12: CMC exposed MUTZ-LCs are used as a positive control and represent maturing cells which preferentially migrate towards CXCL12. Unexposed or vehicle exposed MUTZ-LC are used as a negative control and represent immature cells with low migratory capacity towards CXCL12 (Fig. 3D).

CCL5: Unexposed or vehicle exposed MUTZ-LCs are used as a positive control and represent immature cells which preferentially migrate towards CCL5. CMC exposed MUTZ-LCs are used as a negative control and represent maturing cells with low migratory capacity towards CCL5 (Fig. 3C).

2.9. Statistical analysis

The results are shown as the mean $\pm$ standard error of the mean. Variations in surface expression of CD34, CD14, CD1a, and Langerin

Table 3

Summary of inter and intra laboratory variation in CXCL12/CCL5 ratio of sensitizers.

Chemical	Laboratory	Experiment	Chemical concentration ^a	CXCL12/CCL5 ratio ^b	Positive experiments ^c	Classification ^d
NiSO ₄	NL	1	150 µM	1.30	4/4	Sensitizer
		2	300 µM	1.23		
		3	600 µM	1.62		
		4	600 µM	1.46		
	UK	1	600 µM	1.09	2/3	Sensitizer
		2	600 µM	1.18		
		3	300 µM	1.22		
	Italy	1	300 µM	1.17	2/3	Sensitizer
		2	600 µM	1.10		
		3	600 µM	1.28		
DNCB	NL	1	6 µM	1.12	6/8	Sensitizer
		2	12 µM	1.55		
		3	12 µM	0.97		
		4	6 µM	1.12		
		5	12 µM	1.08		
		6	12 µM	1.23		
		7	12 µM	1.36		
		8	6 µM	1.40		
	UK	1	12 µM	1.24	3/3	Sensitizer
		2	12 µM	1.34		
		3	12 µM	1.18		
	Italy	1	12 µM	1.15	2/3	Sensitizer
		2	12 µM	1.44		
		3	12 µM	1.09		
		3	12 µM	1.09		
CIN	NL	1	50 µM	1.12	3/5	Sensitizer
		2	50 µM	1.45		
		3	12.5 µM	1.07		
		4	25 µM	1.10		
		5	50 µM	1.11		
	UK	1	100 µM	1.53	3/3	Sensitizer
		2	100 µM	1.43		
		3	50 µM	1.13		
		3	50 µM	1.13		
TMTD	NL	1	0.2 µM	0.99	2/3	Sensitizer
		2	0.1 µM	1.11		
		3	0.2 µM	1.24		
HCPT	NL	1	200 µM	1.18	3/4	Sensitizer
		2	300 µM	1.08		
		3	300 µM	1.11		
		4	200 µM	1.27		
		4	200 µM	1.27		
<i>Pre-pro haptens</i> EU	UK	1	900 µM	1.60	3/3	Sensitizer
		2	450 µM	1.23		
		3	450 µM	1.32		
IE	UK	1	950 µM	1.41	3/3	Sensitizer
			950 µM	1.38		
			950 µM	1.31		
CA	UK	1	50 µM	1.25	4/4	Sensitizer
		2	50 µM	1.31		
		3	50 µM	1.21		
			50 µM	1.96		
PPD	UK	1	200 µM	1.1.32	3/3	Sensitizer
		2	200 µM	1.1.39		
		3	200 µM	1.1.76		

Each experiment represents a totally independent biological experiment performed with a different passage number of MUTZ-3 on a different day. The average CXCL12/CCL5 ratio of the intra-experiment duplicate is shown.

^a The chemical concentration which resulted in the highest CXCL12/CCL5 ratio and >75% viability in the dose response

^b highest CXCL12/CCL5 ratio reached at non toxic concentration (>75% viability) in dose response of 50% stepwise decreases in chemical concentration starting at the chemical concentration which correlates to no more than 25% cytotoxicity, 75% cell viability.

^c number of positive experiments in which a CXCL12/CCL5 ratio >1.10 is reached

^d if ≥ 2 of 3 experiments CXCL12/CCL5 ratio >1.10 chemical is a sensitizer; if <2 of 3 experiments CXCL12/CCL5 ratio >1.10 then chemical is a non-sensitizer (applies also to multiple numbers of experiments).

or the CXCL12/CCL5 ratios of migrated MUTZ-LC after chemical treatment are compared using the one-way ANOVA test followed by Dunnett's multiple comparison posttest (where the ratio of the treated cells is compared to that of the control vehicle or untreated cells), $p < 0.05$ was considered significant.

Differences between immature MUTZ-LC and matured MUTZ-LC are compared using the students t -test where values $p < 0.05$ are noted as being significant.

3. Results

Tables 3 and 4 present a new prediction model and summarize all results obtained until present and which have been extensively described by Ouwehand et al. (2010b); Rees et al. (2011). In the prediction model, the 9 sensitizers and 4 non sensitizers tested were assessed according to their ability to stimulate MUTZ-LC migration in totally independent biological experiments. Each indi-

Table 4
Summary of inter and intra laboratory variation in CXCL12/CCL5 ratio of non-sensitizers.

Chemical	Laboratory	Experiment	Chemical concentration ^a	CXCL12/CCL5 ratio ^b	Positive experiments ^c	Classification ^d
SDS	NL	1	300 μ M	1.29	2/6	Non sensitizer
		2	150 μ M	0.98		
		3	600 μ M	1.03		
		4	150 μ M	1.10		
		5	150 μ M	1.11		
		6	600 μ M	1.06		
	UK	1	400 μ M	0.92	0/3	Non sensitizer
		2	300 μ M	0.98		
		3	400 μ M	0.98		
	Italy	1	150 μ M	0.97	0/3	Non sensitizer
		2	300 μ M	0.98		
		3	300 μ M	0.97		
SA	NL	1	2 mM	1.04	0/3	Non sensitizer
		2	2 mM	0.90		
		3	1 mM	0.84		
	UK	1	100 μ M	0.74	0/3	Non sensitizer
		2	100 μ M	0.94		
		3	50 μ M	0.90		
	Italy	1	2 mM	1.02	0/3	Non-sensitizer
		2	4 mM	1.03		
		3	1 mM	0.98		
Phenol	NL	1	6 mM	0.95	0/5	Non-sensitizer
		2	6 mM	0.95		
		3	3 mM	1.01		
		4	3 mM	1.01		
		5	6 mM	1.02		
Octanoic acid	UK	1	1.5 mM	0.84	0/4	Non-sensitizer
		2	2 mM	0.98		
		3	2 mM	0.90		
		4	2 mM	0.94		

Each experiment represents a totally independent biological experiment performed with a different passage number of MUTZ-3 on a different day. The average CXCL12/CCL5 ratio of the intra-experiment duplicate is shown.

^a The chemical concentration which resulted in the highest CXCL12/CCL5 ratio and >75% viability) in the dose response.

^b highest CXCL12/CCL5 ratio reached at non toxic concentration (>75% viability) in dose response of 50% stepwise decreases in chemical concentration starting at the chemical concentration which correlates to no more than 25% cytotoxicity, 75% cell viability.

^c number of positive experiments in which a CXCL12/CCL5 ratio >1.10 is reached.

^d if ≥ 2 of 3 runs CXCL12/CCL5 ratio >1.10 chemical is a sensitizer; if <2 of 3 runs CXCL12/CCL5 ratio >1.10 then chemical is a non-sensitizer (applies also to multiple numbers of runs).

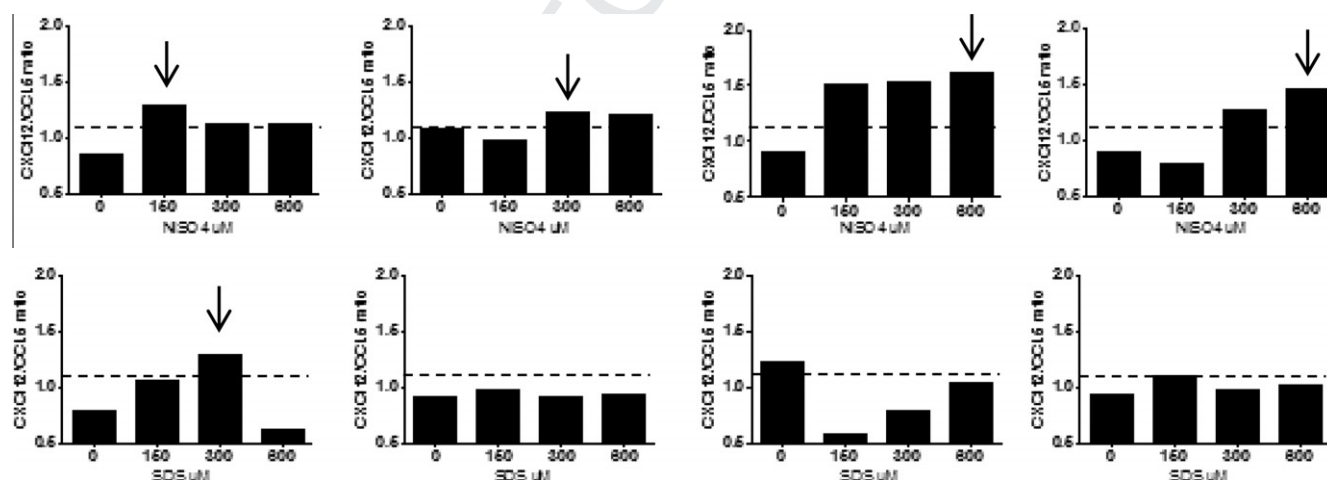


Fig. 5. The dose response used to identify the maximum CXCL12/CCL5 ratio in the prediction model. If in ≥ 2 of 3 experiments the CXCL12/CCL5 ratio >1.10 then the chemical is classed as a sensitizer; if in <2 of 3 experiments the CXCL12/CCL5 ratio >1.10 then the chemical is a non-sensitizer (applies also to multiple numbers of experiments). Four typical independent experiments are shown for NiSO₄ and SDS. Each experiment is shown as the average of an intra-experiment duplicate and each experiment is performed with a different passage number of MUTZ-LC. Arrow indicates value >1.1 used in the prediction model. Note 4/4 positive experiments for the sensitizer NiSO₄ and only 1/4 positive experiments for the non sensitizer SDS.

vidual dose response experiment represents a different passage number of MUTZ-3/LC and the average of an intra-experiment duplicate (Fig. 5). The maximum CXCL12/CCL5 migration ratio and the chemical concentration at which it was obtained in the dose response is shown in Tables 3 and 4. Of note, this chemical

concentration could vary slightly between independent experiments which emphasises the necessity of the dose response in order to identify the maximum CXCL12/CCL5 migration ratio. The prediction model classed a chemical as a sensitizer if 2 out of 3 independent experiments have a CXCL12/CCL5 ratio >1.1 and as a non

sensitizer if 2 out of 3 independent experiments have a CXCL12/CCL5 ratio ≤ 1.1 . All 9 sensitizers and 4 non sensitizers were identified correctly. Four of the sensitizers were pre-pro haptens and 1 was a respiratory sensitizer (HCPT). Five of the chemicals were tested in an inter-laboratory study with 3 different EU partners (NL = lead lab; UK and Italy). The MUTZ-3 maintenance, differentiation and maturation methods were transferable from the lead laboratory (NL) to the UK and Italy laboratories. Results were reproducible between the laboratories. All 3 laboratories identified the chemicals correctly. Tables 3 and 4 show that the variation between the independent experiments within a laboratory and between laboratories was low.

4. Discussion

This manuscript describes in detail the methodology of the MUTZ-LC migration assay which until now has been able to distinguish nine sensitizers from four non sensitizers. This assay is a functional assay in that it is dependent on the migration of dendritic cells (MUTZ-LC) towards a chemokine gradient created by either CXCL12 or CCL5 pipetted into the lower transwell compartment only (Fig. 1). The migration is dependent on the differential chemokine receptor expression on the surface of MUTZ-LC. Upon exposure to a sensitizer the CXCR4/CXCL12 migration axis is dominant. Upon exposure to a non sensitizer the CCR2/CCR5/CCL5 migration axis is dominant.

Since the DC migration assay addresses a physical change in the behavior of the cell i.e. cell migration towards CXCL12, the assay is unique compared to other assays under development. These other assays address changes in biomarker expression (GARD, hCLAT, MUSST), promoter activity (KeratinSens) or peptide binding and reactivity (Direct Protein Reactivity Assay) (Ashikaga et al., 2006; Gerberick et al., 2007; Gerberick et al., 2009; Johansson et al., 2011; Natsch et al., 2009, 2011; Sakaguchi et al., 2006). The NCTC/IL-18 assay distinguishes contact sensitizers from respiratory sensitizers and non sensitizers and therefore was unable to identify HCPT as a sensitizer (Corsini et al., 2009). This makes the DC migration assay complementary to these other assays and therefore a suitable candidate for incorporation into the battery of assays aimed at distinguishing sensitizers from non sensitizers. The complexity involved in performing the DC migration assay however suggests that it would be best implemented in a tiered strategy below the initial first round screening. For example, GARD which assesses chemicals according to their genomic biomarker signature, or the Direct Protein Reactivity Assay would permit large numbers of chemicals to be screened in a cost effective manner and therefore one of these assays could be anticipated in a first round screen. Potential sensitizers emerging from either of these assays could then be further tested in the DC migration assay. The DC migration assay and T-cell assays (Martin et al., 2010) are the only assays under development at the moment which address a physical change in behavior of the cell.

Currently MUTZ-LCs are used in the DC migration assay. This physiologically relevant cell line exhibits exactly the same mechanism of LC migration via CXCR4/CXCL12 and CCR2/CCR5/CCL5 as was observed after freshly excised intact human skin was exposed to sensitizers and non sensitizers (Ouweland et al., 2008, 2010a, 2010b). It is possible that other DC like cell lines can be incorporated into the DC migration assay. However, it is possible that migration will not be directed by the CXCL12/CXCR4 axis as with MUTZ-LC and ex vivo LC and therefore this would first need confirming. If the CXCL12/CXCR4 axis is not used then other chemokine receptor/ligand pairs would need to be identified which would be regulated differentially upon sensitizer or non sensitizer exposure. Such anticipated cell lines could be U937 or THP-1 which

are considerably easier to culture than MUTZ-3 since their growth and differentiation is not cytokine dependent. Currently positive and negative controls in the DC migration assay are based on untreated or cytokine cocktail treated MUTZ-LC. Our results indicate that in the final assay set up a chemical allergen (e.g. Nickel sulfate) could be most suitable for a positive control to represent maturing cells which migrate preferentially to CXCL12 and a chemical non sensitizer (e.g. octanoic acid) could be most suitable for a positive control to represent immature cells which migrate preferentially to CCL5.

As with all current *in vitro* cell based assays, the DC migration assay does have a number of limitations. The major limitation being the MUTZ-3 cell line is a notoriously demanding and slow growing cell line. The methodology used to differentiate MUTZ-3 into MUTZ-LC and then to perform the migration assay does require a certain amount of DC expertise. However, we do show here that these most physiologically relevant cells did grow to provide sufficient numbers to perform the assay in all laboratories and the entire protocol was indeed transferable. Another major limitation, as with most other *in vitro* assays, is the fact that chemicals have to dissolve in the culture medium. Chemicals can be added directly to the culture medium or introduced via a vehicle e.g. DMSO (0.1% final concentration). However, many chemicals cannot be dissolved in DMSO and for chemicals where this is possible it remains unsure whether the chemical remains in solution once added to the culture medium. More extensive research aimed at identifying different vehicles (e.g. cyclodextran, liposomes) may reduce this limitation. Also, the use of integrated immune-competent skin equivalents containing DC may provide another alternative for certain chemicals since this model would permit water insoluble chemicals to be applied topically to the stratum corneum in for example acetone: olive oil 4:1 (Ouweland et al., 2011; dos Santos et al., 2011). In addition to solubility limitations potential chemical stability/reactivity issues may exist. Currently not enough data are available to determine whether this assay will give information on sensitizer potency. Another limitation of the assay is its low throughput and complexity which go together with high costs.

Critical points in the performance of the DC migration assay are (i) the passage number of the MUTZ-3 after thawing (passage 6–40 should be used); (ii) cell viability, after chemical exposure MUTZ-LC should be at least 80% viable otherwise migration will not take place; (iii) optimization of the cell number to be introduced into the upper transwell chamber (determined from standard curve correlation of fluorescence with cell number); and (iv) optimization of the amount of recombinant CXCL12 and CCL5 used in the lower transwell chamber (determined by titration). The values obtained in criteria (iii) and (iv) may vary slightly between different laboratories. However by following the protocol described in detail in this manuscript, optimal working conditions can be identified for each laboratory. The mixed population of cells present after differentiation of MUTZ-3 into MUTZ-LC does not influence the final readout of the assay although there should be a significant increase CD1a and Langerin expression indicating that the cells had indeed differentiated into LC. Slight variations between laboratories are acceptable as indicated by the UK-lab having lower CD14 expression after maturation of MUTZ-3 to MUTZ-LC than either the NL lab or Italy lab without this affecting the outcome of the assay (Rees et al., 2011).

It is generally accepted that a single *in vitro* assay will not be able to replace the LLNA and that a battery of assays will be required which cover the different areas of sensitization in a cost effective manner (Corsini and Roggen, 2009; Maxwell et al., 2011; Roggen et al., 2008). The DC migration assay is a potential candidate assay for incorporation into such a battery of assays. Until date only nine sensitizers and four non sensitizers have been tested. Our results suggest MUTZ-LC may be metabolically active

and that the assay may be suitable for identifying pre-, pro-haptens which until now have been a considerable challenge for *in vitro* assay development. The DC migration assay was also able to classify correctly SDS as a non sensitizer, indicating a good correlation of the *in vitro* data with clinically relevant human data. Notably, SDS is a well known false positive in the mouse LLNA (Basketter and Kimber, 2011). The technology has been shown to be transferable and results reproducible between three EU laboratories despite the complexity of the assay (Rees et al., 2011). This physiologically relevant DC migration assay is at the stage where it requires extensive testing with coded chemicals within a ring-study setting in order to expand on the current results and identify the applicability domain. Taking into account the limitations, advantages and preliminary results we can conclude that the DC migration assay is ready to enter pre-validation studies.

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