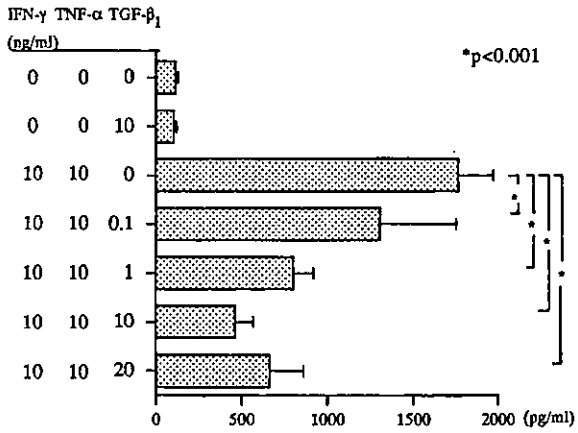


Fig. 1. TGF-$\beta_1$ inhibits TARC secretion by HaCaT cells after stimulation with IFN-γ and TNF-α. The concentration of TARC produced by HaCaT cells was measured after stimulation with TGF-$\beta_1$ (10 ng/ml) alone or stimulation with IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) with or without TGF-$\beta_1$ (0–20 ng/ml) for 24 h. When the concentration of TGF-$\beta_1$ added to the medium was increased, TARC production decreased. The maximum inhibition achieved at a concentration of 10 ng/ml of TGF-$\beta_1$. The data are shown from four different experiments. The bars indicate the standard deviation.

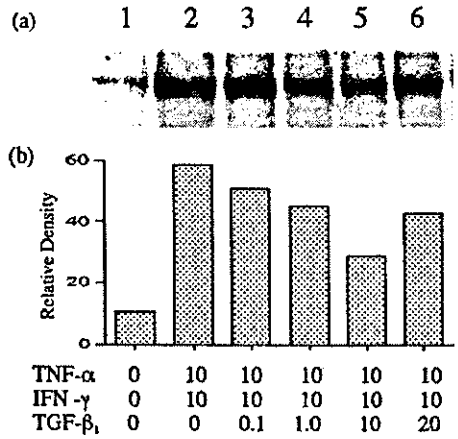


Fig. 2. Western blot analysis of TARC produced by HaCaT cells. (a) The panel shows TARC protein of HaCaT cells: for lane 1, un-stimulated; lane 2, stimulated with both IFN-γ (10 ng/ml) and TNF-α (10 ng/ml); lanes 3–6, stimulated with IFN-γ, TNF-α and TGF-$\beta_1$ (0.1 ng/ml (lane 3), 1.0 ng/ml (lane 4), 10 ng/ml (lane 5), 20 ng/ml (lane 6)) for 24 h. A representative data is shown from three different experiments. (b) The relative density of the blots was calculated as follows: the density of the blots—the density of background of the same lane. The maximum inhibition was achieved at a concentration of 10 ng/ml of TGF-$\beta_1$. These data are based on three different experiments.

production by TGF-$\beta_1$ increased gradually with increasing concentration up to a maximum at 10 ng/ml, which was 73.8% inhibition compared with control. TGF-$\beta_1$ at 20 ng/ml was less effective than at 10 ng/ml. Spearman's analysis also clarified the statistical correlations between down-regulation of TARC levels and TGF-$\beta_1$ concentrations ($r = -0.87$, $p < 0.00$). To confirm the ELISA results, Western blot analyses were carried out and the results are shown in Fig. 2. The TGF-$\beta_1$ modulation of TARC synthesis by HaCaT cell was obvious after densitometer analysis using NIH Image, similar to the results form ELISA. The maximum inhibition of TARC production by TGF-$\beta_1$ was 50.7% at a concentration of 10 ng/ml. This means that the increased concentration of TARC in the culture supernatant was due to newly synthesized TARC by HaCaT cells and that TGF-$\beta_1$ inhibited the synthesis of TARC by HaCaT cells co-stimulated with IFN-γ and TNF-α.

To clarify whether TARC mRNA expression was also modulated by TGF-$\beta_1$, RNA was extracted from HaCaT cells and Northern blot analysis was performed. The results (Fig. 3) demonstrated that HaCaT cells expressed TARC mRNA spontaneously at a low level, and that TARC mRNA greatly increased after the co-stimulation with IFN-γ and TNF-α. Furthermore, when TGF-$\beta_1$ was added to the medium with IFN-γ and TNF-α, the expression of TARC mRNA in HaCaT cells was inhibited by TGF-$\beta_1$. The maximum inhibitory effects of TGF-$\beta_1$ on TARC mRNA expression were also detected at 10 ng/ml, same as the results of Western blot analysis.

To further elucidate that the down-regulation of TARC production from HaCaT cells by TGF-$\beta_1$, anti-TGF-$\beta_1$ Ab was added to the culture medium with IFN-γ (10 ng/ml), TNF-α (10 ng/ml) and TGF-$\beta_1$ (10 ng/ml). As shown in Fig. 4, additional anti- TGF-$\beta_1$ mAb greatly reversed the inhibitory effects of TGF-$\beta_1$ on TARC secretion by HaCaT cells. This data indicate the direct effect of TGF-$\beta_1$ on TARC secretion by HaCaT cells co-stimulated with and IFN-γ and TNF-α.

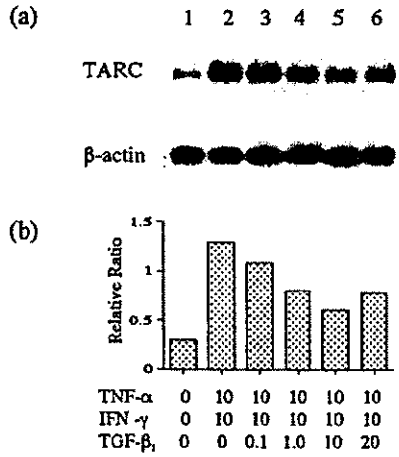


Fig. 3. TGF-$\beta_1$ inhibits TARC mRNA expression by HaCaT cells after the co-stimulation with IFN-γ and TNF-α. (a) The panel shows Northern blot of TARC mRNA (upper) and β-actin mRNA (lower) of HaCaT cells. Lane 1, un-stimulated; lane 2, stimulated with both IFN-γ and TNF-α; lanes 3–6, stimulated with IFN-γ, TNF-α and various concentrations of TGF-$\beta_1$ (0.1 ng/ml (lane 3), 1.0 ng/ml (lane 4), 10 ng/ml (lane 5) and 20 ng/ml (lane 6)) for 24 h. The maximum inhibitory effect of TGF-$\beta_1$ on TARC mRNA expression was obtained at 10 ng/ml. A representative data are shown from three different experiments. (b) The relative density of TARC mRNA to β-actin mRNA ratio was analyzed using densitometer (NIH image).

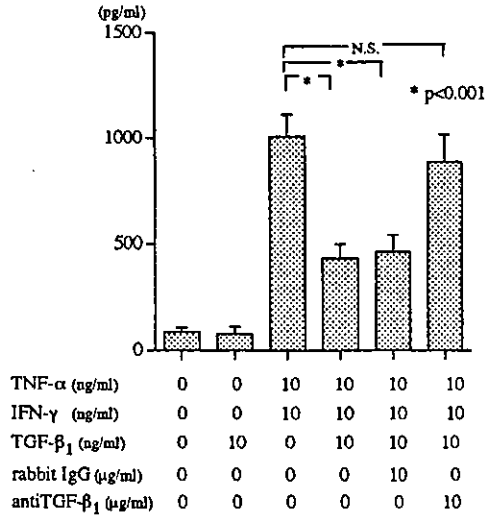


Fig. 4. Anti-TGF-$\beta_1$ mAb restores the inhibitory effect of TGF-$\beta_1$ on TARC secretion of HaCaT cells co-stimulated with IFN-γ and TNF-α. The first column shows TARC production by unstimulated HaCaT cells. The second column shows that stimulated with TGF-$\beta_1$ (10 ng/ml) alone for 24 h. The third column shows that co-stimulated by both IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) for 24 h. The fourth column shows that co-stimulated by IFN-γ, TNF-α and TGF-$\beta_1$ (10 ng/ml) for 24 h. The fifth column shows that co-stimulated with IFN-γ, TNF-α, TGF-$\beta_1$ (10 ng/ml) and rabbit IgG (10 μg/ml) for 24 h. The sixth column shows that co-stimulated with IFN-γ, TNF-α and TGF-$\beta_1$ (10 ng/ml) and anti-TGF-$\beta_1$ mAb (10 μg/ml) for 24 h. Anti-TGF-$\beta_1$ mAb restored the immunosuppressive effect of TGF-$\beta_1$ on TARC production by HaCaT cells co- stimulated by IFN-γ and TNF-α, however isotype matched control (rabbit IgG) did not restore this. Data are based on four different experiments.

## 4. Discussion

Chemokines and their receptors are important for the selective attraction of various subsets of leukocytes [2,3]. It has been shown TARC/CCL17 and its receptors (CCR4) participated in immunological processes such as AD and bronchial asthma [2,8,9]. AD is a chronically relapsing skin disorder, and in the early stage of the disease lymphocytes infiltrating in the acute skin lesion produce mostly Th2 cytokines, such as IL-4 and IL-5 [15]. We have reported that the serum TARC levels and CCR4 expression of $CD4^+$ $CD45RO^+$ T cells were correlated with the activity of AD, and that the lesional epidermal KCs expressed TARC in AD [11,16]. Therefore, the local production of TARC plays an important role in the induction and maintenance of inflammatory process as a result of the promotion of Th2 cell infiltration. As epidermal KCs can produce TARC, we explored the expression and modulation of TARC by HaCaT cells, a human KCs cell line. We confirmed that the synthesis and secretion of TARC by HaCaT cells, when co-stimulated with TNF-α and IFN-γ, greatly increased, as previous reported [12].

To expand our fundamental understanding of the modulation of TARC expression of HaCaT cells, we investigated whether TGF-$\beta_1$ could regulate the expression of TARC. We clearly showed that TGF-$\beta_1$ decreased both TARC protein production and secretion by HaCaT cells stimulated with both IFN-γ and TNF-α. Because TARC mRNA expressions were also inhibited by TGF-$\beta_1$, these data strongly suggest that the inhibitory effect of TGF-$\beta_1$ on TARC production

by HaCaT cells occur at pre-transcriptional level. In addition, the neutralizing experiments of anti TGF-$\beta_1$ Ab further confirmed the inhibitory effect of TGF-$\beta_1$. TGF-$\beta_1$ has various functions of cell differentiation, growth and regulation of immunological function [15,16]. It has recently been reported that polymorphism of the TGF-$\beta_1$ (+ 915C allele) gene was frequently identified in childhood in AD [14]. The frequency of this TGF-$\beta_1$ genotype, known to be a low producer of TGF-$\beta_1$, was well correlated with severe group compared with mild, and moderate group of AD or healthy controls. These data suggest that the low production of TGF-$\beta_1$ in AD might be related to the maintenance and exaggeration of the skin eruption of AD. Our data suggest that TGF-$\beta_1$ might modulate Th2 cytokines in AD by suppressing TARC production and secretion by epidermal KCs, and show that TGF-$\beta_1$ might play an important role for modulating Th1 and Th2 imbalance in AD by modulating Th2 chemokines.

TGF-$\beta_1$ can be produced by KCs as well as fibroblasts or $CD4^+$ $CD25^+$ T cells by various stimuli [15]. Although the origin of TGF-$\beta_1$ in AD is not fully understood, it has been reported that eosinophils secrete TGF-$\beta_1$ and that this modulates fibrosis in asthma [17]. We showed that a subpopulation of $CD4^+$ $CCR4^+$ T cells in PBMC expressed CD25 in AD patients [16] and the secretion of cytokines from $CD4^+$ $CD25^+$ T cells is currently being investigated. Our studies also present the question of how TGF-$\beta_1$ regulates the production of TARC from HaCaT cells. Berlin et al. reported that the inhibition of the activation of nuclear factor (NF)-κB completely abrogated TARC production in bronchial epithelial cells [18]. The mechanism by which TGF-$\beta_1$ modulated the expression of TARC mRNA and protein of HaCaT cells should be elucidated in the future.

In summary, we have shown that TGF-$\beta_1$ can down-regulate the secretion and synthesis of TARC protein and the expression of TRAC mRNA of HaCaT cells. This is a novel finding of the modulation of chemokine, TARC, by TGF-$\beta_1$. This finding may clarify the role of TGF-$\beta_1$ in the pathogenesis of Th2 related disease, and could lead to a new therapeutic approach for allergic diseases such as AD.

## Acknowledgements

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## References

[1] Kaplan AP. Chemokines, chemokine receptors and allergy. Int Arch Allergy Immunol 2001;124:423–31.
[2] Baggiolini M. Chemokines and leukocyte traffic. Nature 1998;392:565–8.
[3] Zlontik A, Yoshie O. The biology of chemokines and their receptors. Immunity 2000;12:121–7.
[4] Vestergaard C, Bang K, Gesser B, Yoneyama H, Matsushima K, Larsen CG. A Th2 chemokine, TARC, produced by keratinocytes may recruit CLA+ CCR4+ lymphocytes into lesional atopic dermatitis skin. J Invest Dermatol 2000;115:640–6.
[5] Imai T, Baba M, Nishimura M, Kakizaki M, Takagi S, Yoshie O. The T cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. J Biol Chem 1997;272:15036–42.
[6] Sallusto F, Palermo B, Lenig D, Miettinen M, Matikainen S, Julkunen I, Forster R, Burgstahler R, Lipp M, Lanzavecchia A. Distinct patterns and kinetics of chemokine production regulate dendritic cell function. Eur J Immunol 1999;29:1617–25.
[7] Rottman JB, Smith TL, Ganley KG, Kikuchi T, Krueger JG. Potential role of the chemokine receptors CXCR3, CCR4, and the integrin alphaEbeta7 in the pathogenesis of psoriasis vulgaris. Lab Invest 2001;8:335–47.
[8] Vestergaard C, Yoneyama H, Murai M, Nakamura K, Tamaki K, Terashima Y. Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. J Clin Invest 1999;104:1097–110.
[9] Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, Gray PW, Matsushima K, Yoshie O. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokine thymus and activation regulated chemokine and macrophage-derived chemokine. Int Immunol 1999;11:81–8.
[10] Kakinuma T, Nakamura K, Wakugawa M, Mitsui H, Tada Y, Saeki H, Torii H, Asahina A, Onai N, Matsushima K, Tamaki K. Thymus and activation-regulated chemokine in atopic dermatitis: serum thymus and activa-

tion-regulated chemokine level is closely related with disease activity. J Allergy Clin Immunol 2001;107:535–41.

[11] Kawasaki S, Takizawa H, Yoneyama H, Nakayama T, Fujisawa R, Izumizaki M, Imai T, Yoshie O, Homma I, Yamamoto K, Matsushima K. Intervention of thymus and activation-regulated chemokine attenuates the development of allergic airway inflammation and hyperresponsiveness in mice. J Immunol 2001;166:2055–62.

[12] Vestergaard C, Kirstejn N, Gesser B, Mortensen JT, Matsushima K, Larsen CG. IL-10 augments the IFN-gamma and TNF-alpha induced TARC production in HaCaT cells: a possible mechanism in the inflammatory reaction of atopic dermatitis. J Dermatol Sci 2001;26:46–54.

[13] Kumagai N, Fukuda K, Nishida T. Synergistic effect of TNF-alpha and IL-4 on the expression of thymus- and activation-regulated chemokine in human corneal fibroblasts. Biochem Biophys Res Commun 2000;279:1–5.

[14] Arkwright PD, Chase JM, Babbage S, Pravica V, David TJ, Hutchinson IV. Atopic dermatitis is associated with a low-producer transforming growth factor beta (1) cytokine genotype. J Allergy Clin Immunol 2001;108:281–4.

[15] Nakamura K, Kitani A, Strober W. Cell contact—dependent immunosuppression by CD4+ CD25+ regulatory T cells is mediated by cell surface-bound transforming growth factor β. J Exp Med 2001;194:629–44.

[16] Wakugawa M, Nakamura K, Kakinuma K, Onai N, Matsushima K, Tamaki K. CC chemokine receptor 4 expression on peripheral blood CD4+ T cells reflects disease activity of atopic dermatitis. J Invest Dermatol 2001;117:188–96.

[17] Minshall EM, Leung DY, Martin RJ, Song YL, Cameron L, Ernst P, Hamid Q. Eosinophil-associated TGF-beta 1 mRNA expression and airway fibrosis in bronchial asthma. Am J Respir Cell Mol Biol 1997;17:326–33.

[18] Berin MC, Eckmann L, Broide DH, Kagnoff MF. Regulated production of the T helper 2-type T-cell chemoattractant TARC by human bronchial epithelial cells in vitro and in human lung xenografts. Am J Respir Cell Mol Biol 2001;24:382–9.

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**Xueyi ZHENG**
**Koichiro NAKAMURA**
**Michiko TOJO**
**Hitoshi AKIBA**
**Noritaka OYAMA**
**Akiko NISHIBU**
**Fumio KANEKO**
**Yuichiro TSUNEMI***
**Takashi KAKINUMA***
**Hidehisa SAEKI***
**Kunihiko TAMAKI***

Department of Dermatology, Fukushima Medical University School of Medicine, Hikarigaoka 1, Fukushima 960-1295, Japan
* Department of Dermatology, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

Reprints: K Nakamura
Fax: (+ 81)-24-548-5412.
E-mail: nakamu-k@fmu.ac.jp

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# Ultraviolet A irradiation inhibits thymus- and activation-regulated chemokine (TARC/CCL17) production by a human keratinocyte HaCaT cell line

Ultraviolet A (UVA) irradiation modulates the immunological functions of skin. We examined the effect of UVA irradiation on the basal and the IFN-γ-and TNF-α-stimulation-induced production of thymus-and activation-regulated chemokine (TARC/CCL17) using HaCaT cells. UVA irradiation inhibited the basal levels of both TARC mRNA expression and TARC protein production. UVA irradiation also significantly inhibited TARC mRNA expression and TARC protein secretion that were induced by co-stimulation with IFN-γ and TNF-α. A time course study showed that: the significant suppression of TARC mRNA expression was detected 8 hours after irradiation and continued for 36 hours; the strongest inhibition of TARC protein secretion occurred in the first 8 hours after UVA irradiation and continued for 36 hours. Our data provide the first evidence that UVA inhibits TARC mRNA expression and TARC protein production by keratinocytes in a dose-dependent manner. These results may suggest an explanation for the UV-induced therapeutic effect.

*Key words: chemokines, keratinocytes, thymus- and activation-regulated chemokine (TARC/CCL17), ultraviolet A (UVA).*

Thymus- and activation-regulated chemokine (TARC/CCL17) is a CC chemokine secreted by dendritic cells, monocytes and by keratinocytes as well [1]. TARC functions as a selective chemoattractant for activated T cells which belong to the Th2 subset and express CC chemokine receptor (CCR4), the receptor for TARC [2]. TARC can be produced locally and recruit CCR4 + T cells and it may play an important role in the immune responses in which CCR4 + Th2 cells participate [3]. Recently, it has been shown that high levels of TARC were expressed in various allergic diseases [4-6]. In particular, TARC levels were markedly elevated in serum from patients with atopic dermatitis and TARC proteins were expressed strongly by their epidermal keratinocytes [6]. The concentration of TARC in the serum was also related to the disease activity of atopic dermatitis [6]. It has been suggested that TARC could be a useful target for therapeutic approaches to allergic disease in which TARC and CCR4 are involved [1, 7].

UV irradiation has been shown to modulate the immune function. [8, 9]. One of the possible mechanisms might be the direct and/or indirect effect of UV irradiation on the modulation of cytokines produced by immune cells [9, 10]. However, to our knowledge, the effects of UV irradiation on TARC expression by keratinocytes have not been examined. In this study, we examined the effects of UVA irradiation on the expression of TARC mRNA and the release of TARC protein by HaCaT cells, a human keratinocyte cell line.

**Abbreviations:** IFN-γ (interferon-γ), TARC/CCL17 (thymus and activation-regulated chemokine), TNF-α (tumor necrosis factor-α), UVA (ultraviolet A).

## Materials and methods

### Reagents and cell culture

The cytokines used were recombinant human (rh) tumor necrosis factor (TNF)-α (Peprotech Inc. London, UK), and rh interferon (IFN)-γ (R&D Systems Inc. Minneapolis, MN). HaCaT cells which were kindly provided by Prof. NDEDFusenig (German Cancer Research Center, Heidelberg, Gemany) were cultured at 37°C, 5% $CO_2$ in RPMI 1640 containing 10% fetal calf serum.

### UV irradiation

The UV source was a DERMARAY Medical Ultraviolet Irradiation Apparatus (Clinical Supply, Tokyo, Japan). This machine can be adjusted to radiate UVA or UVB specifically. The emission spectrum of the lamp used for UVA irradiation is 310-410nm, and the peak emission is at 360nm. The irradiation dose was measured with a UV radiometer (Clinical Supply, Tokyo, Japan). The UVA irradiation doses used were 1, 4 and 7J/cm$^2$. Cultures were fed fresh culture medium, with or without TNF-α (10ng/ml) and IFN-γ (10ng/ml), 24 hours before UVA irradiation. Just before UVA exposure, the medium was collected. The cells were washed twice with PBS (37°C) and then irradi-

ated in the presence of PBS without the plastic lid. For a 6-cm diameter plate 3-ml PBS were added. After UV irradiation, the PBS was removed, the collected medium was added and the cells were incubated for the indicated times. Sham-irradiated controls were placed under aluminum foil while irradiation was being carried out. The irradiation distance was 40 cm.

### Enzyme-linked immunosorbent assay (ELISA)

The concentration of TARC in culture supernatants was measured using a commercially available kit (Genzyme, Minneapolis, USA). In brief, samples were added to wells onto which a monoclonal antibody specific for TARC had been pre-coated. After washing, an enzyme-linked polyclonal antibody conjugated to horseradish peroxidase was added. Following washing, color was developed. The optical density (OD) was measured using an ELISA reader (Spectra and Rainbow Readers). The minimum detectable concentration was less than 7pg/ml. The protein level in the supernatant was normalized to total cell number. Each supernatant from four different experiments was analyzed in duplicate.

### Northern hybridization [11]

Total mRNA was extracted from the cells using a lysis buffer (Invitrogen Co., CA, USA). Total mRNA (20μg) was fractionated on a 1.5% agarose-formaldehyde gel and transferred to a nylon membrane (Bio-Rad, Hercules, CA, USA). The membrane was UV cross-linked, incubated in 10ml of pre-hybridization solution (0.25M $Na_2HPO_4$ pH 7.2 and 7% SDS) for 2 hours at 65°C and then hybridized for 20 hours with a cDNA probe labeled with digoxigenin(DIG)-dUTP using PCR amplification [9]. After washing stringently, the cDNA-mRNA hybrids were visualized using a DIG nucleic acid detection kit (Roche, Germany). The probe was a 511bp TARC cDNA fragment.
For cDNA probe synthesis, polyA$^+$ mRNA was extracted using a Micro-FastTrack$^{TM}$ 2.0 kit (Invitrogen Co., CA, USA). Approximately 1μg of polyA$^+$ mRNA was reverse-transcripted using a First cDNA kit (Invitrogen Co., CA, USA). The PCR labeling mixture included cDNA, each primer (25-50pmol), $MgCl_2$, dNTP (the ratio of dTTP to DIG-dUTP was 19) and Taq polymerase (TaKaRa, Otsu, Japan). The cycling condition consisted of 40 cycles of denaturation at 95°C, annealing at 57°C, and extension at 72°C. After PCR, the probe was purified. The relative blot intensities of the TARC mRNA were quantified by densitometric scanning and analyzed using NIH Image computer software. Because UV irradiation might influence the expression of GAPDH mRNA expression [12] and our preliminary results also indicated that β – actin mRNA expression may be affected by UVA irradiation, the amounts of RNA loaded were normalized to 28S RNA.

### Cell viability [13]

After UVA irradiation (1, 4 and 7J/cm$^2$), cell viability was evaluated by trypan blue dye exclusion [13]. Cells floating in the supernatant and cells adhering to the dish were collected 24 hours or 48 hours after UVA irradiation together using 0.02% EDTA and 0.25% trypsin. The viable and dead cells were counted.

### Statistical analysis

ANOVA was used for comparing the differences in TARC mRNA or protein levels between the UVA-irradiated and sham-irradiated groups. Non-parametric Spearman's correlation test was used to evaluate the correlation between the doses of UVA irradiation and their effects on TARC mRNA or protein levels. Student's t-test was used to compare the differences in TARC mRNA or protein levels at different time points between the UVA-irradiated and sham-irradiated groups. The values shown are means ± standard deviation (SD). Differences were considered significant at $p < 0.05$.

## Results

### Effects of UVA irradiation dose on cell viability

The percentages of viable cells, based on three counts per plate, and three plates per treatment, were not significantly different for UVA doses of 0, 1, 4 and 7J/cm$^2$ at either the 24 or 48 hours post-UVA irradiation. There was no difference between the UVA irradiated and non-irradiated groups ($P > 0.2$) (Data not shown).

### Effects of UVA irradiation on the baseline level of TARC mRNA expression and TARC protein secretion in non-stimulated HaCaT cells

We first examined the effects of UVA irradiation on the basal level of the expression of TARC mRNA and the secretion of TARC proteins. Non-stimulated HaCaT cells expressed a low level of TARC mRNA and secreted a very small amount of TARC protein, as has been reported [14]. Under our experimental conditions, per $10^6$ cells secreted 64 ± 12.9pg/ml TARC proteins. Culture supernatants and /or total mRNAs were harvested at 24 hours after UVA irradiation or sham-irradiation. The concentrations of TARC protein in the culture supernatants were 53.8 ± 11.8, 24.7 ± 10.5, and 8.63 ± 4.2pg/ml at UVA irradiation doses of 1, 4, and 7 J/cm$^2$, respectively *(Fig. 1)*. The level of TARC protein secretion was significantly inhibited at doses of 4 and 7 J/cm$^2$, but not at 1 J/cm$^2$, when the TARC protein concentration was measured at 24 hours after UVA irradiation. Because the minimum detectable dose was about 7pg/ml, we think that the basal levels of TARC protein secretion were almost completely inhibited by UVA irradiation at a dose of 7J/cm$^2$. There was a significant correlation between the inhibitory effects of UVA on TARC secretion and the UVA irradiation doses ($r = 0.57$, $P = 0.003 < 0.01$). Northern hybridization showed the same pattern of suppression of TARC mRNA expression. At UVA irradiation doses of 1, 4 and 7J/cm$^2$, TARC mRNA expression was suppressed by 22%, 58% and 79%, respectively compared to controls (0J/cm$^2$) *(Fig. 2)*.

### Effects of UVA irradiation on the levels of TARC mRNA expression and TARC protein secretion by HaCaT cells co-stimulated with IFN-γ and TNF-α

High levels of TARC production can be induced in HaCaT cells by co-stimulating them with IFN-γ and TNF-α [14]. Moreover, it has been shown the both IFN-γ and TNF-α were involved in the pathogenesis of inflammatory skin disease such as atopic dermatitis [15]. Thus co-stimulating HaCaT cells with IFN-γ and TNF-α mimics the inflammatory condition. Further research was conducted to examine whether UVA irradiation could regulate IFN-γ and TNF-α stimulation induced TARC mRNA expression and TARC protein secretion by HaCaT cells. *Fig. 3* shows the inhibi-

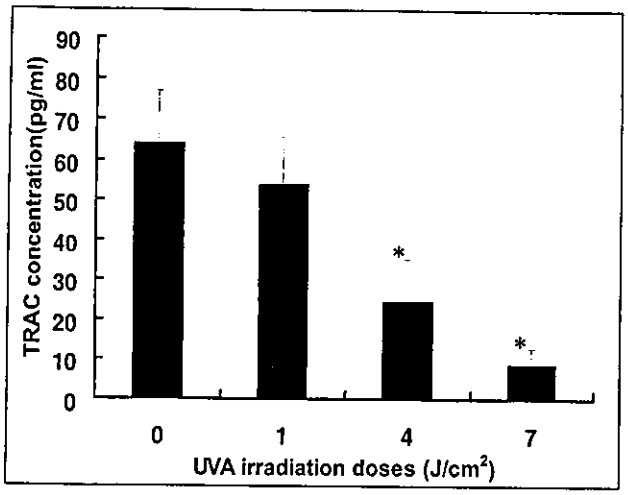


Figure 1. *Inhibitory effects of UVA irradiation on TARC protein secretion by non-stimulated HaCaT cells. To examine the effects of UVA irradiation on the baseline levels of TARC protein production, non-stimulated HaCaT cells were irradiated with UVA (0, 1, 4, and 7J/cm²), and culture supernatants were harvested 24 hours later. There was a significant correlation between the inhibitory effects and the UVA irradiation doses (r = 0.57, P = 0.003<0.01). There were significant differences in the TARC protein levels between the sham-irradiated controls (0J/cm²) and the HaCaT cells irradiated with UVA at doses as 4 and 7J/cm² (* p < 0.05).*

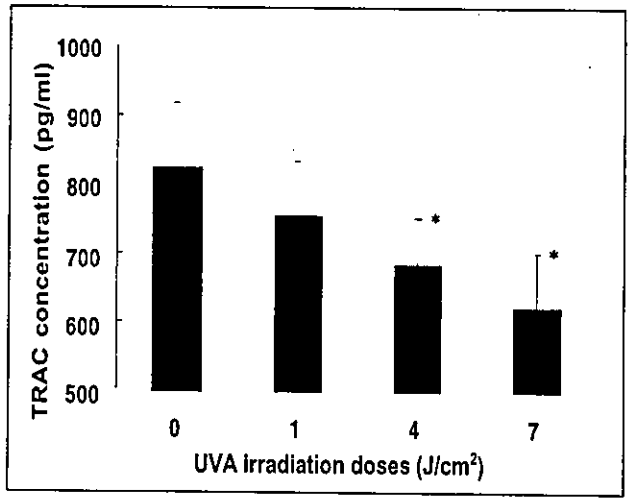


Figure 3. *Inhibitory effects of UVA irradiation on TARC secretion by HaCaT cells co-stimulated by IFN-γ and TNF-α. HaCaT cells were irradiated with UVA (1, 4 and 7J/cm²), and the culture supernatants were harvested 24 hours later. There was a significant correlation between the inhibitory effects and the UVA irradiation doses (r = 0.59, P = 0.003<0.01). There were significant differences between the controls and the UVA irradiated HaCaT cells (* p < 0.05, ** p < 0.01).*

tory effects of UVA irradiation on IFN-γ and TNF-α stimulation induced TARC protein secretion by HaCaT cells. The TARC protein levels in the culture supernatants which were collected at 24 hours after UVA irradiation were 825.1 ± 92.8, 755.5 ± 79.6, 685.3 ± 68.3, and 623.2 ± 76.2pg/ml at UVA irradiation doses of 0, 1, 4 and 7J/cm² respectively. There was significant suppression after exposure to UVA irradiation dose of 1, 4 and 7J/cm². There was a significant correlation between the inhibitory effects of UVA on TARC secretion and the UVA irradiation doses (r = 0.59, P = 0.003<0.01). Northern hybridization showed that, compared to sham-irradiated controls, UVA irradiation inhibited IFN-γ and TNF-α stimulation induced TARC mRNA expression by 24%, 49% and 74% at dose of 1, 4 and 7J/cm² respectively *(Fig. 4)*.

**Time course of the effects of UVA irradiation on IFN-γ and TNF-α stimulation induced TACR mRNA expression and TARC protein secretion by HaCaT cells**

Confluent HaCaT cells was co-stimulated with IFN-γ-and TNF-α 24 hours before UVA irradiation as stated at materials and methods. After one exposure to UVA irradiation (7J/cm²), total RNA was harvested at different time points and was subjected to Northern hybridization *(Fig. 5)*. Significant inhibitory effect on TARC mRNA expression was detected 8 hours after UVA irradiation. The inhibitory effect on TARC mRNA expression was the most effective at 16-24 hours after UVA irradiation. At about 36 hours after UVA irradiation, the inhibitory effect of UVA irradiation on

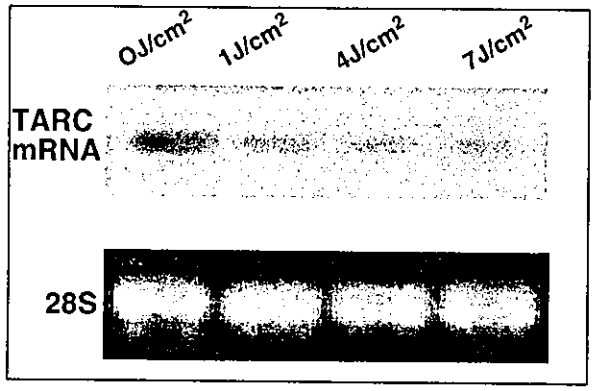


Figure 2. *Inhibitory effects of UVA irradiation on TARC mRNA expressions by non-stimulated HaCaT cells. Northern hybridization results. Upper lane, TARC mRNA; lower lane, 28S mRNA. A representative result from three different experiments is shown.*

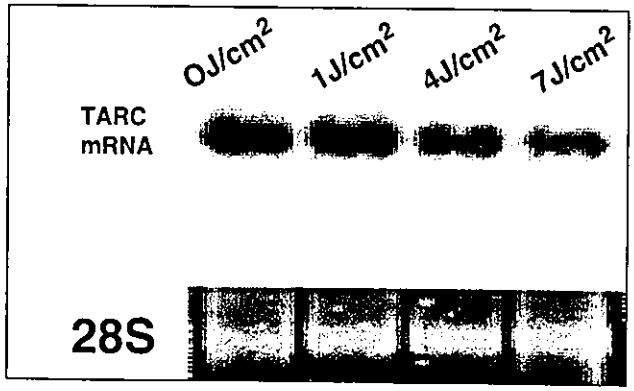


Figure 4. *Inhibitory effects of UVA irradiation on TARC mRNA expressions by HaCaT cells co-stimulated by IFN-γ and TNF-α. Northern hybridization results. Upper lane, TARC mRNA; lower lane, 28S RNA. A representative result from three different experiments is shown.*

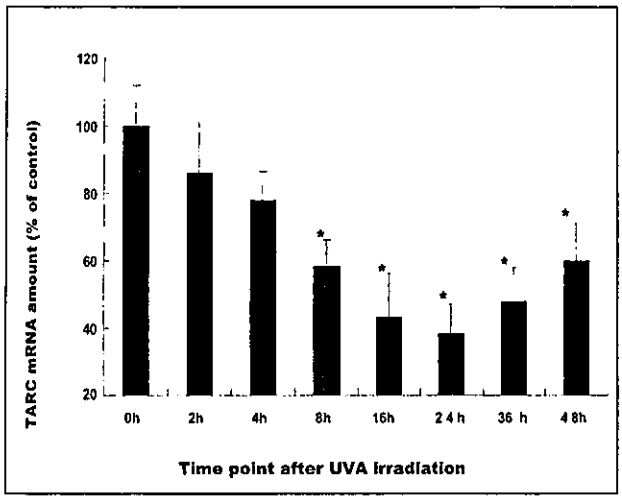


Figure 5. *A time course study of the effects of UVA irradiation on TARC mRNA expression by HaCaT cells. The relative amount of TARC mRNAs. Data are based on three different Northern hybridization experiments. After UVA irradiation 2 h, the amount of TARC mRNA began to reduce. At 8 h after UVA irradiation, TARC mRNAs were significantly inhibited. At 16-24 h after UVA irradiation, the inhibitory effects of UVA became most effective. The inhibitory effects of UVA irradiation began to decrease 36 h after UVA irradiation.*

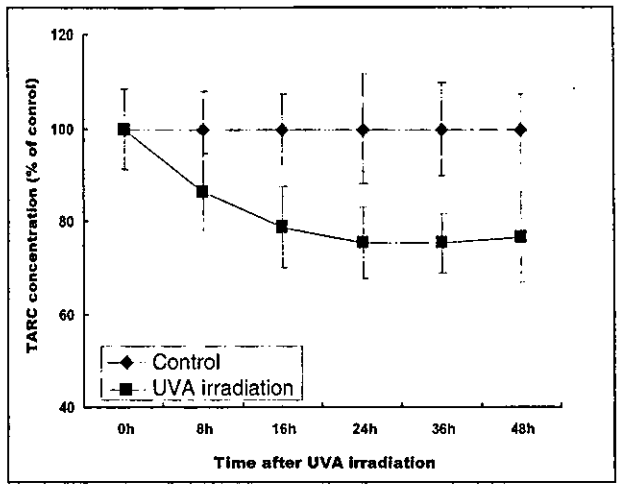


Figure 6. *A time course study of the effects of UVA irradiation on TARC protein secretion by HaCaT cells. Twenty-four hours after co-stimulation by IFN-γ and TNF-α, HaCaT cells were UVA irradiated (7J/cm²). The culture supernatants were collected 0, 8, 16, 24, 36 and 48 hours after UVA irradiation. For all experiments n = 4, and all measures were made in duplicate. The bars indicate the standard deviations (* p < 0.05). TARC concentration in the culture supernatant of UVA irradiated groups were significantly lower than that of controls. We also found that the inhibitory effect of UVA on the secretion rate of TARC was the strongest at the first 8 hours and the secretion rate of TARC was not significantly different from that of control 36 hours after UVA irradiation.*

TARC mRNA expression became less effective at 36-hour time point than that of 24-hour time point. This suggests that the inhibitory effects of UVA irradiation on IFN-γ and TNF-α stimulation induced TARC mRNA expression began to reduce. *Fig. 6* shows the inhibitory effects of UVA irradiation on TARC protein secretion. Culture supernatant was collected at indicated time points. UVA irradiation partially inhibited IFN-γ-and TNF-α co-stimulation induced TARC protein secretion by HaCaT cells under our experimental conditions *(Table I)*. TARC protein concentration in the culture supernatant after 24-hour time point was significantly lower than that of controls. The most effective inhibition of UVA irradiation on TARC protein secretion occurred during the first 8 hours after UVA irradiation because the rate of TARC protein secretion was 43 ± 4.9% of the control which was the smallest compared to that of any other periods after UVA irradiation (not shown). During the period of from 36 hours to 48 hours after UVA irradiation, the rate of TARC protein secretion was 83 ± 9.4% of controls. This secretion rate was not significantly different to the control groups, even through the TARC concentrations at 36-hour and 48-hour time point were significantly lower than control. Our results also shows the inhibitory effect of UVA irradiation on TARC secretion gradually became weaker and at about 36 hours after UVA irradiation the rate of TARC secretion is not significantly different from the controls. Thus, 36 hours after UVA irradiation, the inhibitory effects of UVA irradiation on TARC protein secretion could not countercheck the IFN-γ-and TNF-α-induced TARC secretion by HaCaT cells nor it is likely that the inhibitory effect of UVA irradiation ceased because of the cellular self-repair function. Another interesting finding is that the inhibitory effects of UVA irradiation on TARC mRNA expression and the inhibitory effects of UVA irradiation protein secretion did not parallel each other. This may suggest that multiple points might be affected by UVA irradiation in the process of TARC production.

## Discussion

Keratinocytes are considered an important component of the skin immune system and actively participate in various kinds of immune responses [16]. In humans, because of their anatomical location, keratinocytes are the natural targets of UV irradiation. Therefore, keratinocytes have been used as a model in studies on the effects of UV irradiation

Table I. *The time course results of TARC protein concentration (pg/ml)*

| Group | 0 h | 8 h | 16 h | 24 h | 36 h | 48 h |
|---|---|---|---|---|---|---|
| Control | 411.0 ± 85 | 535.3 ± 80 | 684.7 ± 74 | 825.1 ± 110 | 1015.2 ± 99 | 1261.7 ± 78 |
| UVA irradiated | 410.9 ± 86 | 463.3 ± 83 | 540.4 ± 87 | 623.2 ± 76 | 766.6 ± 64 | 933.8 ± 98 |

*Twenty-four hours after co-stimulation by IFN-γ and TNF-α, HaCaT cells were UVA irradiated (7J/cm²). The culture supernatants were collected 0, 8, 16, 24, 36 and 48 hours after UVA irradiation. For all experiments n = 4, and all measures were made in duplicate.*

[17]. In this study, we showed that UVA irradiation inhibited both the basal level and the IFN-γ and TNF-α stimulation-induced TARC mRNA expression and TARC protein secretion by HaCaT cells. The UVA irradiation doses (1, 4 and 7J/cm$^2$) we used, did not affect cell viability, in agreement with what others have reported [18]. This excluded the possibility that the inhibition of TARC mRNA expression and TARC protein secretion was caused by UVA irradiation-induced cytotoxicity. To our knowledge, this is the first report showing that UVA irradiation can inhibit TARC mRNA expression and TARC protein secretion by a human keratinocyte line, HaCaT cells. We believe that this finding is important to understand the underlying mechanism of UVA irradiation in the process of modulating immune responses.

High levels of TARC production can be induced in HaCaT cells through co-stimulating with IFN-γ and TNF-α [14]. Because it has been shown that both IFN-γ and TNF-α were involved in the pathogenesis of many inflammatory diseases [15], the stimulation of HaCaT cells with IFN-γ and TNF-α mimics the inflammatory condition, as mentioned before [19]. We found that UVA irradiation could inhibit the IFN-γ- and TNF-α-induced TARC mRNA expression and TARC protein secretion by HaCaT cells, indicating that UVA irradiation might suppress the IFN-γ- and TNF-α-induced TARC-involved inflammation. Our time course experiments also show that the inhibitory effect of UVA irradiation on TARC secretion gradually became weaker and that at about 36 hours after UVA irradiation, the rate of TARC secretion is not significantly different from the controls. Thus, 36 hours after UVA irradiation, the inhibitory effects of UVA irradiation on TARC protein secretion could not countercheck the IFN-γ-and TNF-α-induced TARC secretion by HaCaT cells nor it is likely that the inhibitory effect of UVA irradiation ceased because of the cellular self-repair function.

It has been proven that UVA irradiation affects the immune function [20-23]. One proposed mechanism was the modulation of immuno-modulatory cytokines of keratinocytes [9, 10, 23]. Substantial evidence suggests that UVA irradiation is selective in its modulating of the induction of cytokines, such as interleukin-12, that promotes Th1 responses and inhibits Th2 responses [20, 21]. TARC acts specifically on Th2 lymphocytes and promotes Th2 response. Our findings that UVA irradiation inhibited TARC mRNA expression and TARC protein secretion by HaCaT cells are consistent with previous findings that UVA irradiation modulates Th2 response [21]. Based on our results, we suggest that UVA irradiation affects the human cutaneous immune function, at least in part, by modulating the capacity of keratinocytes to produce TARC. It has been shown that high production of TARC was detected by lesional keratinocytes in atopic dermatitis and that a high level of serum TARC concentration is correlated with the disease severity of atopic dermatitis [6]. In this study, we found that UVA irradiation inhibits TARC mRNA expression and TARC protein secretion by human HaCaT keratinocyte cell lines. Thus, we consider that the inhibitory effects of UVA irradiation on TARC production might be an underlying mechanism responsible for the effectiveness of UVA irradiation on immune modulation.

The mechanism by which UVA irradiation inhibited TARC mRNA expression and TARC protein secretion needs to be investigated. Berlin *et al* showed that nuclear factor (NF)-kB participated in epithelial cell TARC mRNA expression [24]. Djavaheri-Mergny M *et al* reported that UVA could induce a decrease in NF-kB activity in human keratinocytes [25]. Whether UVA inhibited TARC mRNA expression and TARC protein secretion through regulating NF-kB remains to be clarified. Also, we found that the inhibitory effect of UVA irradiation on TARC mRNA expression and the inhibitory effect of UVA irradiation protein secretion which were induced by co-stimulation with IFN-γ-and TNF-α did not parallel each other. This may suggest that multiple points might be affected by UVA irradiation in the process of TARC production. Further research on the underlying mechanism may lead to important findings.

In summary, our data provide the first evidence that UVA inhibits TARC mRNA expression and its protein production by HaCaT cells in a dose-dependent manner. This observation indicates that UVA-irradiation might modulate the skin immune function through regulating TARC production by keratinocytes. ■

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## References

**1.** Kaplan AP. Chemokines, chemokine receptors and allergy. *Int Arch Allergy Immunol* 2001; 124: 423-31.

**2.** Imai T, Baba M, Nishimura M, Kakizaki M, Takagi S, Yoshie O. The T cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. *J Biol Chem* 1997; 272: 15036-42.

**3.** Vestergaard C, Bang K, Gesser B, Yoneyama H, Matsushima K, Larsen CG. A Th2 chemokine, TARC, produced by keratinocytes may recruit CLA$^+$ CCR4$^+$ lymphocytes into lesional atopic dermatitis skin. *J Invest Dermatol* 2000; 115: 640-6

**4.** Vestergaard C, Yoneyama H, Murai M, Nakamura K, Tamaki K, Terashima Y. Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. *J Clin Invest* 1999; 104: 1097-10.

**5.** Panina-Bordignon P, Papi A, Mariani M, Di Lucia P, Casoni G, Bellettato C, Buonsanti C, Miotto D, Mapp C, Villa A, Arrigoni G, Fabbri LM, Sinigaglia F. The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *J Clin Invest* 2001; 107: 1357-64.

**6.** Kakinuma T, Nakamura K, Wakugawa M, Mitsui H, Tada Y, Saeki H, Torii H, Asahina A, Onai N, Matsushima K, Tamaki K. Thymus and activation-regulated chemokine in atopic dermatitis: Serum thymus and activation-regulated chemokine level is closely related with disease activity. *J Allergy Clin Immunol* 2001; 107: 535-41.

**7.** Kawasaki S, Takizawa H, Yoneyama H, Nakayama T, Fujisawa R, Izumizaki M, Imai T, Yoshie O, Homma I, Yamamoto K, Matsushima K. Intervention of thymus and activation-regulated chemokine attenuates the development of allergic airway inflammation and hyperresponsiveness in mice. *J Immunol* 2001; 166: 2055-62.

**8.** Kondo S. The roles of keratinocyte-derived cytokines in the epidermis and their possible responses to UVA-irradiation. *J Investig Dermatol Symp Proc* 1999; 4:177-83.

**9.** Simon JC, Pfieger D, Schopf E. Recent advances in phototherapy. *Eur J Dermatol* 2000; 10: 642-5.

**10.** Clingen PH, Berneburg M, Petit-Frere C, Woollons A, Lowe JE, Arlett CF, Green MH. Contrasting effects of an ultraviolet B and an ultraviolet A tanning lamp on interleukin-6, tumour necrosis factor-alpha and intercellular adhesion molecule-1 expression. *Br J Dermatol* 2001; 145: 54-62.

**11.** Kevil CG, Walsh L, Laroux FS, Kalogeris T, Grisham MB, Alexander JS. An improved, rapid Northern protocol. *Biochem Biophys Res Commun* 1997; 238: 277-9.

**12.** Garmyn M, Yaar M, Holbrook N, Gilchrest BA. Immediate and delayed molecular response of human keratinocytes to solar-simulated irradiation. *Lab Invest* 1991; 65: 471-8.

**13.** Sasaki H, Akamatsu H, Horio T. Protective role of copper, zinc superoxide dismutase against UVB-induced injury of the human keratinocyte cell line HaCaT. *J Invest Dermatol* 2000; 114: 502-7.

**14.** Vestergaard C, Kirstejn N, Gesser B, Mortensen JT, Matsushima K, Larsen CG. IL-10 augments the IFN-yamma and TNF-alpha induced TARC production in HaCaT cells: a possible mechanism in the inflammatory reaction of atopic dermatitis. *J Dermatol Sci* 2001; 26: 46-54.

**15.** Farrell AM, Antrobus P, Simpson D, Powell S, Chapel HM, Ferry BL. A rapid flow cytometric assay to detect CD4 + and CD8 + T-helper (Th) 0, Th1 and Th2 cells in whole blood and its application to study cytokine levels in atopic dermatitis before and after cyclosporin therapy. *Br J Dermatol* 2001; 144: 24-33.

**16.** Uchi H, Terao H, Koga T, Furue M. Cytokines and chemokines in the epidermis. *J Dermatol Sci* 2000; 24: S29-38.

**17.** Barr RM, Walker SL, Tsang W, Harrison GI, Ettehadi P, Greaves MW, Young AR. Suppressed alloantigen presentation, increased TNF-alpha, IL-1, IL-1Ra, IL-10, and modulation of TNF-R in UV-irradiated human skin. *J Invest Dermatol* 1999; 112: 692-8

**18.** Otto AI, Riou L, Marionnet C, Mori T, Sarasin A, Magnaldo T. Differential behaviors toward ultraviolet A and B radiation of fibroblasts and keratinocytes from normal and DNA-repair-deficient patients. *Cancer Res* 1999; 59: 1212-8.

**19.** Rahat MA, Chernichovski I, Lahat N. Increased binding of IFN regulating factor 1 mediates the synergistic induction of CIITA by IFN-yamma and tumor necrosis factor-alpha in human thyroid carcinoma cells. *Int Immunol* 2001; 13:1423-32.

**20.** Kondo S, Jimbow K. Dose-dependent induction of IL-12 but not IL-10 from human keratinocytes after exposure to ultraviolet light A. *J Cell Physiol* 1998; 177: 493-8.

**21.** Shen J, Bao S, Reeve VE. Modulation of IL-10, IL-12, and IFN-yamma in the epidermis of hairless mice by UVA (320-400 nm) and UVB (280-320 nm) radiation. *J Invest Dermatol* 1999; 113: 1059-64.

**22.** Dumay O, Karam A, Vian L, Moyal D, Hourseau C, Stoebner P, Peyron JL, Meynadier J, Cano JP, Meunier L. Ultraviolet A 1 exposure of human skin results in Langerhans cell depletion and reduction of epidermal antigen-presenting cell function: partial protection by a broad-spectrum sunscreen. *Br J Dermatol* 2001; 144: 1161-8.

**23.** Grewe M, Gyufko K, Schopf E, Krutmann J. Lesional expression of interferon-gamma in atopic eczema. *Lancet* 1994;343(8888): 25-6.

**24.** Berin MC, Eckmann L, Broide DH, Kagnoff MF. Regulated production of the T helper 2-type T-cell chemoattractant TARC by human bronchial epithelial cells in vitro and in human lung xenografts. *Am J Respir Cell Mol Biol* 2001; 24: 382-9

**25.** Djavaheri-Mergny M, Gras MP, Mergny JL, Dubertret L. UV-A-induced@decrease in nuclear factor-kappaB activity in human keratinocytes. *Biochem J* 1999; 338: 607-13.

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# Differential regulation of thymus- and activation-regulated chemokine induced by IL-4, IL-13, TNF-α and IFN-γ in human keratinocyte and fibroblast

Bin Yu [a,*], Tetsuya Koga [a], Kazunori Urabe [a], Yoichi Moroi [a], Shoko Maeda [b], Yukiyoshi Yanagihara [b], Masutaka Furue [a]

[a] *Department of Dermatology, Graduate School of Medical Sciences, Kyushu University, 3-1-1, Maidashi, Higashi-ku, J-812-8582 Fukuoka, Japan*

[b] *Clinical Research Center for Allergy, National Sagamihara Hospital, Sagamihara, Kanagawa 228-8522, Japan*

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**Abstract**

The CC chemokine thymus- and activation-regulated chemokine (TARC/CCL17) acts on CC chemokine receptor 4 (CCR4), which is known to be selectively expressed in Th2 cells. In order to compare the regulatory profiles of TARC production by tumor necrosis factor-α (TNF-α), IFN-γ, interleukin-4 (IL-4) and IL-13 in keratinocytes and fibroblasts, HaCaT cells, a human keratinocyte cell line, and NG1RGB cells, a human skin fibroblast cell line, were used. The expression of TARC protein was measured using enzyme-linked immunosorbent assay (ELISA), and the mRNA level was detected by reverse transcriptase polymerase chain reaction (RT-PCR). The spontaneous expression of TARC protein and mRNA levels were augmented by TNF-α and IFN-γ and were inhibited by IL-4 and IL-13 in the keratinocytes. The fibroblasts expressed the TARC protein and mRNA only in the presence of IL-4+TNF-α or IL-13+TNF-α stimulation. IFN-γ further enhanced the IL-4+TNF-α or IL-13+TNF-α-induced TARC production in the fibroblasts. Thus, TNF-α and IFN-γ -induced TARC production was differentially regulated by IL-4 and IL-13 in human keratinocytes and fibroblasts. 

*Keywords:* Keratinocytes; Fibroblasts; Thymus-and activation-regulated chemokine; TNF-α; IFN-γ; IL-4; IL-13

## 1. Introduction

Chemokines, a family of low-molecular-weight proteins that induce specific types of leukocyte chemotaxis, play essential roles in regulating extravasation and tissue accumulation of lymphocytes during immune and inflammatory responses [1,2]. Thymus-and activation-regulated chemokine (TARC/CCL17) was identified by cloning the

*Abbreviations:* CCR4, CC chemokine receptor 4; TARC, thymus-and activation-regulated chemokine.

* Corresponding author. Tel.: +81-92-642-5585; fax: +81-92-642-5600

*E-mail address:* yubin@dermatol.med.kyushu-u.ac.jp (B. Yu).

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D3A gene from peripheral blood mononuclear cells (PBMCs) after stimulation with PHA [3]. TARC is a basic protein with a predicted molecular weight of 8 kDa, and is produced by dendritic cells [3–5], keratinocytes [6,7] and bronchial epithelial cells [8]. TARC acts as a specific ligand for CC chemokine receptor 4 (CCR4), which is predominantly expressed on Th2-type CD4+T cells [2,3]. Thus, TARC selectively chemoattracts Th2 cells into inflammatory sites. In a murine study, a monoclonal antibody against TARC inhibited Th2-mediated bacteria-induced fulminant hepatic failure [9].

Predominant infiltration of Th2 cells has been found in allergic disorders such as atopic dermatitis and asthma [10–12]. TARC has been shown to be overproduced in keratinocytes of NC/Nga mice exhibiting atopic dermatitis-like lesions [4]. Lymphocytes that infiltrate acute or early lesional skin of atopic dermatitis are mainly Th2 cells, which produce interleukin-4 (IL-4), IL-5, IL-10 and IL-13. However, it has been reported that Th1 cells, which produce IFN-γ, increase in number in chronic lesions [13,14]. Keratinocytes also secrete various kinds of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) [15]. Thus, it would be interesting to know how these cytokines regulate the production of TARC. In this study, we examined the production of TARC in keratinocytes and fibroblasts in the presence of IL-4, IL-13 or TNF-α, IFN-γ, and we found that IL-4 and IL-13 reciprocally regulated the production of TARC in keratinocytes and in fibroblasts.

## 2. Materials and methods

### 2.1. Reagents

Dulbecco's modified eagle's medium (DMEM), penicillin–streptomycin and trypsin–EDTA were purchased from Gibco-BRL (Grand Island, NY). RITC 80-7 medium was purchased from IWAKI Glass (Chiba, Japan). Fetal bovine serum (FBS) was obtained from HyClone (Logan, USA). Recombinant human IL-4 and IL-13 were purchased from PeproTech (Rocky Hill, NJ). Recombinant human TNF-α and IFN-γ, and agarose were purchased from Sigma (Life Science Research, USA). Human TARC was obtained from R&D Systems (Minneapolis, MN). An RNA isolation reagent, Isogen, and ethidium bromide were purchased from Nippon Gene (Tokyo, Japan). Superscript™ II RNase reverse transcriptase was purchased from Gibco-BRL.

### 2.2. Cell culture

HaCaT cells, a spontaneously immortalized, nontumorigenic human skin keratinocyte cell line (kindly provided by Dr N.E. Fusenig, DKFZ Heidelberg), and NG1RGB cells, a normal human skin fibroblast cell line (kindly provided by Dr H. Nagamune, Tokushima University, Japan), were used for the experiments. HaCaT cells were maintained in DMEM with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml). NG1RGB cells were maintained in RITC 80-7 with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml). HaCaT cells ($4 \times 10^5$ cells per well) were plated into 24-well plates, and NG1RGB cells ($2 \times 10^6$ cells per well) were plated into six-well plates, respectively. They were then cultured for 24 h. Before stimulation, all of the culture medium in each well was replaced by identical formation containing 0.1% BSA in place of FBS. The cells were treated with the medium only or with various concentrations (0.1–100 ng/ml) of TNF-α, IFN-γ, IL-4 and IL-13. After incubation for the indicated periods of time, the supernatant was collected from each culture dish and centrifuged at 15 000 rpm for 1 min and then stored at −80 °C until further analysis.

### 2.3. TARC enzyme-linked immunosorbent assay (ELISA)

Concentrations of TARC in the supernatant were measured with an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's instructions using an ELISA reader, Labsystems Multiskan MS (DAINIPPON, Tokyo). This ELISA method enabled detection of TARC concentrations of greater than 15.6 pg/ml.

### 2.4. Detection of TARC by reverse transcriptase polymerase chain reaction (RT-PCR)

Human keratinocyte HaCaT cells and fibroblast NG1RGB cells were cultured and stimulated as described above, and then the cells were washed with PBS and total RNA was extracted. Extracted RNA was subjected to reverse transcription using a superscript™ II RNase reverse transcriptase kit. Transcripts of the constitutively expressed gene for β-actin served as control in each sample. The sequences of the PCR primers were as follows: TARC sense, 5′-ATGGCCCCACTGAAGATGCT-3′; TARC antisense, 5′-TGAACACCAACGGTGGAGGT-3′; and β-actin sense, 5′-CCTCGCCTTTGCCGATCCGC-3′; β-actin antisense, 5′-AGGTAGTCAGTCAGGTCCCG-3′. These primers yielded PCR products of the expected sizes of 332 bp for TARC mRNA and 598 bp for β-actin mRNA. The initial stage of the PCR protocol consisted of denaturation at 94 °C for 7 min, annealing at 58 °C for 2 min and elongation at 72 °C for 3 min. The second stage, followed by 34 cycles of TARC cDNA amplification, consisted of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and elongation at 72 °C for 2 min. For the amplification of β-actin cDNA, the cycles included one step of denaturation at 94 °C for 7 min, annealing at 56 °C for 2 min and elongation at 72 °C for 3 min, and then 29–34 cycles of the second step by the same procedure as that of the second stage of TARC cDNA PCR. The results were visualized on a 2% agarose gel, which was then stained with ethidium bromide (1 μg/ml) and examined with PrintgraphFX (ATTO, Tokyo) consisting of a charge-coupled device camera, an ultraviolet transilluminator, and an analysis program.

### 2.5. Statistical analysis

Data are expressed as means ± S.E.M. Statistical significance of differences was analyzed using an analysis of variance (ANOVA) method ANOVA. A $P$-value of less than 0.05 was considered to be statistically significant.

## 3. Results

### 3.1. Production of TARC protein and mRNA expression were augmented by TNF-α and IFN-γ but were inhibited by IL-4 and IL-13 in human keratinocytes

We first studied the effects of TNF-α, IFN-γ, IL-4 and IL-13 on the production of TARC by human keratinocytes. HaCaT cells were cultured with medium only or with 0.1–100 ng/ml of TNF-α, IFN-γ, IL-4 and IL-13 for 72 h, and the concentrations of TARC in the supernatants were quantified by ELISA. TNF-α (Fig. 1A) and IFN-γ Fig. 1B) significantly augmented the spontaneous production of TARC from HaCaT cells in a dose-dependent manner. In contrast, IL-4 (Fig. 1C) and IL-13 (Fig. 1D) dose-dependently inhibited the TARC production. Time-course experiments showed that the up- or down-regulatory effects of these cytokines were time-dependent for 72 h (Fig. 2). To confirm the results, we next examined the expression levels of TARC mRNA in HaCaT cells incubated with medium only, IL-4, IL-13, TNF-α or IFN-γ for 24 h. In accordance with the protein levels, the TARC mRNA expression was enhanced by TNF-α and IFN-γ and inhibited by IL-4 and IL-13 (Fig. 3). In each experiment, the inhibitory effects of IL-4 and IL-13 on TARC production by keratinocytes were similar, but the enhancing effect of IFN-γ was stronger than that of TNF-α.

In order to determine whether similar regulatory mechanisms operate in fibroblasts, we examined the TARC production by NG1RGB cells, a human fibroblast cell line. However, NG1RGB cells did not produce detectable amounts of TARC even in the presence of TNF-α, IFN-γ, IL-4 and IL-13 (Table 1).

### 3.2. Reciprocal regulation by IL-4 and IL-13 of TARC production by keratinocytes and fibloblasts in the presence of TNF-α

To further elucidate the regulatory effects, we next examined the co-stimulatory response of TARC production in the keratinocytes and the fibroblasts under various combinations of these

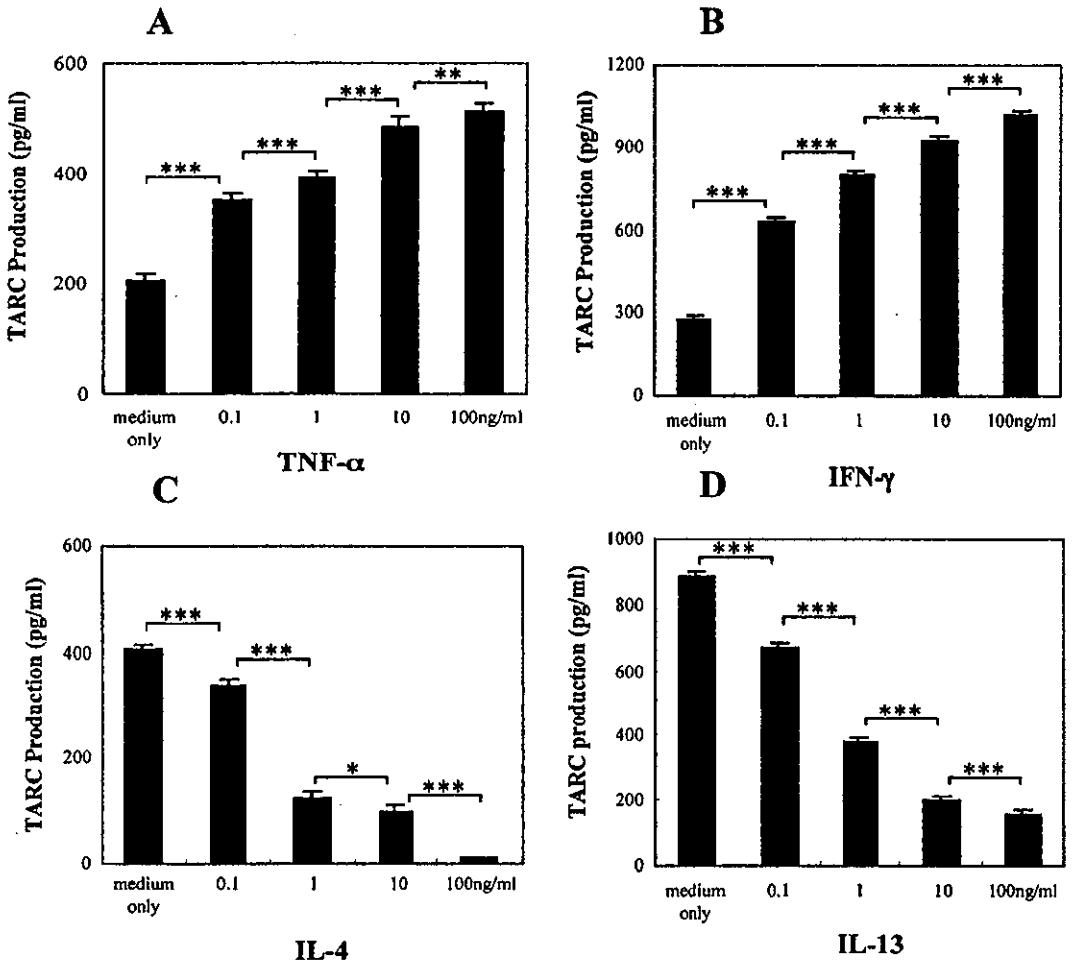


Fig. 1. Production of TARC by HaCaT keratinocytes stimulated with various concentrations of TNF-α (0–100 ng/ml), IFN-γ (0–100 ng/ml), IL-4 (0–100 ng/ml) or IL-13 (0–100 ng/ml). HaCaT cells were cultured for 72 h with the indicated concentrations of cytokines. Data are presented as means ± S.E.M. Representative data are shown ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

four cytokines. The augmenting effects of TNF-α and IFN-γ were neutralized by IL-4 and IL-13 in the keratinocytes. IFN-γ did not further enhance the up-regulatory effect of TNF-α in the keratinocytes (Table 1).

In sharp contrast, IL-4, IL-13, TNF-α and IFN-γ did not induce the TARC production in the fibroblasts. The significant production of TARC was observed only in the dual presence of IL-4 and TNF-α or IL-13 and TNF-α (Table 1). Interestingly, IFN-γ? synergistically enhanced the TARC production induced by TNF-α+IL-4 or TNF-α+IL-13 in the fibroblasts (Table 1).

To confirm the effects of TNF-α+IL-4 or TNF-α+IL-13 on TARC production by the fibroblasts, we next examined the mRNA levels of TARC in the fibroblasts stimulated with various combinations of cytokines. As shown in Fig. 4, TNF-α induced detectable mRNA accumulation for TARC only in the presence of IL-4 or IL-13. In

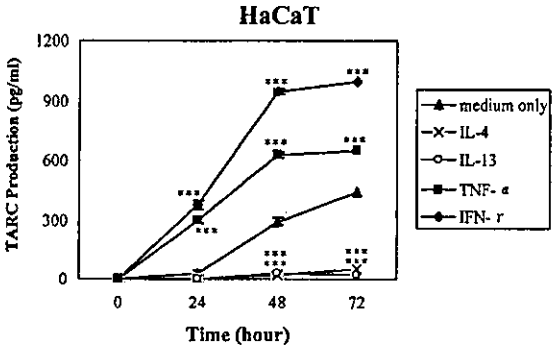


Fig. 2. Time courses of TARC production from HaCaT keratinocytes sitmulated with TNF-α (50 ng/ml), IFN-γ (100 ng/ml), IL-4 (10 ng/ml) or IL-13 (50 ng/ml) for 24, 48 or 72 h. Data are presented as means±S.E.M. Representative data are shown ($n=3$). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, compared with representative medium only.


Fig. 3. Comparison of TARC mRNA levels by HaCaT keratinocytes incubated with IL-4 (10 ng/ml), IL-13 (50 ng/ml), TNF-α (50 ng/ml) or IFN-γ (100 ng/ml) for 24 h. Representative data of two independent experiments with similar results are shown.

addition, IFN-γ further enhanced the accumulation of TARC mRNA induced by TNF-α+IL-4 or TNF-α+IL-13 stimulation in the fibroblasts.

## 4. Discussion

We examined the production of TARC by keratinocytes and fibroblasts, two major cell populations of skin, in a mixture of proinflammatory (TNF-α), Th1 (IFN-γ) and Th2 (IL-4 and IL-13) cytokines. In keratinocytes, the synthesis of TARC was strongly enhanced by TNF-α and IFN-γ but was inhibited by IL-4 and IL-13 both in protein and in mRNA levels. In contrast, IL-4 and IL-13 induced the production of TARC protein and mRNA by fibroblasts in the presence

Table 1
Levels of TARC protein expression by human kerationcyte HaCaT cells and fibroblast NG1RGB cells

| Stimulation | TARC Production (pg/ml) | |
|---|---|---|
| | HaCaT cells | NG1RGB cells |
| Medium only | 291.45 | 0 |
| IL-4 | 25.05[a] | 0 |
| IL-13 | 27.10[a] | 0 |
| TNF-α | 628.02[a] | 0 |
| IFN-γ | 946.54[a] | 0 |
| TNF-α+IFN-γ | 865.19[a] | 0 |
| IL-4+TNF-α | 154.06[b] | 286.22[b] |
| IL-13+TNF-α | 128.56[b] | 246.45[b] |
| IL-4+IFN-γ | 377.83[c] | 0 |
| IL-13+IFN-γ | 442.24[c] | 0 |
| IL-4+TNF-α+IFN-γ | 682.20[d] | 797.75[d] |
| IL-13+TNF-α+IFN-γ | 625.60[d] | 755.28[d] |

HaCaT cells and NG1RGB cells were incubated for 48 h with IL-4 (10 ng/ml), IL-13 (50 ng/ml), TNF-α (50 ng/ml), IFN-γ (100 ng/ml) or various combinations of these cytokines. Representative data are shown ($n=3$). [a], $P<0.001$ in comparison with Medium only; [b], $P<0.001$ with TNF-α; [c], $P<0.001$ with IFN-γ; [d], $P<0.001$ with TNF-α+IFN-γ.

of TNF-α. IFN-γ per se did not stimulate the fibroblasts to synthesize TARC protein but it synergistically augmented the IL-4 +TNF-α- or IL-13+TNF-α-induced TARC synthesis in the fibroblasts.

Various types of inflammatory cells and their cyto-chemokines play important roles in the pathomechanisms of atopic dermatitis. Many investigators have demonstrated a predominant emergence of Th2 cells both in peripheral blood and in skin lesions of patients with atopic dermatitis [16–18]. The infiltration of Th2 cells in patients with atopic dermatitis is clearly demonstrable in vivo, particularly in atopic patch test sites, with allergens. Yamada et al. reported that infiltration of IL-4-producing lymphoid cells was observed in the perivascular area of the dermis even at 2 h after application of mite allergen [19]. However, in later stages of the disease, Th1-type IFN-γ ?is produced in the lesions of atopic dermatitis [13,14]. TNF-α has been reported to be up-regulated in mast cells in the lesional skin of atopic dermatitis [20]. In the present study, we demonstrated that both human skin keratinocytes and fibroblasts are potent producers of TARC in a

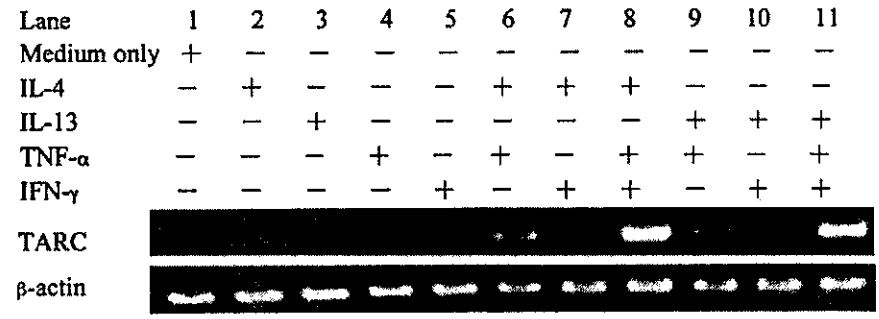


Fig. 4. Levels of TARC mRNA expression by human fibroblast NG1RGB cells incubated for 24 h with IL-4 (10 ng/ml), IL-13 (50 ng/ml), TNF-α (50 ng/ml), IFN-γ (100 ng/ml) or various combinations of these cytokines. Representative data of two independent experiments with similar results are shown.

certain combination with TNF-α, IFN-γ, IL-4 and IL-13. Vestergaard et al. reported that TARC was highly expressed in the basal epidermis of lesional skin of NC/Nga atopic dermatitis model mice [6]. Kakinuma et al. recently reported serum TARC levels of patients with atopic dermatitis were significantly higher than those of healthy control subjects and patients with psoriasis. The serum TARC levels were significantly correlated with clinical severity scores and with levels of eosinophils and serum soluble E-selectin. Kakinuma et al. also immunohistologically detected the expression of TARC in the lesional epidermis of atopic dermatitis [21].

Since TARC is a specific chemoattractant for Th2 cells, it would be interesting to know the differential regulation of TARC synthesis by keratinocytes and fibroblasts. PAM 212 cells, a murine keratinocyte cell line, produced TARC after stimulation with TNF-α or IFN-γ [6]. In the previous study, IL-10 greatly augmented the TNF-α+IFN-γ-induced TARC production by human keratinocyte HaCaT cells [22]. An obvious difference between keratinocytes and fibroblasts is their responses to IL-4 and IL-13. In our experiments, IL-4 and IL-13 inhibited the production of TARC by HaCaT cells but synergistically induced the de novo synthesis of TARC by NG1RGB fibroblasts only in the presence of TNF-α. Human corneal fibroblasts have also been shown to produce TARC only in the presence of both IL-4 and TNF-α [23]. These results indicate (1) that fibroblasts require dual stimulation with IL-4 and TNF-α for the production of TARC, (2) that TNF-α and IFN-γ act as enhancers of TARC production by keratinocytes and fibroblasts and (3) that IL-4 and IL-13 act as inhibitors of TARC production by keratinocytes but as enhancers of TARC production by fibroblasts. Sekiya et al. have recently reported the regulation of TARC production by bronchial epithelial cells. Two bronchial cell lines, A549 and BEAS-2B, reacted to produce TARC protein by dual stimulation with TNF-α+IL-4 [8]. Moreover, IFN-γ synergistically up-regulated the TNF-α+IL-4-induced TARC production, manifesting a similar regulatory pattern to that observed in the NG1RGB skin fibroblasts in the present study.

Campbell et al. reported that CCR4 and TARC were important in the recognition of skin vasculature by circulating T cells and in directing lymphocytes that were involved in systemic immunity to their target tissues [24]. It is known that the much higher level of mononuclear cell infiltration is observed in the dermis than in the epidermis of a chronic lesion of atopic dermatitis [25].

Although the precise molecular mechanisms remain unclear, the differential regulation by IL-4 and IL-13 in the TARC production by keratinocytes and fibroblasts is a very interesting evidence.

## Acknowledgements

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## References

[1] Baggiolini M. Chemokine and leukocyte traffic. Nature 1998;392:565–8.
[2] Qin S, Rottman JB, Myers P, Kassam M, Weinblatt M, Loetscher M, et al. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. J Clin Invest 1998;101:746–54.
[3] Imai T, Yoshida T, Baba M, Nishimura M, Kakizaki M, Yoshie O. Molecular cloning of a novel T cell-directed CC chemokine expressed in thymus by signal sequence trap using Epstein Barr virus vector. J Biol Chem 1996;271:21514–21.
[4] Imai T, Baba M, Nishimura M, Kakizaki M, Yoshie O. The T cell-derived CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. J Biol Chem 1997;272:15036–42.
[5] Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, et al. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokine thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol 1999;11:82–8.
[6] Vestergaard C, Yoneyama H, Murai M, Nakamura K, Tamaki K, Terashima Y, et al. Overproduction of Th2 CC chemokines TARC and MDC in the skin of the NC/Nga mouse correlates with exacerbation of atopic dermatitis like lesions. J Clin Invest 1999;104:1097–105.
[7] Vestergaard C, Bang K, Gesser B, Yoneyama H, Matsushima K, Larsen CG. A Th2 chemokine, TARC, produced by keratinocytes may recruit CLA+CCR4+lymphocytes into lesional atopic dermatitis skin. J Invest Dermatol 2000;115:640–6.
[8] Sekiya T, Miyamasu M, Imanishi M, Yamada H, Nakajima T, Yamagucji M, et al. Inducible expression of a Th2-type CC chemokine thymus and activation-regulated chemokine by human bronchial epithelial cells. J Immunol 2000;165:2205–13.
[9] Yoneyama H, Harada A, Imai T, Baba M, Yoshie O, Higashi H, et al. Pivotal role of TARC, a CC chemokine, in bacteria-induced fulminant hepatic failure in mice. J Clin Invest 1998;102:1933–41.
[10] Furue M. Atopic dermatitis—immunological abnormality and its background. J Dermatol Sci 1994;7:159–68.
[11] Grewe M, Bruijnzeel-Koomen CA, Schopf E, Thepen T, Langeveld-Wilschut AG, Ruzicka T, et al. A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. Immunol Today 1998;19:359–61.
[12] Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. New Engl J Med 1992;326:298–304.
[13] Grewe M, Gyufko K, Schopf E, Krutmann J. Lesional expression of interferon-γ in atopic eczema. Lancet 1994;343:25–6.
[14] Werfel T, Morita A, Grewe M, Renz H, Wahn U, Krutmann J, et al. Allergen specificity of skin-infiltrating T cells is not restricted to a type-2 cytokine pattern in chronic skin lesions of atopic dermatitis. J Invest Dermatol 1996;107:871–6.
[15] Uchi H, Terao H, Koga T, Furue M. Cytokines and chemokines in the epidermis. J Dermatol Sci 2000;24(suppl 1):29–38.
[16] Van der Heijden FL, Wierenga EA, Bos JD, Kapsenberg ML. High frequency of IL-4-producing CD4+ allergen-specific T lymphocytes in atopic dermatitis lesional skin. J Invest Dermatol 1991;97:389–94.
[17] Sager N, Feldmann A, Schilling G, Kreitsch P, Neumann C. House dust mite-specific T cells in the skin of subjects with atopic dermatitis: frequency and lymphokine profile in the allergen patch test. J Allergy Clin Immunol 1992;89:801–10.
[18] Van Reijsen FC, Bruijnzeel-Koomen CAFM, Kalthoff FS, Maggi E, Romagnani S, Westland JKT, et al. Skin-derived aeroallergen-specific T cell clones of Th2 phenotype in patients with atopic dermatitis. J Allergy Clin Immunol 1992;90:184–92.
[19] Yamada N, Wakugawa M, Kuwata S, Yoshida T, Nakagawa H. Chronologic analysis of in situ cytokine expression in mite allergen-induced dermatitis in atopic subjects. J Allergy Clin Immunol 1995;96:1069–75.
[20] Ackermann L, Harvima T. Mast cells of psoriasis and atopic dermatitis skin are positive for TNF-α and their degranulation is associated with expression of ICAM-1 in the epidermis. Arch Dermatol Res 1998;290:353–9.
[21] Kakinuma T, Nakamura K, Wakugawa M, Mitsui H, Tada Y, Saeki H, et al. Thymus and activation-regulated chemokine in atopic dermatitis: serum thymus and activation-regulated chemokine level is closely related with disease activity. J Allergy Clin Immunol 2001;107(3):535–41.
[22] Vestergaard C, Kirstejn N, Gesser B, Mortensen JT, Matsushima K, Larsen CG. IL-10 augments the IFN-γ and TNF-α induced TARC production in HaCaT cells: a possible mechanism in the inflammatory reaction of atopic dermatitis. J Dermatol Sci 2001;26(1):46–54.
[23] Kumagai N, Fukuda K, Nishida T. Synergistic effect of TNF-a and IL-4 on the expression of thymus- and activation-regulated chemokine in human corneal fibroblasts. Biochem Biophys Res Commun 2000;279(1):1–5.
[24] Champbell JJ, Haraldsen G, Pan J, Rottman J, Qin S, Ponath P, Andrew DP, Warnke R, Ruffing N, Kassam N, Wu L, Butcher EC. The chemokine receptor CCR4 in

vascular recognition by cutaneous but not intestinal memory T cells. Nature 1999;40:776–80.
[25] Cohen LM, Skopicki DK, Harrist TJ, Clark WH, Jr. Noninfectious vesiculobullous and vesiculopustular diseases. In: Elder D, Elenitsas R, Jaworsky C, Johnson B, editors. Lever's histopathology of the skin, 8th ed. Philadelphia: Lippincott-Raven Publishers, 1997:209–15.

THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

# Cutting Edge: Profile of Chemokine Receptor Expression on Human Plasma Cells Accounts for Their Efficient Recruitment to Target Tissues[1]

*Takashi Nakayama,* Kunio Hieshima,* Dai Izawa,* Youichi Tatsumi,† Akihisa Kanamaru,† and Osamu Yoshie[2]**

*We systematically examined the repertoire of chemokine receptors expressed by human plasma cells. Fresh bone marrow plasma cells and myeloma cells consistently expressed CXCR4, CXCR6, CCR10, and CCR3. Accordingly, plasma cells responded to their respective ligands in chemotaxis and very late Ag-4-dependent cell adhesion to fibronectin. Immobilized CXC chemokine ligand (CXCL)16, a novel transmembrane-type chemokine and CXCR6 ligand, also directly induced adhesion of plasma cells without requiring $G_{\alpha i}$ signaling or divalent cations. Furthermore, we revealed consistent expression of CXCL12 (CXCR4 ligand), CXCL16 (CXCR6 ligand), and CC chemokine ligand 28 (CCR10 and CCR3 ligand) in tissues enriched with plasma cells including bone marrow, and constitutive expression of CXCL12, CXCL16, and CC chemokine ligand 28 by cultured human bone marrow stromal cells. Collectively, plasma cells are likely to be recruited to bone marrow and other target tissues via CXCR4, CXCR6, CCR10, and CCR3. CXCR6 may also contribute to tissue localization of plasma cells through its direct binding to membrane-anchored CXCL16. The Journal of Immunology,* 2003, 170: 1136–1140.

Chemokines play important roles in innate and acquired immunity by inducing directed migration of various types of leukocytes through interactions with a group of seven-transmembrane, G protein-coupled receptors (1). Accumulating evidence has demonstrated that chemokines and their receptors are crucial in trafficking and tissue microenvironmental localization of various lymphocyte classes and subsets (1). Thus, upon differentiation, maturation, and activation, cells of the lymphoid lineages dynamically change their expression profiles of chemokine receptors, leading to their specific migration programs to new sets of chemokines (1).

Plasma cells represent the end stage of B cell differentiation and function as the factories for Ab production. Plasma cells from immunized mice demonstrated up-regulation of CXCR4 and down-regulation of CXCR5 and CCR7 (2, 3). Furthermore, plasma cells in chimeric mice reconstituted with CXCR4-deficient fetal liver cells were mislocalized within the spleen, found in elevated numbers in the blood, and failed to accumulate in the bone marrow (2). Thus, CXCR4 and its ligand CXC chemokine ligand (CXCL)[3]12 play a major role in the localization of plasma cells within splenic red pulp and lymph node medullary cords as well as in the bone marrow (2). Furthermore, IgA-producing cells but not those producing IgG or IgM in mice express CCR9 and efficiently respond to its ligand CC chemokine ligand (CCL)25, which is selectively expressed by cryptic epithelial cells in the small intestine (4).

Recently, we have found that EBV-immortalized human B cells consistently up-regulate CCR6 and CCR10 and down-regulate CXCR4 and CXCR5 (5). We have further shown that the EBV-encoded latent proteins are responsible for up-regulation of CCR6 and down-regulation of CXCR4 (5). However, the up-regulation of CCR10, whose expression in normal B cells has not been reported so far (6), or the down-regulation of CXCR5 could not be explained by the effects of the EBV-encoded latent proteins (5). Because EBV-immortalized B cells resemble plasma cells, we speculated that their differentiation stages fixed by immortalization with EBV may be responsible for CCR10 up-regulation and CXCR5 down-regulation. Indeed, recent studies have consistently shown CXCR5 down-regulation in mouse plasma cells (2, 3). These considerations prompted us to examine the full repertoire of chemokine receptors expressed by human plasma cells. In this study, we report that human bone marrow plasma cells and myeloma cells selectively express CXCR4, CXCR6, CCR10, and CCR3, and that tissues known to be enriched with plasma cells as well as cultured human bone marrow stromal cells constitutively express CXCL12 (CXCR4 ligand) (7), CXCL16 (CXCR6 ligand) (8, 9), and CCL28 (CCR10 and CCR3 ligand) (10, 11).

Departments of *Microbiology and †Internal Medicine III, Kinki University School of Medicine, Osaka, Japan

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[2] Address correspondence to Dr. Osamu Yoshie, Department of Microbiology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan. E-mail address: o.yoshie@med.kindai.ac.jp

[3] Abbreviations used in this paper: CXCL, CXC chemokine ligand; CCL, CC chemokine ligand; $CX_3CL$, $CX_3C$ chemokine ligand; VLA-4, very late Ag-4; SEAP, secreted form of placental alkaline phosphatase.



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# Materials and Methods

*Cells and cytokines*

RPMI8226 (JCRB0034), KMS-12BM (JCRB0429), KMS-12PE (JCRB0430), and KHM-1B (JCRB0133) were human myeloma cell lines obtained from Health Science Research Resources Bank (Sennan, Osaka, Japan). Human bone marrow irradiated stromal cells were purchased from Takara Biomedicals (Kyoto, Japan) ($n = 2$). Peripheral blood samples were obtained from healthy adult donors ($n = 3$) and patients with multiple myeloma ($n = 3$). Bone marrow samples were obtained from adult donors ($n = 7$) and also purchased from Takara Biomedicals ($n = 5$). Informed consents were obtained from all donors. Mononuclear cells were isolated by centrifugation on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) and stored at −80°C until use. All human recombinant cytokines were purchased from PeproTech (Rocky Hill, NJ).

*RT-PCR*

This was conducted as described previously (5). cDNA samples from various human tissues were purchased from Clontech (Palo Alto, CA). Primers for the chemokine receptors and G3PDH were described previously (5). Primers for chemokines were as follows: +5′-CCCTCTGTGAGATCCGTCTTTG GCCT-3′ and −5′-TCTGATTGGAACCTGAACCCCTGCTG-3′ for CXCL12; +5′-CGTCACTGGAAGTTGTTATTGTGGT-3′ and −5′-TG GTAGGAAGTAAATGCTTCTGGTG-3′ for CXCL16; +5′-ACCACC TCTCACGCCAAAGCTCACAC-3′ and −5′-CGGCACAGATATCCTT GGCCAGTTTG-3′ for CCL11; +5′-CAACCTTCTGCAGCCTCCTG-3′ and −5′-CCATTTTCCTTAGCATCCCA-3′ for CCL27; and +5′-AGAAGCCATACTTCCCATTGC-3′ and −5′-AGCTTGCACTTTCAT CCACTG-3′ for CCL28. Real-time PCR was performed using TaqMan assay and 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Conditions for PCR included 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 s (denaturation) and 60°C for 1 min (annealing/extension). The primers for chemokines were as follows: +5′-CCATGCCGATTCTT CGAAAG-3′ and −5′-TTCAGCCGGGCTACAATCTG-3′ for CXCL12; +5′-CGCCATCGGTTCAGTTCAT-3′ and −5′-ACACACGCTCCAGG AAAGGA-3′ for CXCL16; and +5′-CAGAGAGGACTCGCCATCGT-3′ and −5′-TGTGAAACCTCCGTGCAACA-3′ for CCL28. The probes for chemokines were as follows: +5′-CATCTCAAAATTCTCAACACTCCAAACT GTGCC-3′ for CXCL12; +5′-ACCATCGGTGTCTATACTACAC GAGGTTCCAG-3′ for CXCL16; and +5′-CTTGGCTGTCTGTGCG GCCCTACAT-3′ for CCL28. The probes were labeled with reporter fluorescent dye 6-FAM at the 5′ end. Primers and fluorogenic probes for G3PDH were from TaqMan kit (Applied Biosystems). Quantification of chemokine expression was obtained using sequence detector system software (Applied Biosystems).

*Flow cytometric analysis*

The following murine mAbs were purchased from R&D Systems (Minneapolis, MN): anti-CXCR3 (clone 49801.111), anti-CXCR6 (clone 56811.11), anti-CCR3 (clone 444.11), anti-CCR6 (clone 53103.111), anti-CXCR4 (clone 44717.111), and anti-CXCR5 (clone 51505.111). Anti-CCR7 (2H7), PE-labeled anti-CD38 (HIT2), and Cy5-labeled CD45 (HI30) were purchased from BD Biosciences (Mountain View, CA). Rabbit anti-CCR10 was purchased from BIOCARTA (San Diego, CA). Isotype controls were purchased from DAKO (Kyoto, Japan). Cells were washed with PBS containing 2% FBS and reacted for 30 min with each mAb. After washing, cells were reacted with FITC-conjugated sheep $(F(ab')_2)$ anti-mouse IgG (Sigma-Aldrich, St. Louis, MO). In some experiments, cells were double stained with PE-labeled anti-CD38 and Cy5-labeled anti-CD45. For intracellular staining of CCR10, cells fixed and permeabilized with 2% paraformaldehyde and 0.1% Triton X-100 were indirectly stained with anti-CCR10 and FITC-labeled goat anti-rabbit IgG (Sigma-Aldrich). After staining, cells were analyzed on FACSCalibur (BD Biosciences) with appropriate gatings and quantitated in comparison with isotype control Abs. Dead cells were gated out by staining with propidium iodide.

*Chemotaxis assay*

All recombinant chemokines were purchased from R&D Systems. Migration assays for fresh human bone marrow mononuclear cells were conducted using Transwell plates with 8-$\mu$m pore size (Corning, Corning, NY) as described previously (5).

*Cell adhesion assays*

Cell adhesion to immobilized fibronectin was determined as described previously (12). The extracellular domain of human CXCL16/SR-PSOX (13) was subcloned into pDREF-SEAP $(His)_6$-Hyg expression vector (14), and CXCL16 fused at the C terminus with secreted form of placental alkaline phosphatase (SEAP), or CXCL16-SEAP, was generated by transfection to HEK293 cells. Cell adhesion to immobilized CXCL16 was determined essentially as described previously (14).

*ELISA*

Human bone marrow stromal cells were seeded in 24-well plates at a density of $1 \times 10^5$ cells/well and cultured without or with 10 ng/ml IL-1$\beta$. Measurement of CXCL12 and CCL28 in the culture supernatants was conducted using ELISA kits purchased from R&D Systems. For standardization of assay, serially diluted recombinant CXCL12 or CCL28 was included in each ELISA plate.

# Results

*Consistent expression of CXCR4, CXCR6, CCR10, and CCR3 in plasma cells and myeloma cells*

To gain an insight into the full repertoire of chemokine receptors expressed by human plasma cells, we first examined chemokine receptor expression in a panel of four human myeloma cell lines. RT-PCR analysis using specific primer sets for all known 18 chemokine receptors (CXCR1~6, CCR1~10, XCR1, and $CX_3CR1$) (1) revealed that the myeloma cell lines were consistently positive for CXCR4, CXCR6, CCR10, and CCR3. Staining of these myeloma cell lines with specific Abs for various chemokine receptors and flow cytometric analysis verified the RT-PCR results (data not shown).

After getting a consensus profile of chemokine receptor expression in human myeloma cell lines, we proceeded to examine the expression of selected chemokine receptors on human bone marrow plasma cells. Plasma cells in bone marrow mononuclear cells could be identified by the expression of high levels of CD38 on their surface (15, 16). We confirmed that $CD38^{high}$ cells sorted from bone marrow mononuclear cells had the typical plasma cell morphology (Fig. 1*a*). As shown in Fig. 1*a*, plasma cells in the bone marrow expressed CXCR4 and CXCR6 at high levels, CCR10 at intermediate levels, CCR3 at low levels, and CXCR5, CCR6, and CCR7 at marginal levels. We found no significant differences in the expression levels of these chemokine receptors between $CD38^{high}CD45^{+}$ immature and $CD38^{high}CD45^{-}$ mature plasma cells (15, 16) (data not shown).

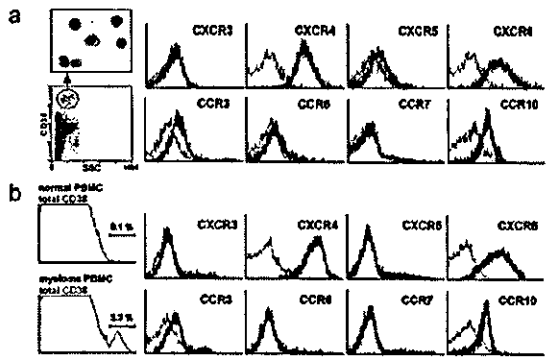


**FIGURE 1.** Flow cytometric analysis for expression of chemokine receptors in fresh plasma cells and myeloma cells. *a*, Bone marrow mononuclear cells obtained from adult donors were triple stained for CD38, CD45, and indicated chemokine receptors, and analyzed by flow cytometry. Representative results from three donors are shown. May-Giemsa staining of $CD38^{high}$ cells sorted from bone marrow mononuclear cells was also shown. *b*, PBMC obtained from healthy adult donors and myeloma patients were double stained for CD38 and indicated chemokine receptors, and analyzed by flow cytometry. Representative results from three patients with multiple myeloma are shown.