
Original Article

Role of TNF- α and extracellular ATP in THP-1 cell activation following allergen exposure

Masaaki Miyazawa¹, Yuichi Ito¹, Nanae Kosaka¹, Yuko Nukada¹, Hitoshi Sakaguchi¹,
Hiroyuki Suzuki² and Naohiro Nishiyama¹

¹Kao Corporation, Safety Science Research Laboratories,
2606 Akabane Ichikai-Maich Haga-Gun Tochigi 321-3497, Japan

²Kao Corporation, Product Quality Management Division,
2-1-3 Bunka Sumida-Ku Tokyo 131-8501, Japan

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ABSTRACT — Dendritic cells (DCs), including Langerhans cells (LCs), play a critical role in the induction phase of allergic contact hypersensitivity. Following exposure to chemical allergens in the skin, LCs undergo a maturation process leading to the up-regulation of expression of co-stimulatory molecules, such as CD86, CD54 and CD40. Our previous study revealed that chemical allergens induce phenotype alterations (e.g., CD86, CD54 and CD40) and cytokine production (TNF- α and IL-8) in THP-1 cells that possibly reflect the maturation of dendritic cells during skin sensitization. However, the physiological signals for phenotypic alterations by chemical allergens are still not fully understood. Therefore, in this study, we investigated the effect of TNF- α and extracellular ATP on THP-1 cell activation induced by chemical allergens. Kinetic studies revealed that TNF- α and IL-8 release occurred in a time-dependent manner with release of two cytokines beginning at 3 hr post-exposure to well-known haptens, DNCB and NiSO₄. While recombinant human TNF- α augmented CD54 and CD40 expression in a dose-dependent manner, rhTNF- α did not increase CD86 expression. Furthermore, neutralization of TNF- α activity strongly inhibited CD54 and CD40 expression induced by allergens. On the contrary, extracellular ATP induced the up-regulation of both CD86 and CD54 expression. In the presence of the P2 receptor antagonist suramin, the up-regulation of CD86 and CD54 expression by allergens was in part suppressed. Therefore, we postulate that not only TNF- α but also extracellular ATP may contribute to cell activation following allergen stimulation, which might reflect the mechanism by which DCs respond to allergens.

Key words: Dendritic cell; THP-1; TNF- α ; ATP; Allergen

INTRODUCTION

Following primary contact with chemical allergens in the skin, DCs, including LCs present in the epidermis, become activated and migrate to regional lymph nodes where they trigger specific T cell activation and proliferation (Banchereau and Steinman, 1998). During the activation or migration, DCs undergo an allergen-induced maturation process that includes phenotypic and functional changes characterized by their increased expression of co-stimulatory molecules (e.g., CD86 and CD54) (Ozawa *et al.*, 1996) and production of several pro-inflammatory cytokines (e.g., IL-1) (Enk and Katz, 1992). Recently, sev-

eral investigations reported that DCs derived from human donor-derived peripheral blood CD14⁺ monocytes (Mo-DCs) (Sallusto and Lanzavecchio, 1994) and CD34⁺ hematopoietic progenitor cells (CD34-DCs) (Caux *et al.*, 1992) following exposure to chemical allergens had an up-regulation of co-stimulatory molecules and cytokine production (Aiba *et al.*, 1997, 2003; Coutant *et al.*, 1999; De Smedt *et al.*, 2002). These features of DCs provide a promising tool for identifying the sensitizing potential of chemicals *in vitro*. However, the investigators for these experiments had technical hurdles while trying to obtain the DCs (e.g., low levels of progenitor cells from the source, heterogeneity, and donor-to-donor variability expression)

(Coutant *et al.*, 1999; Tuschl *et al.*, 2000). To overcome these issues, the human myeloid cell lines, THP-1, U937 and MUTZ-3, have been evaluated as surrogate DC (Ashikaga *et al.*, 2002; Ade *et al.*, 2006; Azem *et al.*, 2006; Python *et al.*, 2007). In our previous study, we revealed that THP-1 cells can display allergen-induced changes in a similar fashion as those shown to be provoked in DCs including LCs, by measuring changes in markers reflecting DC maturation (CD86, CD54, CD40, TNF- α , and IL-8) (Yoshida *et al.*, 2003; Miyazawa *et al.*, 2007). Therefore, the sensitizing potential of chemicals could be detected by evaluating THP-1 cell activation following exposure to test chemicals. Recently, based on the above findings, we have proposed the human Cell Line Activation Test (h-CLAT) as a novel *in vitro* skin sensitization test system using THP-1 cells (Sakaguchi *et al.*, 2006). The h-CLAT correctly identified six known allergens (e.g., DNCB) and three non-allergens (e.g., SLS) tested by using CD86 and CD54 augmentation, as indicators of sensitization potential (Sakaguchi *et al.*, 2006).

However, questions still remain about pathways leading to up-regulation of surface molecule expression on DCs upon allergen stimulation. Mechanistically, TNF- α , IL-8, and ATP play key roles in the regulation of the inflammatory and immune response. TNF- α is known to play important roles in the migration of DCs (Wang *et al.*, 1999). Also, Salgado *et al.* (1999) showed that TNF- α up-regulated the expression of CD40 and CD80 on murine purified LCs *in vitro*. The authors suggest that TNF- α plays a role in the regulation of DC maturation, such as increasing expression of surface molecules. IL-8 is known to be involved in the inflammation reaction. Although the role of IL-8 in the skin sensitization process is currently being investigated, Mo-DC, THP-1 and U937 cells will strongly increase production and release of IL-8 following exposure to allergens (Aiba *et al.*, 2003; Miyazawa *et al.*, 2007; Python *et al.*, 2007).

Extracellular nucleotides also play a role as crucial regulator of inflammatory and immune response. Among them, ATP is stored in the cytosol of most cells in a concentration of 5-10 mM and can be released following damage to plasma membrane or passive leakage from damaged cells (Di Virgilio *et al.*, 1996). Extracellular ATP can act as extracellular signaling molecules through the activation of plasma membrane receptors known as P2 purinergic receptors (Di Virgilio *et al.*, 2001). Two P2 receptor subfamilies have been characterized: P2X receptors, which are identified as membrane channels (Abbracchio and Burnstock, 1994), and P2Y receptors, which are coupled to G proteins (Fredholm *et al.*, 1994). Recently, DCs have been shown to express functional P2 receptors (Ferrari *et al.*, 2000) and extracellular ATP has been associated with the up-regulation of activation markers, such as CD86, CD83 and CD54, via the activation of P2 receptors on DCs (La Sala *et al.*, 2001). Recent evidence showed that THP-1 cells constitutively expressed mRNAs for some P2 receptors, especially P2X receptors, and released ATP, producing extracellular concentrations of approximately 4 nM when assayed at 1×10^6 cells in 1 mL (Into *et al.*, 2002).

Therefore, in this study, to further elucidate the mechanism of augmentation of surface molecules expression on THP-1 cells, we investigated the effects of released TNF- α , IL-8 and extracellular ATP on the up-regulation of surface molecule expression induced by allergens.

MATERIALS AND METHODS

Cell line and medium

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

Chemical treatment of THP-1 cells

Cells (1×10^6) were cultured for 24 hr in 24-well plates in 1 mL of culture medium with or without the test chemicals described below. Exposure times of 1, 3, 6, 9, 12 and 24 hr were used in the kinetic study. Test allergens were: 2,4-dinitrochlorobenzene (DNCB), nickel sulfate (NiSO₄), formaldehyde (FA), propyl gallate (PG), and eugenol (EU). The non-allergen was sodium lauryl sulfate (SLS). All test chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Test concentrations for each chemical were determined to exhibit 70-95% cell viability. The concentrations used were: 2.5 and 5 μ g/mL of DNCB; 85 and 170 μ g/mL of NiSO₄; 15 and 16.5 μ g/mL of FA; 29 and 45 μ g/mL of PG; 130 and 150 μ g/mL of EU; and 54 μ g/mL of SLS. NiSO₄, FA, and SLS were prepared in physiological saline, while DNCB, PG and EU were dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich) and added to the medium. The final concentration of DMSO in culture media was less than 0.2%. At least three independent experiments were done for each chemical tested.

In some experiments, THP-1 cells were treated for 24 hr with 9.8 to 2500 pg/mL of recombinant human TNF- α (R&D Systems, Abingdon, UK), 80 to 50000 pg/mL of recombinant human IL-8 (R&D Systems) or 30 and 250 μ M of ATP (Molecular Probes, Eugene, OR, USA), and

followed by collection of the supernatants or flow cytometric analysis. In other experiments, to examine the effects of the inhibition of TNF- α and whether the up-regulation of CD86 and CD54 expression is mediated by the activation of P2 receptors, THP-1 cells were pre-cultured for 1 hr with or without 0.625 μ g/mL of anti-TNF- α antibody (R&D Systems), or 100 or 200 μ M suramin, a competitive inhibitor of P2 receptors. Then, cells were incubated with allergens for 24 hr before collection of the supernatants or flow cytometric analysis.

Phenotypic analysis by flow cytometry

After treatment with chemicals, cells were recovered and expression of cell surface markers was analyzed by flow cytometry. Cell staining was performed using the following FITC-conjugated monoclonal antibodies (mAbs): anti-human CD54 (clone; 6.5B5) from DAKO (Glostrup, Denmark) and anti-human CD86 (clone; Fun-1), and anti-human CD40 (clone; 5C3) from BD PharMingen (San Diego, CA, USA). Isotypic controls were FITC labeled-mouse IgG1 from DAKO and FITC labeled-IgG1, κ from BD PharMingen. Using the manufacturer's recommended dilutions, cells were incubated with the above mAbs at 6 μ L/ 3×10^5 cells/50 μ L for the anti-human CD86 mAb, the anti-human CD40 mAb and FITC labeled-mouse IgG1, κ , and 3 μ L/ 3×10^5 cells/50 μ L for the anti-human CD54 mAb and FITC labeled-mouse IgG1. Cells were incubated with mAbs and appropriated isotypic controls for 30 min at 4°C after blocking Fc receptor with Globlins Cohn fraction II, III (SIGMA-Aldrich) for 10 min at 4°C. After washing and resuspending with phosphate buffered saline supplemented with 0.1% bovine serum albumin, stained cells were analyzed by FACSCalibur and CellQuest software (Beckton Dickinson, San Jose, CA, USA). Dead cells were gated out after staining with 0.625 μ g/mL propidium iodide (PI) solution and cell viability was determined. A total of 10,000 living cells were analyzed. Results of phenotypic analysis are expressed as relative fluorescent intensity (RFI), which is calculated by the following formula:

$$\text{RFI (\%)} = \frac{\text{MFI of chemical-treated cells} - \text{MFI of chemical-treated Isotype control cells}}{\text{MFI of vehicle control cells} - \text{MFI of vehicle Isotype control cells}} \times 100$$

MFI = (Geometric) Mean fluorescence intensity

Analysis of cytokine production

Culture supernatants were recovered after exposure to

various chemicals. The production of cytokines (TNF- α and IL-8) was measured with an ELISA kit (R&D Systems, Minneapolis, MN, USA) using 96-well plates according to the manufacturer's instructions.

Statistical analysis

Student's *t*-test was used to evaluate a statistical significance between control cells and chemical-treated cells in cytokine production and surface markers. *p*-values less than or equal to 0.05 were considered statistically significant.

RESULTS

Kinetics study of cytokine production and phenotypic alterations induced by DNCB, NiSO₄ and SLS

We studied time-dependent effects of DNCB (2.5 and 5 μ g/mL), NiSO₄ (85 and 170 μ g/mL) and SLS (54 μ g/mL) on TNF- α and IL-8 production and CD86, CD54 and CD40 expression on THP-1 cells. Although cell viability of non-treated cells did not decrease below 98% even after 24 hr, cells treated with 5 μ g/mL DNCB or 170 μ g/mL NiSO₄ had a decrease to about 82% after 24 hr (Table 1). The decreases in cell viability were not observed until after 12 hr. Cells treated with 2.5 μ g/mL DNCB, 85 μ g/mL NiSO₄ or 54 μ g/mL SLS had decreases to 91% or 93% in cell viability in a similar time-dependent manner; the decrease was mostly noted between 12 hr and 24 hr measurements.

Time-dependent effects on TNF- α and IL-8 production on THP-1 cells induced by DNCB, NiSO₄ and SLS are shown in Fig. 1. The higher concentrations of DNCB and NiSO₄ induced a higher level of TNF- α production with maximum production between 6 and 12 hr. Increases in TNF- α production were also observed with the other test concentrations, except for the SLS and control treatments. Both concentrations of NiSO₄ appeared to have caused a similar time-dependent pattern in IL-8 production : a steep increase in IL-8 production within the first 3 hr post-exposure, followed by a slower increase in product after 6 hr post-treatment. For DNCB, both doses caused a two-phase time-dependent increase in IL-8 production. First, a fast phase occurred for the first 6 hr, and then a slow phase (plateau) beginning at 9 hr post-treatment and ending at 12 hr. Cells treated with NiSO₄ significantly augmented TNF- α and IL-8 production starting at least 3 hr compared with relevant vehicle control cells (Table 2). The non-allergen, SLS, had no effects in TNF- α and IL-8 production at each time point.

Time-dependent effects on THP-1 cell surface marker

expression (i.e., CD86, CD54 and CD40) induced by DNCB, NiSO₄ and SLS are shown in Fig. 1. Both DNCB and NiSO₄ caused an up-regulation of CD86, CD54 and CD40 expression at later time points compared to induction of TNF- α and IL-8. Increases in each surface marker expression began between 3 and 5 hr post-treatment and

continued to the last time point measured, 24 hr. Statistically significant up-regulation was observed in CD86, CD54 and CD40 expression by DNCB and NiSO₄ following a 24 hr exposure (Table 2). Both doses of NiSO₄ statistically significantly induced the up-regulation of CD54 expression from 3 hr ($p<0.05$) to 24 hr and CD40 from at 9

Table 1. Time-dependent effects on cell viability induced by DNCB, NiSO₄ and SLS.

Chemicals	Tested concentration (μg/mL)	Mean percent viability $\pm$ S.D. (%)					
		1 hr	3 hr	6 hr	9 hr	12 hr	24 hr
non-treated		98 $\pm$ 1	98 $\pm$ 1	98 $\pm$ 1	98 $\pm$ 1	98 $\pm$ 1	98 $\pm$ 1
DNCB	2.5	98 $\pm$ 1	97 $\pm$ 1	96 $\pm$ 1	96 $\pm$ 1	95 $\pm$ 1	91 $\pm$ 1
	5	98 $\pm$ 1	96 $\pm$ 1	96 $\pm$ 1	96 $\pm$ 1	95 $\pm$ 1	83 $\pm$ 1
NiSO ₄	85	98 $\pm$ 1	97 $\pm$ 1	97 $\pm$ 1	96 $\pm$ 1	96 $\pm$ 2	91 $\pm$ 2
	170	98 $\pm$ 1	96 $\pm$ 1	96 $\pm$ 1	95 $\pm$ 2	94 $\pm$ 2	82 $\pm$ 3
SLS	54	98 $\pm$ 1	96 $\pm$ 1	97 $\pm$ 1	96 $\pm$ 1	96 $\pm$ 1	93 $\pm$ 3

Cells were treated with chemicals for 1, 3, 6, 9, 12 and 24 hr. After treatment, cell viability was measured by flow cytometry using PI staining. Mean percent viability ($\pm$ S.D.) was given for at least three independent experiments and is shown for each test concentration for each chemical.

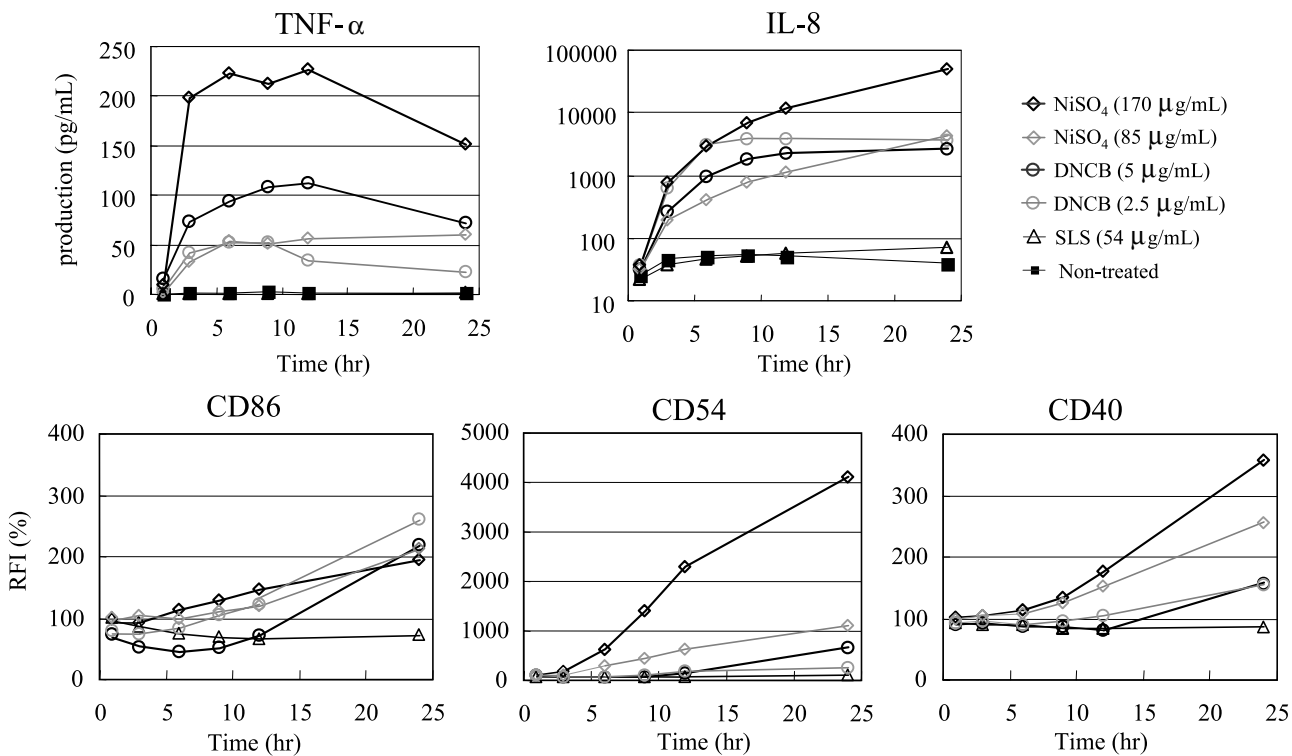


Fig. 1. Time-dependent effects on TNF- α , IL-8, CD54, CD86 and CD40 expression induced by DNCB, NiSO₄, and SLS. Cells were treated with chemicals for 1, 3, 6, 9, 12 and 24 hr. After treatment, the culture supernatant was collected to measure TNF- α and IL-8 secretion by ELISA. The expression of CD86, CD54 and CD40 was measured by flow cytometry and RFIs were calculated. The mean of six independent experiments is shown for each chemical.

Role of TNF- α and extracellular ATP.**Table 2.** Statistical analysis of surface cell marker up-regulation induced by DNCB, NiSO₄ and SLS.

Chemicals	Tested concentration ($\mu\text{g/mL}$)	p value (TNF- α , n=6)						p value (IL-8, n=6)					
		1 hr	3 hr	6 hr	9 hr	12 hr	24 hr	1 hr	3 hr	6 hr	9 hr	12 hr	24 hr
DNCB	2.5	<0.05	<0.05	<0.01	<0.01	<0.01	<0.01	ns ^a	<0.05	<0.01	<0.01	<0.01	<0.05
	5	<0.05	<0.01	<0.01	<0.01	<0.01	<0.01	ns ^a	<0.05	<0.05	<0.05	<0.01	<0.01
NiSO ₄	85	ns ^a	<0.01	<0.01	<0.01	<0.01	<0.01	ns ^a	<0.01	<0.05	<0.05	<0.05	<0.01
	170	ns ^a	<0.01	<0.01	<0.01	<0.01	<0.01	ns ^a	<0.01	<0.01	<0.05	<0.01	<0.01
SLS	54	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a

Chemicals	Tested concentration ($\mu\text{g/mL}$)	p value (CD86, n=3)						p value (CD54, n=3)						p value (CD40, n=3)					
		1 hr	3 hr	6 hr	9 hr	12 hr	24 hr	1 hr	3 hr	6 hr	9 hr	12 hr	24 hr	1 hr	3 hr	6 hr	9 hr	12 hr	24 hr
DNCB	2.5	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	<0.01	ns ^a	ns ^a	ns ^a	ns ^a	<0.01	<0.01	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	<0.01
	5	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	<0.01	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	<0.01	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	<0.01
NiSO ₄	85	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	<0.05	ns ^a	<0.05	<0.05	<0.01	<0.05	<0.01	ns ^a	ns ^a	ns ^a	<0.01	<0.05	<0.01
	170	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	<0.01	ns ^a	<0.01	<0.01	<0.01	<0.01	<0.01	ns ^a	ns ^a	ns ^a	<0.05	<0.01	<0.01
SLS	54	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a	ns ^a

p values were calculated with a Student's *t*-test in order to evaluate a statistical significance between the relevant vehicle control cells and chemical-treated cells. p values were calculated for each time.

^anot significant.

hr ($p < 0.05$) to 24 hr. On the contrary, SLS did not augment RFI and did not show a trend in augmentation.

Effect of rhTNF- α on CD86, CD54 and CD40 expression

To examine if TNF- α can trigger THP-1 cell activation, we studied the effects of recombinant human TNF- α (rhTNF- α) on CD86, CD54 and CD40 expression on THP-1 cells. Cells were treated with rhTNF- α at five concentrations for 24 hr. As shown in Fig. 2, cell viability after rhTNF- α treatment was similar to that of non-treated cells. rhTNF- α significantly up-regulated CD54 expression with a test concentration of 9.8 pg/mL and CD40 expression with a dose of 39.1 pg/mL. The dose-dependent up-regulation of CD54 expression was more profound than CD40

up-regulation. Although the dose-dependent effect by rhTNF- α was observed in CD54 and CD40 expression, rhTNF- α was superfluous for CD86 expression.

Effect of rhIL-8 on phenotypic alterations and TNF- α production

To examine if IL-8 is involved in THP-1 cell activation, we studied the effects of recombinant human IL-8 (rhIL-8) on phenotypic changes and TNF- α production in THP-1 cells. Cells were treated with rhIL-8 at five concentrations for 24 hr. As shown on Fig. 3 and Table 3, 24 hr treatment of rhIL-8 did not affect cell viability at test doses. rhIL-8 did not induce CD86 expression and TNF- α production. Similar findings were observed with CD54 expression (data not shown).

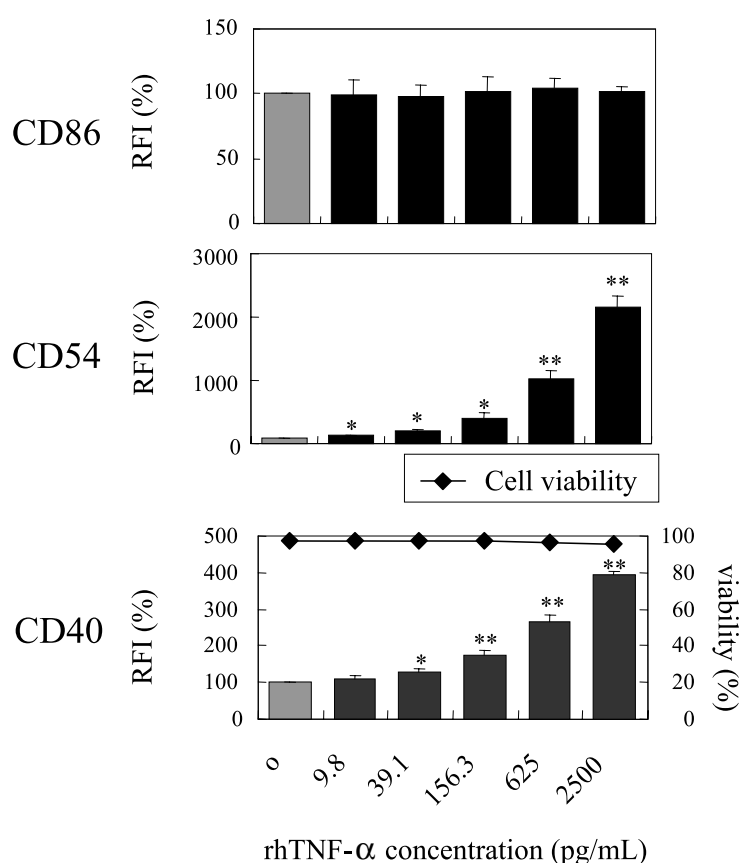


Fig. 2. Effect of rhTNF- α on CD86, CD54 and CD40 expression. Cells were treated with rhTNF- α alone with five concentrations for 24 hr. The line graph represents cell viability. Mean $\pm$ S.D. of CD86, CD54 and CD40 expression, as indicated by percent RFI increase, for three independent experiments is shown for each concentration of rTNF- α tested.

Neutralization effect of TNF- α bioactivity induced by chemical allergens

To investigate the role of TNF- α released by THP-1 cells, we examined the neutralization effect of TNF- α bioactivity on CD54 and CD40 expression induced by DNCB and NiSO₄. Cells were pre-cultured with or without anti-TNF- α antibody (0.625 μ g/mL) 1 hr before treatment with DNCB (2.5 and 5 μ g/mL) or NiSO₄ (85 and 170 μ g/mL). To confirm the neutralization effect, we quantified TNF- α production by ELISA. As shown in Fig. 4, TNF- α production induced by DNCB or NiSO₄ was not completely but rather strongly abrogated due to the addition of anti-TNF- α . A statistically significant inhibition of TNF- α was observed in the 5 μ g/mL DNCB ($p < 0.01$), and 85 ($p < 0.05$) and 170 μ g/mL ($p < 0.01$) NiSO₄ treatment groups. Furthermore, the neutralization of TNF- α significantly inhibited

the up-regulation of CD54 expression induced by DNCB at two doses ($p < 0.01$). The up-regulation of CD54 expression by NiSO₄ was also significantly inhibited at 85 ($p < 0.01$) and 170 μ g/mL ($p < 0.05$). In addition to inhibition of CD54 expression, significant inhibition of CD40 expression was observed in the 5 μ g/mL DNCB-treated group ($p < 0.01$), and the 85 ($p < 0.05$) and 170 μ g/mL ($p < 0.01$) NiSO₄ -treated groups. To further confirm the specific effect of TNF- α , we examined TNF- α production, the changes of CD54 expression, and the neutralization effect of TNF- α following treatment with other chemical allergens (FA, PG and EU) (Fig. 5). Although TNF- α production was significantly up-regulated in the 29 μ g/mL ($p < 0.05$) and 45 μ g/mL ($p < 0.01$) PG treated groups and the 16.5 μ g/mL ($p < 0.05$) FA treated group when compared with the relevant vehicle, no effect was observed in TNF- α production in the EU treated groups. On the other hand, a statistically significance in up-regulation of CD54 expression ($p < 0.05$) was observed in EU, PG and FA treated cells. The neutralization of TNF- α strongly inhibited TNF- α production and CD54 expression induced by FA and PG. Since the amount of TNF- α production by EU was similar to the level of the vehicle, anti-TNF- α antibody did not affect CD54 expression induced by EU.

Table 3. Effect of rhIL-8 on TNF- α production.

rhIL-8 concentration	TNF- α production (pg/mL)
0	0.8
80	0.7
400	0.9
2000	1.0
10000	1.0
50000	0.9

Cells were treated with rhIL-8 with five concentrations for 24 hr. After treatment, the culture supernatant was measured for TNF- α secretion by ELISA.

Effect of extracellular ATP on CD86 and CD54 expression on THP-1 cells

THP-1 cells were cultured for 24 hr with ATP in order to study effect of extracellular ATP on CD86 and CD54

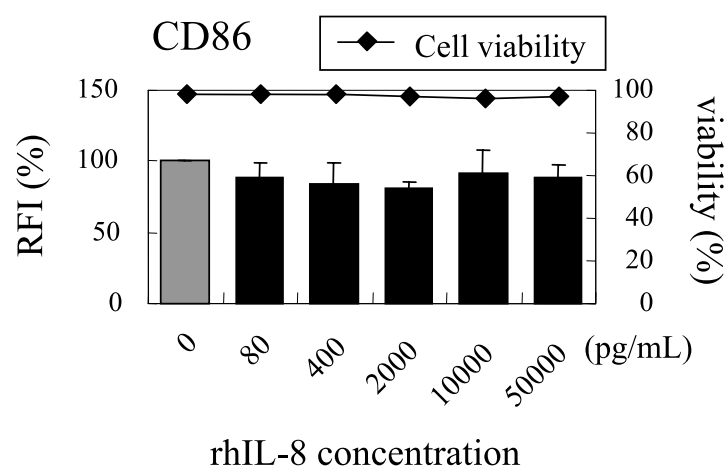


Fig. 3. Effect of rhIL-8 on CD86 expression.

Cells were treated with rhIL-8 at five concentrations for 24 hr. The line graph represents cell viability. Mean $\pm$ S.D. of three independent experiments is shown for each concentration of rhIL-8.

expression. ATP was treated at low concentrations (30 and 250 μ M), which did not affect cell viability (data not shown). Fig. 6 showed representative fluorescence histograms for non-treated or cells treated with ATP (30 and 250 μ M). The external addition of ATP enhanced the expression of CD86 and CD54 in a dose-dependent manner.

Effect of suramin on THP-1 cell CD86 and CD54 expression induced by DNCB, NiSO₄ and EU.

To examine whether CD86 and CD54 expression induced by allergens is mediated by ATP, THP-1 cells were pre-cultured with or without suramin. The P2 receptor antagonist, suramin, was added to cell cultures at 100 and 200 μ M 1 hr before treatment with DNCB (2.5 and 5 μ g/mL), NiSO₄ (85 and 170 μ g/mL), and EU (130 and 150 μ g/mL). In the presence of suramin, CD86 and CD54 expression induced by NiSO₄ were statistically significantly inhibited (Fig. 7). Although a statistically significant inhibition of CD86 expression was not obtained with

suramin, a trend in the inhibition of CD86 expression was observed in the two DNCB treated groups. A similar trend in inhibition of CD86 expression was noted for the EU treated groups. CD86 expression was almost completely inhibited by suramin, but no effect was observed on CD54 expression.

DISCUSSION

Recent investigations from our and other laboratories have demonstrated that THP-1 cells have a high capacity to undergo changes reflective of the DC maturation process (i.e., expression or induction of CD86, CD54, MHC class II, CD40, CD83, TNF- α , and IL-8) (Ashikaga *et al.*, 2002; Yoshida *et al.*, 2003; Miyazawa *et al.*, 2007). Furthermore, these cells distinguished between allergens and non-allergens in the same manner as those reported for DCs (e.g., Mo-DCs, CD34-DCs) (Ashikaga *et al.*, 2006; Sakaguchi *et al.*, 2006, 2007).

In this study, we examined a series of well-character-

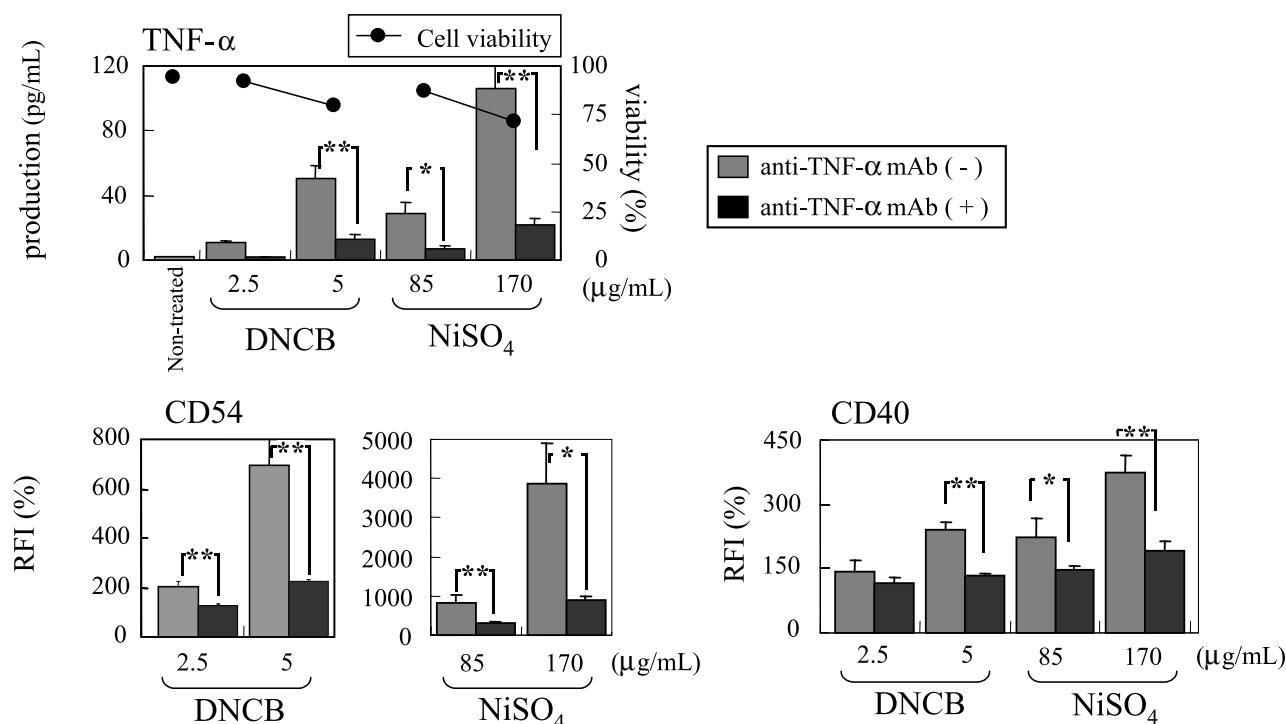


Fig. 4. Neutralization effect of TNF- α bioactivity in THP-1 cells induced with DNCB and NiSO₄. Cells were pre-treated with and without anti-TNF- α antibody for 1 hr and then treated with DNCB and NiSO₄ for 24 hr. After culture, cells were analyzed for TNF- α by ELISA and for CD54 and CD40 expression by flow cytometry. The line graphs represent cell viability. Mean $\pm$ S.D. of TNF- α production and CD54 and CD40 expression for three independent experiments is shown for each chemical. Statistical significance was calculated with a Student's *t*-test (** p <0.01; * p <0.05).

Role of TNF- α and extracellular ATP.

ized contact allergens including DNCB, NiSO₄, FA, PG and EU (Ashby *et al.*, 1995; Gerberick *et al.*, 2004). Each of these chemicals is known to be positive in animal skin sensitization tests as well as to cause allergic contact hypersensitivity in human (Fisher, 1986). Kinetic studies using THP-1 cells demonstrated that a statistically significant TNF- α and IL-8 release occurs 3 hr after DNCB and NiSO₄ stimulation, and is followed with the augmentation of CD54, CD86 and CD40 expression.

TNF- α is known to have an essential role in DC migration. TNF- α can be largely produced from keratinocytes (KC) in the skin during the skin inflammation process. Freshly prepared LCs also express the mRNA for TNF- α . In the murine system, the migration of epidermal LCs induced by allergens was inhibited by neutralizing antibody against TNF- α (Cumberbatch and Kimber, 1995). Moreover, fresh immature LCs purified from human skin expressed up-regulated levels of co-stimulatory molecules, such as CD40 and CD54 following treatment with rhTNF- α (Berthier-Vergnes *et al.*, 2005). This suggests that TNF- α modulates the activation status of human LCs.

In this study, we studied the effects of TNF- α upon allergen-induced THP-1 cell activation. Treatment with

rhTNF- α augmented CD54 and CD40 expression in a dose-dependent manner, but did not affect CD86 expression. This result suggests that TNF- α , released by allergens, might be involved in the up-regulation of CD54 and CD40 expression on THP-1 cells. Since TNF- α can be largely produced from KC or freshly prepared LCs, it would be necessary to obtain highly purified LCs to examine the precise effect of TNF- α on LC maturation, excluding the participation of KC-derived cytokines. Salgado *et al.* (1999) demonstrated that TNF- α markedly up-regulated CD40 expression, but not CD86 on highly purified LCs from murine skin. Thus, the response to TNF- α on phenotypic alterations in THP-1 cells is similar to that of DCs.

Furthermore, following pre-incubation with anti-TNF- α antibody, neutralization of TNF- α activity strongly inhibited CD54 and CD40 expression on THP-1 cells induced by allergens. These results revealed that the autocrine effect of TNF- α plays a crucial role in up-regulation of CD54 and CD40 expression on THP-1 cells following exposure to allergens. Boislève *et al.* (2004) have recently reported that CCR7 expression, which is related to DC maturation, was increased by the autocrine loop of TNF- α

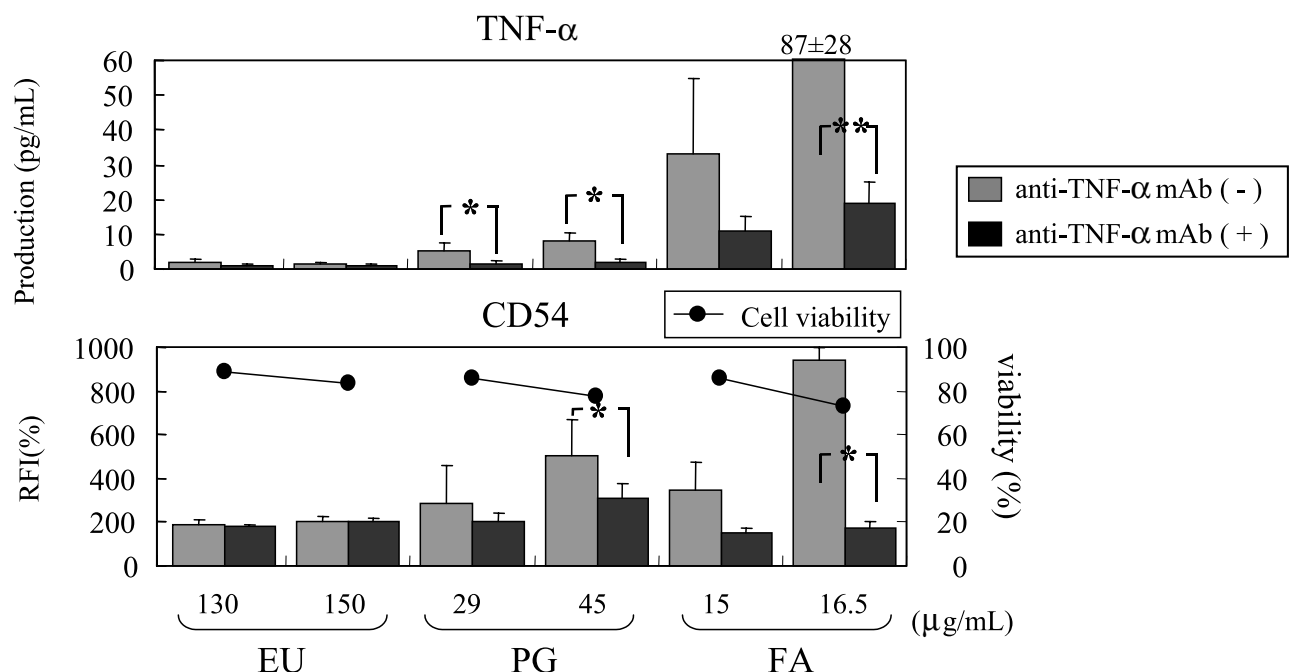


Fig. 5. Neutralization effect of TNF- α bioactivity in THP-1 cells induced by EU, PG and FA. Cells were pre-treated with and without anti-TNF- α antibody for 1 hr and then treated with EU, PG and FA for 24 hr. The line graphs represent cell viability. Mean $\pm$ S.D. of TNF- α production and CD54 expression for three independent experiments is shown for each chemical. Statistical significance was calculated with a Student's *t*-test (** p <0.01; * p <0.05).

following treatment with DNCB. The above data indicate that phenotypic alterations caused by allergens through the release of TNF- α occur not only in THP-1 cells but also DCs.

Next, we observed that the extremely marked expression of CD54 induced by DNCB and NiSO₄ was not completely but strongly dependent on secreted TNF- α . Also, the amount of TNF- α production for the allergen EU was very low and similar to that of the vehicle control. Thirdly, anti-TNF- α antibody did not affect CD54 expression. This suggests that there might be mechanisms irrelevant to TNF- α to regulate CD54 expression, as shown with EU. Recently, Boislève *et al.* (2004) has shown that CCR7 expression on CD34-DCs induced by NiSO₄ was not dependent on TNF- α release but directly mediated through p38 mitogen-activated protein kinases (MAPK) and c-jun N-terminal kinase (JNK) activation. Therefore, the intracellular signaling pathway directly leading to up-regulation

of CD54 expression might be present in THP-1 cells. Further work is needed to address mechanisms of DC maturation.

In addition, we studied whether IL-8 was involved in cell activation since a large amount of IL-8 was produced after a short allergen stimulation period. Although rhIL-8 was cultured with cells at higher concentrations compared with the amount of IL-8 released after exposure to tested allergens (e.g., DNCB or NiSO₄), no effect was observed in the expression of any marker analyzed. Therefore, we suggest that IL-8 might be produced as a result of cell activation induced by allergens, but causes no effect on surface marker expression.

Recently, some investigations demonstrated that human DCs express mRNA for several P2X and P2Y receptors and up-regulate costimulatory molecules, such as CD86, CD80, CD54 and CD83 when incubated with extracellular ATP (Berchtold *et al.*, 1999; Schnurr *et al.*, 2000). These

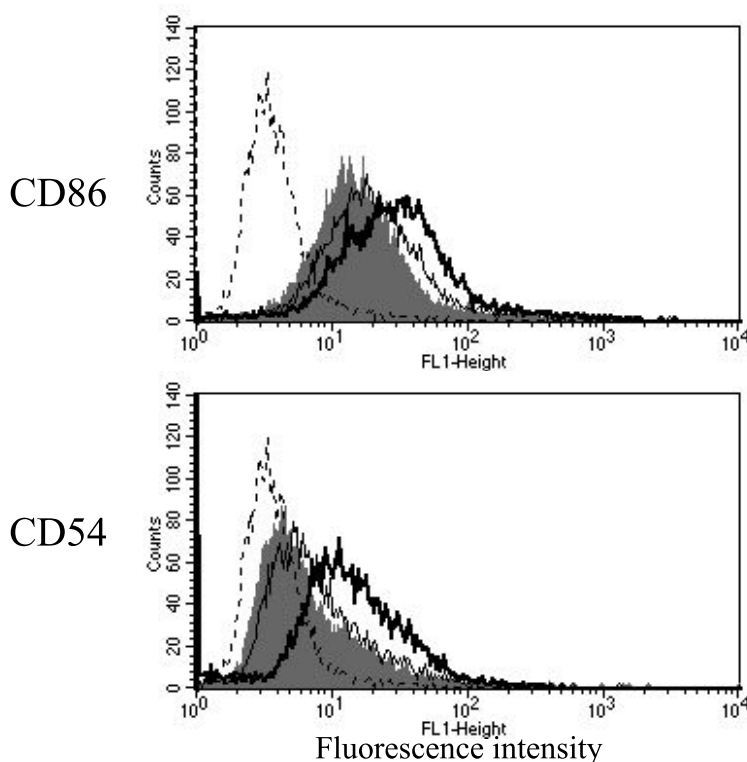


Fig. 6. Effect of extracellular ATP on CD86 and CD54 expression. Cells were treated with ATP for 24 hr. CD86 and CD54 expression was analyzed with flow cytometry. The histograms are for isotype control (dotted line), non-treated (shaded peak), 30 μ M of ATP-treated (thick solid line), or 250 μ M of ATP-treated (dark solid line). These are representative data of three independent experiments.

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data suggest that ATP acts as a costimulatory factor by activating DC. In this study, we found that THP-1 cells exposed to extracellular ATP enhanced CD86 and CD54 expression. Furthermore, the data suggest that the up-regulation of CD86 and CD54 expression on THP-1 cells induced by allergens might be mediated by P2 receptors, since the augmentation was partially inhibited by suramin, a competitive antagonist of P2 receptors. Recent evidence also showed that THP-1 cells constitutively released intracellular ATP (Into *et al.*, 2002). ATP is stored in the cytosol of most cells in a concentration of 5-10 mM and release of ATP has been observed by plasma membrane damage or passive leakage from damaged cells (Di Virgilio *et al.*, 1996). These results suggest that extracellular ATP might play important roles in cell activation induced by allergens.

Earlier, we reported that a sub-toxic concentration was needed for up-regulation of surface marker expression and cytokine production following 24 hr exposure to allergens (Yoshida *et al.*, 2003; Sakaguchi *et al.*, 2007; Miyazawa *et al.*, 2007). Hulet *et al.* (2005) reported that up-regulation of CD86 expression in Mo-DCs was observed when cells were treated with allergens at concentrations which induced 10-15% cytotoxicity at 48 hr. These results indicated a close association between phenotypic alterations or cytokine production, and cell viability. In the present kinetic study, the significant up-regulation of all CD86, CD54 and CD40 expression with both doses of DNCB and NiSO₄ was observed only at 24 hr, when some cytotoxicity was induced. However, a significant increase in TNF- α and IL-8 production was induced by non-toxic concentrations of DNCB and NiSO₄ at 3 hr post treatment and con-

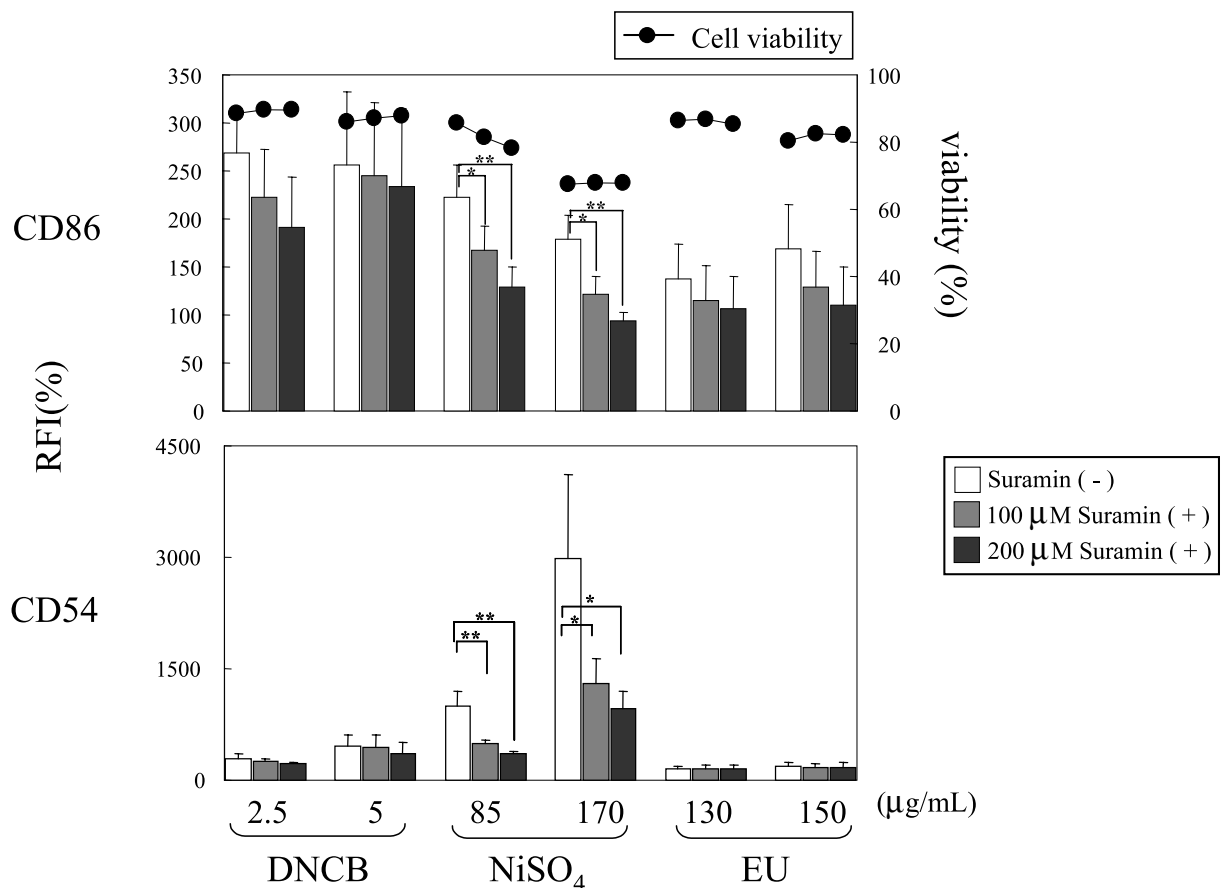


Fig. 7. Effect of suramin on CD86 and CD54 expression induced by DNCB, NiSO₄ and EU.

Cells were pre-cultured with or without P2 receptor antagonist suramin 1 hr before treatment with DNCB, NiSO₄ and EU. The line graphs represent cell viability. Mean $\pm$ S.D. of three independent experiments is shown for each chemical. Statistical significance was calculated with a Student's *t*-test (**p<0.01; *p<0.05).

tinuing 12 hr post treatment. In contrast, SLS did not have any effect on up-regulation of TNF- α and IL-8 production at each time point. Therefore, the kinetic data shows that cytokine production might not be necessarily associated with cell viability. That is, cytokine production and cytotoxicity following 24 hr exposure to allergens might occur as independent cellular events. Recently, Ade *et al.* (2006) have also demonstrated that in U937, myeloid cell lines, apoptosis is specifically related to allergens exposure and there is no possible interdependence between CD86 expression and apoptosis induced by allergens. Since the mode of death induced by chemicals is different among chemicals, more detailed understanding between cell activation, such as cytokine production and phenotypic alterations, and cytotoxicity might be needed.

In conclusion, this work indicates that, on THP-1 cells, the up-regulation of CD54 and CD40 expression induced by chemical allergens might be regulated through the auto-crine effect of released TNF- α . Furthermore, extracellular ATP plays a role for the up-regulation of CD86 and CD54 expression on THP-1 cells. This suggests that THP-1 cell activation induced by allergens might be regulated through the mechanism similar to that of DC maturation. Therefore, THP-1 would be a potentially useful model to elucidate DC maturation in the induction phase of contact hypersensitivity.

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