

Original Article

Production of IL-8 in THP-1 cells following contact allergen stimulation via mitogen-activated protein kinase activation or tumor necrosis factor- α production

Yuko Nukada, Masaaki Miyazawa, Nanae Kosaka, Yuichi Ito, Hitoshi Sakaguchi
and Naohiro Nishiyama

Kao Corporation, Safety Science Research Laboratories, 2606 Akabane Ichikai-Maich Haga-Gun, Tochigi
321-3497, Japan

(Received November 22, 2007; Accepted December 21, 2007)

ABSTRACT — Contact allergens induce *in vitro* and *in vivo* the activation of dendritic cells (DC) and Langerhans cells (LC), which includes the up-regulation of surface marker expression (e.g. CD86, CD54) and cytokine production (e.g. TNF- α , IL-1 β , IL-8). The mitogen-activated protein kinase (MAPK) pathway also has a crucial role in this activation. However, the extent of MAPK involvement in the IL-8 production during DC/LC activation is not well understood. Earlier, we reported that contact allergens activated THP-1 cells, human monocytic cell line, like LC/DC *in vitro*. In this study, we further characterize the mechanism of IL-8 production using THP-1 cells as surrogate DCs. First, we evaluated the potential of 23 chemicals with different skin sensitization potencies to predominantly induce IL-8 production *in vitro*. Next we investigated the role of MAPK signaling and TNF- α , which is known to have autocrine effects on DC activation (e.g., IL-8 production). Inhibition of extracellular signal-regulated kinase (ERK), one of the MAPK pathways, suppressed the IL-8 production induced by both 2,4-dinitrochlorobenzene (DNCB) and nickel sulfate (NiSO₄), and inhibition of p38 MAPK, a second MAPK pathway, significantly suppressed IL-8 production induced by only DNCB. Additionally, neutralization of TNF- α activity suppressed IL-8 production in THP-1 cells exposed to DNCB and NiSO₄. In conclusion, IL-8 production was predominantly induced in THP-1 cells following allergen stimulation, and MAPK pathways and TNF- α were involved in the IL-8 production induced by DNCB and NiSO₄. A better understanding of the mechanism of DC activation *in vitro* might lead to the clarification of the *in vivo* skin sensitization mechanism.

Key words: IL-8, Dendritic cell, THP-1 cell, allergen-induced activation, p38 MAPK, ERK

INTRODUCTION

Allergic contact dermatitis (ACD) is a delayed type of immune response mainly caused by low molecular weight compounds binding to an endogenous protein to form a “hapten”. In the induction phase of contact allergy, dendritic cells (DCs) in epidermis, including Langerhans cells (LCs), will recognize the contact allergen, become activated, and migrate to the regional lymph node.

During DC activation, augmentations of cell surface molecules (e.g. CD86, CD54, CD40, and CD83) occur and the pro-inflammatory cytokines represented by TNF- α , IL-8, IL-1 β and IL-6 are produced (Enk and Katz, 1992; Cumberbatch *et al.*, 1996; Ozawa *et al.*, 1996; Verrier *et al.*, 1999). These phenotypic alterations and

changes of cytokine production were confirmed *in vitro* using the monocyte-derived dendritic cells (Mo-DC) or CD34+-progenitor cells-derived dendritic cells (CD34-DC) (Aiba *et al.*, 1997, 2003; Coutant *et al.*, 1999; De Smedt *et al.*, 2001, 2002; Staquet *et al.*, 2004). Moreover, the human myeloid cell lines, for example THP-1, U937 and MUTZ-3, have also been found to undergo analogous alternations, which indicate a similar potential to behave like a LC/ DC (Ashikaga *et al.*, 2002; Ade *et al.*, 2006; Azam *et al.*, 2006; Python *et al.*, 2007). We have reported that the expression of surface markers (CD86, CD54, and CD40) and cytokine production (TNF- α and IL-8) were preferentially induced following exposure to contact allergens such as 2,4-dinitrochlorobenzene (DNCB) and nickel sulfate (NiSO₄) using THP-1 cells as surro-

Correspondence: Yuko Nukada (E-mail: nukada.yuko@kao.co.jp)

gate DCs (Yoshida *et al.*, 2003; Miyazawa *et al.*, 2007a). Because the Mo-DC and surrogate cells, U937, THP-1, and MUTZ-3, have similar phenotypic alterations and cytokine responses, the surrogate cells might be more widely accepted as tools to investigate the key intracellular mechanisms involved in DC activation.

The intracellular mechanisms for DC activation have been analyzed using Mo-DCs or CD34-DCs *in vitro* previously. It has been reported that the representative contact allergen, NiSO₄, induced p38 MAPK phosphorylation on Mo-DCs, and that p38 MAPK inhibitor almost completely suppressed the augmentation of CD80 and CD83 expression induced by NiSO₄ (Arrighi *et al.*, 2001). Similarly, Aiba *et al.* (2003) reported that p38 MAPK and extracellular signal-regulated kinase (ERK) play distinct roles in the DCs activation following stimulation with two representative contact allergens, nickel dichloride (NiCl₂) and DNCB. It has also been reported that p38 MAPK and c-Jun N-terminal kinase (JNK) were phosphorylated on CD34-DCs following stimulation with DNCB and NiSO₄, and that p38 MAPK participated in the TNF- α production induced by DNCB (Boislève *et al.*, 2004).

TNF- α is known to play important roles in the migration of mature DCs (Wang *et al.*, 1999). Salgado *et al.* (1999) showed that TNF- α up-regulated the expression of CD40 and CD80 on murine purified LCs *in vitro*. The author suggested that TNF- α plays a role in the regulation of DC activation, such as increasing expression of surface markers or production of cytokines.

Recently, several investigators reported that mRNA expression or production of IL-8 in DCs (Mo-DCs or surrogate cell lines) was induced following exposure to contact allergens (Toebak *et al.*, 2006; Miyazawa *et al.*, 2007a; Python *et al.*, 2007). IL-8 is a chemokine that mainly has the role as inducer of neutrophils or T-lymphocyte migration (Sebastiani *et al.*, 2002). In skin sensitization, augmentation of IL-8 production has been shown to occur after poison ivy/oak extract was applied topically to the skin *in vivo* (Griffiths *et al.*, 1991). However, the mechanism for IL-8 production in DCs following stimulation with contact allergens is still less known. It is proposed that by understanding the specificity and the mechanism for augmenting IL-8 production, a greater comprehension will not only be made of the mechanism of LCs activation but will also provide insight into the entire skin sensitization process. Thereupon, in this study, we first confirmed the alteration of IL-8 production in THP-1 cells following stimulation with several chemicals (allergens and non-allergens) having different skin sensitization potencies. After which, to elucidate further the mechanism for induction of IL-8 production,

we examined the effect of MAPK pathways and TNF- α on IL-8 production following stimulation with two representative contact allergens DNCB and NiSO₄ using THP-1 cells as surrogate DCs. The latter work was conducted in order to elucidate further the mechanism for induction of IL-8 production by looking at intracellular/extracellular induction mechanisms.

MATERIALS AND METHODS

Cell line and Medium

THP-1 cells from American Type Culture Collection (Manassas, VA, USA) were cultured in RPMI1640 containing 25mM HEPES buffer and L-glutamine (Invitrogen Corp., Carlsbad, CA USA), supplemented with 10% fetal bovine serum (FBS; Invitrogen Corp.), 0.05mM 2-mercaptoethanol and 1% penicillin-streptomycin (Invitrogen Corp.).

Test chemicals and preparation of chemicals

The 16 contact allergens employed in the experiment were: 1-benzoylacetone (1-BA), 5-chloro-2-methyl-4-isothiazolin-3-one/2-methyl-4-isothiazolin-3-one (MCI/MI), DNCB, glutaraldehyde (GA), benzoyl peroxide (BP), phthalic anhydride (PA), 2-mercaptobenzothiasole (2-MBT), ethylenediamine (ED), methyl-2-nonynoate (MN), NiSO₄, diethyl maleate (DEM), hexylcinnamaldehyde (HCA), citral (CI), eugenol (EU), aniline (AN) and geraniol (GE). The 7 non-allergens were; 1-bromobutane (1-BB), diethyl phthalate (DEP), benzalkonium chloride (BKC), vanillin (VA), 2-hydroxypropyl methacrylate (2-HPM), acetoanisole (AA) and 6-methylcoumarin (6-MC). All chemicals have been evaluated in the local lymph node assay (LLNA) by other authors, and these authors have published the EC3 values for the allergens (Table 1; Gerberick *et al.*, 1992, 2005; Kimber *et al.*, 1998). All tested chemicals except 2-HPM were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-HPM was purchased from Wako Pure Chemicals (Osaka, Japan). Water-soluble test chemicals (MCI/MI, GA, ED and NiSO₄ and BKC) were initially prepared in saline, while water-insoluble test chemicals (1-BA, DNCB, PA, BA, 2-MBT, DEM, MN, HCA, CI, EU, AN, GE, 1-BB, DEP, VA, 2-HPM, AA and 6-MC) were dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich). Final concentration of DMSO in culture medium was less than 0.2 %.

Determination of test concentrations by cytotoxicity

Test concentrations of each test chemical to be used for confirming the augmentation of IL-8 production were

IL-8 production by contact allergen via MAPK and TNF- α

determined based on cell viability (Table 1). In detail, THP-1 cells were cultured in 24-well plates (1.0×10^6 cells/ml/well) with various concentrations of chemicals for 24 hr. After that, cells were washed twice with phosphate buffer saline supplemented with 0.1 % bovine serum albumin, then stained with propidium iodide (PI; 0.625 μ g/ml as the final concentration, Sigma-aldrich), and cell viability was measured by flow cytometry (FACSCalibur and CellQuest software; Beckton Dickinson, San Jose, CA, USA). Total events for living cell counting were 10,000. The concentrations that inhibited cell viability (CV) to 75 % (CV75) were calculated from the cell viability data. CV75 of each chemical is shown in Table 1. Based on the value of CV75, we selected 5 concentrations. These are at 1.2x, 1x, 1/1.2x, 1/1.2²x,

and 1/1.2³ x CV75, and covered the range of concentrations from approximately 50 to 95 % cell viability, which equaled from moderate to almost no cytotoxicity. Maximum dose of test chemicals did not exceed 1 mg/ml (if solvent was DMSO) or 5 mg/ml (if solvent was saline). Test concentrations of DNCB and NiSO₄ for the MAPK or TNF- α inhibition assay were 5 μ g/ml and 170 μ g/ml, respectively.

Chemicals and cytokines exposure of THP-1 cells

THP-1 cells (1×10^6 cells) were cultured for 24 hr in 24-well plates in 1 ml of culture medium with or without the test chemicals prepared as described above. To investigate the involvement of MAPK pathways, cells were

Table 1. The list of chemicals tested and the value of CV75.

Chemicals	Abbreviation	CAS		LLNA Potency category*	CV75 (μ g/ml)**
1-Benzoylacetone	1-BA	93-91-4		Extreme	82
5-Chloro-2-methyl-4-isothiazolin-3-one Solution	MCI/MI	26172-55-4		Extreme	2.1
2,4-Dinitrochlorobenzene	DNCB	97-00-7		Extreme	5
Glutaraldehyde	GA	111-30-8		Strong	11
Benzoyl peroxide	BP	94-36-0		Strong	42
Phthalic anhydride	PA	85-44-9		Strong	ND***
2-Mercaptobenzothiasole	2-MBT	149-30-4		Moderate	150
Ethylene diamine	ED	107-15-3	Allergens (16)	Moderate	273
Methyl-2-nonynoate	MN	111-80-8		Moderate	230
Nickel sulfate	NiSO ₄	10101-97-0		Moderate	150
Diethyl maleate	DEM	141-05-9		Moderate	120
Hexylcinnamaldehyde	HCA	101-86-0		Weak	37
Citral	CI	5392-40-5		Weak	20
Eugenol	EU	97-53-0		Weak	150
Geraniol	GE	106-24-1		Weak	167
Aniline	AN	62-53-3		Weak	930
1-Bromobutane	1-BB	109-65-9			500
Diethyl phthalate	DEP	84-66-2			600
Benzalkonium chloride	BKC	63449-41-2	Non-allergens (7)	Non-sensitizer	3
Vanillin	VA	121-33-5			650
2-hydroxypropyl methacrylate	2-HPM	923-26-2			ND***
Acetoanisole	AA	100-06-1			320
6-methylcoumarin	6-MC	92-48-8			277

* The EC3 value and the potency of skin sensitization were estimated using the LLNA (Gerberick *et al.*, 1992, 2005; Kimber *et al.*, 1998). All chemicals were ranked from extreme to non-sensitizer.

** CV75 of each chemical was estimated by flow cytometry analysis as described in the section "Materials and Methods".

*** ND; not decided

cultured with various concentrations of inhibitors for ERK or p38 MAPK for 1 hr before exposure to DNCB or NiSO₄ for 24 hr. PD98059, a MEK1 (MAPK Kinase) inhibitor, blocks the phosphorylation of ERK and equally inhibits ERK pathway. SB203580 is a specific inhibitor of p38 MAPK. Both were purchased from Calbiochem (La Jolla, CA, USA). PD98059 and SB203580 were used at the concentration of 0.3 or 3 μ M, and 0.03 or 0.3 μ M, respectively. To investigate the effects of TNF- α , cells were cultured with 0.625 μ g/ml anti-TNF- α antibodies (Abs) (R&D systems, Minneapolis, MN, USA) in the same manner as described above in the inhibition of MAPK pathways experiment. In some experiments, THP-1 cells were cultured for 24 hr with 9.8 to 2,500 pg/ml of recombinant human TNF- α (rhTNF- α ; R&D systems) and the amount of IL-8 produced was measured. Also, the amount of TNF- α produced in THP-1 cells treated with 80 to 50,000 pg/ml of recombinant human IL-8 (rhIL-8; R&D systems) for 24 hr was obtained to assess the role of IL-8 in TNF- α production.

Analysis of cytokine production and cell viability

After exposure to test chemicals, culture supernatants were collected and used to evaluate the extent of IL-8 production and TNF- α production with an enzyme-linked immunosorbent assay (ELISA) kit obtained from R&D Systems. The kits were used according to the manufacturer's instructions. Cell viability was determined using flow cytometry as described above.

Statistics

Student's *t*-test was used to evaluate a statistical significance. *P*-values less than or equal to 0.05 and 0.01 were considered statistically significant.

RESULTS

Stimulation of IL-8 production in THP-1 cells with 23 chemicals

We investigated the alteration of IL-8 production in THP-1 cells following exposure to a total of 23 chemicals with different reported skin sensitization potencies as assessed by LLNA. Fig. 1 shows the changes in IL-8 production and cell viability following exposure to 16 contact allergens and 7 non-allergens. Baseline levels of 47.1 ± 21.3 pg/ml and 40.8 ± 22.0 pg/ml of IL-8 were obtained for non-treated cells and DMSO-only treated cells, respectively. When the allergens were evaluated, we found that 13 out of 16 contact allergens induced statistically significant augmentation of IL-8 production. Ten of these allergens (1-BA, MCI/MI, DNCB, GA, BP, 2-

MBT, NiSO₄, DEM, CI, and AN) had over a 10-fold IL-8 production increase in THP-1 cells as compared to medium and DMSO control. In particular, extreme and strong contact allergens, as determined in the LLNA, tended to be able to significantly increase IL-8 production at lower concentrations. Meanwhile, the cell viability following exposure to chemicals changed inversely with IL-8 production in a dose-dependent manner. The statistically significant augmentations of IL-8 production were induced at the sub-toxic concentration, specifically at the range of 60 to 90%. Thirty out of 35 concentrations used for 13 chemicals that showed statistically significant augmentation of IL-8 production were included in this range. Three of the contact allergens (PA, ED, and GE) did not significantly induce augmentation of IL-8 production and only ED and GE cause cytotoxicity while PA did not cause cytotoxicity.

In contrast to contact allergens, no significant change in IL-8 production was detected in cells exposed to the non-allergens (BKC, VN, 2-HPM, AA, and 6-MC). These chemicals, though, were able to cause cytotoxicity akin to the contact allergens, with the exception of 1-BB and DEP. 1-BB and DEP had a potential to induce the augmentation of IL-8 production. In particular, 1-BB had over a 10-fold IL-8 production increase as compared to DMSO control, which was similar to most of the allergens.

Involvement of MAPK pathway in THP-1 cell IL-8 production

To investigate whether MAPK pathways contribute to IL-8 production following exposure to allergens, we used specific inhibitors for the ERK and p38 MAPK pathways. First, we found that THP-1 cells treated with both inhibitors had little influence on cell viability. Cell viability range was 84 to 85 % after 24 hr exposure to DNCB with or without both ERK inhibitor (PD98059) and p38 MAPK inhibitor (SB203580). Cell viability range was 74 to 76 % after 24 hr exposure to NiSO₄ with or without both inhibitors. Treatment of THP-1 cells with 0.3 or 3 μ M PD98059 partially suppressed IL-8 production after exposure to both DNCB (5 μ g/ml) and NiSO₄ (170 μ g/ml) in a dose-dependent manner (Fig. 2a). A statistically significant difference was observed only between the 3 μ M PD98059 inhibitor treatment group and the non-inhibitor treated group. On the other hand, treatment of THP-1 cells with 0.03 or 0.3 μ M SB203580 prior to exposing cells with 5 μ g/ml DNCB suppressed IL-8 production, but did not suppress IL-8 production when cells were treated with NiSO₄ (Fig. 2b). These results revealed that DNCB could induce IL-8 production via both ERK

IL-8 production by contact allergen via MAPK and TNF- α

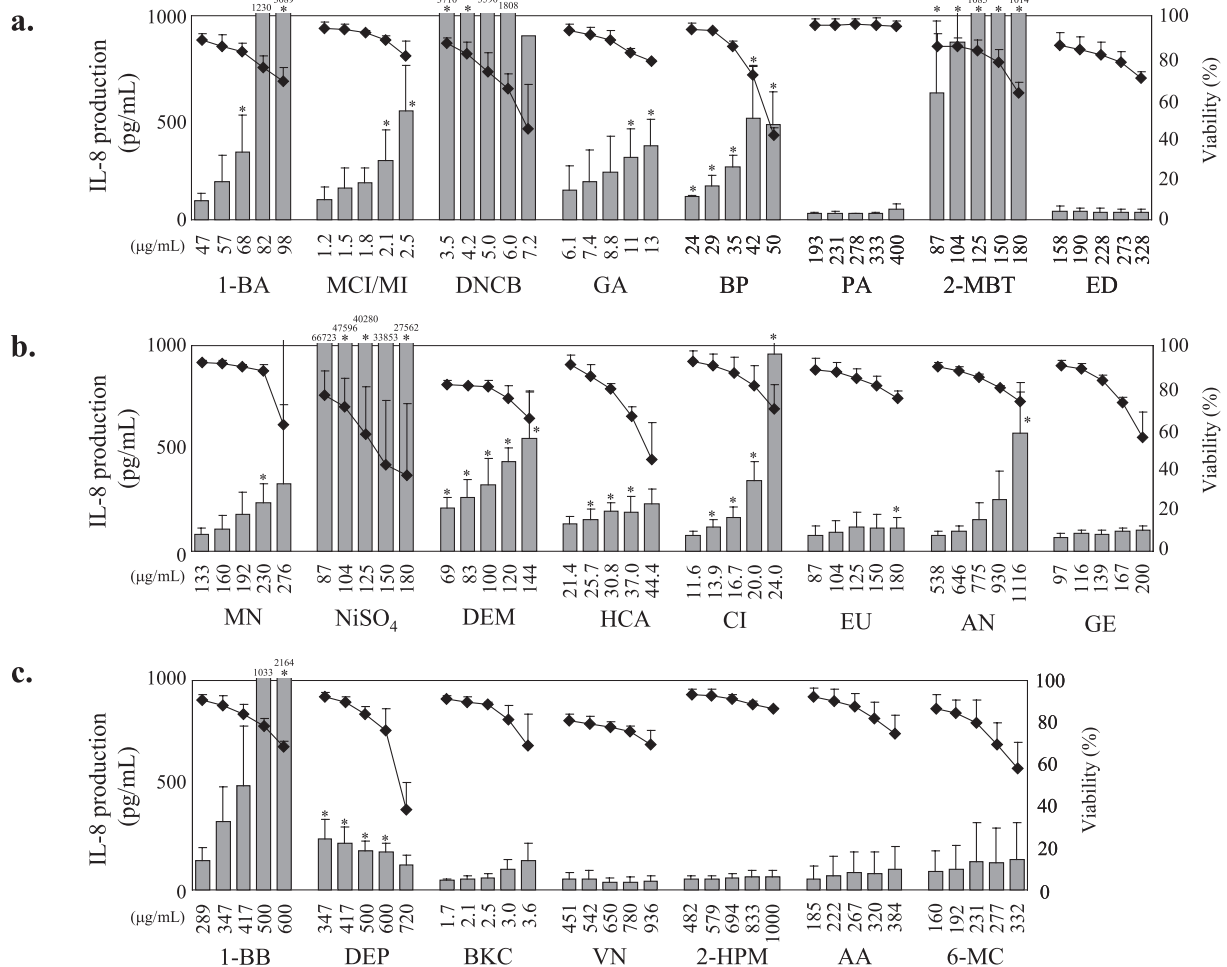


Fig. 1. The alteration of IL-8 production in THP-1 cells exposed to 16 contact allergens (a, b) and 7 non-allergens (c). Each data point represents the mean $\pm$ S.D. for three independent experiments. Bars indicate extent of IL-8 production and squares provide cell viability. Non-treated cells served as negative control for water-soluble test chemicals and DMSO-treated cells were negative controls for water-insoluble test chemicals. Values for these groups were also measured at each experiment. (cf. Non-treated cells; 47.1 ± 21.3 pg/ml, DMSO-treated cells; 40.8 ± 22.0 pg/ml, $n=18$). Statistical significance was calculated by Student's t-test (* $P<0.05$, ** $P<0.01$).

and p38 MAPK pathways, and that NiSO₄ induced the IL-8 production through the ERK pathway but not the p38 MAPK pathway.

Involvement of TNF- α in THP-1 cell IL-8 production

To demonstrate whether TNF- α regulates IL-8 production in THP-1 cells, cells were treated with various concentrations of rhTNF- α for 24 hr and the extent of IL-8 production was determined. rhTNF- α clearly induced augmentation of IL-8 production in a dose-dependent manner (Fig. 3a). In contrast, cells treated with various concentrations of rhIL-8 did not induce the augmentation of TNF- α

production at all (Fig. 3b). We also observed that rhTNF- α and rhIL-8 treatments had little influence on cell viability; cell viability range was 96 to 97%. These results showed that TNF- α is one of the upstream regulators of IL-8 production in THP-1 cells.

We next investigated whether TNF- α produced by THP-1 cells following exposure to contact allergens regulates IL-8 production. We examined the neutralization effect of TNF- α bioactivity using specific antibodies for TNF- α on IL-8 production following exposure to 5 μ g/ml DNCB and 170 μ g/ml NiSO₄. As before, we found that the additional presence of inhibitors had little influence on cell viability (data not shown). As shown in Fig. 3c,

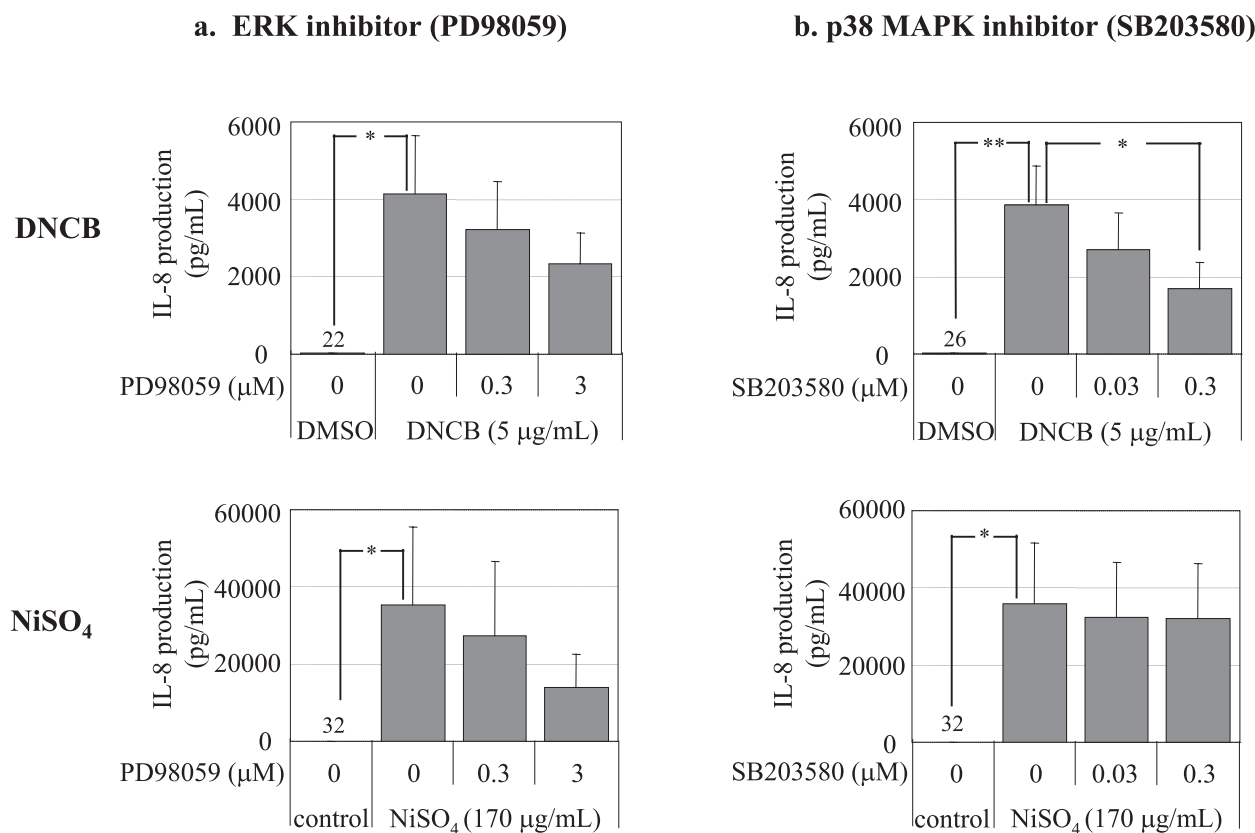


Fig. 2. The involvement of MAPK pathway in IL-8 production was demonstrated when THP-1 cells were treated with MAPK-specific inhibitors prior to exposing cells to DNCB or NiSO₄. Cells were cultured with inhibitors for ERK (PD98059) or p38 MAPK (SB203580) pathway for 1 hr following exposure to DNCB (5 μg/ml) or NiSO₄ (170 μg/ml) for 24 hr. After exposure to test chemicals, culture supernatants were collected and extent of IL-8 production was measured using an ELISA kit. Mean $\pm$ S.D. was given for three independent experiments. Statistical significance was calculated by Student's t-test (* P <0.05, ** P <0.01). Fig. 2a provides the extent of IL-8 production when PD98059 is present prior to exposing cells with DNCB (upper panel) or NiSO₄ (lower panel). Data for SB203580 is provided in Fig. 2b when cells are exposed to DNCB (upper panel) or NiSO₄ (lower panel).

inhibition of TNF- α suppressed IL-8 production induced by both DNCB and NiSO₄. These data indicate that the marked production of IL-8 induced by DNCB and NiSO₄ can be partly dependent on TNF- α autocrine effect.

DISCUSSION

At the beginning of contact hypersensitivity, a cascade of morphological and functional changes will occur in DCs and LCs following recognition of the contact allergen. Some of the changes include augmentation of cell surface marker expression (e.g. CD86, CD54, and CD40) and cytokine production (e.g. TNF- α , IL-8, IL-1 β and IL-6), which have been found to occur in DCs and LCs both *in vitro* as well as *in vivo* (Aiba *et al.*, 1997, 2003; Coutant *et al.*, 1999; De Smedt *et al.*, 2001, 2002;

Staquet *et al.*, 2004). Moreover, some of the intracellular mechanisms for these alterations have also been analyzed (Arrighi *et al.*, 2001; Aiba *et al.*, 2003; Boislève *et al.*, 2004). Recently, Toebak *et al.* (2006) reported that representative contact allergens could specifically induce IL-8 production while irritant chemicals did not induce IL-8 production. Python *et al.* (2007) also found that the augmentation of IL-8 mRNA expression was predominantly induced in U937 cells following exposure to contact allergens. Likewise, we reported that representative contact allergens, for example DNCB and NiSO₄, have the high capacity to induce IL-8 production in THP-1 cells (Miyazawa *et al.*, 2007a). However, the mechanism for augmentation of IL-8 production in cells following exposure to contact allergens is not well understood.

In this study, we have attempted to further clarify the

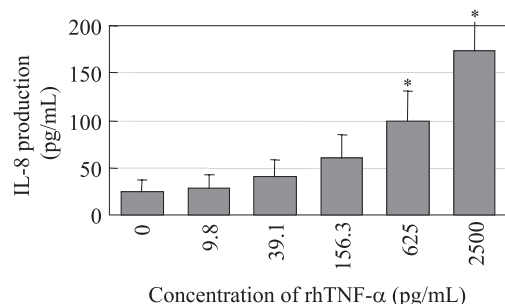
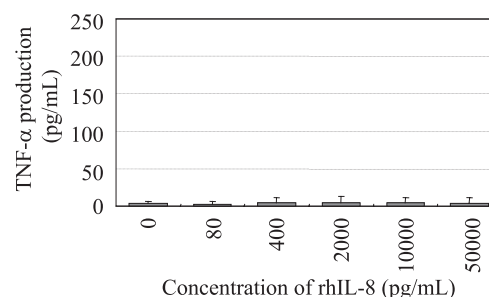
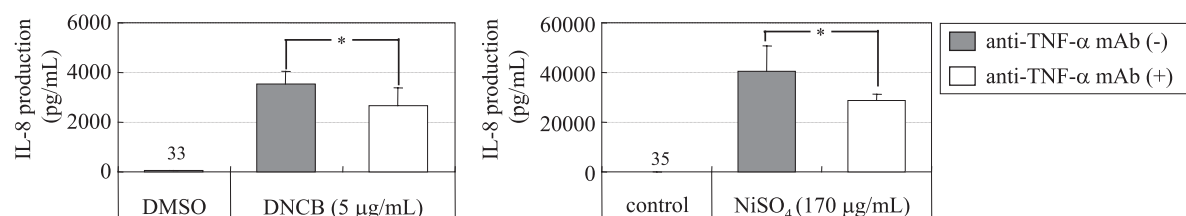
IL-8 production by contact allergen via MAPK and TNF- α **a. IL-8 production in presence of varying rhTNF- α concentrations****b. TNF- α production in presence of varying rhIL-8 concentrations****c. Neutralization of TNF- α activity using specific antibody**

Fig. 3. The effect of either TNF- α or IL-8 on the production of the other cytokine/chemokine is presented. In Fig. 3a, cells were cultured with various concentrations of rhTNF- α for 24 hr. The culture supernatants were collected and extent of IL-8 production was determined. In Fig. 3b, cells were cultured with various concentrations of rhIL-8 for 24 hr. The culture supernatants were collected and extent of TNF- α production was determined. In Fig. 3c, cells were cultured with 0.625 μ g/ml anti-TNF- α Abs for 1 hr followed by exposure to DNCB (5 μ g/ml) or NiSO₄ (170 μ g/ml) for 24 hr. After exposure to test chemicals, culture supernatants were collected and extent of IL-8 production was determined. Mean $\pm$ S.D. was given for three independent experiments for Figs. 3a and b and for six independent experiments for Fig. 3c. Statistical significance was calculated by Student's t-test (* P <0.05).

mechanism for IL-8 production *in vitro* following allergen stimulation. We first examined the changes in IL-8 production when THP-1 cells are treated with a total of 23 chemicals in order to confirm allergen specificity on IL-8 production. After which, we demonstrated the induction mechanism for IL-8 production.

From our results, we found that 16 contact allergens, except PA, ED, and GE, induced statistically significant augmentation of IL-8 production. Moreover, 11 out of 13 chemicals (1-BA, MCI/MI, GA, BP, 2-MBT, MN, DEM, HCA, CI, EU, and AN) induced the augmentation of IL-8 production in a dose-dependent manner. For DNCB and NiSO₄, it was reported that DNCB and NiSO₄ induced statistically significant augmentation of IL-8 production on THP-1 cells at the lower concentrations than those tested in this study (Miyazawa *et al.*, 2007a). We also found that THP-1 cells produced over a 10-fold increase in stimulation with 10 contact allergens (1-BA, MCI/MI, DNCB, GA, BP, 2-MBT, NiSO₄, DEM, CI, and AN) as compared to medium or DMSO control. NiSO₄ espe-

cially had a strong induction in IL-8 production. These results suggested that the augmentation of IL-8 production is a unique phenomenon of contact allergens in DC activation. Toebak *et al.* (2006) has reported that IL-8 production in Mo-DCs was induced specifically by stimulation with representative contact allergens, for example DNCB, p-phenylenediamine, and metal salts including NiSO₄, in a dose-dependent manner, with NiSO₄ also having the highest effect in inducing IL-8 production. Meanwhile, Python *et al.* (2007) analyzed the extent of IL-8 mRNA expression following exposure to eight contact allergens, three non-allergens, and five oxidative hair dye precursors using U937 cells as surrogate DCs. The authors revealed that IL-8 mRNA expression was predominantly induced by contact allergens at the non-toxic (85-90 % of cell viability) concentration. The authors defined a scoring system based on the relative induction levels of IL-8 and exposure time point (24, 48, and 72 hr). This score was the index of whether chemicals have the potential to strongly induce and maintain IL-8 mRNA

expression. According to the author's scoring criteria, DNCB, 2-MBT, EU, and NiSO₄ got the higher scores while HCA and CI got the lower scores. We had a similar result. In our study, DNCB and 2-MBT had over a 10-fold IL-8 production increase as compared with vehicle at a non-toxic concentration. For NiSO₄, the lowest concentration had over a 1,000-fold increase in IL-8 production compared with control. But the concentration tested was a slightly toxic concentration (76±12 % of cell viability). In contrast, a non-toxic concentration of HCA and CI had only about a 2-fold increase in IL-8 production as compared with control. The results from all of these investigations suggest that IL-8 production increased in THP-1 cells, like the Mo-DCs, U937 cell line, and other surrogate DC cell lines, following stimulation with contact allergens. In addition, the data suggest that the skin sensitization potency of a chemical can be related to the extent of IL-8 production/expression.

As briefly mentioned earlier, IL-8 production following stimulation with contact allergens was observed more strongly at sub-toxic concentrations (60-90 % cell viability) than non-toxic concentrations. In our study, we found a significantly increased level of IL-8 production at the sub-toxic concentration. Similarly, Toebak *et al.* (2006) found that concentrations of contact allergens giving a cell viability of at least 75% caused an induction of IL-8 production in Mo-DCs. This finding is almost identical to our findings. Moreover, this relationship between cytotoxicity and IL-8 production was also observed for CD86 and CD54 expressions following treatment with contact allergens in THP-1 cells (Yoshida *et al.*, 2003; Sakaguchi *et al.*, 2006, 2007; Miyazawa *et al.*, 2007a). Thus the data suggest that the augmentation of cell surface marker expression or cytokine production involves cytotoxicity. In order to better understand the relationship between cytotoxicity (including stress response, apoptosis and necrosis) and phenotypic alterations, further detailed work is needed.

Of the 16 reported skin sensitizers tested in our study, three contact allergens (PA, ED, and GE) did not show the capacity to induce IL-8 production. For PA, no cytotoxicity was observed even at the highest soluble concentration. Cell viability of non-treated cells was 95 ± 2 % (n=18) while the cell viability of PA-treated cells was 95 ± 3 % (n=3) at the highest test dose. PA exhibited a low solubility in water and DMSO compared to other chemicals tested in this study. The lack of effect, cytotoxicity and augmentation of IL-8 production, observed for PA may be due to the limitations in solubility; THP-1 cells were not exposed to enough chemical to induce the various cellular responses. For GE, monoterpenes such as

GE have the potential to auto-oxidize upon air exposure and form highly allergenic compounds. In brief, the auto-oxidation of GE greatly influenced the sensitizing effect of GE (Hagvall *et al.*, 2007). Under the test conditions, augmentation of IL-8 production by GE may not have occurred because of a lower exposure to the oxidized form of GE. For ED, the lack of IL-8 production in THP-1 cells may be due to the lack of metabolism of ED by the THP-1 cells. ED is postulated to be oxidized and hydrolyzed to the sensitising dialdehyde, glyoxal, by the enzyme monoamine oxidase (Foussereau *et al.*, 1983). However, this theory remains to be proven. Further work is needed to test these hypotheses for the lack in IL-8 production in THP-1 cells following exposure to PA, GE, or ED.

For the reported non-contact allergens used in our study, 5 of the 7 non-allergens did not cause an increase of IL-8 production. Two, 1-BB and DEP, however, caused an augmentation of IL-8 production. Both chemicals were ranked as 'non-sensitizers' when evaluated in the LLNA (Gerberick *et al.*, 2005). But for 1-BB, we have obtained positive *in vivo* results using the guinea pig maximization method (unpublished data). In addition, the potential of 1-BB to be a skin sensitizer has been evaluated in the peptide reactivity assay (PRA), which tests the binding between chemicals and protein using specific peptides, including cysteine or lysine (Gerberick *et al.*, 2007). Gerberick *et al.* (2007) reported that 1-BB was 'positive' for skin sensitization and classified as 'low' ranking sensitizer. For DEP, *in vivo* human data reveals that DEP has the potential to be a very weak sensitizer (NIH Publication, 1999). Given the additional information for each chemical, both chemicals have the potential to be skin sensitizers, although weak, and are capable of causing an augmentation of IL-8 production in THP-1 cells. We propose that the observed augmentation of IL-8 production in THP-1 cells is not false-positive results but due to the intrinsic properties of the chemical.

A key role in the sensitization process is the activation of DCs/LCs with the characteristic phenotypic and cytokine changes. One part of that process is IL-8 augmentation. In the second phase of our study, we attempted to further understand the mechanism by which augmentation of IL-8 production occurs. Many researchers have reported that MAPK pathways, especially p38 MAPK, are activated when DCs are exposed to contact allergens. MAPK pathways, including ERK, p38 MAPK, and JNK, are well known for having important roles in the induction of the proliferation, activation, differentiation, and migration of several immune cells (Flavell, 1999; Nakahara *et al.*, 2006). These pathways

IL-8 production by contact allergen via MAPK and TNF- α

regulated the phenotypic alteration and cytokine production in Mo-DCs (Arrighi *et al.*, 2001; Aiba *et al.*, 2003; Boislève *et al.*, 2004). We also evaluated the role of MAPK pathways and TNF- α on augmentation of IL-8 production following stimulation with DNCB or NiSO₄. Our results suggest that IL-8 production is induced by both contact allergens. DNCB induces both ERK and p38 MAPK pathways, while the ERK pathway is induced by NiSO₄. Aiba *et al.* (2003) reported that the DNCB induction of IL-8 production in Mo-DCs involved the p38 MAPK pathway. Aiba *et al.* (2003) also found that NiCl₂ induction of IL-8 mRNA expression was suppressed only when ERK was inhibited but not when p38 MAPK was inhibited. These are the same results we observed in our study using THP-1 cells. These results indicate that ERK and p38 MAPK pathways regulate induction of IL-8 production when these cells are exposed to contact allergens and the MAPK pathway involved will depend on the chemical stimuli.

TNF- α is well known as the cytokine that positively regulates the activation of DCs. Salgado *et al.* (1999) demonstrated that TNF- α markedly up-regulated CD40 expression, but not CD86 on highly purified LCs from murine skin. Moreover, fresh immature LCs purified from human skin expressed increased levels of co-stimulatory molecules, such as CD40 and CD54 following treatment with rhTNF- α (Berthier-Vergnes *et al.*, 2005). In our study, we found that TNF- α up-regulated IL-8 production in THP-1 cells following stimulation with DNCB and NiSO₄. We also confirmed TNF- α production following stimulation with DNCB and NiSO₄ before IL-8 production by these stimulations (Miyazawa *et al.*, 2007b). Since it has been reported that TNF- α production following exposure to contact allergens regulate MAPK signaling (Aiba *et al.*, 2003; Boislève *et al.*, 2004), IL-8 production might be regulated by TNF- α via MAPK pathways. But on the other hand, we found that IL-8 production was not completely suppressed after neutralizing TNF- α with antibodies prior to contact allergen exposure. These data suggest that there are other pathways of IL-8 production following exposure to contact allergens, except TNF- α . Boislève *et al.* (2004) has reported that the CCR7 expression induced by exposure to DNCB or NiSO₄ was not inhibited by neutralization of TNF- α effect, but was inhibited by inhibition of p38 MAPK and JNK, one of the MAPK. These findings suggest that MAPK pathways and TNF- α independently regulate the IL-8 production following exposure to chemical allergens. Further investigation on how IL-8 production is regulated downstream of MAPK is needed. Overall, these findings suggest that there are differences in induction pathways for phenotyp-

ic alteration and cytokine production following chemical stimuli.

IL-8 is the chemokine that functions as an inducer of migration to neutrophils or other immune cells at the inflammation site. *In vivo*, IL-8 is involved in the delayed-type hypersensitivity (DTH), including contact hypersensitivity (Larsen *et al.*, 1995). Larsen *et al.* (1995) analyzed the influence on pathophysiology of DTH using a tuberculin skin reaction and they revealed that IL-8 plays an important role for the development of a tuberculin skin reaction in rabbits, especially for migration of lymphocytes, as judged by histologic, biochemical and clinical examinations. On the other hand, it has been well known that keratinocytes (KCs), which account for a large share of epidermis cells, secrete IL-8 in skin sensitization. When the contact allergen enters the skin, LCs are not the only ones involved but also KCs. Given the greater presence of KCs, KCs might appear to be the source of most of the IL-8 observed at the exposure site. The role of LCs in IL-8 production *in vivo* is currently being investigated.

In conclusion, this study revealed some of the induction pathways for the augmentation of IL-8 production in THP-1 cells following exposure to DNCB and NiSO₄. Moreover, the response observed in the THP-1 cells was effectively similar to those reported for the Mo-DCs. Because of the similarities, THP-1 cells would be expected to have the same or similar functional pathways as LCs or DCs *in vivo*. Therefore, our results provide further knowledge to the understanding of the mechanism for DC activation *in vitro*. We expect that the better understanding of the mechanism of DC activation *in vitro* might lead to the clarification of the *in vivo* skin sensitization mechanism.

ACKNOWLEDGMENT

We thank Dr. Javier Avalos for his critical review of this manuscript.

REFERENCES

- Ade, N., Martinozzi-Teissier, S., Pallardy, M. and Rousset, F. (2006): Activation of U937 cells by contact sensitizers: CD86 expression is independent of Apoptosis. *J. Immunotoxicol.*, **3**, 189-197.
- Aiba, S., Terunuma, A., Manome, H. and Tagami, H. (1997): Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. *Eur. J. Immunol.*, **27**, 3031-3038.
- Aiba, S., Manome, H., Nakagawa, S., Mollah, Z.U.A., Mizuashi, M., Ohtani, T., Yoshino, Y. and Tagami, H. (2003): p38 mitogen-activated protein kinase and extracellular signal-regulated kinas-

- es play distinct roles in the activation of dendritic cells by two representative haptens, NiCl₂ and 2,4-dinitrochlorobenzene. *J. Invest. Dermatol.*, **120**, 390-399.
- Arrighi, J.F., Rebsamen, M., Rousset, F., Kindler, V. and Hauser, C. (2001): A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF- α , and contact sensitizers. *J. Immunol.*, **166**, 3837-3845.
- Ashikaga, T., Hoya, M., Itagaki, H., Katumura, Y. and Aiba, S. (2002): Evaluation of CD86 expression and MHC class II molecule internalization in THP-1 human monocyte cells as predictive endpoints for contact sensitizers. *Toxicol. In Vitro.*, **16**, 711-716.
- Azam, P., Peiffer, J.L., Chamousset, D., Tissier, M.H., Bonnet, P.A., Vian, L., Fabre, I. and Ourlin, J.C. (2006): The cytokine-dependent MUTZ-3 cell line as an *in vitro* model for the screening of contact sensitizers. *Toxicol. Appl. Pharmacol.*, **212**, 14-23.
- Berthier-Vergnes, O., Bermond, F., Flacher, V., Massacrier, C., Schmitt, D. and Péguet-Navarro, J. (2005): TNF- α enhances phenotypic and functional maturation of human epidermal Langerhans cells and induces IL-12 p40 and IP-10/CXCL-10 production. *FEBS Lett.*, **579**, 3660-3668.
- Boislève, F., Kerdine-Romer, S., Rougier-Larzat, N. and Pallardy, M. (2004): Nickel and DNCB induce CCR7 expression on human dendritic cells through different signalling pathways: role of TNF- α and MAPK. *J. Invest. Dermatol.*, **123**, 494-502.
- Coutant, K.D., de Fraissinette, A.B., Cordier, A. and Ulrich, P. (1999): Modulation of the activity of human monocyte-derived dendritic cells by chemical haptens, a metal allergen, and a staphylococcal superantigen. *Toxicol. Sci.*, **52**, 189-198.
- Cumberbatch, M., Dearman, R.J. and Kimber, I. (1996): Constitutive and inducible expression of interleukin-6 by Langerhans cells and lymph node dendritic cells. *Immunology*, **87**, 513-518.
- De Smedt, A.C.A., Van Den Heuvel, R.L., Berneman, Z.N. and Schoeters, G.E.R. (2001): Modulation of phenotype, cytokine production and stimulatory function of CD34⁺-derived DC by NiCl₂ and SDS. *Toxicol. In Vitro.*, **15**, 319-325.
- De Smedt, A.C., Van Den Heuvel, R.L., Van Tendeloo, V.F., Berneman, Z.N., Schoeters, G.E., Weber, E. and Tuschl, H. (2002): Phenotypic alterations and IL-1 beta production in CD34⁺ progenitor- and monocyte-derived dendritic cells after exposure to allergens: a comparative analysis. *Arch. Dermatol. Res.*, **294**, 109-116.
- Enk, A.H. and Katz, S.I. (1992): Early molecular events in the induction phase of contact sensitivity. *Proc. Nat. Acad. Sci. USA*, **89**, 1398-1402.
- Flavell, R.A. (1999): The molecular basis of T cell differentiation. *Immunol. Res.*, **12**, 159-168.
- Foussereau, J., Samsoen, M. and Hecht, M.T. (1983): Occupational dermatitis to Ampholyt G in hospital personnel. *Contact Dermatitis*, **9**, 233-234.
- Gerberick, G.F., House, R.V., Fletcher, E.R. and Ryan, C.A. (1992): Examination of the local lymph node assay for use in contact sensitization risk assessment. *Fundam. Appl. Toxicol.*, **19**, 438-445.
- Gerberick, G.F., Ryan, C.A., Kern, P.S., Schlatter, H., Dearman, R.J., Kimber, I., Patlewicz, G.Y. and Basketter, D.A. (2005): Compilation of Historical local lymph node data for evaluation of skin sensitization alternative methods. *Dermatitis*, **16**, 157-202.
- Gerberick, G.F., Vassallo, J.D., Foertsch, L.M., Price, B.B., Chaney, J.G. and Lepoittevin, J.P. (2007): Quantification of chemical peptide reactivity for screening contact allergens; a classification tree model approach. *Toxicol. Sci.*, **97**, 417-427.
- Griffiths, C.E.M., Barker, J.N.W.N., Kunkel, S. and Nickoloff, B.J. (1991): Modulation of leucocyte adhesion molecules, a T-cell chemotaxin (IL-8) and a regulatory cytokine (TNF- α) in allergic contact dermatitis (rhys dermatitis). *Br. J. Dermatol.*, **124**, 519-526.
- Hagvall, L., Bäcktorp, C., Svensson, S., Nyman, G., Börje, A. and Karlberg, A.T. (2007): Fragrance compound Geraniol forms contact allergens air exposure. Identification and quantification of oxidation products and effect on skin sensitization. *Chem. Res. Toxicol.*, **20**, 807-814.
- Kimber, I., Hilton, J., Dearman, R.J., Gerberick, G.F., Ryan, C.A., Basketter, D.A., Lea, L., House, R.V., Ladics, G.S., Loveless, S.E. and Hastings, K.L. (1998): Assessment of the skin sensitization potential of topical medicaments using the local lymph node assay: an interlaboratory evaluation. *J. Toxicol. Environ. Health*, **53**, 563-579.
- Larsen, C.G., Thomsen, M.K., Gesser, B., Thomsen, P.D., Deleuran, B.W., Nowak, J., Skodt, V., Thomsen, H.K., Deleuran, M., Thestrup-Pedersen, K., Harada, A., Matsumura, K. and Menne, T. (1995): The delayed-type hypersensitivity reaction is dependent on IL-8. Inhibition of a tuberculin skin reaction by an anti-IL-8 monoclonal antibody. *J. Immunol.*, **155**, 2151-2157.
- Miyazawa, M., Ito, Y., Yoshida, Y., Sakaguchi, H. and Suzuki, H. (2007a): Phenotypic alterations and cytokine production in THP-1 cells in response to allergens. *Toxicol. In Vitro.*, **21**, 428-437.
- Miyazawa, M., Ito, Y., Kosaka, N., Nukada Y., Sakaguchi, H., Suzuki, H. and Nishiyama, N. (2007b): Role of TNF- α and extracellular ATP in THP-1 cell activation following allergen exposure. *J. Toxicol. Sci.* (in press)
- Nakahara, T., Moroi, Y., Uchi, H. and Furue, M. (2006): Differential role of MAPK signaling in human dendritic cell maturation and Th1/Th2 engagement. *J. Dermatol. Sci.*, **42**, 1-11.
- NIH Publication (1999): The murine local lymph node assay: A test method for assessing the allergic contact dermatitis potential of chemicals/compounds. No. 99-4494.
- Ozawa, H., Nakagawa, S., Tagami, H. and Aiba, S. (1996): Interleukin-1 β and granulocyte macrophage colony-stimulating factor mediate Langerhans cell maturation differently. *J. Invest. Dermatol.*, **106**, 441-445.
- Python, F., Goebel, C. and Aebly, P. (2007): Assessment of the U937 cell line for the detection of contact allergens. *Toxicol. Appl. Pharmacol.*, **220**, 113-124.
- Sakaguchi, H., Ashikaga, T., Miyazawa, M., Yoshida, Y., Ito, Y., Yoneyama, K., Hirota, M., Itagaki, H., Toyoda, H. and Suzuki, H. (2006): Development of an *in vitro* skin sensitization test using human cell lines; human Cell Line Activation Test (h-CLAT) II. An inter-laboratory study of the h-CLAT. *Toxicol. In Vitro.*, **20**, 774-784.
- Sakaguchi, H., Miyazawa, M., Yoshida, Y., Ito, Y. and Suzuki, H. (2007): Prediction of preservative sensitization potential using surface marker CD86 and/or CD54 expression on human cell line, THP-1. *Arch. Dermatol. Res.*, **298**, 427-437.
- Salgado, C.G., Nakamura, K., Sugaya, M., Tada, Y., Asahina, A., Fukuda, S., Koyama, Y., Irie, S. and Tamaki, K. (1999): Differential effects of cytokines and immunosuppressive drugs on CD40, B7-1, and B7-2 expression on purified epidermal Langerhans cells. *J. Invest. Dermatol.*, **113**, 1021-1027.
- Sebastiani, S., Albanesi, C., De, P.O., Puddu, P., Cavani, A. and Girolomoni, G. (2002): The role of chemokines in allergic contact dermatitis. *Arch. Dermatol. Res.*, **293**, 552-559.

IL-8 production by contact allergen via MAPK and TNF- α

- Staquet, M.J., Sportouch, M., Jacquet, C., Schmitt, D., Guesnet, J. and Péguet-Navarro, J. (2004): Moderate skin sensitizers can induce phenotypic changes on *in vitro* generated dendritic cells. *Toxicol. In Vitro.*, **18**, 493-500.
- Toebak, M.J., Pohlmann, P.R., Sampat-Sardjoepersad, S.C., von Blomberg, B.M.E, Bruynzeel, D.P., Scheper, R.J., Rustemeyer, T. and Gibbs, S. (2006): CXCL8 Secretion by dendritic cells predicts contact allergens from irritants. *Toxicol. In Vitro.*, **20**, 117-124.
- Verrier, A.C., Schmitt, D. and Staquet, M.J. (1999): Fragrance and contact allergens *in vitro* modulate the HLA-DR and E-cadherin expression on human epidermal Langerhans cells. *Int. Arch. Allergy Immunol.*, **120**, 56-62.
- Wang, B., Amerio, P. and Sauder, D.N. (1999): Role of cytokines in epidermal Langerhans cell migration. *J. Leukoc. Biol.*, **66**, 33-39.
- Yoshida, Y., Sakaguchi, H., Ito, Y., Okuda, M. and Suzuki, H. (2003): Evaluation of the skin sensitization potential of chemicals using expression of co-stimulatory molecules, CD54 and CD86, on the naive THP-1 cell line. *Toxicol. In Vitro.*, **17**, 221-228.