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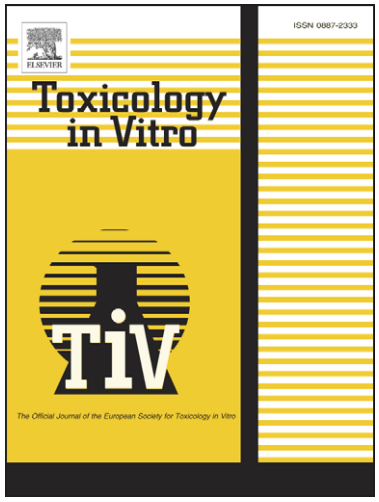

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Identification of PDL-1 as a novel biomarker of sensitizer exposure in dendritic-like cells

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Abstract.

The development of novel *in vitro* methods to assess risks of allergic sensitization are essential in reducing animal testing whilst maintaining consumer safety. The main research objectives of this study were to identify novel biomarkers to assess the sensitization predictability of chemicals. Phenotypic and cytokine responses of moDCs and MUTZ-3 cells were investigated following application of contact sensitizers; Dinitrochlorobenzene (DNCB), Cinnamaldehyde (Cin), Eugenol (E), Isoeugenol (IE), P- Phenylenediamine (PPD) and non-sensitizers; Salicyclic Acid (SA) and Sodium Lauryl Sulphate (SLS). CD86 was up-regulated on MUTZ-3 cells in response to DNCB, Cin and PPD, however, moDCs only modulated CD86 in response to DNCB and E. PDL-1 (Programmed death receptor ligand-1) proved a promising sensitization biomarker in MUTZ-3 cells where up-regulation occurred in response to DNCB, Cin, IE and PPD. Additionally, moDC-expressed PDL-1 was modulated in response to Cin, IE and E thus demonstrating improved sensitizer predictability when compared with CD86. MCP-1 and RANTES were identified as biomarkers of DNCB exposure but MCP-1 did not show any change in expression above controls for the other sensitizers investigated. However, RANTES was increased in MUTZ-3 cells by both DNCB and Cin. Our findings highlight novel biomarkers which, in MUTZ-3 cells, could be taken forward within a multiple biomarker *in vitro* assay ensuring strong and reliable predictability.

Keywords:

Dendritic cells, MUTZ-3 cells, sensitization, PDL-1, *in vitro* model

1. Introduction.

Dendritic cells (DCs) form a sentinel network and are specialised for the uptake, transport, processing and presentation of antigens to T cells. It is well established that DCs play an essential role in antigen recognition and priming of naive T cells in the initial phase of the sensitization reaction (Sasaki and Aiba, 2007). Allergic contact dermatitis (ACD) represents the clinical manifestation of a T cell mediated inflammatory reaction occurring in sensitized individuals upon allergen challenge. The mechanism of ACD sensitization occurs in two phases which firstly comprises sensitization, occurring upon first contact with the sensitizer and is subsequently followed by an elicitation phase upon secondary exposure to the sensitizer resulting in clinical manifestation of eczematous symptoms (Python et al., 2007).

A European Union ban on *in vivo* testing of cosmetic and toiletry ingredients will be enforced in 2013, therefore, there is a need to develop novel and accurate alternative methods for assessing hazard risks and potential potencies of sensitizing chemicals (Divkovic et al., 2005). Several contact sensitizers, known to cause hypersensitivity responses, have been shown to directly induce DC maturation *in vitro* (Arrighi et al., 2001). Researchers have focused on identifying reliable cytokine or phenotypic biomarkers in DCs which could function as a predictive test to assess the sensitization potential of a chemical. A range of potential biomarkers have been reported including maturation markers; CD86, Major Histocompatibility Complex-II (MHC II), CXCR4 (fusin), generation of interleukin (IL)-8, intracellular IL-1β, mRNA of MCP-1 *(*Monocyte chemoattractant protein 1) and intracellular signalling components (Coutant et al., 1999). However, despite promising data there are technical issues for the routine use of these cells such as donor variability, low cell numbers and availability of human blood (Rougier et al., 2000). To avoid such issues, human cell lines, with characteristics similar to DCs, such as THP-1 and MUTZ-3 cells have been utilised (Sakaguchi et al., 2009). MUTZ-3 comprises the most physiologically similar cell line to its *in vivo* DC counterpart and represents the immortalised equivalent of $CD34^+$ DC precursors (Larsson et al., 2006). Current assay development utilises a test panel of chemicals consisting of extreme, strong, intermediate and weak sensitizers with non-sensitizers all of which have been classified by the local lymph node assay (LLNA) in order to correlate *in vitro* data with known *in vivo* data. When exposed to weak, moderate and strong haptens such

as dinitrochlorobenzene (DNCB), MUTZ-3 cells respond by up-regulating phenotypic expression of CD40, CD54 and CD86 (Azam et al., 2006). However, although CD86, a key costimulatory receptor which activates T cells, appears a promising biomarker, the response varies according to the sensitizer and cell type utilised. This reinforces the view that integrated multiple *in vitro* assays are required in order to provide an accurate and robust sensitization model (Jowsey et al., 2006). Two new members of the B7 family have been identified; programmed death ligand-1 (PDL-1) and PDL-2 which comprise ligands for the T cell expressed programmed death receptor-1 (PD-1) (Matsumoto et al., 2004). Several groups have independently revealed dual functions of PDL-1 in regulating T cell responses (Kuipers et al., 2006). Firstly, PDL-1 signals negatively regulate activated T cell function and survival (Freeman et al., 2000). Secondly, PDL-1 signals are able to co-stimulate early T cell priming and differentiation *in vivo* and *in vitro* (Dong et al., 1999). Thus PDL-1 may be a hypothesised to be a putative biomarker for DC-induced sensitisation.

The sensitizer-specific secretion of cytokines may comprise additional markers for inclusion in a combined *in vitro* assay. Researchers have identified a number of cytokines of importance in the sensitization process which may constitute an important aspect of *in vitro* test development. Recent studies suggested that IL-8 detection following sensitizer or non-sensitizer application to THP-1 cells could function as a promising *in vitro* model to assist with sensitization screening (Mitjans et al., 2008, Miyazawa et al., 2008, Toebak et al., 2006). In addition, IL-18 has also been proposed as a relevant biomarker (Wang et al., 2002). Thus, the current study used cytokine array technology to identify a sensitizer-specific profile in moDCs and MUTZ cells.

Currently, it is thought that no single *in vitro* assay will meet all the necessary requirements and assays consisting of multiple biomarkers should be developed for use concurrently. The aim of this study, therefore, was to identify novel biomarkers which may aid *in vitro* assay development.

2. Materials and Methods.

2.1. Chemical compounds.

The skin sensitizers Dinitrochlorobenzene (DNCB), Eugenol (E), Isoeugenol (IE), P-Phenylenediamine (PPD) and the non-sensitizer Salicyclic Acid (SA) were dissolved in dimethyl sulphoxide (DMSO) (Sigma Aldrich, UK). The skin sensitizer Cinnamaldehyde (Cin) and the non-sensitizer Sodium Lauryl Sulphate (SLS) were dissolved in the appropriate cell culture medium. All chemicals were supplied by the EU Framework VI Sens-It-Iv Consortium (LSHP-CT-2006-018681).

2.2. Cell culture.

MUTZ-3, a human acute myeloid leukaemia cell line was obtained from DSMZ (Germany). MUTZ-3 cells were cultured in α-MEM medium (including ribonucleosides, deoxyribonucleosides [Fisher Scientific, UK]) supplemented with 20% Foetal Calf Serum (Invitrogen, UK), 100U/ml and 100µg/ml Penicillin/Streptomycin solution, 2mM L-Glutamine (Fisher Scientific, UK) and 10ng/ml rhGM-CSF (Peprotech EC, UK). Twice weekly cells were harvested and re-cultured at $2x10^5$/ml.

2.3. Generation of monocyte derived DC (moDCs).

All experiments were carried out in accordance with local ethical guidelines. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh peripheral blood, from healthy volunteers, by density centrifugation. PBMCs, present in the buffy layer, were aspirated and washed (x3) in sterile PBS. Monocytes were isolated from the PBMC population using the Monocyte Isolation Kit II (Miltenyi Biotec, Germany) according to the manufacturer's instructions and subsequently cultured following an established method (Zheng et al., 2004). Briefly, cells were cultured at $2x10^6$/ml at 37°C, 5%CO_2 for 5 days in RPMI-1640 (Fisher Scientific, UK) supplemented with 10% FCS, 100U/ml and 100µg/ml Penicillin/Streptomycin solution and L-Glutamine (2mM) in the presence of GM-CSF (800U/ml) and IL-4 (500U/ml [Peprotech EC, UK]). Cells were fed every other day through

the replenishment of half the volume of fresh medium and cytokines. At day 5, cells were characterised and considered to be iDC by the low expression of CD14, CD40, CD80, CD86 and CD83 (Williams et al., 2008).

2.4. Optimisation of concentration ranges used.

MoDCs and MUTZ-3 cells were seeded at $1x10^6$ cells/well and incubated in 24 well plates with appropriate concentrations of the sensitizers and non-sensitizers for 24 hours prior to phenotypic analysis. Cell supernatants were also harvested for cytokine analysis. To determine the maximum sensitizer concentration for cell application, a dose-response study was performed, in which both cell types were exposed to a range of concentrations to ensure maximum cell death did not exceed 25%. Cell viability was determined by propidium iodide (PI) staining (20µg/ml [Sigma Aldrich, UK]) and evaluated by flow cytometry. The solvents and concentrations of the sensitizers/non-sensitizers utilised within this study are listed in Table 1.

2.5. Immunophenotyping of moDCs and MUTZ-3 cells.

Following 24 hours incubation, cells were harvested and stained for flow cytometric analysis. Cells were washed and re-suspended in 50µl of either control medium, CD14 (5µl purified antibody in 0.01M PBS containing 1% BSA and 15mM sodium azide [Sigma Aldrich, UK]), CD34 (0.5µg/ml), CD80 (0.5µg/ml), CD86 (4µg/ml [BD Biosciences, UK]) or PDL-1 (5µg/ml [Insight Biotech, UK]) and incubated for 30 min at 4°C. Cells were washed and incubated with 50µl polyvalent anti-mouse IgG FITC antibody (Sigma Aldrich, UK) for 30 minutes at 4°C. Phenotypic expression was analysed using the BD FACSVantage and BD CellQuest software. Cells were gated against FSC/SSC and 10,000 events collected. Geometric mean fluorescence intensity (GMFI) changes from isotype control were assessed and mean $\pm$ SEM presented.

2.6. Determination of MCP-1 and RANTES secretion.

Cell supernatants were harvested following 24 hour exposure of moDCs and MUTZ-3 cells to the sensitizers and non-sensitizers in complete medium. Cytokine array analysis was performed in triplicate from three independent repeats by Millipore, UK. Results are demonstrated as mean pg/ml.

2.7. Statistical Analysis.

All data has been expressed as the mean ± SEM. Data was statistically analysed using a two sample student's *t* test where P values ≤ 0.05 were considered as significant. Cells treated with sensitizers which were prepared in medium are compared to the untreated cell control and those prepared in DMSO are compared to the DMSO cell control.

3. Results.

3.1. CD86 expression on MUTZ-3 cells and moDCs.

Firstly, the key identification markers of MUTZ-3 cells; CD14, CD34, CD80 and CD86, were analysed by flow cytometry to confirm the presence of a progenitor MUTZ-3 population. Phenotypic analyses indicated a typical MUTZ-3 progenitor phenotype comprising moderate levels of CD14 (33±1.8%), high levels of CD34 (62±0.8%) with lower expression of CD80 (2±0.8%) and CD86 (19±1.5%) (data not shown).

In this study, CD86 expression was studied to further investigate and validate this marker as a sensitization biomarker but most importantly CD86 expression was used as a comparative tool to assess the novel biomarker PDL-1. When compared to basal expression of the appropriate control, CD86 was highly significantly up-regulated on MUTZ-3 cells in response to all concentrations tested of DNCB, Cin and PPD (Fig. 1). DNCB and PPD application induced a dose-dependent CD86 up-regulation with Cin consistently up-regulated in all tested concentrations. Modulation of CD86 was not observed following treatment with the non-sensitizers SLS and SA, therefore, confirming the usefulness of CD86 as a sensitization biomarker.

Figure 2 demonstrated that moDC-expressed CD86 was up-regulated consistently and significantly in response to DNCB (5µM) and E (900, 920µM) application when compared with the DMSO control. No significant modulation of CD86 expression on moDCs occurred following exposure to Cin and IE or to the non-sensitizers, SLS and SA thus demonstrating a specific up-regulation of CD86 in response to some sensitizers.

3.2. Investigating the expression of PDL-1 as a novel sensitization biomarker in MUTZ-3 cells and moDCs.

To evaluate PDL-1 as a biomarker of sensitization, firstly, MUTZ-3 cells were exposed to a panel of sensitizers and non-sensitizers. As demonstrated in figure 3, treatment of MUTZ-3 with DNCB (5µM), Cin (50, 75, 100µM), IE (930, 970µM) and PPD (150, 200µM) significantly up-regulated PDL-1 expression above basal expression on the untreated and

DMSO treated controls, although no dose-related effect was observed. There were no changes following SLS or SA treatment, therefore, PDL-1 was predominantly sensitizer-specific and demonstrates potential as a novel biomarker of sensitization.

In order to verify PDL-1 as a potential tool for discriminating between sensitizers and non-sensitizers, modulations of PDL-1 expression was further investigated in moDCs. The expression of PDL-1 was significantly up-regulated in response to Cin (90, 120µM), IE (950, 970µM) and E (900, 920µM) when compared to basal expression of the appropriate control. No significant changes were observed upon DNCB and PPD treatment of moDCs. Non-sensitizers, SLS and SA, did not influence the levels of PDL-1 from the basal expression of the controls as illustrated in figure 4.

3.3. MCP-1 and RANTES secretion from MUTZ-3 cells and moDCs following sensitizer exposure.

This study also addressed the detection of soluble factors secreted from MUTZ-3 cells and moDCs as possible novel biomarkers. Resulting from initial cytokine microarray analyses, two cytokines, MCP-1 and RANTES, were selected for further evaluation (Table. 2). As demonstrated in figure 5a the secretion of MCP-1 from MUTZ-3 cells upon sensitizer and non-sensitizer treatment was very inconsistent and as such the use of MCP-1 is neither reliable nor robust in predicting sensitization in MUTZ-3 cells. The secretion of RANTES, however, may present a potentially useful biomarker for detecting DNCB and Cin sensitization. In response to these sensitizers, RANTES secretion was significantly modulated when compared to the respective controls (Fig. 5b).

MCP-1 secretion from moDCs was significantly reduced in response to DNCB (8µM), E (900µM) and IE (950µM) treatment for 24 hours. The application of Cin, PPD and the non-sensitizer, SLS, did not induce a change from basal MCP-1 levels secreted by moDCs (Fig. 6a). However, no significant differences were observed in RANTES secretion with any of the sensitizers or non-sensitizer tested when compared with basal moDC secretion (Fig. 6b).

4. Discussion.

The development and validation of novel *in vitro* methods for assessing the potential of chemicals to cause allergic sensitization is of ultimate importance in reducing animal tests and maintaining product safety. Predictive testing to assess the ability of chemicals to induce ACD is a major part of the safety assessments performed on new ingredients in topically applied cosmetics and drugs (Ashikaga et al., 2002). The main research objectives of the current study were, therefore, to identify novel biomarkers to evaluate the sensitization predictability of a chemical. Previous research has demonstrated that chemical allergens, such as DNCB, can induce changes in the expression of DC surface markers including CD54, CD80 and CD86, suggesting that phenotypic analyses could constitute the basis for an *in vitro* predictive test (Hulette et al., 2002). Cytokine analyses also indicated that following allergen treatment there were modifications in cytokine production (Aiba et al., 1997). The current research, therefore, aimed to further validate CD86 as a sensitization biomarker and evaluate the effects of contact sensitizer and non-sensitizer application on the phenotypic expression of PDL-1 and secretion of MCP-1 and RANTES from moDCs and the DC-like cell line MUTZ-3 as potentially novel biomarkers for inclusion in *in vitro* sensitization assays.

It has been frequently documented that *in vitro* use of DCs are associated with research limitations such as inter-donor variability (Ryan et al., 2005). As a consequence of these limitations, research is currently focusing on the development of DC-like cell lines, which display characteristics similar to their *in vivo* counterparts (Ashikaga et al., 2002). In the current research, the myelomonocytic cell line, MUTZ-3, was assessed for the ability to respond to sensitizing agents and the expression of biomarkers investigated. When compared to other DC-like cell lines used in current research, such as THP-1 cells, MUTZ-3 represent the most physiologically similar cells to *in vivo* DCs (Larsson et al., 2006).

Firstly, the response of the classic co-stimulatory marker, CD86, was analysed on MUTZ-3 cells and moDCs. Myriad reports have highlighted CD86 as a suitable marker for developing an *in vitro* test system (Aiba et al., 1997; Tuschl and Kovac, 2001; De Smedt et al., 2002; Aeby et al., 2004; Boisleve et al., 2004; Sakaguchi et al., 2006; Pepin et al., 2007), although Hulette et al. (2005) reported that CD86 was an unreliable biomarker in primary DCs due to differences in donor variability and responsiveness. In contrast to these findings, in the present study, CD86 was found to be consistently and specifically up-regulated on MUTZ-3 cells in response to DNCB, Cin and PPD, although, moDCs only showed a response to the sensitizers DNCB and E. Thus, although CD86 may be a useful biomarker, marked

differences were observed in the phenotypic responses to the sensitizers between the two cell types. Sakaguchi et al. (2006) also observed variable responses depending on both the sensitizer and cell type used suggesting that sensitizers act differently depending on the cellular system (Sakaguchi et al., 2006). Recent work has indicated that the cell responsiveness to sensitizers is affected by external 'danger signals' (Lavergne et al., 2009) and hapten:protein interactions (Jenkinson et al., 2009) thus there are a range of factors which impact on overall predictability of DCs to sensitizers in *in vitro* culture. Overall, CD86 does respond specifically to certain sensitizers but clearly does not act as a strong predictive biomarker thus new biomarkers are required to extend the predictability of a mono-cell culture *in vitro* test. Recently identified markers eg. PDL-1, expressed on DCs, have been characterised as belonging to the B7 receptor family, which includes CD86. PDL-1 comprises the ligand for the T cell expressed PD-1 receptor and is of importance in regulating the balance between T cell activation, tolerance and immunopathology (Keir et al., 2008). Therefore, the phenotypic response of PDL-1 to sensitizers and non-sensitizers was investigated on MUTZ-3 cells and moDCs**.** PDL-1 proved a promising sensitization biomarker in MUTZ-3 cells where sensitizer-specific modulation of PDL-1 occurred in response to DNCB, Cin, IE and PPD. In addition, moDC expressed PDL-1 was specifically up-regulated in response to the sensitizers Cin, IE and E. Intriguingly, no response was observed with DNCB. Further work by us (unpublished data) suggests that this outcome may have a functional underpinning as T cell proliferation studies with sensitiser (DNCB, Cin, E, IE and PPD)-induced DC's only showed a significantly increased proliferation in response to DNCB thus suggesting that DNCB induces a strongly stimulatory T cell outcome and thus may not upregulate the inhibitory PDL-1. Thus, as previously observed, a difference in the response of PDL-1 was evident between cell types, however, PDL-1 showed a better predictability of sensitizer exposure than CD86. PDL-1 has been previously reported to be induced on DCs through sensitizer exposure *in vivo* (Kim et al., 2006) and thus is a good candidate as a novel biomarker in MUTZ-3 cells.

Phenotypic markers comprise only one source from which to discover novel sensitization biomarkers. The current research also addressed the detection of soluble factors secreted from MUTZ-3 cells and moDCs**.** The secretion of cytokines and chemokines from DCs and MUTZ-3, upon antigenic challenge, is a key factor in the initiation of the immune response (Cumberbatch et al., 2005). Supernatants were collected following sensitizer/non-sensitizer exposure and tested for a range of cytokines and chemokines (IL-12, IL-15, IL-1β, IL-7, IP-

10, MCP-1, MIP-1α, TNF-α, RANTES) which had been documented to be of importance in the sensitization process. Following these initial studies, MCP-1 and RANTES were further investigated for their potential to act as useful, accurate and reliable biomarkers.

Analysis of the secretion of MCP-1 from sensitizer-exposed MUTZ-3 cells revealed no consistent change from solvent control or irritant control, therefore, negating the use of MCP-1 as a sensitization biomarker in MUTZ-3 cells. However, moDCs showed a specific decrease in MCP-1 for all sensitizers tested except PPD. The reduction in MCP-1 secretion levels does not appear to be logical in the context of a sensitization immune response, as even during the initial sensitization phase, increased numbers of DCs and other APCs are recruited to interact with the allergen, mature and migrate to the lymph nodes for T cell presentation and activation. However, with regards to moDCs, it may be possible that the MCP-1 effects illustrated in the current study may not have a functional role. It appears possible that the expression of MCP-1 may have peaked at an earlier time point than that investigated within this study. Published research has shown, albeit from various cell lines, that upon stimulation with TNF-α or IL-1β, MCP-1 was detectable at 4 hours following stimulation with maximal secretion observed around 8 hours post stimulation. Levels had decreased significantly by 24 hours (Goldstein et al., 1996; Pype et al., 1999). Therefore, with regards to moDC MCP-1 secretion, optimisation of the time point for maximal detection may be required.

Studies of cytokine secretion from MUTZ-3 cells demonstrated that RANTES secretion was significantly and consistently increased in response to both DNCB and Cin, although no change in E, IE and PPD was observed. However, the latter results may be related to the metabolic factors highlighted previously reducing the sensitizer capacity of these pro-haptens. The effects of Octanoic Acid and Oxazolone on MUTZ-3-secretion of RANTES has also been investigated and significant increases above irritant controls (Glycerol and Lactic acid [data not shown] were found thus RANTES does appear a novel biomarker on MUTZ-3 cells. However, this outcome is not seen in moDCs where no significant changes from irritant or solvent control was observed. RANTES has been previously identified *in vivo* as being a key cytokine in the murine ear test to DNCB (Baumer et al., 2004) thus supporting its relevance as an *in vitro* biomarker. In order to compare the sensitizer predictability of RANTES with a currently used cytokine (Mitjans et al., 2008, Toebak et al., 2006, Miyazawa et al., 2008) we investigated the generation of IL-8 on MUTZ-3 cells. Our unpublished data showed that IL-8 was increased comparably to the sensitizers seen for RANTES, thus,

RANTES can be considered as extending the *in vitro* biomarkers available for sensitizer testing.

Many investigations are currently underway to develop *in vitro* sensitization assays and novel biomarkers resulting from phenotypic, cytokine, genomic and proteomic studies are being revealed. It is difficult to envisage that an accurate single *in vitro* method of prediction could be developed as many factors contribute to the manifestation of ACD such as chemical structure, binding affinity and immune responsiveness. It is likely that combined multiple tests which correlates with known *in vivo* data derived from the LLNA and human data is the most likely direction in which research will focus. Our findings, amongst others, clearly demonstrates large variation in the expression of phenotypic or cytokine markers, therefore, highlighting that biomarker responses in moDCs or MUTZ-3 is not consistent or sensitizer specific in all cases which further strengthens the case for development of an *in vitro* assay comprising multiple biomarkers to ensure strong and reliable predictability. This research has demonstrated novel phenotypic and chemokine biomarkers of MUTZ-3 cells which will be a useful addition to further *in vitro* sensitization research.

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Figure 1. Modulation of CD86 expression on MUTZ-3 cells following treatment with sensitizers and non-sensitizers. **a**. CD86 was highly significantly up-regulated on MUTZ-3 cells in response to all concentrations tested of DNCB, Cin and PPD. There was no change from basal levels following treatment with the non-sensitizers, SLS and SA. Data is expressed as GMFI ± SEM. n≥3. **p<0.01, ***p<0.001 compared to the DMSO control. xxx p<0.001 compared to the untreated cells. **b.** CD86 expression by MUTZ-3 cells with grey overlay demonstrating CD86 up-regulation in response to DNCB (8µM) **c**. in response to Cin (100µM) and **d**. in response to PPD (200µM). The shaded histogram depicts the appropriate control cells stained with CD86. One representative histogram of n≥3 is shown.
DNCB Cin IE E PPD SLS SA

Figure 2. Modulation of CD86 expression on moDCs. a. moDC-expressed CD86 was up-regulated significantly in response to DNCB (5µM) and E (900, 920µM) application when compared with the DMSO control. The non-sensitizers, SLS and SA, did not increase CD86 expression above basal levels. Data is expressed as GMFI ± SEM. n≥3. *p<0.05 compared to the DMSO control. **b.** CD86 expression by moDCs with grey overlay demonstrating CD86 up-regulation in response to DNCB (5µM) and **c**. in response to E application (920µM). The shaded histogram depicts the appropriate control cells stained with CD86. One representative histogram of n≥3 is shown. DNCB Cin IE E PPD SLS SA

Figure 3. PDL-1 expression as a novel sensitization biomarker in MUTZ-3 cells. a. Treatment of MUTZ-3 cells with DNCB (5µM), Cin (50, 75, 100µM), IE (930, 970µM) and PPD (150, 200µM) significantly up-regulated the expression of PDL-1 above basal expression of the appropriate control. There were no changes following SLS or SA treatment. Data is expressed as GMFI ± SEM. n≥3. *p<0.05 compared to the DMSO control. x p<0.05, xx p<0.01 compared to the untreated control. **b.** PDL-1 expression on MUTZ-3 cells with grey overlays demonstrating PDL-1 up-regulation in response to DNCB (5µM) **c.** in response to Cin (100µM), **d**. in response to IE (970µM) and **e**. in response to PPD application (200µM). The shaded histogram depicts the appropriate control cells stained with PDL-1. One representative histogram of n≥3 is shown. DNCB Cin IE E PPD SLS SA

Figure 4. PDL-1 expression in moDCs following sensitizer exposure. a. The expression of PDL-1 on moDCs was significantly up-regulated in response to Cin (90, 120µM), IE (950, 970µM) and E (900, 920µM) when compared to basal expression levels. The application of SLS and SA did not influence the levels of PDL-1. Data is expressed as GMFI ± SEM. n≥3. *p<0.05, **p<0.01, ***p<0.001 compared to the DMSO control. xxx p<0.001 compared to

DNCB Cin IE E PPD SLS SA

the untreated control. **b.** PDL-1 expression on moDCs with grey overlays demonstrating PDL-1 up-regulation in response to Cin (120µM) application, **c.** IE application (970µM). **d.** E application (920µM). The shaded histogram depicts the appropriate control cells stained with PDL-1. One representative histogram of n≥3 is shown.

Figure 5. MCP-1 and RANTES secretion from MUTZ-3 cells. a. MCP-1 secretion from MUTZ-3 cells was inconsistent following sensitizer application. b. Exposure of MUTZ-3 cells to DNCB (10µM) and Cin (100µM) significantly increased RANTES secretion when compared with the appropriate control. Other sensitizers and non-sensitizers tested did not influence the secretion of RANTES from MUTZ-3 cells. Data is expressed as mean pg/ml ± SEM. n=3 in triplicate. *p<0.05, **p<0.01, ***p<0.001 when compared with the DMSO control. x p<0.05 compared with the untreated cell control.

DNCB Cin IE E PPD SLS

Figure 6. Cytokine response of moDCs. a. MCP-1 analysis revealed significantly reduced secretion specific to DNCB (8µM), E (900µM) and IE (950µM). MCP-1 secretion was not modulated by the application of Cin or PPD. b. RANTES secretion from moDCs in response to sensitizers. There were no significant changes in the secretion of RANTES upon treatment of moDCs with either sensitizer or non-sensitizer application. Data is expressed as mean pg/ml ± SEM. n=3 in triplicate. *p<0.05 when compared with the DMSO control.

DNCB Cin IE E PPD SLS

Figure 1.

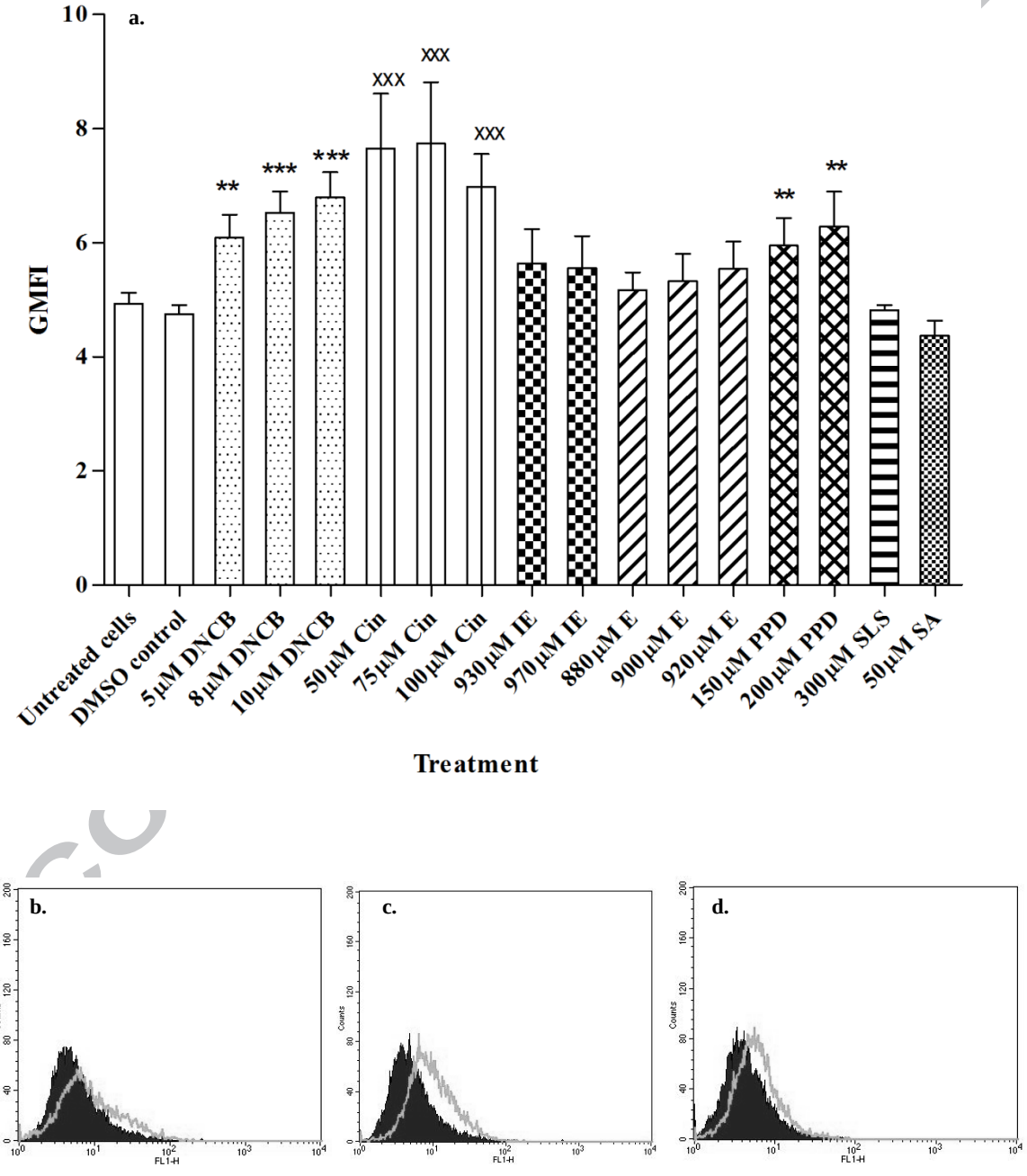
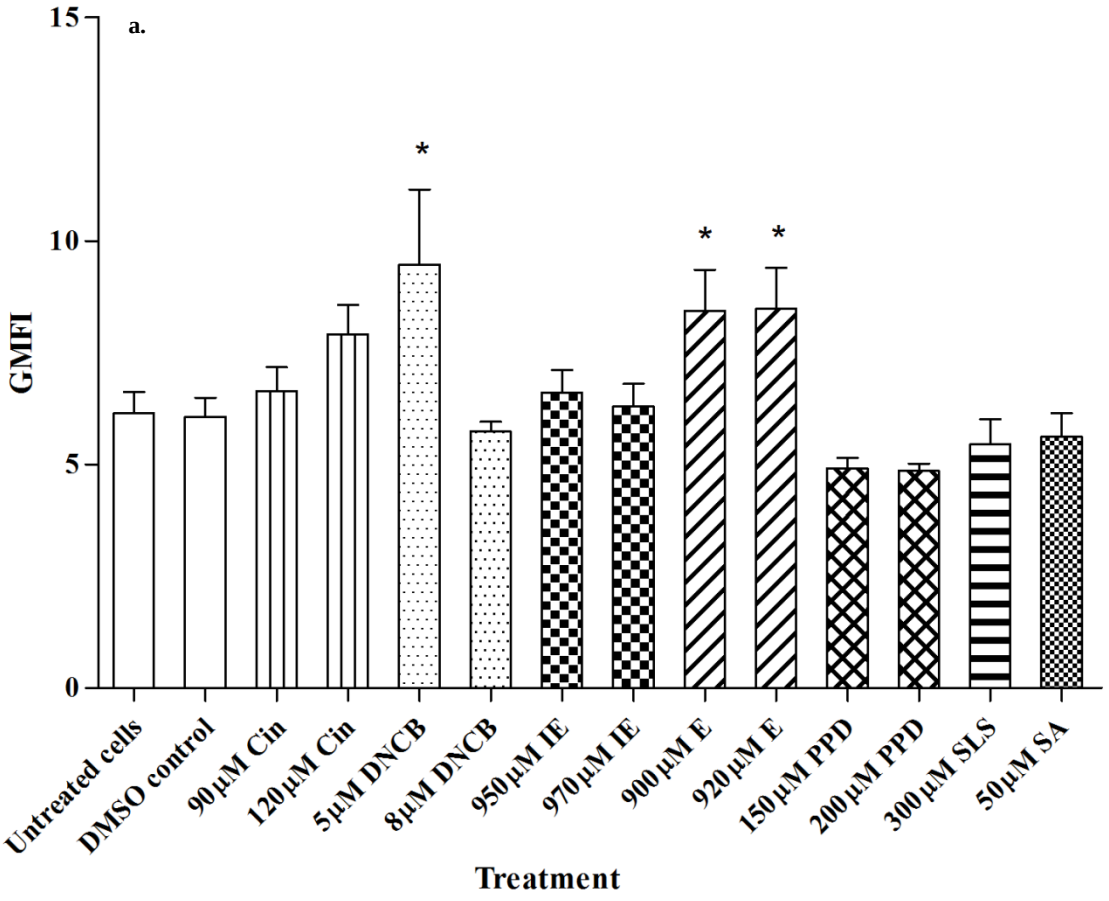


Figure 2.



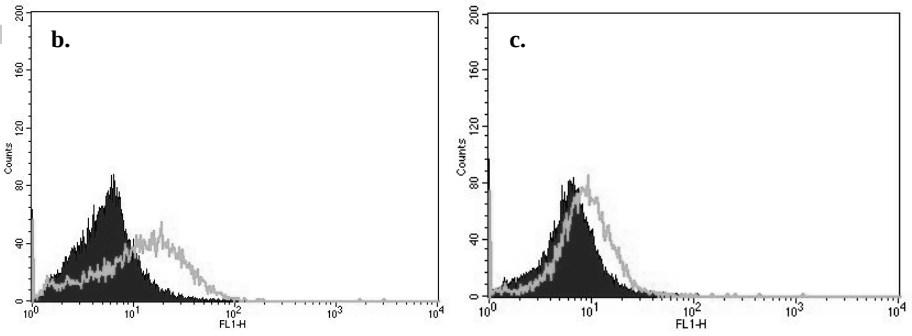
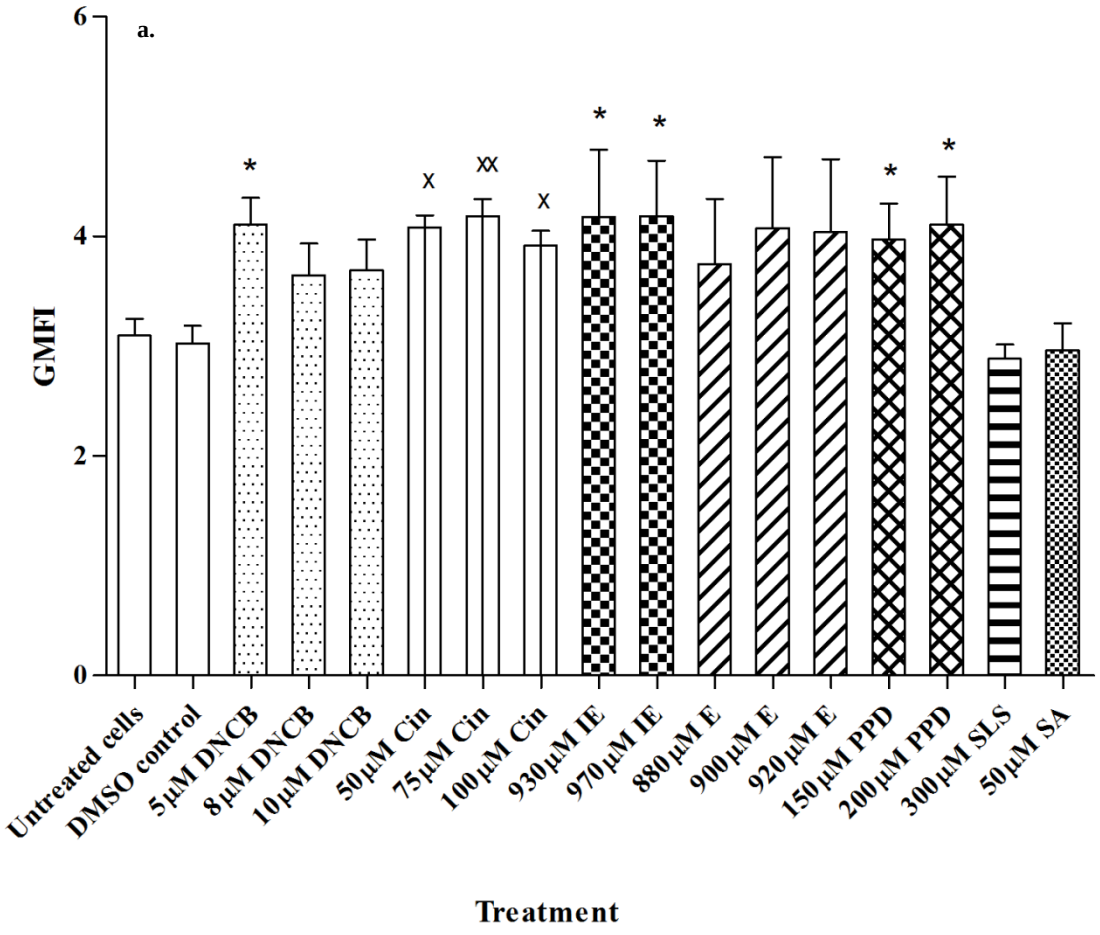


Figure 3.



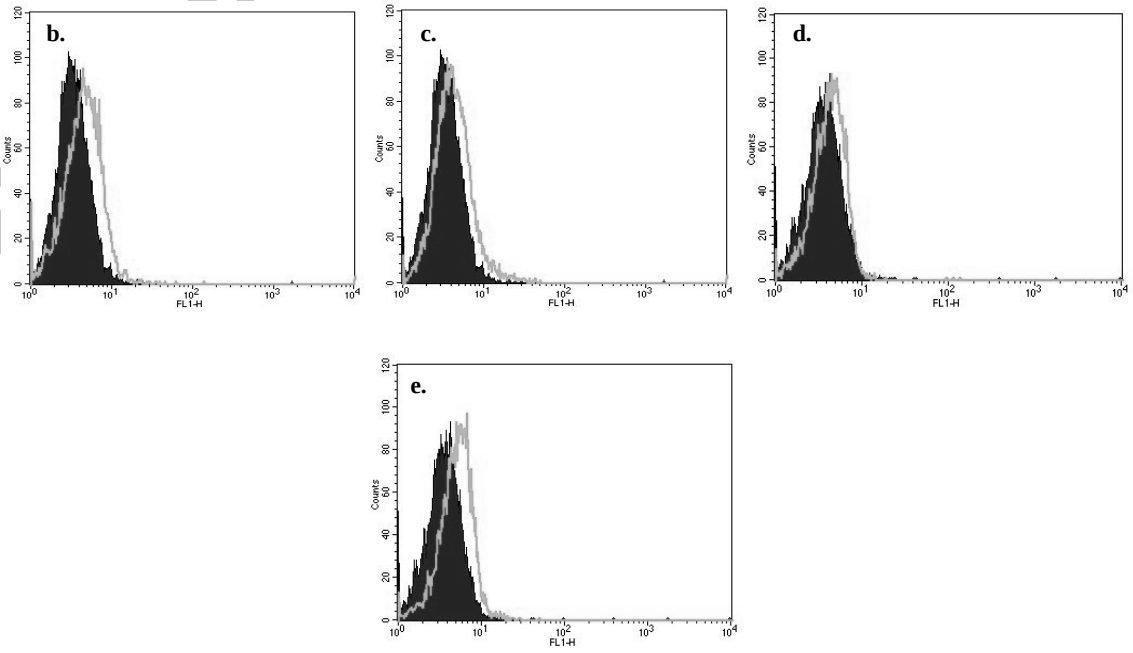
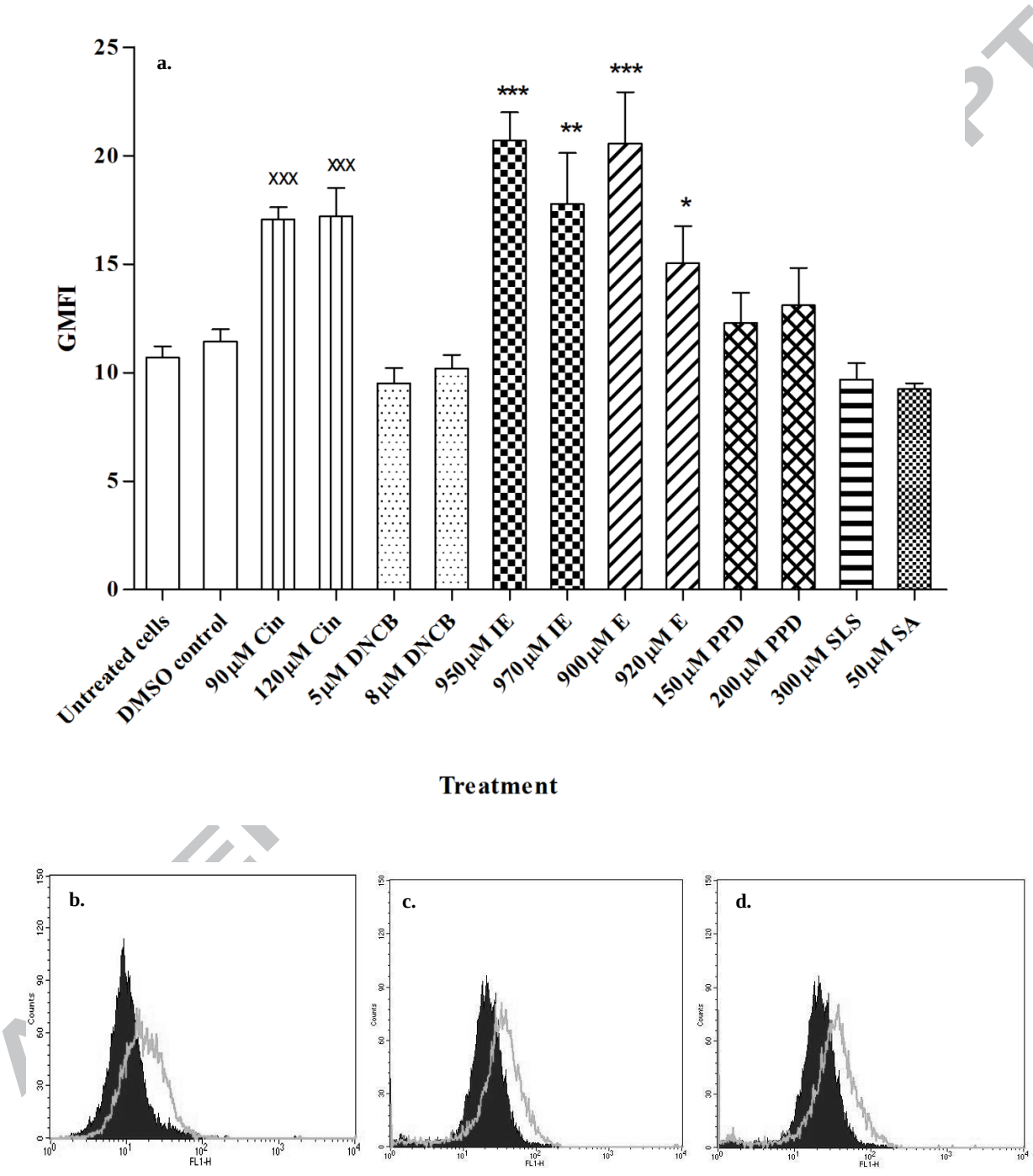


Figure 4.



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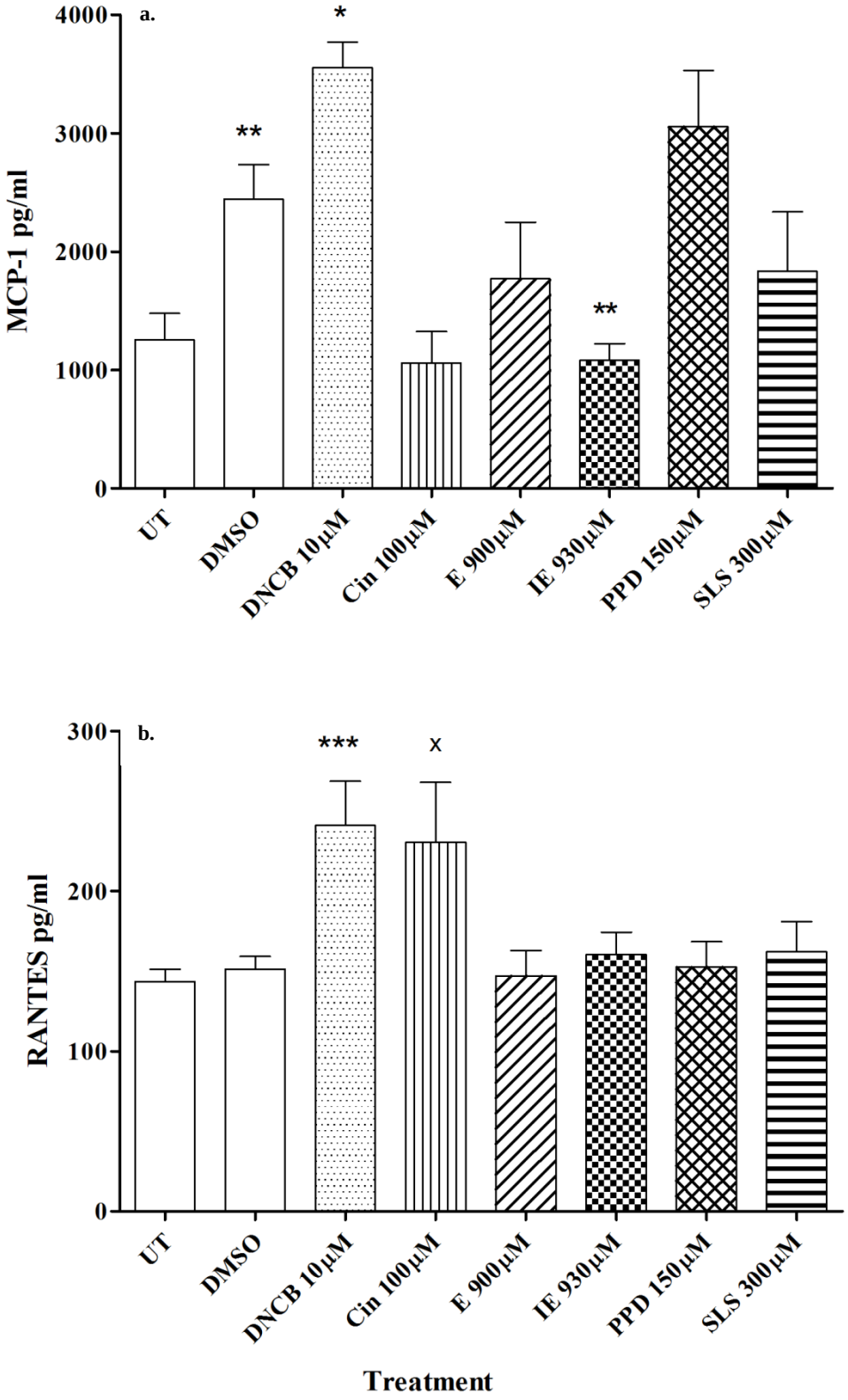

Figure 6.

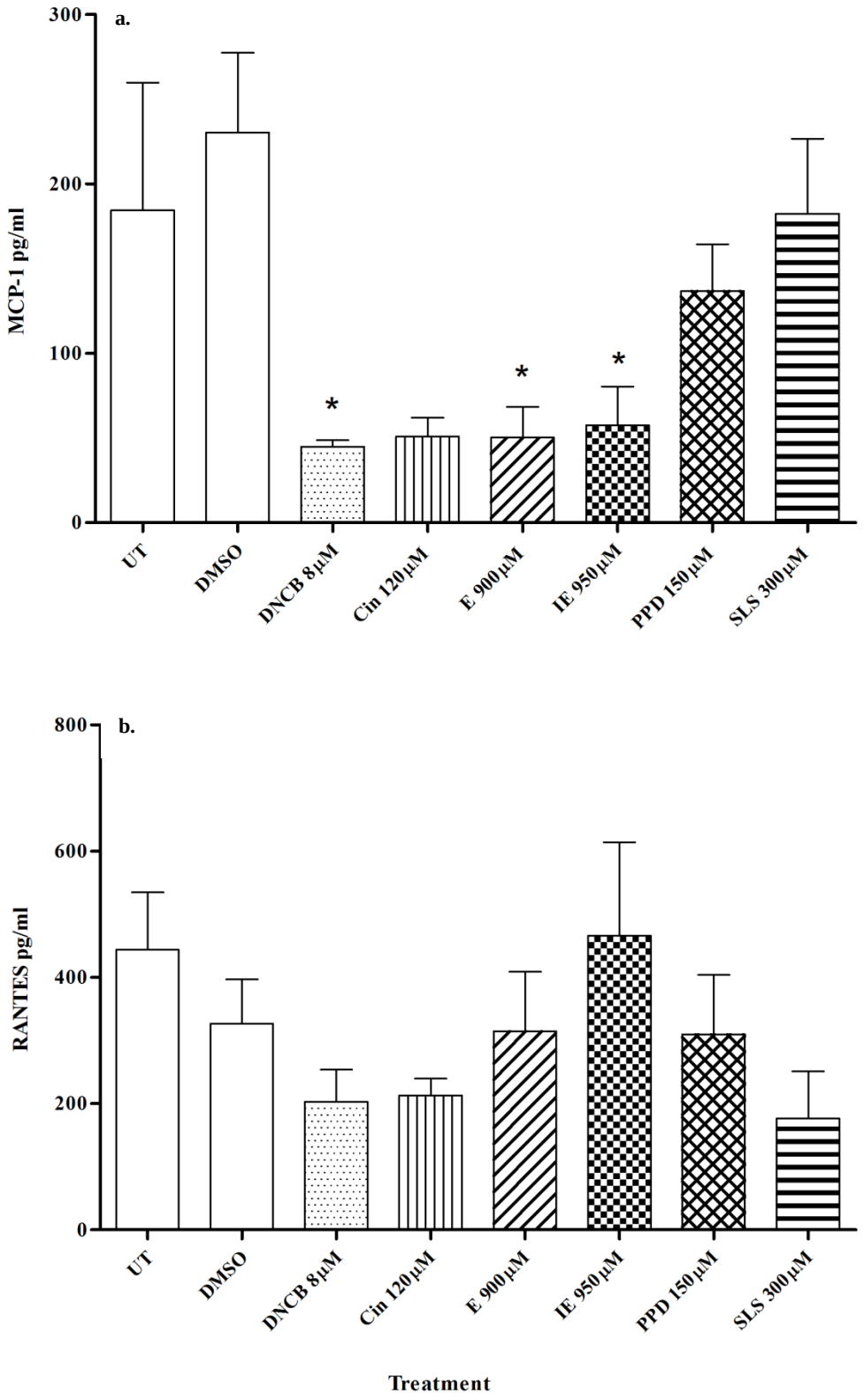

Table 1. Range of sensitizer/non-sensitizer concentrations and solvents utilised.

Sensitizers/non-sensitizers	moDCs	MUTZ-3	Solvent
DNCB	5, 8µM	5, 8, 10µM	DMSO[a]
Cin	90, 120µM	50, 75, 100µM	Medium
IE	950, 970µM	930, 970µM	DMSO[a]
E	900, 920µM	880, 900, 920µM	DMSO[a]
PPD	150, 200µM	150, 200µM	DMSO[a]
SA	50µM	50µM	DMSO[a]
SLS	300µM	300µM	Medium

[a] cells exposed to a final concentration of 0.1% DMSO.

Cell type	IL-12	IL-15	IL-1β	IL-7	MCP-1	MIP-1α	TNF-α	RANTES	IP-10
DC + DMSO	**12.2**	**nd**	**nd**	**2.8**	**214.5**	**32.9**	**46.4**	**2300.0**	**1630.0**
DC + DNCB 8µM	**nd**	**nd**	**nd**	**7.8**	**133.5**	**35.6**	**14.5**	**465.0**	**nd**
DC + Cin 120µM	**nd**	**nd**	**nd**	**4.7**	**79.0**	**29.1**	**7.2**	**465.0**	**2.11**
DC + SLS 300µM	**nd**	**nd**	**nd**	**4.7**	**106.1**	**161.5**	**22.4**	**345.5**	**5.4**
MUTZ + DMSO	**3.58**	**nd**	**nd**	**6.68**	**1555.0**	**14.4**	**nd**	**270.0**	**2.63**
MUTZ + DNCB 10µM	**6.07**	**nd**	**1.3**	**9.0**	**3110.0**	**23.3**	**0.1**	**203**	**1.17**
MUTZ + Cin100µM	**3.58**	**1.29**	**1.5**	**9.9**	**815.5**	**20.4**	**nd**	**369.5**	**nd**

Cytokines = pg/ml

nd: Both replicates below detectable limits.

Table 2. **Cytokine profile of MUTZ-3 and moDCs in response to sensitizer and non-sensitizer application**. Exposure of MUTZ-3 cells induced MCP-1 and RANTES secretion only. Of all cytokines investigated predominant secretion of MCP-1 and RANTES was observed from moDCs. Data is expressed as mean pg/ml. n=1 in duplicate.