

Inter-laboratory study of the *in vitro* dendritic cell migration assay for identification of contact allergens

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ABSTRACT

The aim of this study was to investigate the transferability of technology and reproducibility of MUTZ-3 derived Langerhans Cell (MUTZ-LC) migration assay. The protocol was transferred from the NL-lab to two Sens-it-iv project partners (UK-lab, Italy-lab). Intra- and inter-laboratory variation with regards to MUTZ-3 progenitor culture, differentiation to MUTZ-LC, maturation and migration assay were investigated. In the transwell-migration-assay, preferential migration of sensitizer-exposed MUTZ-LC towards CXCL12 was observed (three sensitizers), whereas non-sensitizer-exposed MUTZ-LC only migrated towards CCL5 (two non-sensitizers). Four pre-pro-haptens were also identified by UK-lab. When taking the arbitrary criteria of at least two of three independent repetitions per laboratory having to have a CXCL12/CCL5 ratio >1.1 for classification as a sensitizer, all sensitizers tested in all labs were easily distinguished from all non-sensitizers. The number of repetitions giving false negative or false positive was very low (only 7 out of a total of 54 repetitions), indicating that both intra- and inter-laboratory variation was extremely low. Even though only a few chemicals were tested in this study, we show clearly that the *in vitro* DC migration assay is transferable between laboratories. The results were consistent between the laboratories, and the dose response data were reproduced in the three laboratories.

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1. Introduction

Potentially sensitising chemicals used as ingredients in the manufacture of cosmetics and toiletries are currently tested using *in vivo* animal models e.g. the murine local lymph node assay (LLNA) (Kimber et al., 1989). However, the introduction of a European ban (Directive 76/768/EEC) on animal testing which is due to be implemented in 2013, together with the REACH (Registration, Evaluation and Authorization of Chemicals) legislation, has made it imperative to develop alternative *in vitro* methodologies (Adler et al., 2011). Product safety is of paramount importance to both the consumer and manufacturer in preventing sensitisation, which could potentially lead to the development of allergic contact dermatitis (ACD).

The aim of the Sens-it-iv EU funded project, (#018681) was to develop and optimise *in vitro* assays to predict the sensitising po-

Abbreviations: MUTZ-LC, MUTZ-3 derived Langerhans Cells; CMC, cytokine maturation cocktail; MUSST, myeloid U937 skin sensitisation test; h-CLAT, human cell line activation test.

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tential of chemicals. The assays should be developed to the stage where they are ready for possible selection into pre-validation studies in the future. One of the assays developed within Sens-it-iv is the dendritic cell (DC) migration assay (Ouwehand et al., 2010b). The technology transfer of this assay within a ring study is the subject of this manuscript.

Dendritic cells play an important role in the immune system since they absorb, transport and present antigens to T cells. When a possible sensitizer comes into contact with the skin and enters the epidermis a sequence of events is instigated. The sensitising agent is taken up by the Langerhans cells (LCs) which start to mature and migrate from the epidermis to the dermis. This migration of LCs is one of the first stages in contact sensitisation. The migration is associated with an increase of CXCR4 and a decrease in CCR1/CCR3/CCR5 receptors (Neves et al., 2008; Lin et al., 1998) on the maturing LCs combined with an increase in the secretion of the chemokine CXCL12 (ligand for CXCR4) in the dermis by fibroblasts. The increase in CXCL12 secretion by fibroblasts is a general stress signal since it is induced by TNF- α (Ouwehand et al., 2008) and in burns (Avniel et al., 2006) and is not restricted to sensitizers (Ouwehand et al., 2008). The mature LCs eventually travel in a CXCR4/CCR7 dependent manner to the lymph nodes where they may prime T cells resulting in sensitisation. In contrast

to sensitizer mediated LC migration via the CXCR4/CCL12 axis, non-sensitizer (irritant) mediated LC migration is mediated by maintained CCR1/CCR3/CCR5 (not decreased) expression and low CXCR4 expression on immature LC together with upregulated CCL5 secretion by dermal fibroblasts. Increased levels of CCL5 result in drawing CCR1/CCR3/CCR5 expressing LC from the epidermis into the dermis (Ouweland et al., 2010a). The DC migration assay is based on this differential chemokine receptor expression on LC after exposure to sensitizers (CXCR4) or non-sensitizers (CCR1, CCR3 and/or CCR5) and their ability therefore to migrate preferentially to CXCL12 or CCL5 respectively.

A number of other DC based assays have been described and are in varying stages of development and pre-validation (dos Santos et al., 2009). Aiba et al. (1997) investigated the response of monocyte dendritic cells (moDC's) to a small number of sensitizers and irritants by assessing their ability to increase surface expression of CD54, CD86 and HLA-DR (Aiba et al., 1997). Research carried out by the partners in the Sens-it-iv consortium focused mainly on the phenotypic changes (CD86, CD80 and PDL-1) (Ouweland et al., 2010b; Williams et al., 2010), chemokine production CXCL8 (Ouweland et al., 2010b; Toebak et al., 2006), and proteomic p38 mitogen-activated protein kinase (Mitjans et al., 2010). Cell lines (e.g.: THP-1, U-937 and MUTZ-3) investigated for their application in DC based assays have been extensively reviewed (dos Santos et al., 2009). Notably, these DC based assays, in contrast to the DC migration assay, are not based on changes in functional behaviour (migration) of DC when exposed to sensitizers and non-sensitizers, but are based on changes in expression of surface and intracellular biomarkers. The DC migration assay is the first assay to be based on the functional migration of LC due to their differential chemokine receptor expression.

The use of cell lines is a useful alternative to using monocytes isolated from peripheral blood mononuclear cells (PBMCs) since they are presumed to avoid the variation in results that could potentially be seen between individual donors. Among the different human monocytic cell lines, MUTZ-3 cells (a human acute myeloid leukaemia cell line) are most physiologically similar to *in vivo* DCs (Larsson et al., 2006). When MUTZ-3 cells are stimulated with a specific cytokine cocktail (GM-CSF, TGF- β and TNF- α) they can differentiate, and in doing so acquire a phenotype consistent with LC (Masterson et al., 2001). In a similar manner to their native counterpart, when MUTZ-3 derived LC (MUTZ-LC) are exposed to a sensitising substance, they will start to mature and migrate in a CXCR4/CXCL12 dependent manner. Upon exposure to a non maturing substance (non-sensitizer) they will maintain

their immature phenotype and migrate in a CCR1/CCR3/CCR5/CCL5 dependent manner. This *in vivo* like differential migratory behaviour makes MUTZ-3 derived LC (MUTZ-LC) ideal cells for the DC migration assay (Ouweland et al., 2010b).

Our previous study describes the ability of MUTZ-LC to preferentially migrate to CXCL12 after exposure to five contact sensitizers (CXCL12/CCL5 ratio >1) and to preferentially migrate to CCL5 after exposure to three irritants (CXCL12/CCL5 ratio <1) (Ouweland et al., 2010b). These promising results led us to determine whether the MUTZ-LC migration assay technology was transferable from the laboratory where it was developed in VU University Medical Centre, Amsterdam (NL-lab) to the laboratories of two other Sens-it-iv partners: University of the West of England, Bristol (UK-lab) and Università degli Studi di Milano, (Italy-lab). Intra-laboratory and inter-laboratory variation with regards to MUTZ-3 progenitor culture, differentiation to MUTZ-LC, maturation and assay implementation are described. Furthermore the UK-lab extended the study to include pro- and pre-haptens since correct classification of these chemicals is still a major challenge in *in vitro* assay development.

2. Materials and methods

The transfer of technology occurred at the Dermatology Department, VU Medical Centre, Amsterdam. A member of staff from each of the participating centres (Italy and UK) took part in a three day course where they were trained in the techniques, according to a standard operating procedure, required to perform the migration assay. The protocol used was exactly as described previously (Ouweland et al., 2010b). The VU Medical Centre provided the MUTZ-3 cell line, culture supernatant from the 5637 cell line, and the cryopreserved 5637 cell line. The laboratories initially used the VU Medical Centre generated culture supernatant and then went onto generate their own supernatant. Each laboratory used their own FBS and other culture medium components.

2.1. Maintenance, and differentiation of MUTZ-3 cell line

The MUTZ-3 cell line was cultured as described previously (Ouweland et al., 2008, 2010b). Briefly, the MUTZ-3 progenitor cell line was maintained in alpha-minimal essential medium (α -MEM, including ribonucleosides, deoxyribonucleosides (Gibco, UK)) supplemented with 20% FBS (Invitrogen, UK), penicillin (100 U/ml)/streptomycin (100 μ g/ml) solution, 2 mM L-glutamine (Gibco, UK)

Table 1
Chemicals used in migration assays.

Chemical	CAS Number	LLNA Classification	Human Classification
2,4 Dinitrochlorobenzene	97-007	Extreme ¹	Extreme ³
Paraphenylenediamine	106-50-3	Strong ²	Strong ³
Cinnamaldehyde	137-26-8	Moderate ²	Moderate ⁴
Isoeugenol	97-54-1	Moderate ²	Moderate ⁴
Nickel sulphate	7786-81-4	Moderate ³	Moderate ³
Eugenol	97-53-0	Weak ²	Weak ⁴
Cinnamic alcohol	104-54-1	Weak ²	Weak ⁴
Sodium lauryl sulphate	151-21-3	Weak/irritant ⁵	Non-Sensitizer ⁵
Salicylic acid	69-72-7	Non-sensitizer ³	Non-Sensitizer ³

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 characterised 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) (μ g/cm²) of chemicals). Of note, sodium lauryl sulphate is a well recognised false positive in LLNA. The chemicals had to be available commercially.

¹ Kimber et al. (2003).

² Gerberick et al. (2005).

³ Schneider and Akkan (2004).

⁴ Basketter et al. (2005).

⁵ Basketter and Kimber (2011).

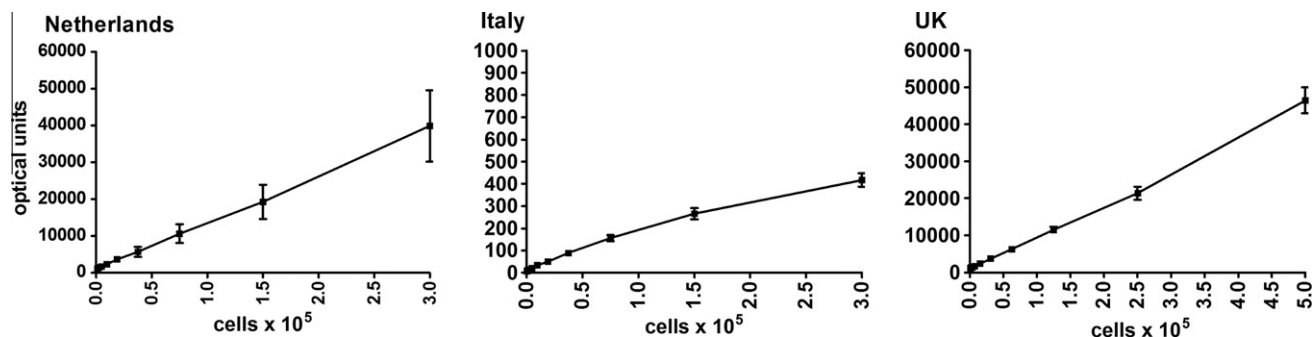


Fig. 1. Standard curves of serial dilutions of CFSE labelled MUTZ-LC up to 3×10^5 cells Netherlands ($n = 8$), Italy ($n = 3$) and 5×10^5 cells for the UK ($n = 8$). n = independent experiment.

and 10% conditioned media obtained by culturing the 5637 human bladder carcinoma cell line (Quentmeier et al., 1997). The cells were passaged every 3–4 days at 2×10^5 cells/ml.

MUTZ-3 cells were differentiated into MUTZ-3 derived Langerhans Cells (MUTZ-LC) in a TGF- β dependent manner. MUTZ-3 cells (2×10^5 /ml) were cultured with 10 ng/ml TGF- β (R&D Systems, UK), 100 ng/ml GM-CSF (PeproTech, UK), 2.5 ng/ml TNF- α (Miltenyi Biotech, UK) and 50 μ M β -mercaptoethanol (Sigma Aldrich, UK) for 10 days with fresh cytokines being added directly to the culture media on days 3, 6 and 9.

2.2. Maturation of MUTZ-LC

MUTZ-LC were exposed to a cytokine maturation cocktail (CMC) for 24 h to obtain mature MUTZ-LC which were used as the positive control in the migration assay. The cytokine cocktail consisted of 100 ng/ml IL-6, 25 ng/ml IL1- β (Miltenyi Biotech, Germany), 50 ng/ml TNF- α , 1 ng/ml Prostaglandin E₂ (Sigma Aldrich, UK).

2.3. Chemical exposure

MUTZ-LC were exposed to chemicals in a dose dependent manner for 24 h. The chemicals were selected by ECVAM based on human data and on validated data collected by the LLNA assay for European research purposes on *in vitro* assay development (Table 1) (Casati et al., 2009). The highest chemical concentration used in the dose response studies correlated to no more than 25% cytotoxicity, 75% cell viability as determined by propidium iodide staining (Invitrogen, Paisley, UK).

The chemicals chosen for the ring study were three sensitizers and two non-sensitizers. Sensitizers were nickel sulphate (NiSO₄), 2,4 dinitrochlorobenzene (DNCB), cinnamaldehyde (CA) and non-sensitizers were sodium lauryl sulphate (SLS) and salicylic acid (SA). DNCB and SA were dissolved in dimethylsulphoxide (DMSO) (Sigma Aldrich, UK) achieving a final concentration of 0.1% DMSO. The other chemicals were dissolved in endotoxin free water and purchased from the Sigma Chemical Company. The UK lab extended the study to include three pro-haptens (eugenol, iso-eugenol, cinnamic alcohol) and the pre-hapten (paraphenylenediamine) (Sigma Aldrich, UK).

2.4. Flow cytometry

Immuno-phenotyping of MUTZ-3 and MUTZ-LC cells before and after CMC exposure was established by flow cytometry.

NL-lab and Italy-lab: Cells were incubated for 30 min with directly conjugated antibodies, washed with PBS containing 0.1% BSA and 0.1% sodium azide and resuspended using the same buffer. NL-lab used CD14-FITC (Miltenyi Biotech, Germany), CD34-PE

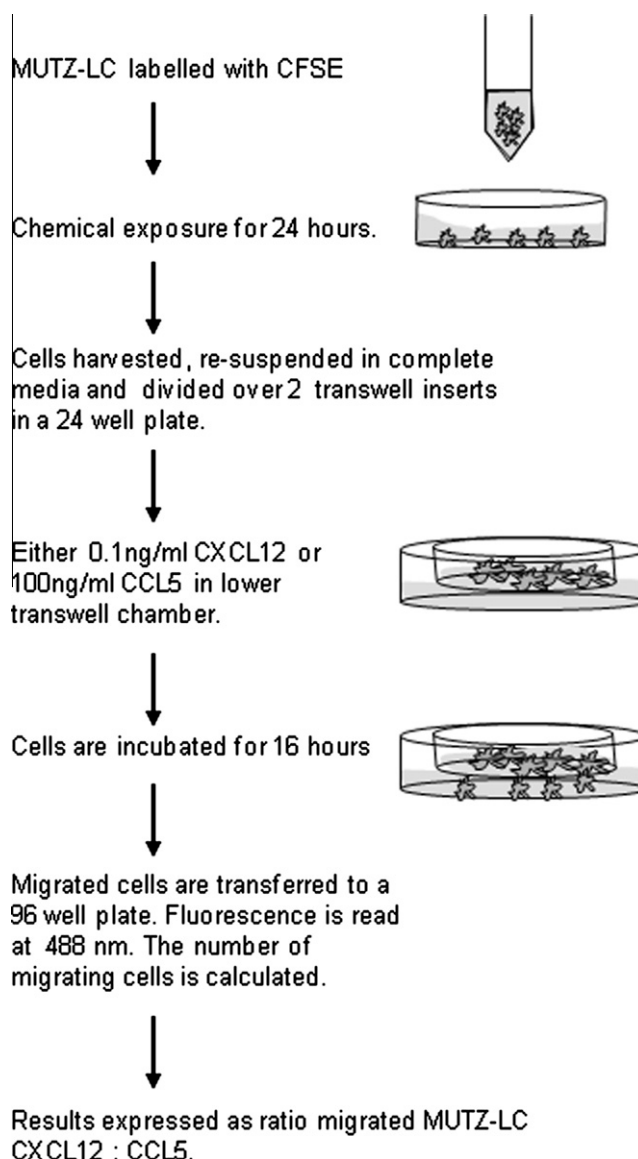


Fig. 2. Schematic overview of the migration assay. MUTZ-LC cells were stained with CFSE. Following incubation for 24 h with chemicals, the cells were re-suspended in complete media and divided over two transwell inserts in two wells of a 24 well plate. CXCL12 at a concentration of 0.1 ng/ml was added to the lower chamber of one well and CCL5 at 100 ng/ml to the lower chamber of the other. The plate was then incubated for 16 h. Cells migrating into the lower chamber were harvested and the fluorescence determined.

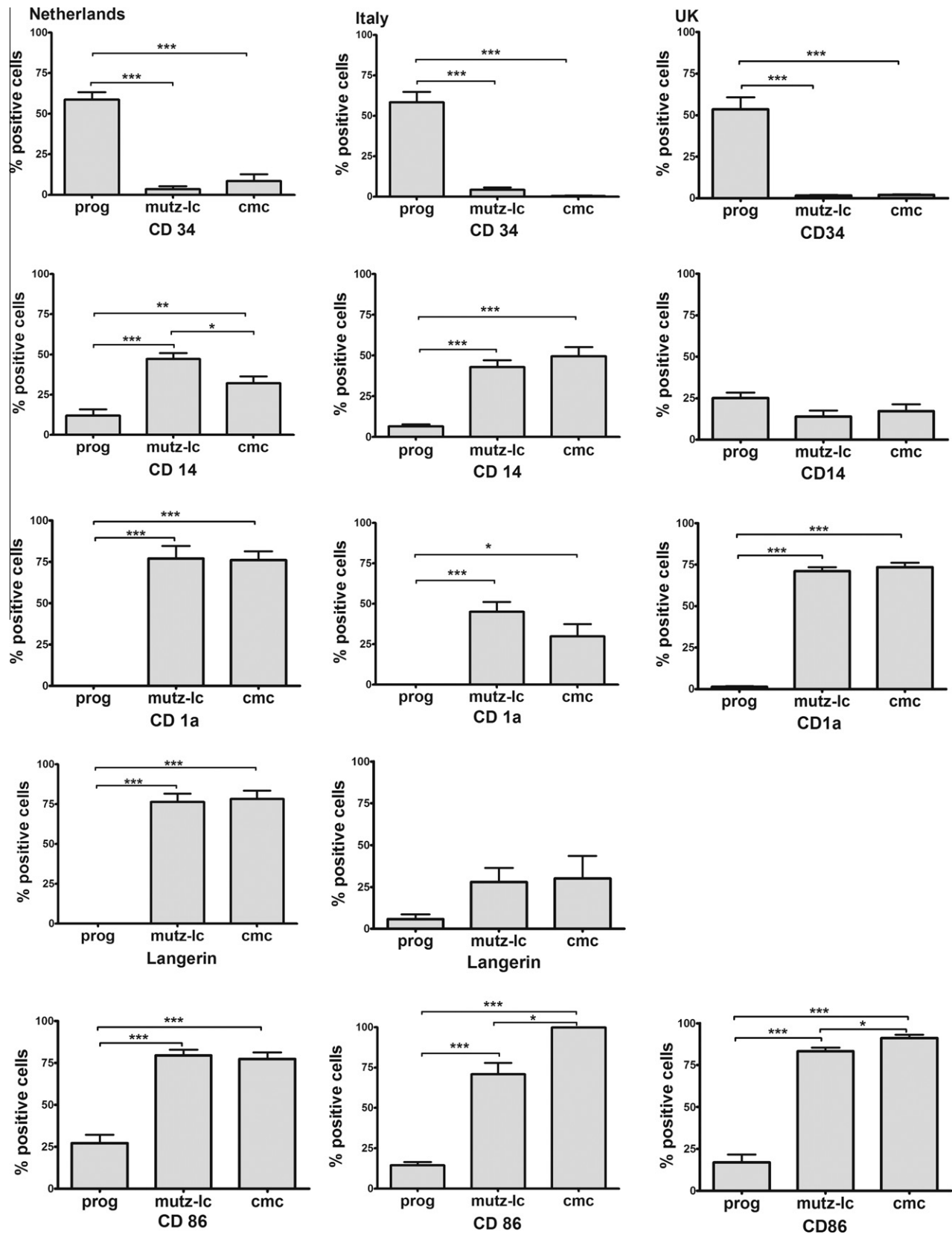


Fig. 3. Comparison of MUTZ-3 progenitor (prog), MUTZ-LC and matured MUTZ-LC (cmc) phenotypes. The changes in % positive cells for CD34 (NL-lab: MUTZ-3 $n = 8$, MUTZ-LC and CMC $n = 7$; Italy-lab: MUTZ-3 $n = 8$, MUTZ-LC $n = 9$ and CMC $n = 3$; UK-lab: MUTZ-3 $n = 5$, MUTZ-LC $n = 8$ and CMC $n = 7$), CD14 (NL-lab: MUTZ-3 $n = 8$, MUTZ-LC and CMC $n = 12$; Italy-lab: MUTZ-3 $n = 8$, MUTZ-LC $n = 9$ and CMC $n = 3$; UK-lab: MUTZ-3 $n = 5$, MUTZ-LC $n = 7$, CMC $n = 6$), CD1a (NL-lab: MUTZ-3 $n = 10$, MUTZ-LC and CMC $n = 9$; Italy-lab: MUTZ-3 $n = 5$, MUTZ-LC $n = 9$ and CMC $n = 3$; UK-lab: MUTZ-3 $n = 7$, MUTZ-LC and CMC $n = 23$), Langerin (NL-lab: MUTZ-3 $n = 8$, MUTZ-LC and CMC $n = 9$; Italy-lab: MUTZ-3 $n = 3$, MUTZ-LC $n = 6$ and CMC $n = 3$) and CD86 (NL-lab: MUTZ-3 $n = 8$, MUTZ-LC and CMC $n = 12$; Italy-lab: MUTZ-3 $n = 7$, MUTZ-LC $n = 9$ and CMC $n = 3$; UK-lab: MUTZ-3 $n = 7$, MUTZ-LC and CMC $n = 22$), are shown. n = independent repetition. Data is shown as mean of independent repetitions $\pm$ SEM and analysed using one way ANOVA with the Dunnett multiple comparison posttest where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

(Sanguin, Netherlands), CD86-PE, CD1a-FITC (BD Systems, San Diego, CA), CD207 (Langerin)-PE (Immunotech, France) and isotype controls IgG1-FITC and IgG2a-PE (BD Systems). Italy-lab used CD14-FITC (Miltenyi Biotech, Germany), CD34-PE, CD86-PE and CD1a-FITC (BD Systems), CD207 (Langerin)-PE (R&D Systems, Minneapolis, MN), IgG FITC and IgG PE isotype controls were used to determine non-specific binding.

UK-lab: The primary antibodies used were CD14 (Sigma Aldrich), CD34 (BD Bioscience), CD86 (BD Bioscience), CD1a (eBioscience). Staining with a secondary antibody, polyvalent anti-mouse IgG-FITC (Sigma Aldrich) followed (Williams et al., 2010). The phenotypic expression was analysed using the Accuri C6 flow cytometer with CFlow software (UK-lab), or FACSCALIBUR (Becton Dickinson) with CellQuest software (NL-lab and Italy-lab). Data are presented as % positive cells.

2.5. Optimisation of cell number

The number of carboxyfluorescein diacetate, succinimidyl ester (CFSE) labelled MUTZ-LC cells which could be detected in the assay was first optimised for each of the labs. The MUTZ-LC were labelled according to the manufacturer's instructions (CFSE cell trace kit, Invitrogen). A standard curve correlating the degree of fluorescence to the number of MUTZ-LC was performed by using doubling dilutions of CFSE (Molecular Probes, Invitrogen) labelled cells starting from 5×10^5 cells (UK-lab) and 3×10^5 cells (NL-lab and Italy-lab). From the linear part of the graph the NL-lab and Italy-lab estimated their optimal number of cells to be 3×10^5 /ml, whereas the UK-lab decided that the minimum number of cells that could be used efficiently in the assay was 1.5×10^5 /ml (Fig. 1). These cell numbers were used for further migration experiments. The fluorescence was read at 488 nm excitation and emission using the Victor² Wallac 1420 Multilabel counter by NL-lab; 485 and 520 nm using the Fluorstar Optima (BMG Labtech) by UK-lab and 485 and 517 nm using the Thermo Scientific Ascent Fluoroskan at Italy-lab (Italy-lab: older multilabel counter model explains lower optical units obtained in Fig. 1 compared to NL- and UK-labs).

2.6. Transwell migration assay

The MUTZ-LC migration assay was carried out exactly as previously described (Ouweland et al., 2010b) and is shown schematically in Fig. 2. Briefly, CFSE labelled MUTZ-LC (1.5×10^5 or 3×10^5 /ml) were exposed to either CMC (positive control) or chemicals for 24 h, at 37 °C in 5% CO₂. The cells were harvested and re-suspended in 100 µl 20% FBS α -MEM with penicillin/streptomycin and glutamine. Cell suspension was added to the 5 µm poresize insert of a transwell plate, (Corning Costar, NY). Either rhCCL5 (100 ng/ml) (R&D Systems) or rhCXCL12 (0.1 ng/ml) (R&D Systems) was added to the lower well. The dilutions of chemokines were prepared in α -MEM with penicillin/streptomycin and glutamine only. The transwell plate was then incubated for 16 h at 37 °C and 5% CO₂. Hereafter, the cells were harvested and transferred to a 96 well flat bottomed plate (Greiner). The fluorescence was 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.

2.7. Statistical analysis

The results are shown as the mean $\pm$ standard error of the mean. Variations in surface expression of CD34, CD14, CD1a, Langerin and CD86 or the CXCL12/CCL5 ratios of migrated MUTZ-LC after chemical treatment were compared using the one-way ANOVA test followed by Dunnett's multiple comparison posttest (where the ratio

of the treated cells was compared to that of the control/untreated cells), $p < 0.05$ was considered significant.

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

3. Results

3.1. Characterisation of MUTZ cells

Since the technology transfer included implementing MUTZ-3 culture, differentiation and maturation protocols from the lead lab (NL-lab) to the UK-lab and Italy-lab, the phenotypes of the MUTZ-3 progenitor cells, the MUTZ-3 cells that had been differentiated into MUTZ-LC and the cytokine matured MUTZ-LC were first compared between the three laboratories (Fig. 3). The MUTZ-3 progenitor cells showed high CD34, and low CD14, CD1a, Langerin, CD86 expression in all labs. As expected CD34 expression decreased after differentiation into MUTZ-LC and maturation. In contrast, and also as expected, CD1a, Langerin and CD86 surface marker expression increased upon differentiation and maturation into MUTZ-LC compared to the progenitor cell. CD14 expression increased as CD34 expression decreased in the NL- and Italy-labs whereas it remained low in the UK-lab. Taken together, these results show that each of the laboratories were able to maintain the culture, and also differentiate and mature MUTZ-3 with negligible intra- and inter-laboratory variation.

3.2. Migration of immature and cytokine matured MUTZ-LC

Having established MUTZ-3 culture in the three laboratories we next determined the transferability of the migration protocol for immature and cytokine matured MUTZ-LC. These function as the negative and positive criteria in the migration assay. Fig. 4 shows that, for all three laboratories, MUTZ-LC cells exposed to CMC preferentially migrated towards the chemokine CXCL12 whereas non-exposed immature MUTZ-LC cells preferentially migrate towards CCL5. This is indicated by the CXCL12/CCL5 ratios of the matured MUTZ-LC cells being >1 and the immature MUTZ-LC having a CXCL12/CCL5 ratio of <1 . The results are expressed as the mean $\pm$ SEM of the CXCL12/CCL5 ratio. The CXCL12/CCL5 ratio of immature MUTZ-LC for NL-lab = 0.95 ± 0.04 ($n = 18$); Italy-lab = 0.89 ± 0.05 ($n = 11$) and UK-lab = 0.87 ± 0.02 ($n = 27$). For matured MUTZ-LC the CXCL12/CCL5 ratio is as follows: NL-lab = 1.24 ± 0.1 ; Italy-lab = 1.11 ± 0.08 and UK-lab = 1.16 ± 0.03 . The low SEM shows that intra-laboratory variation is low. All three laboratories concurred with the expected result of the migration of the MUTZ-LC towards CCL5 and the matured MUTZ-LC towards the CXCL12.

3.3. Migration of chemical exposed MUTZ-LC

In order to further test the transferability of the MUTZ-LC migration assay protocol, MUTZ-LC cells were exposed to the same panel of sensitizers and non-sensitizers in each of the three laboratories (Fig. 5A). Sensitizers were NiSO₄ (NL-lab: $n = 4$, Italy-lab and UK-lab: $n = 3$), DNCB, (NL-lab: $n = 9$, Italy-lab and UK-lab: $n = 3$) and CA (NL-lab: $n = 5$, UK-lab: $n = 3$, Italy-lab: not done). Non-sensitizers were SLS (NL-lab: $n = 6$, Italy-lab and UK-lab: $n = 3$) and SA ($n = 3$ for all labs). The MUTZ-LC exposed to sensitizers display preferential migration towards CXCL12 indicated by a CXCL12/CCL5 ratio of >1 . In contrast MUTZ-LC exposed to non-sensitizers show a preferential migration towards CCL5 (Fig. 5B) indicated by the CXCL12/CCL5 ratio being <1 . For all the chemicals used the viability of the cells in all three laboratories was $>75\%$ with the exception of 24 µM DNCB which had a viability of 50%.

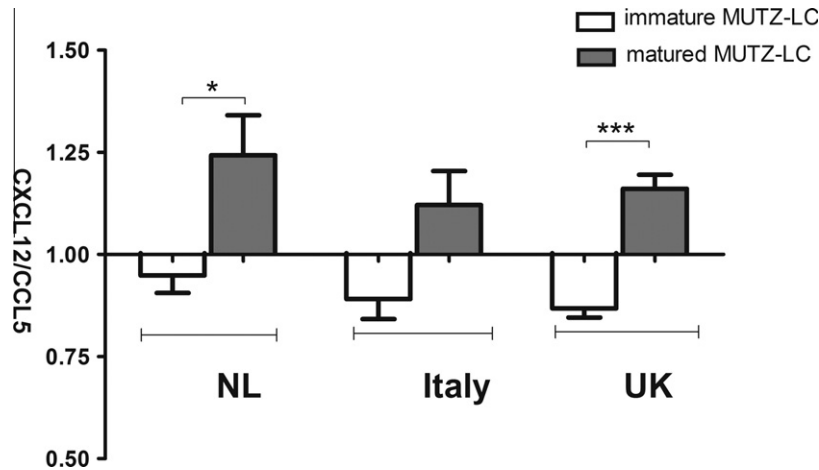


Fig. 4. Migration of immature and cytokine matured MUTZ-LC. Mature MUTZ-LC preferentially migrate towards the chemokine CXCL12 whereas immature MUTZ-LC migrate towards CCL5. NL-lab: $n = 18$; Italy-lab: $n = 11$ and UK-lab: $n = 27$; n = independent repetition (repetition = average of intra-experiment duplicates). Data is shown as mean of independent repetitions $\pm$ SEM (* $p < 0.05$ and *** $p < 0.001$, students paired t -test).

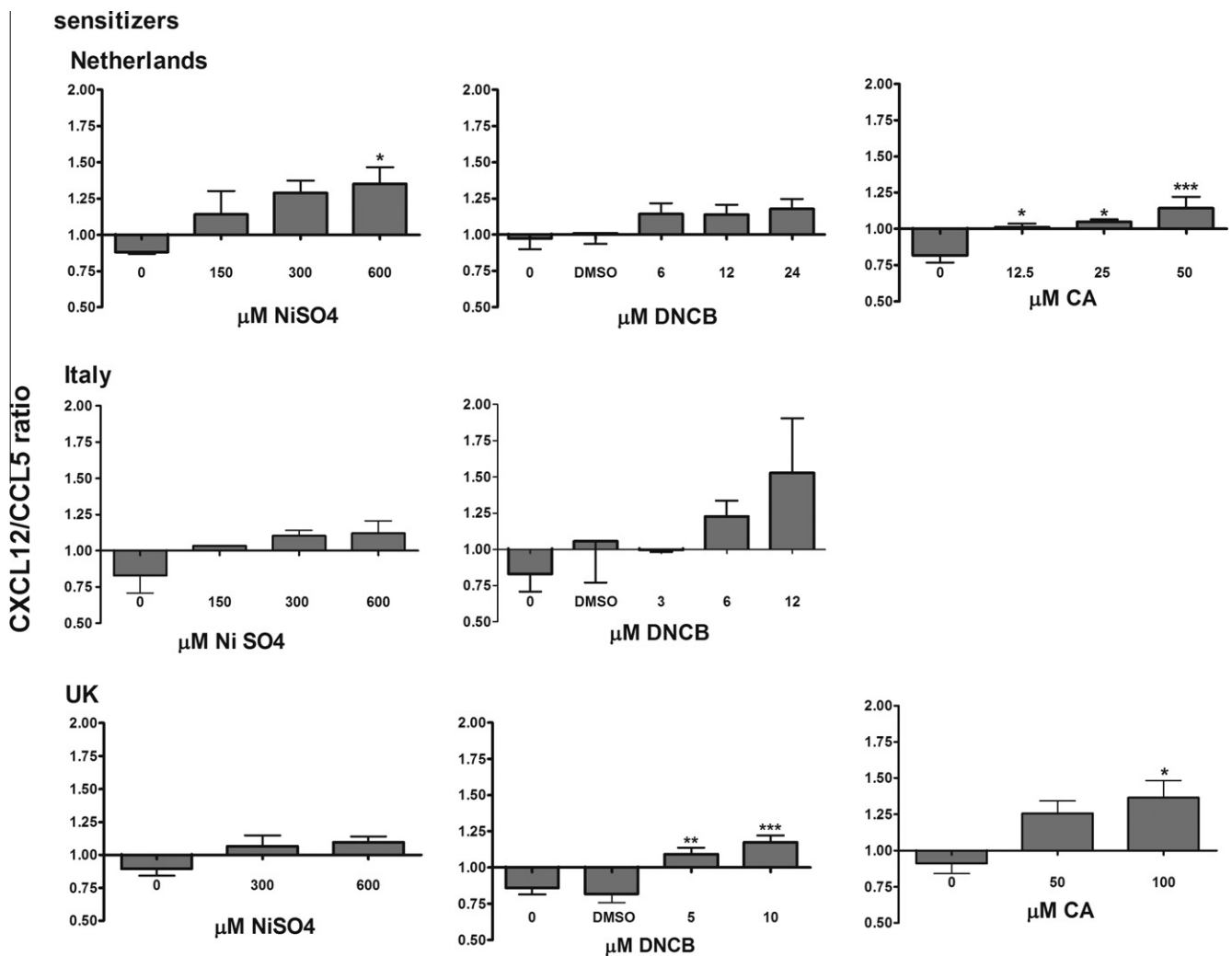


Fig. 5A. Sensitizer exposed MUTZ-LC preferentially migrate towards CXCL12.

In order to assess the intra-laboratory and inter-laboratory variation in more detail the individual experimental repetitions were analysed (Tables 2 and 3). Of note, each repetition consisted of

completely independently cultured, differentiated and matured MUTZ-LC being used in the migration assay on different days. The highest CXCL12/CCL5 ratio obtained at a non-toxic chemical

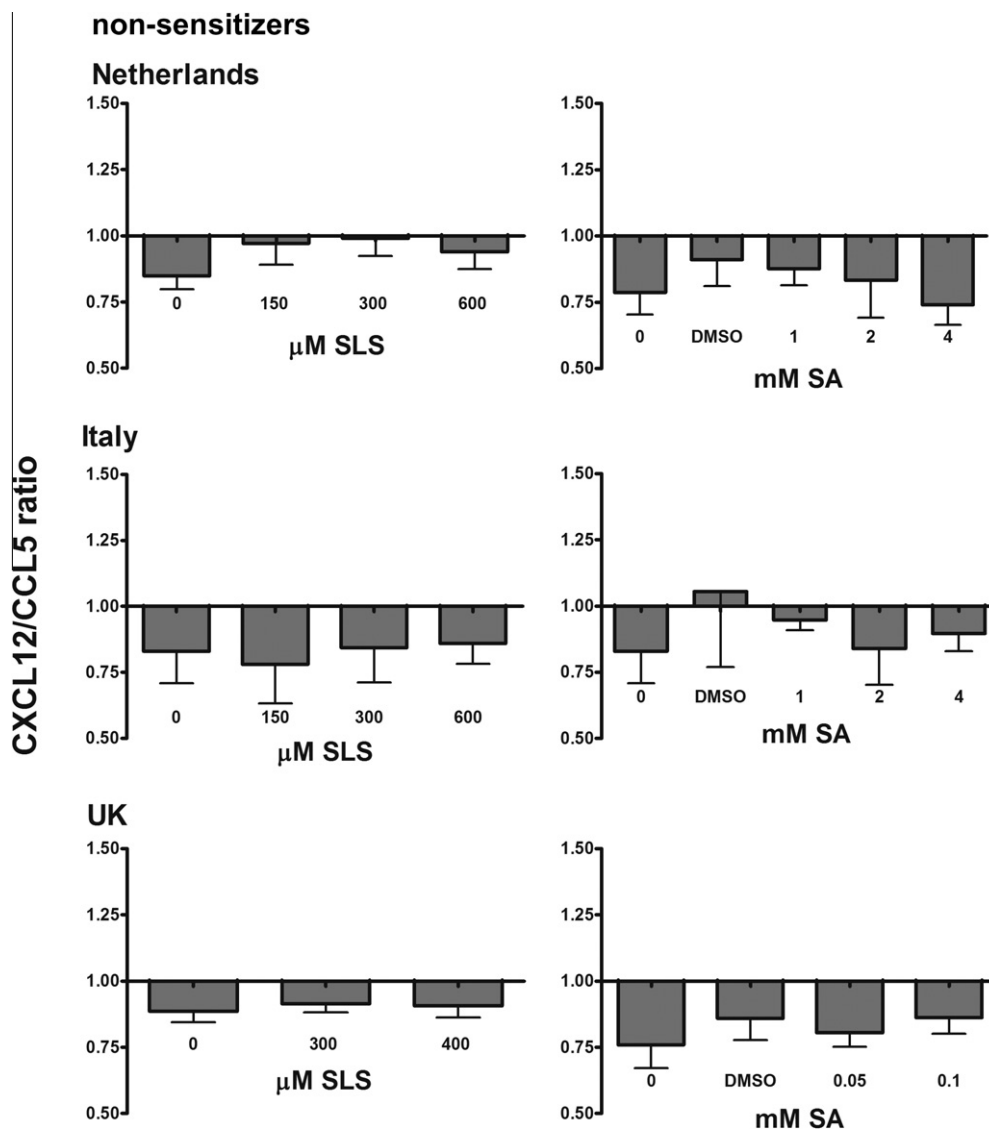


Fig. 5B. Non-sensitizer exposed MUTZ-LC preferentially migrate towards CCL5. For SLS NL-lab: $n = 6$, Italy-lab and UK-lab: $n = 3$; for SA all laboratories $n = 3$. n = independent repetition (repetition = average of intra-experiment duplicates). The data was analysed using the one way ANOVA with the Dunnett posttest and showed no significant difference between the untreated MUTZ-LC cells and those exposed to either SLS or SA.

concentration and the chemical concentration at which this was reached was recorded. A chemical was rated as positive if at least 2 out of 3 independent repetitions showed a CXCL12/CCL5 ratio > 1.1 . The CXCL12/CCL5 ratio > 1.1 was implemented since this indicates that more cells migrate towards CXCL12 than towards CCL5. (A ratio of 1.0 would indicate that the same number of cells migrate towards CXCL12 as towards CCL5 and therefore would be non conclusive.) Using these criteria, all three laboratories clearly and correctly rated the sensitizers as positive and the non-sensitizers as negative.

3.4. Pro-haptens are identified in MUTZ-LC migration assay

Since chemical pro- and pre-haptens are difficult to identify in many *in vitro* assays due to their requirement for metabolic activation, we next determined whether the novel MUTZ-LC migration assay was also suitable for identifying such chemicals. MUTZ-LC were therefore exposed to three pro-haptens (eugenol, iso eugenol and cinnamic alcohol) and the pre-hapten paraphenylenediamine (PPD) in the UK-lab. Remarkably, a preferential migration towards

CXCL12 (ratio of CXCL12/CCL5 > 1) was observed for all pro- and pre-haptens, whereas the immature (untreated) and vehicle (DMSO) treated MUTZ-LC had a CXCL12/CCL5 ratio < 1 (Fig. 6).

4. Discussion

The aim of this study was to investigate the transferability of technology and reproducibility of the assay used in the DC based migration assay. Technology transfer is an important requirement prior to possible (pre-)validation studies in the future. The results of this study indicate that the *in vitro* DC migration assay is transferable between laboratories. The results were consistent between the laboratories, and the dose response data were reproduced in the three laboratories.

Our assay involved a complex technology transfer involving a number of steps including maintenance of MUTZ-3 cell line, differentiation into immature MUTZ-LC, CFSE labelling of immature MUTZ-LC, chemical exposure, migration and finally quantification of fluorescently labelled MUTZ-LC migrating into the lower transwell chamber. Since all three laboratories participating in the DC

Table 2

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

Chemical	Laboratory	Repetition	CXCL12/CCL5 ratio ^a	Chemical concentration ^b	Positive repetitions ^c	Classification ^d
NiSO ₄	NL (lead lab)	1	1.30	150 µM	4/4	Sensitizer
		2	1.23	300 µM		
		3	1.62	600 µM		
		4	1.46	600 µM		
	UK	1	1.09	600 µM	2/3	Sensitizer
		2	1.18	600 µM		
		3	1.22	300 µM		
	Italy	1	1.17	300 µM M	3/3	Sensitizer
		2	1.10	600 µM M		
		3	1.28	600 µM M		
DNCB	NL (lead lab)	1	1.12	6 µM	7/9	Sensitizer
		2	1.55	12 µM		
		3	0.97	12 µM		
		4	1.12	6 µM		
		5	1.08	12 µM		
		6	1.23	12 µM		
		7	1.36	12 µM		
		8	1.40	6 µM		
	UK	1	1.24	12 µM	3/3	Sensitizer
		2	1.34	12 µM		
		3	1.18	12 µM		
	Italy	1	1.15	12 µM	2/3	Sensitizer
		2	1.44	12 µM		
		3	1.09	12 µM		
CA	NL (lead lab)	1	1.12	50 µM	4/5	Sensitizer
		2	1.45	50 µM		
		3	1.07	12.5 µM		
		4	1.10	25 µM		
		5	1.11	50 µM		
	UK	1	1.53	100 µM	3/3	Sensitizer
		2	1.43	100 µM		
		3	1.13	50 µM		
	Italy	1	nd			
		2	nd			
		3	nd			

A repetition is shown as an average of intra-experiment duplicates.

^a Highest CXCL12/CCL5 ratio reached at non toxic concentration (>75% viability) in dose response.^b Chemical concentration at which highest CXCL12/CCL5 ratio was reached.^c Number of positive repetitions in which a CXCL12/CCL5 ratio greater than 1.10 is reached.^d If ≥ 2 of 3 repetitions CXCL12/CCL5 ratio >1.10 chemical is a sensitizer; if <2 of 3 repetitions CXCL12/CCL5 ratio >1.10 then chemical is a non-sensitizer (applies also to multiple numbers of repetitions).

migration assay showed consistent results it indicates that the assay, although being rather complex, is a robust assay suitable for inter-laboratory implementation. In contrast to our assay, most other promising cell based assays described in ring studies have involved a relatively simpler technology transfer procedure involving fewer steps e.g. maintenance of the cell line followed by chemical exposure and assessment of biomarker expression. Promising examples are (i) myeloid U937 skin sensitisation test (MUSST) which determines changes in CD86 expression in the U-937 cell line (Aeby et al., 2010; Python et al., 2007); (ii) the human cell line activation test (h-CLAT) which determines changes in expression of both CD54 and CD86 in the THP-1 cell line (Ashikaga et al., 2006; Sakaguchi et al., 2006) and (iii) the KeratinoSens assay which uses the stable transgenic keratinocyte cell line to assess induction of luciferase gene under control of anti-oxidant response element (ARE) (Natsch et al., 2009, 2011). It should be noted that these assays are based on a readout of biomarker expression whereas the DC migration assay is a functional assay based on the *in vivo* like migration of LC upon sensitizer or non-sensitizer exposure.

In our study, the three partners used the same batch of MUTZ-3 and 5637 epithelial cell line for harvesting supernatant to supplement the MUTZ-3 culture medium. All other reagents (e.g. serum)

and apparatus (e.g. multi-plate readers) used were not co-ordinated and were the standards generally used in the different laboratories. Indeed, the UK-lab used primary and secondary antibodies to stain cells whereas the NL-lab and Italy-lab used conjugated antibodies. Also, flow cytometers were from different manufacturers and therefore it was decided that using percentage positive cells would be the most precise way of expressing changes in MUTZ-3 phenotype upon differentiation rather than fluorescent intensity. For the migration assay, different plate readers were used by the three partners which led to each laboratory having to optimise the number of cells to be used in the migration assay. Both NL-lab and Italy-lab found the optimal cell number to be 3×10^5 cells whereas the UK-lab used 1.5×10^5 cells. For the h-CLAT, great care was taken to co-ordinate all reagents and in particular suppliers of different antibodies was investigated prior to starting the ring studies (Ashikaga et al., 2006). For the KeratinoSens ring study, only the cell line was the same in the three laboratories participating in the ring study. All other reagents were standard lab in house reagents of each laboratory (Natsch et al., 2011).

Our results show that progenitor MUTZ-3 cells were successfully maintained using media from the feeder cell line 5637 and

Table 3
Summary of inter and intra laboratory variation in CXCL12/CCL5 ratio of non-sensitizers.

Chemical	Laboratory	Repetition	CXCL12/CCL5 ratio ^a	Chemical concentration ^b	Positive repetitions ^c	Classification ^d
SLS	NL (lead lab)	1	1.29	300 µM	2/6	Non-sensitizer
		2	0.98	150 µM		
		3	1.03	600 µM		
		4	1.10	150 µM		
		5	1.11	150 µM		
		6	1.06	600 µM		
	UK	1	0.92	400 µM	0/3	Non-sensitizer
		2	0.98	300 µM		
		3	0.98	400 µM		
	Italy	1	0.97	150 µM	0/3	Non-sensitizer
		2	0.98	300 µM		
		3	0.97	300 µM		
SA	NL (lead lab)	1	1.04	2 mM	0/3	Non-sensitizer
		2	0.90	2 mM		
		3	0.84	1 mM		
	UK	1	0.742	100 µM	0/3	Non sensitizer
		2	0.944	100 µM		
		3	0.903	50 µM		
	Italy	1	1.02	2 mM	0/3	Non-sensitizer
		2	1.03	3 mM		
		3	0.98	1 mM		

^a Highest CXCL12/CCL5 ratio reached at non toxic concentration (>75% viability) in dose response.
^b Chemical concentration at which highest CXCL12/CCL5 ratio was reached.
^c Number of positive repetitions in which a CXCL12/CCL5 ratio greater than 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). A repetition is shown as an average of intra-experiment duplicates.

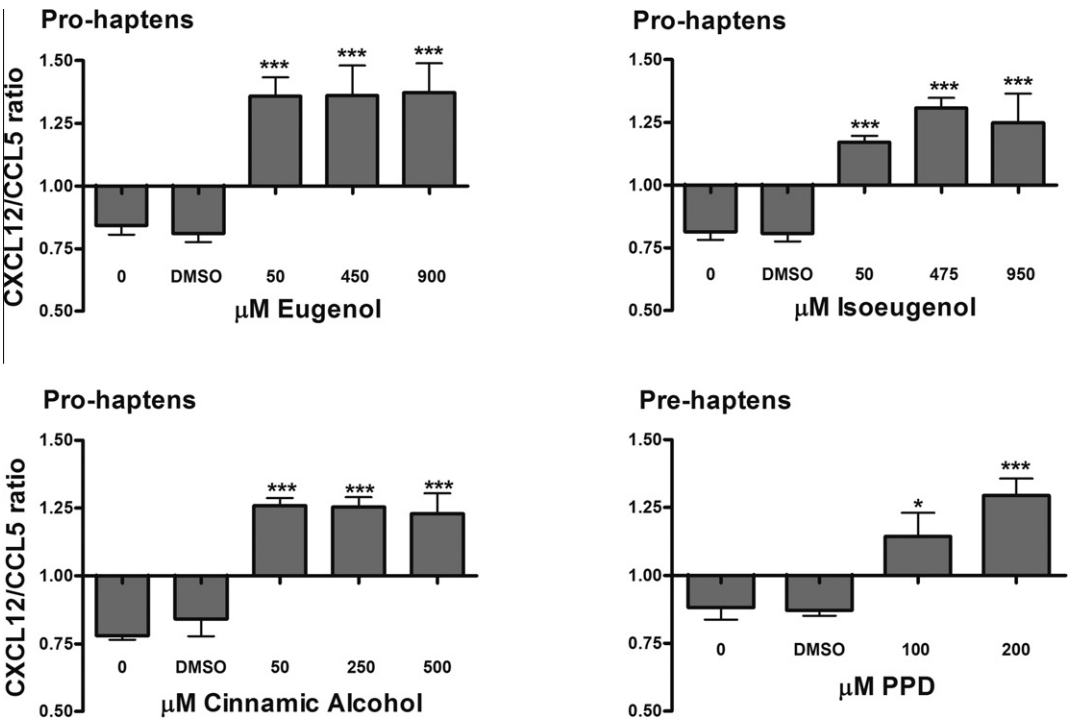


Fig. 6. Pro-haptens and pre-hapten exposed MUTZ-LC preferentially migrate towards CXCL12. Data is shown as the mean of three independent repetitions ± SEM; one way ANOVA with the Dunnett post test (**p* < 0.05, ****p* < 0.001) (repetition = average of intra-experiment duplicates).

cells retained their high CD34, low CD86 expression and either no or very low expression of CD1a. The phenotypes after differentiation from MUTZ-3 progenitor cells to MUTZ-3 LC showed a decrease in CD34 and significant increases in CD86, CD1a and Langerin. Both CD1a and Langerin are typical markers for LC. Only CD14 expression showed variation between the participants. The

results from NL-lab and Italy-lab showed increases in CD14 expression upon differentiation to MUTZ-3 LC whereas the UK-lab showed a small decrease in CD14 expression. However this decrease in CD14 expression did not influence the final outcome of the migration assay. Of note, the MUTZ-3 progenitor pool is described to consist of proliferating CD34+ cells and CD14+ cells

which are committed to differentiate (Masterson et al., 2001). Once fully differentiated they have been known to clearly reduce their CD14 expression which would explain the variability found in this transiently expressed surface marker.

After differentiation, the MUTZ-LC were exposed to a cytokine maturation cocktail (CMC: IL-6, IL1 β , TNF- α and PGE $_2$) or to a dose range of chemicals for 24 h before placing in the migration assay upper transwell. In all laboratories, when averaging the number of intra-experimental runs, maturing stimuli (CMC, sensitizers) resulted in a CXCL12/CCL5 ratio >1 and non-maturing stimuli (irritants) resulted in a CXCL12/CCL5 ratio <1. When looking in more detail at the independent runs it was found the chemical concentration where maximum migration occurred could shift slightly between runs but this did not influence the overall result. For example, the maximum migration resulting from nickel sulphate exposure could occur at 150, 300 or 600 μ M. When taking the arbitrary criteria of at least two of three independent repetitions having to have a CXCL12/CCL5 ratio >1.1 for classification as a sensitizer, all sensitizers in all labs were easily distinguished from all non-sensitizers. The number of repetitions giving false negative or false positive was very low (only 7 out of a total of 54 repetitions) indicating that both intra- and inter-laboratory variation was extremely low (Tables 2 and 3). In comparison, the h-CLAT inter-laboratory study in which both U937 and THP-1 were investigated each in two laboratories testing six sensitizers, three non-sensitizers and combined assessment of CD86 and CD54, the THP-1 cell line showed a 100% inter-laboratory accuracy similar to the DC migration assay and the U-937 cell line showed a 67% inter-laboratory accuracy (Sakaguchi et al., 2006). The extensive KeratinoSens ring study involved five laboratories testing 28 substances and had an accuracy ranging between 85.7% and 92.9% in the different laboratories. Not surprisingly in this extended study there were a few outliers e.g.; hexyl cinnamic alcohol, eugenol, and SLS (Natsch et al., 2011). Interestingly, these chemicals were correctly assessed in the DC migration assay supporting the principle for a strategic battery of assays being implemented in risk assessment of potential sensitizers. Another very promising *in vitro* approach is based on the ability of a chemical to react with proteins containing cysteine and lysine thus forming stable (covalent) bonds (Direct Protein Reactivity Assay). This non-cell based assay classifies minimum reactivity as non-sensitizers and low, moderate and high reactivity as sensitizers (Gerberick et al., 2007, 2009). Following two rounds of ring trials inter-laboratory reproducibility was also acceptable and concordance was 89% with LLNA. In all of these studies as well as ours, the technology transfer was successful and the intra- and inter-laboratory variation acceptable indicating that *in vitro* cell based assays will be able to be implemented on a wide scale in the future.

The chemicals used in our technology transfer experience were directly active and so their sensitising potential could easily be assessed using this *in vitro* assay. However, there are potential chemical sensitizers which require either abiotic (pre-haptens) or enzymatic (pro-haptens) means to activate them. There are possible limitations to *in vitro* methodologies that do not take into account the need for this activation (Gerberick et al., 2009). The UK-lab extended the study to investigate whether the assay could in fact detect the sensitising potential of pre- and pro-haptens and found that indeed the CXCL12/CCL5 ratios for the pre-hapten (PPD) and the three pro-haptens (Eugenol, Isoeugenol and cinnamic alcohol) were >1. The explanation for the positive result obtained from PDD can be explained by the fact that PPD is known to oxidise on exposure to oxygen (Gerberick et al., 2009) so normal handling of the chemical while preparing the assay may have been adequate to cause its activation. A possible explanation as to how the pro-hapten chemicals become activated was determined by Ott who investigated the pro-hapten carboxime (Ott et al., 2009). They

demonstrated upregulation of IL-8 in moDC exposed to the pro-hapten and concluded that this activation may be the result of metabolic activation within the cells. In another study which investigated the up regulation of p38 mitogen-activated protein kinase (p38 MAPK) on THP-1 cells included the chemical isoeugenol (Mitjans et al., 2010). They observed an increase in activation of p38 MAPK on cells incubated with isoeugenol. Therefore we can assume that the isoeugenol was converted into a hapten for it to become reactive and cause the increase in p38 MAPK in the THP-1 cells.

We can conclude that the technology transfer of the *in vitro* DC based assay was successful and results were reproducible between the three laboratories despite the complexity of the assay. Also, importantly we show that the assay may be suitable for detecting pre- and pro-haptens and was able to classify correctly SLS as a non-sensitizer, indicating a good correlation of our *in vitro* data with clinically relevant human data. Notably, SLS is a well known false positive in the mouse LLNA (Basketter and Kimber, 2011). Even though our results are promising, our assay may have a number of limitations. Similar to all *in vitro* cell based assays only water soluble chemicals can be tested since the chemical is delivered (often with the aid of a vehicle e.g.: DMSO) to the cells by supplementing the culture medium. This solubility issue goes hand in hand with potential chemical stability/reactivity issues. Furthermore, not enough data is available yet to determine whether this assay will give information on sensitizer potency. Another potential limitation of the assay is its low throughput and complexity which goes together with high costs. Taken together, it is therefore 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 sensitisation in a cost effective manner (Maxwell et al., 2011; Corsini and Roggen, 2009; Roggen et al., 2008). The DC migration assay may prove to be suitable for incorporation into such a risk assessment strategy but will first require extensive testing with a wide range of different types of chemicals under coded conditions in a ring study setting in order to identify fully its limitations, confirm its robustness and to define its efficacy domain.

5. Conflict of interest

The authors have no financial/commercial conflicts of interest regarding the publication of this manuscript.

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