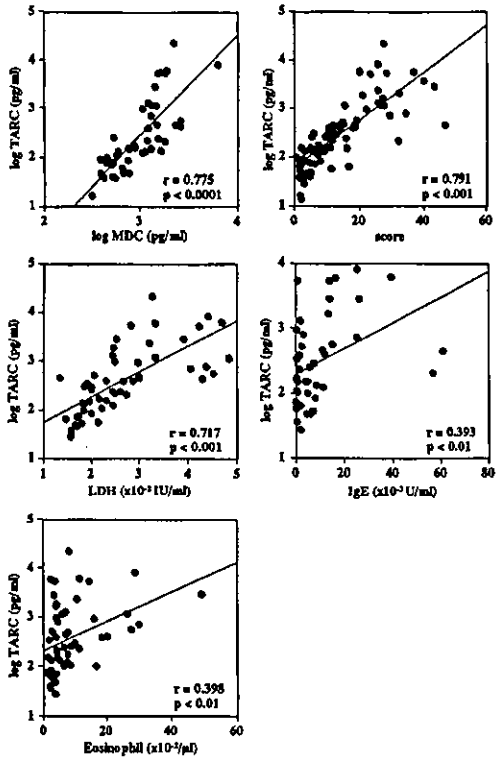

Fig. 2. Correlation analysis. Correlations of plasma TARC levels with plasma MDC levels, EASI scores, serum LDH levels, serum total IgE and blood eosinophil counts were analyzed for the AD patients.

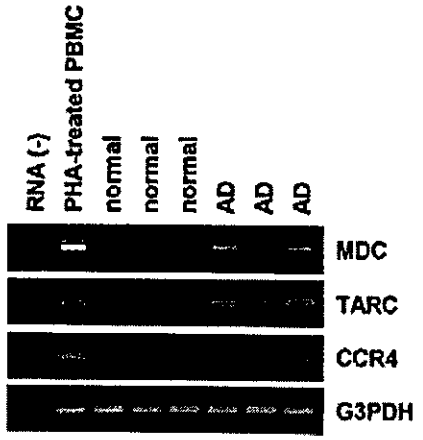

Fig. 3. RT-PCR analysis on expression of TARC, MDC and CCR4 in human skin tissues. Total RNA samples were prepared from peripheral blood mononuclear cells treated with phytohemagglutinin for 24 h (PHA-treated PBMC), skin biopsies of three normal donors (normal) and those from three atopic patients (AD). RT-PCR analysis was carried out for MDC, TARC, CCR4 and G3PDH as described in Methods.

Furthermore, we observed MDC immunoreactivity in epidermal keratinocytes (Fig. 4E and F), which was even stronger and more widely distributed in the epidermal layers than TARC immunoreactivity. We also observed MDC$^+$ cells in the dermis of AD lesional skin (Fig. 4G), which were likely to be mostly dendritic cells as reported previously (6,21). On the other hand, we hardly observed immunoreactivity for TARC and MDC in normal skin (n = 3) (not shown) or psoriatic skin (n = 3) (Fig. 4D and H).

Inducible co-expression of TARC and MDC in primary human epidermal keratinocytes in vitro

Positive TARC and MDC immunoreactivity in epidermal keratinocytes in AD skin lesions led us to examine expression of TARC and MDC by primary human epidermal keratinocytes *in vitro*. As shown in Fig. 5, TARC and MDC were both induced in primary epidermal keratinocytes primarily by IFN-γ. MDC was also weakly induced by the combination of IL-1α and TNF-α. On the other hand, IL-4, the major T_h2-type cytokine, was totally incapable of inducing TARC or MDC alone or in combination with other cytokines. As shown in Fig. 6(A), MDC was dose-dependently secreted from primary keratinocytes upon treatment with IFN-γ. By using the same culture supernatants, however, we hardly detected TARC (<0.6 pg/ml). We also examined production of TARC and MDC by a human

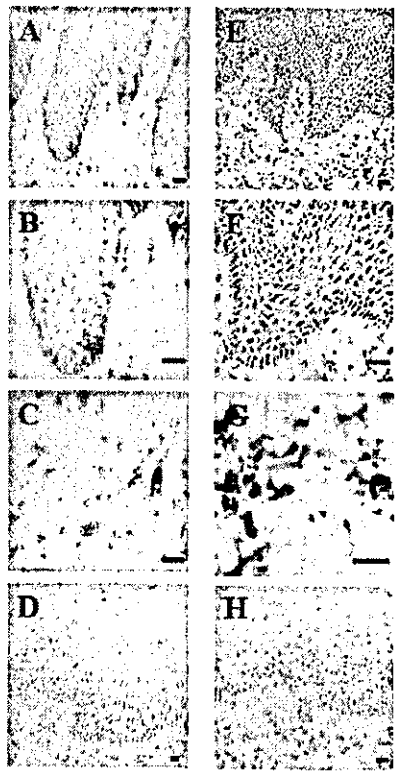

Fig. 4. Immunohistochemical staining of TARC and MDC in lesional skin of AD and psoriasis. Indirect staining of TARC and MDC with monoclonal anti-TARC and anti-MDC antibodies was carried out as described in Methods. (A–D) Staining with anti-TARC; (E–H) staining with anti-MDC. (A–C and E–G) AD skin; (D and H) psoriasis skin. Horizontal bars indicate 50 μm (A–F and H) or 20 μm (G).

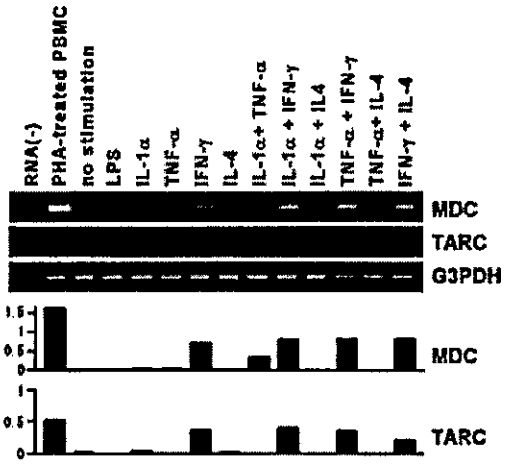

Fig. 5. Induction of TARC and MDC in primary epidermal keratinocytes by IFN-γ. Human primary epidermal keratinocytes were mock-treated or treated with LPS at 30 ng/ml, IL-1α at 10 ng/ml, TNF-α at 50 ng/ml, IFN-γ at 100 ng/ml and IL-4 at 100 ng/ml, alone or in combinations as indicated, for 24 h. Total RNA was prepared and RT-PCR was carried out as described in Methods. Representative results from three separate experiments are shown. The lower panel shows the signal intensity ratio between each chemokine and G3PDH.

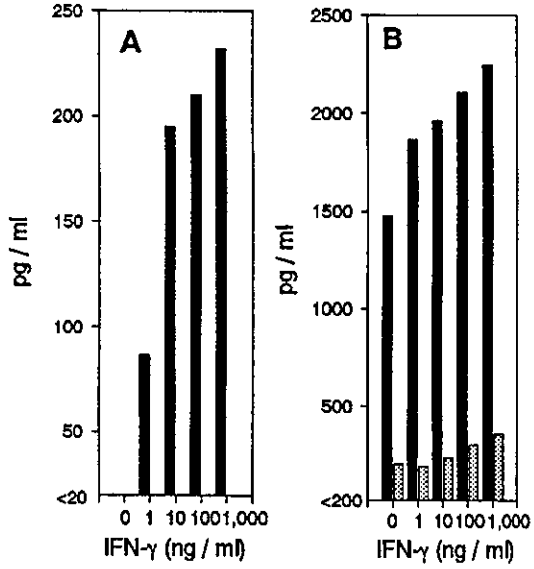

Fig. 6. Secretion of MDC and TARC by epidermal keratinocytes. Primary human epidermal keratinocytes (A) and an immortalized human epidermal keratinocytic cell line HaCaT (B) were cultured in six-well plates to confluency and mock-treated or treated with indicated concentrations of IFN-γ for 24 h. The contents of MDC (filled bars) and TARC (dotted bars) in the culture supernatants were determined with ELISA. Representative results from two separate experiments are shown.

keratinocytic cell line HaCaT. As shown in Fig. 6(B), HaCaT constitutively produced MDC and its production was moderately augmented by IFN-γ. HaCaT also constitutively produced TARC, but at a level much lower than that of MDC and again its production was moderately elevated by IFN-γ.

In vivo induction of TARC and MDC in mouse skin by IFN-γ

To test whether IFN-γ was also capable of inducing TARC and MDC *in vivo*, we injected IFN-γ into mouse skin, and examined tissue expression of TARC and MDC by RT-PCR. As shown in Fig. 7, a low level expression of TARC, but not MDC, was seen in control mouse skin tissues. Upon injection with IFN-γ, both TARC and MDC were strongly induced with a peak at 6 h.

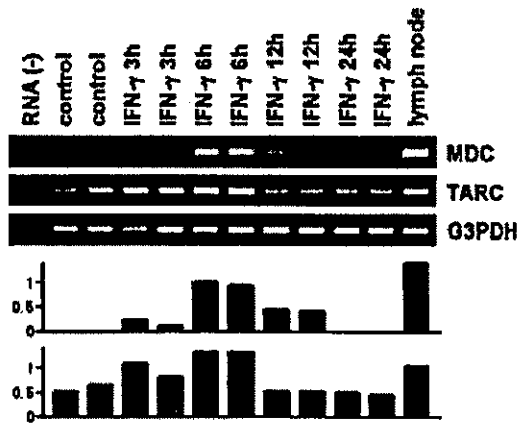

Fig. 7. Induction of MDC and TARC in mouse skin by IFN-γ. BALB/c mice were intradermally injected with 100 μl of PBS alone or containing 10 ng of murine IFN-γ and skin tissues at injected sites were obtained at indicated time points. Total RNA was prepared and RT-PCR analysis was carried out as described in Methods. Representative results from two separate experiments are shown. The lower panel shows the signal intensity ratio between each chemokine and G3PDH: top, HPC; bottom, TARC.

Discussion

Previously, highly elevated 'serum' levels of TARC and MDC were reported in AD patients (9,10). However, chemokine contents in serum samples could be quite different from those in the circulating blood, since (i) some chemokines are known to be actively released from platelets during clotting (14,15), (ii) many chemokines are also passively released from DARC on red blood cells during clotting (17) and (iii) some chemokines are adsorbed to newly formed blood clots (18). In fact, we have recently demonstrated that platelets contain a substantial amount of TARC and release it upon coagulation (Fujisawa *et al.*, submitted). Therefore, in the present study, we re-evaluated circulating levels of TARC and MDC in normal and AD subjects by using 'plasma' samples. We have shown for the first time that plasma levels of TARC and MDC are significantly elevated in AD patients and correlate well with disease severity and other clinical parameters known to be elevated in AD patients (Figs 1 and 2). Thus, plasma levels of TARC and MDC are quite useful for clinical evaluation of AD patients. These results also strongly support that TARC and MDC play important roles in AD pathogenesis.

By RT-PCR analysis, we showed that signals of TARC, MDC and their shared receptor CCR4 were strongly up-regulated in AD lesional skin tissues (Fig. 3). This supports that local production of TARC and MDC in AD skin lesions promoted infiltration of T cells expressing CCR4, which are known to be mostly T_h2-polarized memory T cells and/or those expressing cutaneous lymphocyte antigen (5,22). Recently, Nakatani *et al.* as well as Wakugawa *et al.* indeed demonstrated prominent dermal infiltration of CCR4+ memory T cells in AD lesional skin tissues by immunohistochemical staining (11,12). Previously, Vestergaard *et al.* also demonstrated TARC immunoreactivity in the epidermal basal layers of AD skin lesions (13). They did not, however, observe TARC immunoreactivity in any other cells in the dermis including vascular endothelial cells. On the other hand, Kakinuma *et al.* detected TARC immunoreactivity in epidermal keratinocytes, dermal vascular endothelial cells, infiltrating T cells and dermal dendritic cells in AD skin lesions (10). In the case of MDC, Galli *et al.* reported MDC-immunoreactivity in dermal-infiltrating cells in AD skin, which were identified as T cells and dendritic cells by double-staining techniques (9). They did not, however, mention MDC immunoreactivity in epidermal keratinocytes. In the present study, we showed by immunohistochemical staining using specific mAb that not only TARC but also MDC was produced in the epidermal layer of AD skin lesions (Fig. 4). We also detected TARC immunoreactivity in vascular endothelial cells, while that of MDC in dermal dendritic cells (Fig. 4). Thus, our results on TARC immunoreactivity were very similar to those by Kakinuma *et al.* (10). Furthermore, the present paper is the first to demonstrate a prominent MDC production by epidermal keratinocytes in AD lesional skin. The discrepancies among previous and present studies concerning the staining patterns of TARC and MDC in AD lesional skin may be due to different staining conditions, including use of different antibodies. We did not observe any immunoreactivity for TARC and MDC in normal skin as reported previously (9,10,13) or in psoriasis skin (Fig. 4).

To prove the intrinsic capability of epidermal keratinocytes to produce MDC as well as TARC, we first examined expression of MDC and TARC mRNA in primary epidermal keratinocytes. We demonstrated that not only TARC mRNA, but also that of MDC was induced in primary epidermal keratinocytes upon treatment with IFN-γ (Fig. 5). We also demonstrated *in vivo* induction of MDC and TARC mRNA in mouse skin upon local injection of IFN-γ (Fig. 7). Accordingly, primary epidermal keratinocytes and an immortalized human epidermal keratinocytic cell line HaCaT efficiently secreted MDC upon treatment with IFN-γ (Fig. 6). This is the first report to show the ability of epidermal keratinocytes to produce MDC. Unexpectedly, however, we hardly detected secretion of TARC by IFN-γ-stimulated primary epidermal keratinocytes (Fig. 6). We were also unable to detect TARC protein in IFN-γ-stimulated primary epidermal keratinocytes by immunofluorescent staining (not shown). Previously, Vestergaard *et al.* reported vigorous secretion of TARC by a human keratinocytic cell line HaCaT upon stimulation with cytokines such as IFN-γ and TNF-α (13). We found that HaCaT indeed secreted TARC upon treatment with IFN-γ, but at levels much lower that those of MDC (Fig. 6). Since both TARC and MDC transcripts were induced at more or less similar levels in primary keratinocytes and HaCaT upon stimulation with IFN-γ, production of TARC protein by epidermal keratinocytes may require further conditions such as particular stages of differentiation and/or some other signals from the local milieu. Consistently, TARC immunoreactivity in epidermal keratinocytes in AD skin lesions was much more restricted than that of MDC (Fig. 4).

It is notable that IFN-γ (the T_h1-type cytokine), but not IL-4 (the T_h2-type cytokine), induces epidermal keratinocytes to express MDC and TARC, which are the pair of T_h2-type chemokines acting on CCR4 (Fig. 5). IFN-γ is also the potent inducer of the trio of T_h1-type CXC chemokines Mig/CXCL9, IP-10/CXCL10 and I-TAC/CXCL11 in various types of cells including human keratinocytes (23). Mig, IP-10 and I-TAC commonly act on CXCR3 and selectively attract T_h1 cells (3). Previous studies on the cytokine pattern of AD skin lesions have demonstrated that a T_h2 cytokine profile (IL-4, IL-5 and IL-13) is predominant during the initial phase of skin inflammation, but both T_h2 and T_h1 cytokines (IL-5 and IFN-γ) are up-regulated in chronic lesions (24). Even though most AD patients had undetectable levels of IFN-γ in their plasma samples as control subjects (<8 pg/ml, data not shown), IFN-γ may be the key factor in the chronic phase of AD because of its unique ability to simultaneously induce both T_h1- and T_h2-attracting chemokines from epidermal keratinocytes in AD skin. Indeed, the critical role of IFN-γ in AD pathogenesis has been amply demonstrated (24). Our findings that IFN-γ is a potent inducer of MDC in epidermal keratinocytes may now provide a new clue for its pivotal role in the AD pathogenesis.

Acknowledgements

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Abbreviations

AD	atopic dermatitis
EASI	eczema area and severity index
LDH	lactate dehydrogenase
MDC	macrophage-derived chemokine
TARC	thymus and activation-regulated chemokine
TNF	tumor necrosis factor

References

1 Beltrani, V. S. 1999. The clinical spectrum of atopic dermatitis. *J. Allergy Clin. Immunol.* 104:87.
2 Leung, D. Y. 1999. Pathogenesis of atopic dermatitis. *J. Allergy Clin. Immunol.* 104:99.
3 Yoshie, O., Imai, T. and Nomiyama, H. 2001. Chemokines in immunity. *Adv. Immunol.* 78:57.
4 Nakayama, T., Fujisawa, R., Yamada, H., Horikawa, T., Kawasaki, H., Hieshima, K., Izawa, D., Fujiie, S., Tezuka, T. and Yoshie, O. 2001. Inducible expression of a CC chemokine liver- and activation-regulated chemokine (LARC)/macrophage inflammatory protein (MIP)-3α/CCL20 by epidermal keratinocytes and its role in atopic dermatitis. *Int. Immunol.* 13:95.
5 Imai, T., Nagira, M., Takagi, S., Kakizaki, M., Nishimura, M., Wang, J., Gray, P. W., Matsushima, K. and Yoshie, O. 1999. Selective recruitment of CCR4-bearing T_h2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int. Immunol.* 11:81.

6 Vestergaard, C., Yoneyama, H., Murai, M., Nakamura, K., Tamaki, K., Terashima, Y., Imai, T., Yoshie, O., Irimura, T., Mizutani, H. and Matsushima, K. 1999. Overproduction of T_h2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. *J. Clin. Invest.* 104:1097.

7 Gonzalo, J. A., Pan, Y., Lloyd, C. M., Jia, G. Q., Yu, G., Dussault, B., Powers, C. A., Proudfoot, A. E., Coyle, A. J., Gearing, D. and Gutierrez-Ramos, J. C. 1999. Mouse monocyte-derived chemokine is involved in airway hyperreactivity and lung inflammation. *J. Immunol.* 163:403.

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

9 Galli, G., Chantry, D., Annunziato, F., Romagnani, P., Cosmi, L., Lazzeri, E., Manetti, R., Maggi, E., Gray, P. W. and Romagnani, S. 2000. Macrophage-derived chemokine production by activated human T cells *in vitro* and *in vivo*: preferential association with the production of type 2 cytokines. *Eur. J. Immunol.* 30:204.

10 Kakinuma, T., Nakamura, K., Wakugawa, M., Mitsui, H., Tada, Y., Saeki, H., Torii, H., Asahina, A., Onai, N., Matsushima, K. and Tamaki, K. 2001. 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.* 107:535.

11 Nakatani, T., Kaburagi, Y., Shimada, Y., Inaoki, M., Takehara, K., Mukaida, N. and Sato, S. 2001. CCR4 memory $CD4^+$ T lymphocytes are increased in peripheral blood and lesional skin from patients with atopic dermatitis. *J. Allergy Clin. Immunol.* 107:353.

12 Wakugawa, M., Nakamura, K., Kakinuma, T., Onai, N., Matsushima, K. and Tamaki, K. 2001. CC chemokine receptor 4 expression on peripheral blood $CD4^+$ T cells reflects disease activity of atopic dermatitis. *J. Invest. Dermatol.* 117:188.

13 Vestergaard, C., Bang, K., Gesser, B., Yoneyama, H., Matsushima, K. and Larsen, C. G. 2000. A T_h2 chemokine, TARC, produced by keratinocytes may recruit CLA^+CCR4^+ lymphocytes into lesional atopic dermatitis skin. *J. Invest. Dermatol.* 115:640.

14 Kameyoshi, Y., Dorschner, A., Mallet, A. I., Christophers, E. and Schroder, J. M. 1992. Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J. Exp. Med.* 176:587.

15 Klinger, M. H., Wilhelm, D., Bubel, S., Sticherling, M., Schroder, J. M. and Kuhnel, W. 1995. Immunocytochemical localization of the chemokines RANTES and MIP-1α within human platelets and their release during storage. *Int. Arch. Allergy Immunol.* 107:541.

16 Hadley, T. J. and Peiper, S. C. 1997. From malaria to chemokine receptor: the emerging physiologic role of the Duffy blood group antigen. *Blood* 89:3077.

17 Morita, A., Shimosako, K., Kikuoka, S., Taniguchi, Y., Kitaura, M., Sasakura, K., Tamaki, M., Tsuji, T., Teraoka, H., Yoshie, O., Nakajima, T. and Hirai, K. 1999. Development of a sensitive enzyme-linked immunosorbent assay for eotaxin and measurement of its levels in human blood. *J. Immunol. Methods* 226:159.

18 Nomiyama, H., Hieshima, K., Nakayama, T., Sakaguchi, T., Fujisawa, R., Tanase, S., Nishiura, H., Matsuno, K., Takamori, H., Tabira, Y., Yamamoto, T., Miura, R. and Yoshie, O. 2001. Human CC chemokine liver-expressed chemokine/CCL16 is a functional ligand for CCR1, CCR2 and CCR5, and constitutively expressed by hepatocytes. *Int. Immunol.* 13:1021.

19 Hanifin, J. M. and Rajka, G. 1980. Diagnostic features of atopic dermatitis. *Acta Dermatol. Venereol.* 92:44.

20 Hanifin, J. M., Thurston, M., Omoto, M., Cherill, R., Tofte, S. J. and Graeber, M. 2001. The eczema area and severity index (EASI): assessment of reliability in atopic dermatitis. EASI Evaluator Group. *Exp. Dermatol.* 10:11.

21 Katou, F., Ohtani, H., Nakayama, T., Ono, K., Matsushima, K., Saaristo, A., Nagura, H., Yoshie, O. and Motegi, K. 2001. Macrophage-derived chemokine (MDC/CCL22) and CCR4 are involved in the formation of T lymphocyte-dendritic cell clusters in human inflamed skin and secondary lymphoid tissue. *Am. J. Pathol.* 158:1263.

22 Campbell, J. J., Haraldsen, G., Pan, J., Rottman, J., Qin, S., Ponath, P., Andrew, D. P., Warnke, R., Ruffing, N., Kassam, N., Wu, L. and Butcher, E. C. 1999. The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. *Nature* 400:776.

23 Tensen, C. P., Flier, J., Van Der Raaij-Helmer, E. M., Sampat-Sardjoepersad, S., Van Der Schors, R. C., Leurs, R., Scheper, R. J., Boorsma, D. M. and Willemze, R. 1999. Human IP-9: A keratinocyte-derived high affinity CXC-chemokine ligand for the IP-10/Mig receptor (CXCR3). *J. Invest. Dermatol.* 112:716.

24 Grewe, M., Bruijnzeel-Koomen, C. A., Schopf, E., Thepen, T., Langeveld-Wildschut, A. G., Ruzicka, T. and Krutmann, J. 1998. A role for T_h1 and T_h2 cells in the immunopathogenesis of atopic dermatitis. *Immunol. Today* 19:359.

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IL-4, BUT NOT IL-13, MODULATES TARC (THYMUS AND ACTIVATION-REGULATED CHEMOKINE)/CCL17 AND IP-10 (INTERFERON-INDUCED PROTEIN OF 10 kDa)/CXCL10 RELEASE BY TNF-α AND IFN-γ IN HaCaT CELL LINE

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It is known that both interleukin-4 (IL-4) and IL-13 are produced by Th2-type cells and share similar biological functions with each other. However, recently accumulated evidences have revealed that IL-4 may be involved in the Th1-type response. Both thymus and activation-regulated chemokine (TARC/CCL17), a ligand for CC chemokine receptor 4 that is mainly expressed on Th2-type cells, and interferon-induced protein of 10 kDa (IP-10/CXCL10), a ligand for CXC chemokine receptor 3 that is mainly expressed on Th1-type cells, are produced by keratinocytes after the stimulation with the primary cytokines such as tumor necrotic factor-α (TNF-α) and/or interferon-γ (IFN-γ). In this study, we investigated the regulation of TARC or IP-10 production from HaCaT cells, an immortalized human keratinocyte cell line, after stimulation with TNF-α, IFN-γ, IL-4 and/or IL-13. Without stimulation, HaCaT cells did not produce TARC. When both TNF-α and IFN-γ were added, they increased synergistically ($P<0.003$). In addition, when HaCaT cells were stimulated with IL-4, but not IL-13, in combination with TNF-α and IFN-γ, the supernatant TARC levels significantly decreased compared to those with both TNF-α and IFN-γ ($P<0.009$). This inhibition was completely abolished with the addition of neutralizing anti-IL-4 antibody. The supernatant IP-10 levels also increased synergistically by stimulation with TNF-α and IFN-γ for 24 h ($P<0.001$). When IL-4, but not IL-13, was added to the medium and the cells were co-cultured with these cytokines, the IP-10 levels significantly increased compared to those with both TNF-α and IFN-γ ($P<0.04$). Furthermore, the effects of IL-4 on TARC and IP-10 production in these cells were detected in a dose-dependent manner. These data strongly suggest that IL-4 may act not only as a mediator of Th1-type response but also as a down-regulator of Th2-type response in terms of the regulation of chemokine production by HaCaT cells.

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Chemokines are a large and growing family of 6- to 14-kDa heparin-binding proteins. According to NH2-terminal cysteine-motifs, they are divided into the C, CC, CXC and CX3C subfamilies.[1] These chemokines possess the corresponding receptors respectively, and regulate leukocyte migration and activation through binding to the transmembrane receptors differentially expressed on lymphocyte subsets in the inflammatory sites.[2,3]

Thymus and activation-regulated chemokine (TARC/CCL17) is a functional ligand for CC chemokine receptor 4 (CCR4).[4] Because CCR4 is predominantly expressed on Th2-type cells,[5] it is suggested that TARC is involved in Th2-polarized diseases. Indeed, we recently revealed that the serum TARC levels significantly correlated the disease activity of atopic dermatitis (AD), characterized as a Th2-dominant

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disease, and the strong immunoreactivity of TARC was observed in lesional keratinocytes (KCs) of patients with AD.[6] Interferon-induced protein of 10 kDa (IP-10/CXCL10) is a ligand for CXC chemokine receptor 3 (CXCR3).[7] In contrast to CCR4, CXCR3 is preferentially expressed on Th1-type cells,[7] supporting the previous report that IP-10 was highly detected in lesional skin of psoriasis vulgaris (PsV), characterized as a Th1-dominant disease.[8] Thus, although TARC and IP-10 have divergent effects in terms of Th1/Th2-polarized situation, both chemokines are important for inflammatory skin diseases such as AD and PsV. In vitro, both TARC and IP-10 are secreted from human KCs after stimulation with tumor necrotic factor-α (TNF-α) and interferon-γ (IFN-γ).[9,10] As TARC and IP-10 have distinct roles for the Th1/Th2-type response, it is of interest and importance to investigate the regulation of TARC and IP-10 by various cytokines.

Interleukin-4 (IL-4) and IL-13 share about 20–25% homology with each other,[11] are produced primarily by Th2-type cells and have similar functions such as production of immunoglobulin E from B cells.[12,13] It is reported that both IL-4 and IL-13 can cross-compete for IL-4Rα, but that only IL-4 binds directly to this receptor chain.[14] IL-13 binds to its own primary binding chain (IL-13Rα1) to which IL-4 does not bind, and recruits IL-4Rα into a receptor complex resulting in an increase in binding affinity and the initiation of signal transduction.[14] Furthermore, IL-13 binds to another IL-13 receptor, IL-13Rα2.[15] In contrast to IL-4, IL-13 specifically binds to IL-13Rα2 with a high affinity and co-expression with IL-4Rα not increasing the IL-13 binding.[15] Recent reports revealed that IL-4 up-regulated the production of IP-10/CXCL10,[10] suggesting that IL-4 secreted by Th2-type cells could have an effect on Th1-type inflammatory response.

To elucidate the effect on Th1/Th2-balance by IL-4 and IL-13 in vitro, we used a HaCaT cell line, established as an immortalized human KC cell line, in this study.[16] We investigated the secretion of TARC and IP-10 from HaCaT cells and the effect of IL-4 and IL-13 on these secretions.

RESULTS

TNF-α and IFN-γ enhanced TARC production from HaCaT cells

First, we tested the TARC and IP-10 production by HaCaT cells with the stimulation of different concentrations of TNF-α IFN-γ. When HaCaT cells were co-cultured with 100 ng/ml of TNF-α and IFN-γ, maximal up-regulation of TARC and IP-10 production from HaCaT cells were observed (data not shown). Without stimulation, the supernatant TARC levels were 28.0 ± 4.2 pg/ml after 24 h culture. When HaCaT cells were co-cultured with TNF-α (100 ng/ml) or IFN-γ (100 ng/ml) for 24 h, the supernatant TARC levels slightly increased to 78.5 ± 15.3 pg/ml and 100.8 ± 20.0 pg/ml, respectively. When HaCaT cells were co-cultured with both TNF-α and IFN-γ for 24 h, they increased synergistically to 1225.3 ± 102.4 pg/ml ($P<0.002$), which is consistent with previous report.[9]

IL-4, but not IL-13, inhibited TARC production from HaCaT cells

The HaCaT cells were then stimulated with IL-4 and/or IL-13 in combination with both TNF-α and IFN-γ for 24 h. The supernatant TARC levels were 330.0 ± 26.4 pg/ml when stimulated with combined stimulation with TNF-α, IFN-γ and IL-4, which was significantly lower compared to those with TNF-α and IFN-γ (1225.3 ± 104.2 pg/ml, $P<0.009$). Moreover, this inhibitory effect of IL-4 on TARC production was completely abolished when HaCaT cells were co-cultured with TNF-α, IFN-γ, IL-4 and neutralizing anti-IL-4 monoclonal antibody (mAb) (1200.8 ± 135.0 pg/ml). In contrast, no significant difference was detected when IL-13 was added in the medium and HaCaT cells were cultured with TNF-α, IFN-γ and IL-13 for 24 h. Furthermore, the supernatant TARC levels showed basal levels when HaCaT cells were cultured with IL-4 alone (35.0 ± 3.9 pg/ml) or IL-13 alone (32.8 ± 5.0 pg/ml). These results are summarized in Figure 1.

When HaCaT cells were stimulated with either TNF-α or IFN-γ in addition to IL-4 for 24 h, there was no significant difference compared to those with TNF-α and IFN-γ (data not shown).

IP-10 production from HaCaT cells was enhanced by TNF-α, IFN-γ and IL-4

We also examined the effects of IL-4 or IL-13 on IP-10 production from HaCaT cells co-stimulated with TNF-α and IFN-γ. The supernatant IP-10 levels were 17.6 ± 5.0 pg/ml without stimulation. When the HaCaT cells were stimulated with TNF-α alone, the IP-10 levels were 26.8 ± 11.2 pg/ml. However, when HaCaT cells were stimulated with IFN-γ, the supernatant IP-10 levels were 140.9 ± 21.5 pg/ml, which was significantly higher than those without stimulation ($P<0.01$). When HaCaT cells were cultured with both TNF-α and IFN-γ, they increased synergistically to 1750.0 ± 325.8 pg/ml ($P<0.001$). Moreover, when HaCaT cells were co-cultured with IL-4 in combination with TNF-α and IFN-γ, the supernatant IP-10 levels were 2212.5 ± 550.3 pg/ml, which was significantly higher compared to those with TNF-α and IFN-γ ($P<0.04$). When anti-IL-4 mAb was added in

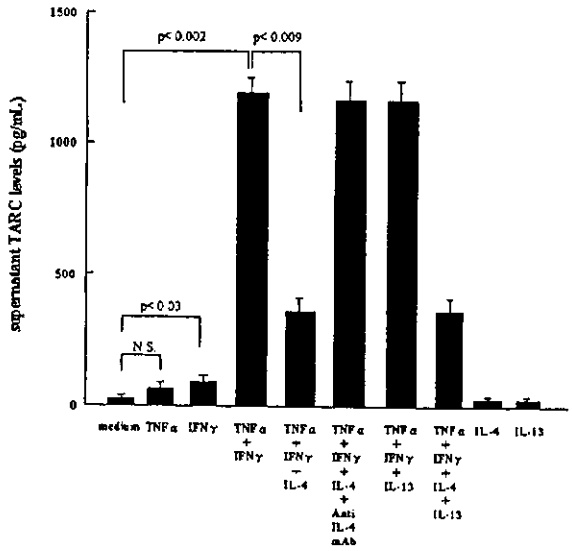

Figure 1. The supernatant TARC levels after stimulation with various cytokines (n=3).

The supernatant TARC levels increased when HaCaT cells were co-cultured with TNF-α (100 ng/ml) and IFN-γ (100 ng/ml) for 24 h. This increased TARC levels were inhibited by IL-4 in combination with TNF-α and IFN-γ. This inhibition by IL-4 was completely blocked when HaCaT cells were co-cultured with TNF-α, IFN-γ, IL-4 and neutralizing anti-IL-4 mAb. In contrast, there was no significant difference of TARC production from HaCaT cells when stimulated with IL-13. Each concentration of the cytokines was as follows; TNF-α: 100 ng/ml, IFN-γ: 100 ng/ml, IL-4: 10 ng/ml, IL-13: 10 ng/ml, Anti-IL-4 mAb: 1 μg/ml.

the culture medium and HaCaT cells were co-cultured with IL-4, anti-IL-4 mAb, TNF-α and IFN-γ for 24 h, the supernatant IP-10 levels restored to 1705.6 ± 380.0 pg/ml. There was no significant difference of the supernatant IP-10 levels by HaCaT cells when HaCaT cells were co-cultured with IL-13 alone or in combination with TNF-α and IFN-γ (Fig. 2).

IL-4 influenced the production of TARC and IP-10 in a dose-dependent manner

As IL-4 inhibited the TARC production and enhanced the IP-10 production from HaCaT cells co-stimulated with TNF-α and IFN-γ, we next investigated the supernatant TARC and IP-10 levels after stimulation with different concentration of IL-4 in combination with TNF-α and IFN-γ. When HaCaT cells were co-stimulated with different concentration of IL-4 (0.01–100 ng/ml), the supernatant TARC levels were detected from 1054.2 ± 85.4 pg/ml to 314.5 ± 29.8 pg/ml. The inhibitory effect of IL-4 on the TARC levels was in a dose-dependent manner. In contrast, when HaCaT cells were co-stimulated with different concentration of IL-4 (0.01–100 ng/ml), the supernatant IP-10 levels were detected from 1830.1 ± 118.5 pg/ml to 2435.0 ± 301.9 pg/ml. Thus,

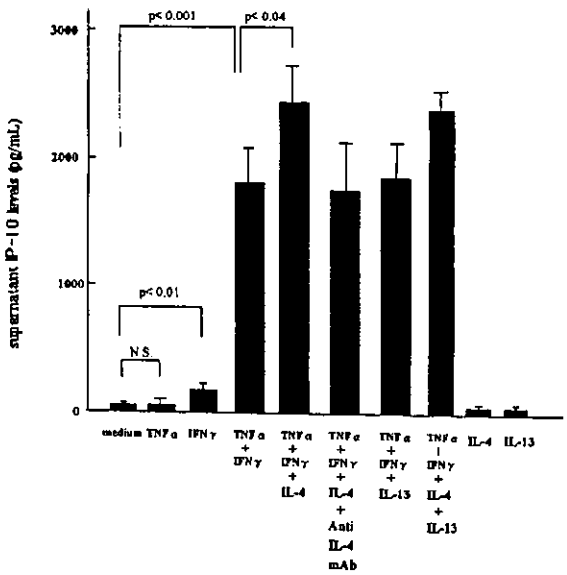

Figure 2. The supernatant IP-10 levels after stimulation with the various cytokines (n=3).

The supernatant IP-10 levels were also up-regulated by TNF-α and IFN-γ, and the increased IP-10 production was augmented by IL-4. In contrast, there was no significant difference of IP-10 production from HaCaT cells when stimulated with IL-13. Each concentration of the cytokines was as follows; TNF-α: 100 ng/ml, IFN-γ: 100 ng/ml, IL-4: 10 ng/ml, IL-13: 10 ng/ml.

the enhancement of IL-4 on the IP-10 levels by HaCaT cells were detected in a dose-dependent manner (Fig. 3). IL-13 did not significantly modulate the TARC or IP-10 production when HaCaT cells were co-stimulated with both TNF-α and IFN-γ (data not shown).

DISCUSSION

In this study, we examined the production of TARC and IP-10 from HaCaT cells and the obtained results were as follows: (1) the production of TARC and IP-10 significantly increased by stimulation with TNF-α and IFN-γ, (2) IL-4 inhibited the TNF-α and IFN-γ-induced TARC production and enhanced the induced IP-10 production from HaCaT cells, (3) IL-13 did not influence the TARC and IP-10 production from HaCaT cells under the same conditions, and (4) the modulation of TARC and IP-10 production from HaCaT cells by IL-4 was seen in a dose-dependent manner.

IL-4 and IL-13 are considered to be produced by Th2-type cells,[12,13,17] and both cytokines are overexpressed in Th2-dominant diseases such as AD[18] and bronchial asthma.[19] However, in contrast to these data, there has recently been some interesting evidence that IL-4 is involved in the Th1-type responses as

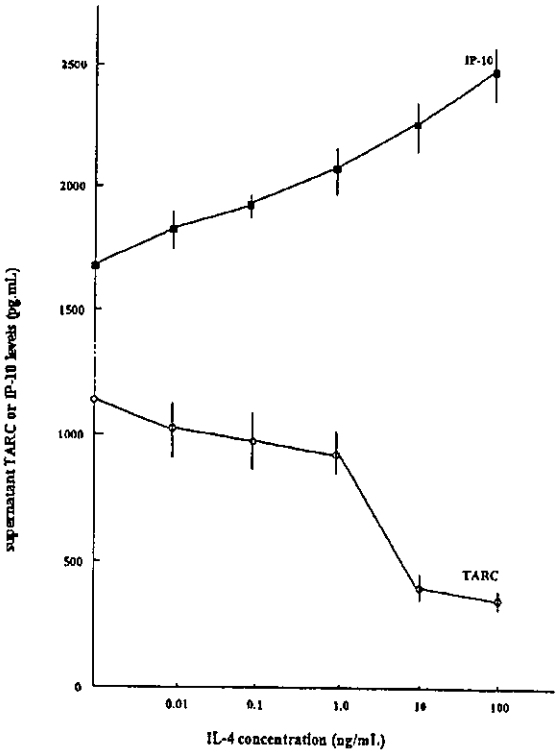

Figure 3. The response of TNF-α (100 ng/ml) and IFN-γ (100 ng/ml)-induced TARC or IP-10 production from HaCaT cells at each concentration for IL-4 (n=3).

The TARC production by IL-4 decreased in a dose-dependent manner. In contrast, the supernatant IL-10 levels increased in a dose-dependent manner.

follows; (1) IL-4-treated rats in the Th1-type autoimmune uveoretinitis showed an exacerabation of general symptoms and IL-4 treatment augmented IFN-γ production.[20] (2) In an antigen-induced arthritis mouse model, the neutralizing anti-IL-4 mAb contributed to control the disease, suggesting that IL-4 may be important for the Th1-type inflammatory response.[21] (3) IL-4 up-regulated the secretion of IP-10/CXCL10, a ligand for CXCR3 that is preferentially expressed on Th1-type cells, from human KCs.[10] (4) IL-4 treatment exacerbated the symptoms of the T cell transfer model of colitis, characterized as Th1-dominant disease.[22]

TARC is a member of the CC chemokines and has an important role for the homing of $CCR4^+CLA^+$ T cells in inflamed skin.[23] Very recently, we showed that TARC was strongly expressed in the lesional KCs of patients with AD, characterized as a Th2-dominant disease, whereas it was weakly expressed in those of patients with psoriasis.[6] Moreover, the serum TARC levels in patients with AD were significantly higher than those in patients with PsV, characterized as a Th1-dominant disease.[6]. Thus, we suggest that TARC may be involved in a pathogenetic role for the Th2-dominant diseases. IP-10 is a functional ligand for CXCR3 which is preferentially expressed on Th1-type cells.[7] In contrast to TARC, IP-10 is highly detected in the lesional skin of patients with psoriasis[8] and is suggested to be involved in the Th1-type responses. Although TARC and IP-10 have divergent effects on the Th1/Th2-type response, both chemokines are important for the pathogenesis of inflammatory skin diseases.

In vitro, a recent paper revealed that the HaCaT cells and the human KCs produced TARC after stimulation with TNF-α and IFN-γ.[9] Furthermore, the human KCs produced IP-10 after the same stimulation.[10] In this study, we confirmed that the TARC production from HaCaT cells increased synergistically after stimulation with TNF-α and IFN-γ. We also confirmed that the IP-10 production from HaCaT cells was enhanced by stimulation with TNF-α, or TNF-α and IFN-γ although the IP-10 production from HaCaT cells by IFN-γ up-regulated less than that from primary human KCs previously reported.[10] Moreover, we clearly showed that TARC production from HaCaT cells was inhibited whereas IP-10 production was enhanced by IL-4 in a dose-dependent manner. These data strongly suggest that IL-4 may act not only as a mediator of Th1-type response but also as a down-regulator of Th2-type chemokines. This is the first report describing the inhibitory regulation of TARC, a Th2-type chemokine, by IL-4.

Previous reports showed that the activation of nuclear factor (NF)-κB is clearly involved in TNF-α-induced TARC and IP-10 expression.[24,25] Moreover, Ohmori and Hamilton[26] showed that NF-κB and an interferon-stimulated response element mediated synergistic induction of IP-10 gene transcription by TNF-α and IFN-γ. However, it is still unclear how the stimulation with TNF-α and IFN-γ works for the synergistic production of TARC. As for the IL-4 signaling, at least two distinct pathways are involved after triggering the ligation of the IL-4Rα: PI3-kinase and signal transducers and activators of transcription-6.[17] In terms of the modulation by IL-4, it is suggested that these two chemokines, TARC and IP-10, may be directly regulated by IL-4, in other words, independent of TNF-α- or IFN-γ-signaling pathways because our results clearly showed that co-stimulation with TNF-α and IFN-γ increased both TARC and IP-10 production, and that IL-4 influenced the reciprocal effect on the production from HaCaT cells. To clarify this, further investigation will be required.

We also examined the involvement of IL-13 for HaCaT cells and there was no significant response for TARC and IP-10 production. Although IL-4 and IL-13 have similar biological functions, our results suggest that this is not the case in the response of

chemokine production. IL-4 binds directly to IL-4Rα whereas IL-13 binds directly to IL-13Rα1 or IL-13Rα2.[14,15] In addition, IL-13Rα2 itself binds to IL-13 with a high affinity while IL-13Rα1 requires IL-4Rα to bind tightly to IL-13.[15,27] Thus, IL-13Rα2 signaling is independent of IL-4, in other words, works specifically for IL-13. This may result in the divergent effect of IL-4 and IL-13 on the production of TARC and IP-10.

In summary, IL-4 modulated the TARC and IP-10 production from HaCaT cells in a dose-dependent manner. Further investigation is required whether or not IL-4 regulates the TARC and IP-10 production in vivo.

MATERIALS AND METHODS

Reagents

The following cytokines were used in this study; human recombinant (r) IFN-γ (Peprotec, Rock Hill, NJ), human rTNF-α (Peprotec), human rIL-4 (R&D Systems, Minneapolis, MN, USA) and human rIL-13 (Peprotec). Goat anti-human IL-4 mAb (TECHNE Co., Minneapolis, MN, USA) was also used for the neutralization of human IL-4 bioactivity, and goat IgG (TECHNE Co.) was used as an isotype matched control.

Cell cultures

HaCaT cells were kindly provided by Dr Toshio Kuroki, Institute of Molecular Oncology, Showa University, Tokyo, Japan. The HaCaT cells were cultured in a 75 ml flask in 30 ml Dulbeccos medium (D-MEM) with glutamine containing 10% fetal bovine serum (FBS) at 37°C and 5% CO_2 until confluence. These cells were then trypsinated, washed and divided into 1×10^6 in 6-well plates. They were incubated overnight in 1 ml of D-MEM containing 10% FBS until attached to the surface of the plates. Subsequently, the medium was replaced with 1 ml of D-MEM without FBS. Simultaneously, various cytokines such as TNF-α, IFN-γ, IL-4 and/or IL-13 were added. The concentration of each cytokine was 0.01, 0.1, 1.0, 10 or 100 ng/ml for IL-4, 100 ng/ml for TNF-α and IFN-γ, and 1.0 or 10 ng/ml for IL-13. When IL-4 mAb was added, a neutralizing concentration of 5 μg/ml was used. These cells were then incubated at 37°C and 5% CO_2 and after 24 h, the supernatants of HaCaT cells were collected and used in this study.

ELISA

We used a 96-well polystyrene coated plate with a murine mAb against human TARC (TECHNE Co) and human IP-10 (R&D Systems). The supernatant levels of TARC or IP-10 were measured according to the standard ELISA protocols. Optical densities were measured at 450 nm with a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). The concentrations of these chemokines were calculated from the standard curve generated by a curve-fitting program. The minimum detectable dose of TARC or IP-10 was 7 pg/ml or 5 pg/ml, respectively. Each sample was tested in triplicate.

Statistical analysis

Data were analyzed using the Mann–Whitney *U* test. A *P*-value of less than 0.05 was considered to be statistically significant.

Acknowledgements

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REFERENCES

1. Zlotnik A, Yoshie O (2000) Chemokines: a new classification system and their role in immunity. Immunity 12:121–127.
2. Baggiolini M, Dewald B, Moser B (1997) Human chemokines: an update. Annu Rev Immunol 15:675–705.
3. Mantovani A (1999) The chemokine system; redundancy for robust outputs. Immunol Today 20:254–257.
4. Imai T, Baba M, Nishimura M, Kakizaki M, Takagi S, Yoshie O (1997) The T cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. J Biol Chem 272:15036–15042.
5. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, Gray PW, Matsushima K, Yoshie O (1999) Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol 11:81–88.
6. Kakinuma T, Nakamura K, Wakugawa M, Mitsui H, Tada Y, Saeki H, Torii H, Asahina A, Onai N, Matsushima K, Tamaki K (2001) 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 107:535–541.
7. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A (1998) Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. J Exp Med 187:875–883.
8. Gottlieb AB, Luster AD, Posnett DN, Carter DM (1988) Detection of a gamma interferon-induced protein IP-10 in psoriatic plaques. J Exp Med 168:941–948.
9. Vestergaard C, Bang K, Gesser B, Yoneyama H, Matsushima K, Larsen CG (2000) A Th2chemokine, TARC, produced by keratinocytes may recruit CLA+CCR4+ lymphocytes into lesional atopic dermatitis skin. J Invest Dermatol 115:640–646.
10. Albanesi C, Scarponi C, Sebastiani S, Cavani A, Federici M, De Pita O, Puddu P, Girolomoni G (2000) IL-4 enhances keratinocyte expression of CXCR3 agonistic chemokines. J Immunol 165:1395–1402.
11. McKenzie ANJ, Li X, Largaespada DA, Sato A, Kaneda A, Zurawski SM, Doyle EL, Milatovich A, Francke U, Copeland NG, Jenkins NA, Zurawski G (1993) Structural comparison and chromosomal localization of the human and mouse IL-13 genes. J Immunol 150:5436–5444.
12. Mosmann T, Coffman R (1989) TH1 and TH2 cells: different patterns of lymphocyte secretion lead to different functional properties. Annu Rev Immunol 5:429–59.
13. Punnonen J, Aversa G, Cocks B, McKenzie A, Menon S, Zurawski G, de Waal Malefyt R, de Vries J (1993) Interleukin-13 induces interleukin-4-independent IgG4 and IgE synthesis and CD23 expression by human B-cells. Proc Natl Acad Sci USA 90: 3730–3734.

14. Zurawski S, Vega F, Huyghe B, Zurawski G (1993) Receptors for interleukin-13 and interleukin-4 are complex and share a novel component that functions in signal transduction. EMBO J 12:3899–3905.

15. Caput D, Laurent P, Kaghad M, Lelias JM, Lefort S, Vita N, Ferrara P (1996) Cloning and characterization of a specific interleukin (IL)-13 binding protein structurally related to the IL-4 receptor α chain. J Biol Chem 271:16921–16926.

16. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 106:761–771.

17. Chomarat P, Banchereau J (1998) Interleukin-4 and interleukin-13: their similarities and discrepancies. Int Rev Immunol 17:1–52.

18. Hamid Q, Boguniewicz M, Leung DY (1994) Differential in situ cytokine gene expression in acute and chronic atopic dermatitis. J Clin Invest 94:870–876.

19. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD (1998) Interleukin-13: central mediator of allergic asthma. Science 282:2258–2261.

20. Ramanathan S, de Kozak Y, Saoudi A, Goureau O, Van der Meide PH, Druet P, Bellon B (1996) Recombinant IL-4 aggravated experimental autoimmune uveoretinitis in rats. J Immunol 157:2209–2215.

21. Jacobs MJ, van der Hoek AE, van Lent PL, van de Loo FA, van de Putte LB, van den Berg WB (1994) Role of IL-2 and IL-4 in exacerbation of murine antigen-induced arthritis. Immunology 83:390–396.

22. Fort MM, Lesley R, Davidson NJ, Menon S, Brombacher F, Leach MW, Rennick DM (2001) IL-4 exacerbates disease in a Th1 cell transfer model of colitis. J Immunol 166:2793–2800.

23. Campbell JJ, Haraldsen J, Pan G, Rottman J, Qin J, Ponath S, Andrew P, Warnke DP, Ruffing R, Kassam N, Wu L, Butcher EC (1999) The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. Nature 400:776–780.

24. Ohmori Y, Hamilton TA (1993) Cooperative interaction between interferon (IFN) stimulus response element and kappa B sequence motifs controls IFN gamma- and liposaccharide-stimulated transcription from the murine IP-10 promoter. J Biol Chem 268:6677–6688.

25. Berin MC, Eckmann L, Broide DH, Kagnoff MF (2001) 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 24:382–389.

26. Ohmori Y, Hamilton TA (1995) The interferon-stimulated response element and a kappa B site mediate synergistic induction of murine IP-10 gene transcription by IFN-gamma and TNF-alpha. J Immunol 154:5235–5244.

27. Aman MJ, Tayebi N, Obiri N, Puri RK, Modi WS, Leonard WJ (1996) cDNA cloning and characterization of the human interleukin-13 receptor-α chain. J Biol Chem 271: 29265–29270.

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Both IL-4 and IL-13 inhibit the TNF-α and IFN-γ enhanced MDC production in a human keratinocyte cell line, HaCaT cells

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KEYWORDS
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MDC;
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Atopic dermatitis

Summary *Background:* Macrophage-derived chemokine (MDC) is a Th2 type chemokine and its receptor CC chemokine receptor 4 (CCR4) is preferentially expressed on Th2 cells. Recent reports demonstrated that MDC is expressed not only by macrophages, dendritic cells and lymphocytes, but also by cultured human keratinocytes (KCs). However, the regulation of MDC production in KCs by various cytokines has not been well documented. *Objective:* In this study, we investigated how Th1/Th2 cytokines regulate MDC production in a human KC cell line, HaCaT cells. *Methods:* HaCaT cells were cultured with or without various cytokines for 24 h and RT-PCR was performed using these cells to evaluate MDC mRNA levels. ELISA was carried out using supernatant of HaCaT cells to calculate secreted MDC protein levels. *Results:* MDC mRNA was weakly expressed in HaCaT cells, and upon stimulation with TNF-α or IFN-γ, MDC expression was strongly upregulated. The supernatant MDC levels when stimulated with TNF-α or IFN-γ were significantly higher than those without stimulation, and were synergistically increased when stimulated with a combination of TNF-α and IFN-γ. Both interleukin-4 (IL-4) and IL-13 inhibited TNF-α and IFN-γ enhanced MDC production in HaCaT cells in a dose-dependent manner. *Conclusion:* Th2-type cytokines IL-4 and IL-13 downregulate the production of MDC, a Th2 type chemokine, by KCs. This may partially contribute to maintaining Th1/Th2 balance in inflammatory skin diseases like atopic dermatitis.
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1. Introduction

Chemokines are small secreted proteins that regulate leukocyte trafficking [1,2]. Based on the

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position of the first two of the four conserved cysteine residues, chemokines are classified into four subfamilies: CXC, CC, C, and CX3C. Keratinocytes (KCs) form the outer component of skin and are known to initiate skin inflammation [3,4]. KCs synthesize many chemokines including members of the CC and CXC subfamilies, such as regulated on activation of normal T cell expressed and secreted (RANTES), gamma-interferon-inducible protein-10 (IP-10), monokine induced by gamma-interferon (MIG) and thymus and activation-regulated chemokine (TARC). They also express some chemokine receptors that mediate the inflammatory or immune response by attracting various kinds of leukocytes [5–7].

Macrophage-derived chemokine (MDC/CCL22) belongs to the CC chemokines and is encoded by a gene on chromosome 16q13 [8]. It is one of the functional ligands for CC chemokine receptor 4 (CCR4) and is a chemoattractant for the CCR4 expressing cells such as Th2-type cells and IL-2 activated NK cells [9–11]. In addition, MDC chemoattracts eosinophils although they have no expression of CCR4 [12]. MDC is constitutively produced by dendritic cells (DCs), B cells, macrophages, and thymic medullary epithelial cells, whereas monocytes, NK cells, and $CD4^+$ T cells produce MDC only with appropriate stimulation [13–16].

A previous report showed that the serum MDC levels in patients with atopic dermatitis (AD), considered to be a Th2-dominant disease especially in acute phase, were higher than those in healthy control subjects, and immunoreactive MDC was observed in dermal DCs and T cells in the lesional skin of patients with AD [16]. We have further shown that the serum MDC levels significantly correlate with the disease activity of AD [17]. These data strongly suggest that MDC is involved in the pathogenesis of Th2-dominant diseases such as AD. Recently, it was reported that MDC was also expressed in the lesional skins of psoriasis and allergic contact hypersensitivity (ACH) [18,19]. Moreover, in vitro, KCs produced MDC after stimulation with TNF-α and IFN-γ, suggesting that KCs are one of the main sources of MDC [20].

Interleukin-4 (IL-4) and IL-13 share about 20–25% homology with each other and have similar biological functions such as the production of IgE from B cells [21,22]. Both cytokines are produced by Th2-type cells and are reported to play a key pathogenetic role in the Th2-dominant diseases AD, bronchial asthma, and bullous pemphigoid [23,24]. However, there are also several interesting pieces of evidence that IL-4 can aid in a Th1-biased situation; in other words, IL-4 secreted by Th2-type cells could have an effect on Th1-type inflammatory response through activation of various cells including KCs [25–28]. In contrast, it is unknown whether IL-13 can also be involved in a Th1-biased situation although both IL-4 and IL-13 share IL-4 receptor α and have similar biological functions.

We have recently reported that IL-4 but not IL-13 downregulates the TNF-α and IFN-γ induced production of TARC, which is another ligand for CCR4 and a kind of Th2-type chemokine in human KCs [29]. However, the regulation of MDC production by these cytokines in KCs has not been fully identified. To clarify this further, we investigated the expression of MDC in HaCaT cells, a human KC cell line. We measured the supernatant MDC levels in HaCaT cells after stimulation with TNF-α, IFN-γ, IL-4, and IL-13, and also investigated the effects of dexamethasone and tacrolimus on MDC production in these cells.

2. Materials and methods

2.1. Samples and reagents

Recombinant TNF-α, IFN-γ, IL-4, and IL-13 were from R&D Systems (Minneapolis, MN). Recombinant MDC, anti-MDC mAb and MDC immunoassay kits were from Genzyme TECHNE (Minneapolis, MN). Dexamethasone was from Wako Pure Chemical Industries, Ltd (Osaka, Japan), and tacrolimus was from Fujisawa Pharmaceutical Co, Ltd (Tokyo, Japan). HaCaT cells were kindly provided by Dr Toshio Kuroki (Institute of Molecular Oncology, Showa University, Japan) with permission from Dr N. Fusenig (Institute Fur Zell- und Tumourbiologie. Deutshes Kresforschungeszentrum, Heidelberg, Germany).

2.2. Cell culture

HaCaT cells were cultured in 150 cm^2 cell culture flasks (Corning, NY) at 37 °C, 5% CO_2 in minimum essential medium eagle (MEME) (Sigma, St. Louis, MO) containing 10% FBS and antibiotics. The cells received fresh medium every 3 d and were subcultured every 10 days. Fortieth- to seventieth-passage cells were used in all experiments. When confluence was achieved, the cells were trypsinized, washed, resuspended in MEME with 10% FBS at 1×10^6 cells/ml, and 1 ml was added to each well of the six-well plates (Becton Dickinson Labware, Franklin Lakes, NJ). When the cells reached confluence, the medium was completely removed and 1 ml MEME without FBS was added to each well.

Simultaneously, cytokines or drugs were added, and the cells were incubated at 37 °C and 5% CO_2. After 6, 12, 24, 36 or 48 h, supernatants were collected, centrifuged to remove cell debris, and stored at −80 °C until analyzed. Cells were trypsinized, pelleted, and stored at −80 °C until use.

2.3. RT-PCR

Total RNA was isolated using a Micro-FastTrack 2.0 Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription and polymerase chain reaction was performed using a GeneAmp RNA PCR Kit and a RNA PCR Core Kit (Applied Biosystems, Foster City, CA). MDC upstream primer: 5′-GCA TGG CTC GCC TAC AGA CT-3′; MDC downstream primer: 5′-GCA GGG AGG GAG GCA GAG GA-3′. The mixture was pre-denaturated for 5 min at 94 °C and then subjected to 35 cycles: 94 °C 1 min, 62 °C 1.5 min, 72 °C 2 min, then 72 °C 7 min. Amplified DNA fragments were judged from 2.5% agarose gel electrophoresis. Human β-actin was used as control primer. Upstream primer: 5′-ACA CTG TGC CCA TCT ACG AGG GG-3′; downstream primer: 5′- ATG ATG GAG TTG AAG GTA GTT TCG TGG AT-3′. The expected fragment length was 497 bp for MDC and 340 bp for β-actin.

2.4. ELISA

The supernatant levels of MDC were measured according to the manufacturer's instructions. OD was measured at 450 nm with a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories Inc., Hercules, CA). The concentrations were calculated from the standard curve generated by a curve-fitting program.

2.5. Statistical analysis

Data were analyzed using the Student's *t*-test. A *P* value less than 0.05 was considered to be statistically significant.

3. Results

3.1. MDC mRNA in HaCaT cells was strongly upregulated by TNF-α or IFN-γ

We investigated the expression of MDC using a human KC cell line HaCaT cells after 24 h culture. RT-PCR revealed that when the cells were cultured without cytokines, MDC mRNA was weakly expressed (Fig. 1). When cultured with TNF-α (10 ng/ml) or IFN-γ (10 ng/ml), this expression was strongly upregulated, however, when cultured with IL-4 (10 ng/ml) or IL-13 (10 ng/ml), it was only slightly downregulated (Fig. 1).

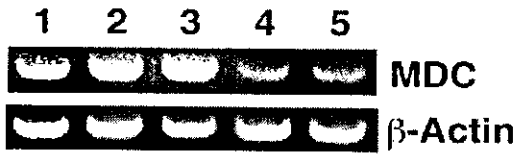

Fig. 1 RT-PCR of MDC in HaCaT cells. Lane 1: no stimulation. Lane 2–5: stimulation with TNF-α (lane 2), IFN-γ (lane 3) IL-4 (lane 4) or IL-13 (lane 5). MDC mRNA in HaCaT cells was strongly upregulated when cultured with TNF-α or IFN-γ, but slightly downregulated when cultured with IL-4 or IL-13. This is representative data from two experiments.

3.2. MDC production in HaCaT cells was enhanced synergistically by TNF-α and IFN-γ

To determine whether MDC protein was secreted from HaCaT cells, ELISA was performed using supernatant of the cultured cells in 24 h with or without cytokines. When HaCaT cells were cultured without cytokines, the supernatant MDC level was 123.8±1.1 pg/ml (mean±standard deviation) (Fig. 2). When cultured with TNF-α (10 ng/ml) or

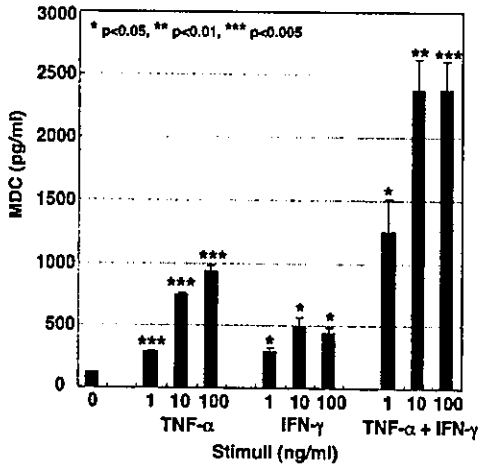

Fig. 2 ELISA of MDC using culture supernatants of HaCaT cells. Each culture condition was tested in duplicate. The error bars indicate standard deviation. This is representative data from three experiments. MDC production in HaCaT cells was significantly upregulated when stimulated by TNF-α or IFN-γ. When stimulated by the combination of TNF-α and IFN-γ, MDC production was synergistically upregulated.

IFN-γ (10 ng/ml), the MDC levels were increased to 745.3±12.8 pg/ml ($P < 0.005$) or 489.2±81.1 pg/ml ($P < 0.05$), respectively. When cultured with the combination of TNF-α (10 ng/ml) and IFN-γ (10 ng/ml), the MDC level was increased synergistically to 2374.0±246.9 pg/ml ($P < 0.01$) (Fig. 2). Culture with IL-4 (10 ng/ml) or IL-13 (10 ng/ml), however, did not result in a change in MDC level (data not shown).

3.3. MDC production induced by TNF-α and IFN-γ in HaCaT cells was increased during the time course, and reached a saturation point at 24 h

To examine whether 24 h is a good time point, we performed a time course study of MDC production. As shown in Fig. 3, the production induced by TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) in HaCaT cells was increased during the time course, and reached a saturation point at 24 h. Therefore, in the following experiments, we cultured HaCaT cells with or without cytokines or reagents for 24 h and measured MDC protein levels in the supernatants by ELISA.

3.4. Both IL-4 and IL-13 inhibited TNF-α and IFN-γ enhanced MDC production in HaCaT cells

To learn whether IL-4 and IL-13 regulate TNF-α and IFN-γ enhanced MDC production in HaCaT cells, MDC levels were measured after adding IL-4 (1–100 ng/ml) or IL-13 (1–100 ng/ml) together with TNF-α (10 ng/ml) and IFN-γ (10 ng/ml). IL-4 inhibited TNF-α and IFN-γ enhanced MDC production in the cells in a dose-dependent manner (Fig. 4). Inhibition rate by IL-4 (100 ng/ml) was about 40% ($P < 0.0001$). Interestingly, IL-13 also inhibited TNF-α and IFN-γ enhanced MDC production in a dose-dependent manner, and the inhibition rate at 100 ng/ml was also about 40% ($P < 0.0001$). However, the combination of IL-4 and IL-13 did not show any additional or synergistic inhibitory effect on TNF-α and IFN-γ enhanced MDC production (Fig. 3).

3.5. Tacrolimus inhibited TNF-α and IFN-γ enhanced MDC production in HaCaT cells

Determination of whether dexamethasone and tacrolimus regulate TNF-α and IFN-γ enhanced MDC production in HaCaT cells was made by measuring MDC levels after adding dexamethasone (10^{-10}, 10^{-8} and 10^{-6} M) or tacrolimus (10^{-10}, 10^{-8} and 10^{-6} M) together with TNF-α (10 ng/ml) and IFN-γ (10 ng/ml). As shown in Fig. 5, tacrolimus, but not dexamethasone, inhibited TNF-α and IFN-γ enhanced MDC production dose-dependently.

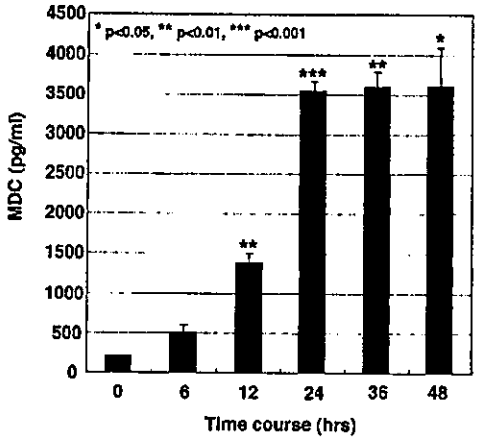

Fig. 3 ELISA of MDC using culture supernatants of HaCaT cells. Each culture condition was tested in duplicate. The error bars indicate standard deviation. This is representative data from two experiments. MDC production induced by TNF-α and IFN-γ in HaCaT cells was increased during the time course and reached a saturation point at 24 h.

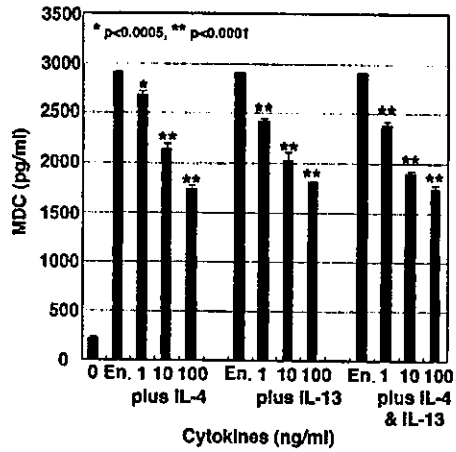

Fig. 4 ELISA of MDC using culture supernatants of HaCaT cells. Each culture condition was tested in triplicate. The error bars indicate standard deviation. This is representative data from three experiments. Both IL-4 and IL-13 inhibited TNF-α and IFN-γ enhanced (En.) MDC production in HaCaT cells in a dose-dependent manner. However, their combination did not show any additional or synergistic inhibitory effect on TNF-α and IFN-γ enhanced MDC production.

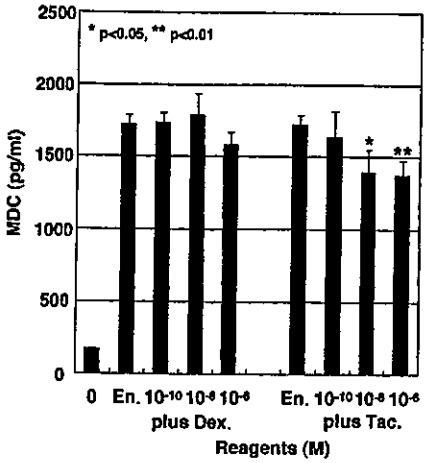

Fig. 5 ELISA of MDC using culture supernatants of HaCaT cells. Each culture condition was tested in triplicate. The error bars indicate standard deviation. This is representative data from two experiments. Tacrolimus (Tac.), but not dexamethasone (Dex.), inhibited TNF-α and IFN-γ enhanced (En.) MDC production in HaCaT cells dose-dependently.

4. Discussion

In the present study, we showed that: (1) MDC mRNA was weakly expressed in HaCaT cells, and upon stimulation with TNF-α or IFN-γ its expression was strongly upregulated; (2) the supernatant MDC levels upon stimulation with TNF-α or IFN-γ were significantly higher than those without stimulation, and were synergistically increased upon stimulation with a combination of both TNF-α and IFN-γ; (3) both IL-4 and IL-13 inhibited TNF-α and IFN-γ enhanced MDC production in HaCaT cells in a dose-dependent manner; (4) tacrolimus inhibited the TNF-α and IFN-γ enhanced MDC production in HaCaT cells. This is the first report describing the regulation of MDC production in a human KC cell line, HaCaT cells by various cytokines.

MDC is a functional ligand for CCR4 which is preferentially expressed on Th2-type cells, and chemoattracts the $CCR4^+$ Th2-type cells into lesional sites [9,10]. It was previously reported that the serum MDC levels in patients with AD were significantly higher than those in healthy control subjects, and both dermal DCs and T cells were strongly immunoreactive for MDC in lesional skin of patients with AD [16]. Recently, we also clarified that the serum MDC levels significantly correlated with the disease activity of AD [17]. These findings strongly suggest that MDC, as well as TARC, another ligand of CCR4 [30,31], may play a pathogenetic role in Th2-dominant diseases. MDC is constitutively produced in DCs, macrophages, and thymic epithelial cells [11,14,16]. In addition, several kinds of leukocytes such as T cells can produce MDC after stimulation with proinflammatory cytokines [16]. As for the regulation of MDC production in leukocytes, previous studies showed that MDC production in DCs was upregulated by IL-4 and IL-13, whereas it was inhibited by IFN-γ [13]. It was further shown that T cells and monocytes produced MDC after stimulation with IL-4 [15,16]. Thus, the upregulation of MDC production in leukocytes depends upon the Th2-type cytokines IL-4 and IL-13. In our study, the expression and production of MDC in HaCaT cells was upregulated after the addition of TNF-α in combination with IFN-γ, a Th1-type cytokine. It was also reported that MDC was expressed in the lesional skins of psoriasis and ACH, which are considered to be Th1-dominant diseases [18,19]. In both these diseases, Th1 chemokines IP-10 and MIG are believed to be selectively involved in the T cell homing to the epidermis, but Th2 chemokines TARC and MDC are also involved in the T cell trafficking to the dermis together with Th1 chemokines [18,19]. This study also showed that TNF-α and IFN-γ enhanced MDC production was inhibited by addition of IL-4 or IL-13, a Th2-type cytokine. These results suggest that MDC production is differently regulated between KCs and leukocytes.

It is well known that IL-4 plays an important role in Th2-type responses [21]. However, interestingly, recent reports revealed that IL-4 also influences the Th1-type responses as follows: (1) In an antigen-induced arthritis mouse model, the neutralizing anti-IL-4 mAb contributed to control of the disease, suggesting that IL-4 may be important for the Th1-type inflammatory response [25]. (2) IL-4-treated rats in the Th1-type autoimmune uveoretinitis showed an exacerabation of general symptoms and IL-4 treatment augmented IFN-γ production [26]. (3) IL-4 upregulated the secretion of IP-10, a ligand for CXCR3 that is preferentially expressed on Th1-type cells, from human KCs [27]. (4) IL-4 treatment exacerbated the symptoms of the T cell transfer model of colitis, characterized as a Th1-dominant disease [28]. Our data that MDC production was inhibited by IL-4 also supports the idea that IL-4 could be involved in Th1-type response. Furthermore, Albanesi et al. reported that KCs appear considerably more sensitive to Th1- than Th2-derived lymphokines in terms of chemokine release [20]. Our result that IL-4 inhibited MDC production by KCs is also consistent with their idea that KCs generally favor Th1 response.

In this study we clearly showed that TNF-α and IFN-γ enhanced MDC production in HaCaT cells. Very recently, Horikawa et al. demonstrated MDC immunoreactivity in the epidermal layer of AD skin, but not in normal or psoriatic skin [32]. Taken together, it is possible that MDC production by KCs in AD skin is partially involved in the pathogenesis of the disease. AD is generally considered to be a Th2-dominant disease especially in the acute phase, but it is reported that in the chronic phase Th1 cells also infiltrate into the lesional skin and play a role in the pathogenesis of the disease [33]. Th2- and Th1-type immune responses are not mutually exclusive, but a human inflammatory disease like AD is based on interactions between different Th-cell subsets [33]. MDC production by T cells and DCs is upregulated by IL-4, however, its production by KCs is downregulated by the same cytokine. Differently regulated MDC production by leukocytes and KCs may partially contribute to maintaining Th1/Th2 balance in an inflammatory skin disease like AD. Leukocytes-mediated Th2 and KCs-mediated Th1 environments are not mutually exclusive, but possibly separated spatially (dermis vs. epidermis) and maybe consequently (acute vs. chronic) in the pathogenesis of AD.

We recently reported that the TNF-α and IFN-γ enhanced TARC production in HaCaT cells was inhibited by IL-4 but not by IL-13 [29]. This study showed that both cytokines inhibited the TNF-α and IFN-γ enhanced MDC production, although there was no synergistic inhibitory effect of these two cytokines, suggesting that IL-13 had the effect on HaCaT cells mainly through IL-4 receptor. The reason there is a difference in the regulatory effect of IL-13 between TARC and MDC production in HaCaT cells remains unclear and further investigation will be necessary. In the present study, tacrolimus inhibited TNF-α and IFN-γ enhanced MDC production in HaCaT cells dose-dependently. When both tacrolimus and IL-4 were added to HaCaT cells simultaneously, they additionally inhibited TNF-α and IFN-γ enhanced MDC production in these cells (data not shown). These results suggest that an immunomodulatory reagent like tacrolimus is effective in treating AD, possibly in part due to the inhibition of MDC production by lesional KCs.

In summary, the Th2-type cytokines IL-4 and IL-13 downregulate the TNF-α and IFN-γ enhanced MDC production by KCs, suggesting the inhibition of Th2 amplification circuit in the skin. This may partially contribute to maintaining Th1/Th2 balance in inflammatory skin diseases like AD.

Acknowledgements

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References

[1] Yoshie O, Imai T, Nomiyama H. Chemokines in immunity. Adv Immunol 2001;78:57–109.
[2] Rossi D, Zlotnik A. The biology of chemokines and their receptors. Annu Rev Immunol 2000;18:217–42.
[3] Sauder DN. The role of epidermal cytokines in inflammatory skin diseases. J Invest Dermatol 1990;95:27S–8S.
[4] Barker JN, Mitra RS, Griffiths CE, Dixit VM, Nicholoff BJ. Keratinocytes as initiators of inflammation. Lancet 1991;337:211–4.
[5] Vestergaard C, Yoneyama H, Murai M, Nakamura K, Tamaki K, Terashima Y, et al. Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. J Clin Invest 1999;104:1097–105.
[6] Wakugawa M, Nakamura K, Akatsuka M, Nakagawa H, Tamaki K. Interferon-gamma-induced RANTES producing by human keratinocytes is enhanced by IL-1 beta, TNF-alpha, IL-4 and IL-13 and is inhibited by dexamethasone and tacrolimus. Dermatology 2001;202:239–45.
[7] Wakugawa M, Nakamura K, Akatsuka M, Kim SS, Yamada Y, Kawasaki H, et al. Expression of CC chemokine receptor 3 on human keratinocytes in vivo and in vitro—upregulation by RANTES. J Dermatol Sci 2001;25:229–35.
[8] Nomiyama H, Imai T, Kusuda J, Miura R, Callen DF, Yoshie O. Human chemokine fractalkine (SCYD1), MDC (SCYA22) and TARC (SCYA17) are clustered on chromosome 16q13. Cytogenet Cell Genet 1998;81:10–1.
[9] Imai T, Chantry D, Raport CJ, Wood CL, Nishimura M, Godiska R, et al. Macrophage-derived chemokine is a functional ligand for the CC chemokine receptor 4. J Biol Chem 1998;273:1764–8.
[10] 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;11:81–8.
[11] Inngjerdingen M, Damaj B, Maghazachi AA. Human NK cells express CC chemokine receptor 4 and 8 and respond to thymus and activation-regulated chemokine, macrophage-derived chemokine, and I-309. J Immunol 2000;164:4048–54.
[12] Bochner BS, Bickel CA, Taylor ML, MacGlashan DW, Gray PW, Raport CJ, et al. Macrophage-derived chemokine induces human eosinophil chemotaxis in a CC chemokine receptor 3- and CC chemokine receptor 4-independent manner. J Allergy Clin Immunol 1999;103:527–32.
[13] Bonecchi R, Sozzani S, Stine JT, Luini W, D'Amico G, Allavena P, et al. Divergent effects of interleukin-4 and interferon-gamma on macrophage-derived chemokine production: an amplification circuit of polarized T helper 2 response. Blood 1998;92:2668–71.
[14] Chantry D, Romagnani P, Raport CJ, Wood CL, Epp A, Romagnani S, et al. Macrophage derived chemokine (MDC) is localized to thymic medullary epithelial cells and is a chemoattractant for CD3+, CD4+, CD8 low thymocytes. Blood 1999;94:1890–8.

[15] Andrew DP, Chang MS, McNinch J, Wathen ST, Rihanek M, Tseng J, et al. STCP-1 (MDC) CC chemokine acts specifically on chronically activated Th2 lymphocytes and is produced by monocytes on stimulation with Th2 cytokines IL-4 and IL-13. J Immunol 1998;161:5027–38.

[16] Galli G, Chantry D, Annunziato F, Romagnani P, Cosmi L, Lazzeri E, et al. Macrophage-derived chemokine production by activated human T cells in vitro and in vivo: preferential association with the production of type 2 cytokines. Eur J Immunol 2000;30:204–10.

[17] Kakinuma T, Nakamura K, Wakugawa M, Mitsui H, Tada Y, Saeki H, et al. Serum macrophage-derived chemokine (MDC) levels are closely related with the disease activity of atopic dermatitis. Clin Exp Immunol 2002;127:270–3.

[18] Rottman JB, Smith TL, Ganley KG, Kikuchi T, Krueger JG. Potential role of the chemokine receptors CXCR3, CCR4, and the integrin alpha beta 7 in the pathogenesis of psoriasis vulgaris. Lab Invest 2001;81:335–47.

[19] Goebeler M, Trautmann A, Voss A, Brocker EV, Toksoy A, Gillitzer R. Differential and sequential expression of multiple chemokines during elicitation of allergic contact hypersensitivity. Am J Pathol 2001;158:431–40.

[20] Albanesi C, Scarponi C, Sebastiani S, Cavani A, Federici M, Sozzani S, et al. A cytokine-to-chemokine axis between T lymphocytes and keratinocytes can favor Th1 cell accumulation in chronic inflammatory skin diseases. J Leukoc Biol 2001;70:617–23.

[21] Minty A, Chalon P, Derocq JM, Dumont JC, Guillemot JC, Kaghad M, et al. Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. Nature 1993;362:248–50.

[22] Punnonen J, Aversa G, Cocks B, McKenzie A, Menon S, Zurawski G, et al. Interleukin-13 induces interleukin-4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. Proc Natl Acad Sci USA 1993;90:3730–4.

[23] Mosmann T, Coffman R. TH1 and TH2 cells: different patterns of lymphocyte secretion lead to different functional properties. Annu Rev Immunol 1989;5:429–59.

[24] McKenzie A, Culpepper J, de Waal Malefyt R, Briere F, Punnonen J, Aversa G, et al. Interleukin-13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. Proc Natl Acad Sci USA 1993;90:3725–9.

[25] Jacobs MJ, van der Hoek AE, van Lent PL, van de Loo FA, van de Putte LB, van den Berg WB. Role of IL-2 and IL-4 in exacerbation of murine antigen-induced arthritis. Immunology 1994;83:390–6.

[26] Ramanathan S, de Kozak Y, Saoudi A, Goureau O, Van der Meide PH, Druet P, et al. Recombinant IL-4 aggravates experimental autoimmune uveoretinitis in rats. J Immunol 1996;157:2209–15.

[27] Albanesi C, Scarponi C, Sebastiani S, Cavani A, Federici M, De Pità O, et al. IL-4 enhances keratinocyte expression of CXCR3 agonistic chemokines. J Immunol 2000;165:1395–402.

[28] Fort MM, Lesley R, Davidson NJ, Menon S, Brombacher F, Leach MW, et al. IL-4 exacerbates disease in a Th1 cell transfer model of colitis. J Immunol 2001;166:2793–800.

[29] Kakinuma T, Nakamura K, Wakugawa M, Yano S, Saeki H, Torii H, Komine M, Asahina A, Tamaki K, IL-4, but not IL-13, modulate TARC (thymus and activation-regulated chemokine)/CCL17 and IP-10 (interferon-induced protein of 10 kDa)/CXCL10 release by TNF-α and IFN-γ in HaCaT cell line. Cytokine 2002;20:1–6..

[30] 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;107:535–41.

[31] Vestergaard C, Bang B, 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.

[32] Horikawa T, Nakayama T, Hikita I, Yamada H, Fujisawa R, Bito T, et al. IFN-γ-inducible expression of thymus and activation-regulated chemokine/CCL17 and macrophage-derived chemokine/CCL22 in epiderma keratinocytes and their roles in atopic dermatitis. Int Immunol 2002;14:767–73.

[33] Grewe M, Bruijnzeel-Koomen CAFM, Schöpf E, Thepen T, Langeveld-Wildschut AG, Ruzicka T, et al. A role for TH1 and Th2 cells in the immunopathogenesis of atopic dermatitis. Immunol Today 1998;19:359–61.

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TGF-β_1-mediated regulation of thymus and activation-regulated chemokine (TARC/CCL17) synthesis and secretion by HaCaT cells co-stimulated with TNF-α and IFN-γ

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Abstract

Thymus and activation-regulated chemokine (TARC/CCL17) contributes not only to the recruitment of leukocytes, but is also involved in immune disorders, such as atopic dermatitis (AD) and bronchial asthma. We have previously reported that the levels of TARC were high in patients with AD and that lesional epidermis were strongly immunoreactive for TARC. In this paper, the effects of transforming growth factor (TGF)-β_1 on the expression of TARC/CCL17 were examined in HaCaT cells, a human keratinocytes (KCs) cell line, co-stimulated with TNF-α and IFN-γ. We found that TGF-β_1 down-regulated the TARC synthesis and secretion of HaCaT cells co-stimulated with TNF-α and IFN-γ in a dose-dependent manner. TGF-β_1 at a concentration of 10ng/ml maximally inhibited this secretion. Northern blot analysis showed a similar inhibitory effect of TGF-β_1 on TARC mRNA expression by HaCaT cells. The TGF-β_1-induced down-regulation of TARC/CCL17 in HaCaT cells suggests that TGF-β_1 might regulate the TARC-related inflammatory processes, which may be important for understanding the pathogenesis of allergic diseases.
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Keywords: Chemokines; Keratinocytes; Thymus and activation-regulated chemokine/CCL17; Transforming growth factor-β_1

1. Introduction

Chemokines have been identified as attractant of different type leukocytes to sites of infection and inflammation [1,2]. They are produced locally in the tissues and act on the leukocytes through specific receptors [3,4]. They also function as regulatory molecules in leukocytes maturation,

Abbreviations: TGF-β_1, transforming growth factor-β_1; TARC, thymus and activation-regulated chemokine; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; KCs, keratinocytes.

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trafficking and homing and in the development of lymphoid tissues [1,3]. Chemokines are a large family of chemotactic cytokines that can be divided into four subfamilies: CXC, CC, CX_3C and C depending on their first two N-terminal cysteine residues [1–3]. Among them, thymus and activation-regulated chemokine (TARC/CCL17) is a member of the CC chemokines, and is expressed constitutively in the thymus and in stimulated peripheral blood mononuclear cells [5]. TARC is produced by monocyte-derived dendritic cells, endothelial cells and keratinocytes as well [6–8]. TARC functions as a selective chemoattractant and serves for the recruitment and migration of T cells that express CC chemokine receptor 4 (CCR4) [9].

Atopic dermatitis (AD) is a chronic relapsing dermatitis which often shows high levels of serum IgE and peripheral blood eosinophilia. Th2 cytokines, IL-4 and IL-5, are thought to contribute to the formation of dermatitis in the acute phase. We have previously shown that TARC was expressed in epidermal keratinocytes (KCs) in the lesional skin of Nc/Nga mice, a mouse model for atopic dermatitis (AD), and in the lesional skin of patients of AD [8,10]. In addition, recent papers have reported that the antibody (Ab) against TARC reduced the developments of allergic airway inflammation and hyper-responsiveness [11]. Thus, these data indicate that the local production of TARC may greatly contribute to the initiation and maintenance of Th2 type inflammation. It has been reported that cytokines such as TNF-α, IFN-γ or IL-10 modulated TARC secretion of KCs [12,13].

TGF-β_1 is an immunosuppressive agent and is efficacious in suppressing a wide variety of immune responses. TGF-β_1 has been reported to regulate CCR4 expression and CLA expression on T cells [6]. It has been reported that TGF-β_1 gene polymorphism (+ 915C allele) was frequently detected in patients with AD, especially in severe cases, indicating the important role of TGF-β_1 in the pathogenesis of AD [14]. There has been no report on the investigation of TGF-β_1-induced regulation of TARC of HaCaT cells. Therefore, to clarify the mechanism by which local TARC secretion regulate the inflammatory process, we determined whether TGF-β_1 modulated the production of TARC by HaCaT cells co-stimulated with TNF-α and IFN-γ.

2. Materials and methods

2.1. Reagents and antibodies (Abs)

The following reagents and antibodies (Abs) were used in the experiments. The cytokines used were recombinant human (rh) TNF-α (Peprotech Inc. London, UK), rhIFN-γ (R&D Systems Inc. Minneapolis, MN) and rhTGF-β_1 (Techne Co, Minneapolis, MN). Goat anti human TARC Ab (Techne Co.) was used in the Western blotting. Rabbit anti TGF-β_1 mAb (R&D systems.) and rabbit IgG (Cedarlane Co. Ontario, Canada) were used in the neutralizing experiment.

2.2. Cell culture

HaCaT cells, a human KCs cell line, were cultured in RPMI 1640 and 10% fetal calf serum with penicillin (5μg/ml) at 37 °C, 5% CO_2. The concentrations of TGF-β_1 added to the culture medium were 0.1–20 ng/ml. The cytokines were added when the cultures reached 75% confluence. The supernatants were harvested 24 h after stimulation and stored at −70 °C until further analysis. The cells were then trypsinized and stored at −70 °C for Western blotting or mRNA analysis.

2.3. ELISA

ELISA was performed using a TARC detecting kit (Techne Co.). In brief, samples were added to each well and were incubated for 2 h at room temperature (RT). After washing, anti-TARC Ab conjugated to horseradish peroxidase (HRP) was added to each well. The plates were incubated for 1 h at RT. After washing, a substrate solution (including tetramethylbenzidine) was added and incubated for 30 min. The optical density of each well was determined using an ELISA reader (Spectra and Rainbow Readers). The minimum amount of TARC detectable was less than 7 pg/ml.

ELISAs were performed at least three times in each experiment.

2.4. Western blot analysis

Cells were suspended in lysis buffer (5 mM Tris–HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.15% Triton X-100, 2 mg/ml aprotitin, 1 mM PMSF, 1 mM pefabloc) and sonicated on ice for 10 s. The cell lysates were then cleared by centrifugation at $12000 \times g$ for 15 min at 4 °C and the supernatants were used for Western blotting. Total protein concentration of each sample was quantified by the Bio-Rad assay method (Bio-Rad, Hercules, CA). Proteins (50 μg/lane) were separated by SDS–polyacrylmide gel electrophoresis and then were transferred to a PVDF membrane. After the transfer, the membrane was blocked overnight at 4 °C. The membrane was incubated with purified goat anti-human TARC mAb (Techne Co.) diluted 1:2000 in blocking buffer for 2 h at room temperature. After washing, the membrane was incubated with alkaline phosphatase (ALP)-conjugated anti-goat IgG (ICN Pharmaceuticals Inc., Auora, OH) diluted 1:2000 for 1 h at RT. The blots were developed using an AP Conjugate Substrate Kit (Bio-Rad, Hercules, CA).

2.5. Northern blot analysis

Total RNA was collected from the cells using lysis buffer (38% phenol, 0.8 M guanidium thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M NaAc). Twenty micrograms of total RNA was mixed with MOPS, formaldehyde, and formamide, and was incubated at 65 °C. After chilling on ice, RNA was fractionated on a 1.5% agarose gel. RNA was then transferred onto nylon-membranes (Zeta-probe membranes, Bio Rad, Hercules, CA) and UV-crosslinked. The membrane was incubated in pre-hybridization solution (0.5 M Na_2HPO_4, pH 7.2 and 7% SDS) for 2 h at 68 °C, and subsequently hybridized for 24 h with a cDNA probe labeled with digoxigenin (DIG)-dUTP using PCR amplification. After washing stringently, the cDNA–mRNA hybrids were visualized using DIG nucleic acid detection kit (Roche, Germany). The probes were 511 bp cDNA for TARC and 504 bp cDNA for β-actin.

For cDNA probe synthesis, polyA$^+$ mRNAs of HaCaT cells were extracted using a Micro-FastTrack^{TM} 2.0 kit (Invitrogen Co., Carlsbad, CA). One microgram of polyA$^+$ mRNA was reverse-transcripted using a cDNA CYCLE^{TM} kit (Invitrogen Co.). PCR labeling mixture included cDNA, each primer (25–50 pmol), $MgCL_2$, dNTP, DIG-dUTP (Roche, Germany), Taq polymerase (TaKaRa, Tokyo, Japan). Cycling condition was as follows: 40 cycles of denaturation at 95 °C, annealing at 57 °C and extenstion at 72 °C. After PCR, the probe was precipitated and purified. The primer sequences used were as follows; TARC sense; 5′ CAC GCA GCT CGA GGG ACC AAT GTG 3′, TARC antisense; 5′ TCA AGA CCT CTC AAG GCT TTG CAG G 3′. β-actin sense; 5′ GAC TAT GAC TTA GTT GCG TTA 3′, β-actin antisense; 5′ GCC TTC ATA CAT CTC AAG TTG 3′.

2.6. Statistics analysis

TARC levels produced by HaCaT cells unstimulated or treated with cytokines were described in mean ± standard deviation (S.D.). The statistical analyses were performed by ANOVA for comparing TARC levels among different groups (such as control and TGF-β_1 groups). For the statistical analysis of the correlation between TARC levels and the TGF-β_1 concentrations, non-parametric Spearman's correlation was applied. $P < 0.05$ was considered to be significant.

3. Results

HaCaT cells were cultured to about 75% confluence and were co-stimulated with IFN-γ (10 ng/ml) and TNF-α (10 ng/ml). After the co-stimulation with IFN-γ and TNF-α for 24 h, the level of TARC in the culture medium was 1108.3 ± 82.7 pg/ml compared to 94.4 ± 2.5 pg/ml for HaCaT cells without stimulation, consistent with a previous report [12]. The TARC levels in the culture supernatants were measured by ELISA after 24 h incubation (Fig. 1). The inhibition of TARC