# Reciprocal Regulation of Thymus and Activation-Regulated Chemokine/Macrophage-Derived Chemokine Production by Interleukin (IL)-4/IL-13 and Interferon-γ in HaCaT Keratinocytes Is Mediated by Alternations in E-cadherin Distribution

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**Keratinocytes produce many cytochemokines that are involved in the pathogenesis of skin disorders. In particular, the CC chemokines thymus and activation-regulated chemokine (TARC)/macrophage-derived chemokine (MDC) play an important role in the infiltration of Th2 cells. This study was undertaken to examine the regulatory effects of interleukin (IL)-4, IL-13, and interferon (IFN)-γ on TARC/MDC production in the human keratinocyte cell line HaCaT. HaCaT cells spontaneously secrete TARC and MDC. The production of TARC/MDC was downregulated by IL-4/IL-13, whereas it was upregulated by IFN-γ. To explore these regulatory mechanisms, we investigated the capacity of cytokines to regulate expression of several adhesion molecules that may affect TARC/MDC production. Of the adhesion molecules examined, the constitutive surface expression of E-cadherin was downregulated by IL-4/IL-13, but was upregulated by IFN-γ. Moreover, disruption of the homophilic adherence of E-cadherin by anti-E-cadherin antibody or calcium chelation abolished the production of TARC/MDC. We further examined the distribution of the adherens junction complex composed of E-cadherin, α-catenin, β-catenin, and γ-catenin. IL-4/IL-13 decreased the levels of membrane staining for adherens junction proteins, whereas IFN-γ increased membrane staining. Taken together, these results suggest that IL-4/IL-13 and IFN-γ induce alternations in the distribution of adherens junctions in a different fashion and thereby contribute to the reciprocal regulation of TARC/MDC production.**

Key words: TARC/E-cadherin/IL-4/IFN-g/Keratinocyte.
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Keratinocytes produce a vast repertoire of cytokines, including interleukins, growth factors, colony-stimulating factors, and chemokines. The pattern of cytochemokines secreted locally plays a critical role in modulating the nature of tissue inflammation (Uchi *et al*, 2000). Thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22) are ligands for the CC chemokine receptor 4 (CCR4), which is expressed selectively on Th2 cells. Ligation of TARC/MDC and CCR4 plays an important role in the migration of Th2 cells into inflamed tissues (Imai *et al*, 1999; Yoshie *et al*, 2001). Very recently, serum levels of TARC and MDC were shown to correlate well with disease severity in Th2-prone skin disorders, such as atopic dermatitis and mycosis fungoides (Kakinuma *et al*, 2001, 2002, 2003). Both chemokines are produced from various cell types, such as dendritic cells, T cells, fibroblasts, and epithelial cells (Alferink *et al*, 2003; Vulcano *et al*, 2001; Hirata *et al*, 2003; Fukuda *et al*, 2003; Albanesi *et al*, 2001).

Immunohistologic staining has revealed that keratinocytes express TARC and MDC in the lesional, but not in the nonlesional, skin of atopic dermatitis *in vivo* (Vestergaard *et al*, 2000; Kakinuma *et al*, 2001; Horikawa *et al*, 2002). In addition, the expression of TARC was demonstrated in the lesional skin of atopic model mice NC/Nga (Vestergaard *et al*, 1999). Moreover, the expression of TARC was detected in the lesional skin of mycosis fungoides (Kakinuma *et al*, 2003). In sharp contrast to these *in vivo* reports, cultured normal human keratinocytes were reported to be capable of secreting only MDC, but not TARC, whereas they expressed mRNA for both cytokines (Albanesi *et al*, 2001; Horikawa *et al*, 2002; Tsuda *et al*, 2003). On the other hand, a spontaneously immortalized, nontumorigenic human HaCaT keratinocyte cell line has been described to synthesize and secrete TARC upon stimulation with interferon (IFN)-γ and tumor necrosis factor (TNF)-α (Vestergaard *et al*, 2000; Tsuda *et al*, 2003). The production of TARC by keratinocytes was also confirmed by culturing a murine keratinocyte cell line (PAM212) in the presence of IFN-γ,

Abbreviations: BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; MDC, macrophage-derived chemokine; p-, phospho-; PBS, phosphate-buffered saline; TARC, thymus and activation-regulated chemokine; TNF, tumor necrosis factor.

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TNF-α, or interleukin (IL)-1β (Vestergaard *et al*, 1999). Although the results, thus far, remain controversial, HaCaT keratinocytes may mimic the lesional keratinocytes of atopic dermatitis and mycosis fungoides.

To understand the regulatory mechanisms for TARC and MDC production in keratinocytes, we investigated the effects of IL-4, IL-13, and IFN-γ on TARC and MDC production by HaCaT keratinocytes. We found that IL-4/IL-13 and IFN-γ induce different alternations in the distribution of adherens junctions and, thereby, contribute to the reciprocal regulation of TARC and MDC production.

## Results

**HaCaT cells, but not normal human keratinocytes, secreted TARC and MDC spontaneously** We examined the production of TARC and MDC from normal human keratinocytes and HaCaT keratinocytes. As previously documented (Albanesi *et al*, 2001; Horikawa *et al*, 2002; Tsuda *et al*, 2003), both normal human keratinocytes and HaCaT keratinocytes expressed mRNA for TARC and MDC (Fig 1*A*). We examined whether normal keratinocytes and HaCaT keratinocytes express the IL-4/IL-13 and IFN-γ receptor components. As shown in Fig 1*A*, mRNA for IL-4Rα, IL-13Rα1, and IL-13Rα2 was expressed, whereas mRNA for IL-2Rγ was undetectable. For IFN-γR1 expression, we examined using flow cytometric analysis. As shown in Fig 1*B*, IFN-γR1 was expressed on the surface of both normal keratinocytes and HaCaT keratinocytes. Only HaCaT keratinocytes produced substantial amounts of TARC and MDC (Fig 1*C*). Because the spontaneous secretion of TARC and MDC by HaCaT keratinocytes achieved a plateau after 48 h of incubation, the following experiments were designed with 48 h of incubation.

**The spontaneous production of TARC/MDC mRNA and protein was suppressed by IL-4/IL-13, but enhanced by IFN-γ** We addressed the effects of IL-4/IL-13 or IFN-γ on TARC/MDC production. HaCaT cells were cultured with or without 0.1 to 10 ng per ml each of IL-4, IL-13, or IFN-γ for 48 h. As shown in Fig 2, both IL-4 and IL-13 downregulated the spontaneous production of TARC/MDC in a concentration-dependent manner. In contrast, IFN-γ augmented the spontaneous production of TARC/MDC. We evaluated whether the regulation of TARC/MDC production by IL-4/IL-13 or IFN-γ accompanied the regulation of TARC/MDC mRNA expression using RT-PCR. As shown in Fig 3*A*, in HaCaT keratinocytes, TARC and MDC mRNA expression was suppressed as early as 1 h after the addition of either IL-4 or IL-13 and gradually decreased thereafter. In contrast, TARC and MDC mRNA expression was enhanced significantly for 24 h by IFN-γ. Like HaCaT cells, TARC/MDC mRNA expression was suppressed by IL-4 and IL-13, but enhanced by IFN-γ for 24 h of incubation in normal human keratinocytes (Fig 3*B*).

**E-cadherin expression was downregulated by IL-4 or IL-13, but was upregulated by IFN-γ** To explore the regulatory mechanisms involved, we investigated the capacity of IL-4/IL-13 or IFN-γ to induce or regulate the expression of several adhesion molecules that may affect TARC and MDC production. After 48 h of incubation with the medium alone or with 10 ng per mL IL-4, IL-13, or IFN-γ, the expression of adhesion molecules was determined by flow cytometry. As indicated in Fig 4, of the adhesion molecules examined, the constitutive expression of E-cadherin and CD54 was downregulated by IL-4, whereas this expression was upregulated by IFN-γ in HaCaT keratinocytes. IL-13 presented similar results as that observed for IL-4 (data not shown). Nevertheless, neither CD11b nor CD18, which are ligands for CD54, was induced, even after cytokine stimulation. In normal human keratinocytes, IFN-γ also upregulated the E-cadherin expression and IL-4/IL-13 downregulated its expression (Table II).

**Disruption of homophilic adhesion of E-cadherins abolished the spontaneous production of TARC and MDC** To determine whether the homophilic interaction between E-cadherins might be involved in the regulation of TARC/MDC production, the production of TARC and MDC was assessed after using an anti-E-cadherin antibody that blocks binding activity. The anti-E-cadherin antibody (1–30 μg/ml) dramatically reduced TARC/MDC production in a concentration-dependent manner (Fig 5). To confirm the participation of E-cadherin in TARC/MDC production, we examined the effect of $Ca^{2+}$ depletion on TARC/MDC production. As indicated in the top part of Fig 6, the constitutive expression of E-cadherin was suppressed by $Ca^{2+}$-free medium treatment and was more potently suppressed by EGTA plus $Ca^{2+}$-free medium treatment. In accordance with the levels of E-cadherin expression, TARC/MDC production was inhibited (bottom part of Fig 6). These data suggest that the levels of E-cadherin expression may contribute to the regulation of TARC/MDC production by HaCaT cells. Thus, we focused our experiments on the adherens junction complex composed of E-cadherin, α-catenin, β-catenin, and γ-catenin.

**IL-4/IL-13 and IFN-γ induced alternations in the distribution of adherens junction proteins** We examined whether IL-4/IL-13 or IFN-γ induced any change on the amount of the each adherens junction protein. As shown in Fig 7, no significant changes in the amount of each adherens junction protein were detected in IL-4/IL-13- or IFN-γ-treated cells. These data indicate that the level of E-cadherin expression by IL-4/IL-13 or IFN-γ is not regulated by changes in the amount of adherens junction proteins. We then examined the distribution of the adherens junction complex by confocal immunofluorescent microscopy. As shown in Fig 8, in nontreated cells, staining for E-cadherin, α-catenin, β-catenin, and γ-catenin was localized to the cell membrane. Nevertheless, membrane staining for E-cadherin and catenins was reduced after IL-4/IL-13 stimulation, whereas the immunoreactivity for surface E-cadherin was increased after IFN-γ stimulation (Fig 9). Taken together, these results may indicate that IL-4 and IL-13 promote the internalization of adherens junction complex, leading to a downregulation of TARC/MDC production. In contrast, IFN-γ inhibits the internalization of adherens junction complex, thus upregulating TARC and MDC production.

We also investigated whether the activation of ERK1/2, p38 MAPK, and JNK was triggered by IL-4, IL-13, and

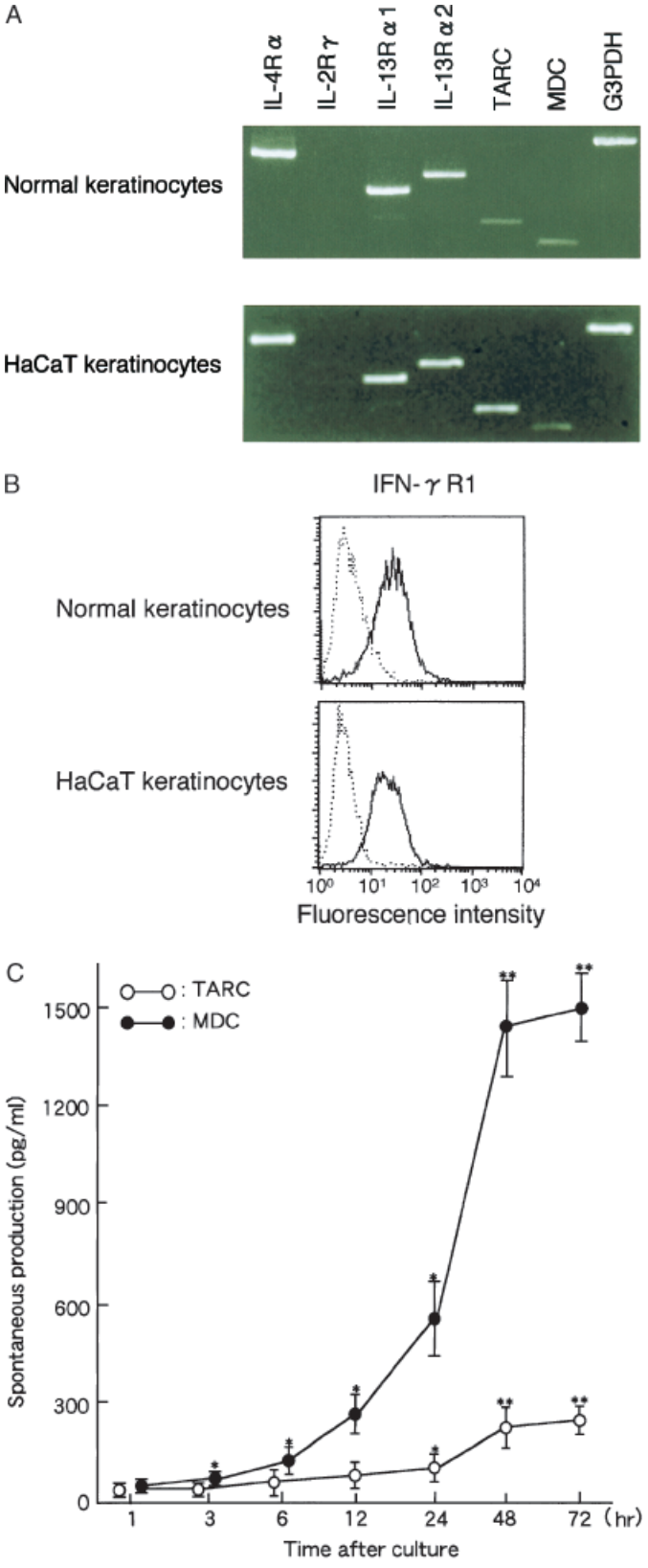



**Figure 1**
Phenotypic and functional profiles. (*A*) The expression of mRNA for IL-4/IL-13 receptor components and TARC/MDC was analyzed by RT-PCR. (*B*) The surface expression of IFN-γR1 was analyzed by flow cytometry. (*C*) A time-course study of spontaneous TARC/MDC production in HaCaT cells. All values are the mean ± SEM of triplicate experiments. $^{*}p<0.05$; $^{**}p<0.005$.

IFN-γ. The activation of p38 MAPK or JNK by any cytokine was not detected (data not shown). In contrast, ERK1/2 was spontaneously activated, but was not regulated by cytokines (Fig 10).

## Discussion

TARC and MDC are very potent chemokines for CCR4 + Th2 cells (Kawasaki *et al*, 2001; Yoshie *et al*, 2001). These chemokines are produced from various cell types such as immunocompetent dendritic cells, T cells, and endothelial cells (Alferink *et al*, 2003; Vulcano *et al*, 2001; Hirata *et al*, 2003; Fukuda *et al*, 2003; Albanesi *et al*, 2001). Because keratinocytes are one of the candidates capable of producing TARC and MDC, the regulatory mechanisms involved in their secretion are of interest. In this study, we compared TARC and MDC secretion from normal human keratinocytes and HaCaT keratinocytes. Consistent with previous findings, HaCaT keratinocytes, but not normal human keratinocytes, secreted TARC and MDC spontaneously. The upregulation of the ERK1/2 signaling pathway in the HaCaT cells may explain this difference. This hypothesis is supported by the fact that an ERK1/2 inhibitor inhibited TARC production by HaCaT keratinocytes (our unpublished data). Further study is necessary to elucidate this point.

HaCaT keratinocytes constitutively express IL-4Rα, IL-13Rα1, and IFN-γR1 on their surface, similar to normal human keratinocytes (David *et al*, 2001; Nickoloff *et al*, 1987; Akaiwa *et al*, 2001). Therefore, we investigated the effect of IFN-γ (Th1 cytokine) and IL-4/IL-13 (Th2 cytokine) on TARC and MDC production. Interestingly, IFN-γ significantly augmented and IL-4/IL-13 inhibited TARC and MDC production in a concentration-dependent manner. Many investigators have reported the regulatory effects of cytokines on TARC/MDC production. PAM 212 cells, a murine keratinocyte cell line, produced TARC after stimulation with either TNF-α or IFN-γ (Vestergaard *et al*, 1999). IL-10 greatly augmented the TNF-α + IFN-γ-induced TARC production by HaCaT cells (Vestergaard *et al*, 2001). In bronchial epithelial cells, Sekiya *et al* (2000) reported that A549 and BEAS-2B cells produce TARC protein after dual stimulation with TNF-α + IL-4. Moreover, IFN-γ synergistically upregulated the TNF-α + IL-4-induced TARC production. Terada *et al* (2001) reported that combined stimulation with IL-4/IL-13 and TNF-α induced TARC expression in nasal epithelial cells. Similarly, human corneal fibroblasts have been shown to produce TARC only in the presence of both IL-4 and TNF-α (Kumagai *et al*, 2000). In monocytes, IL-4/IL-13 induces MDC production, whereas IFN-γ inhibits it (Andrew *et al*, 1998; Bonecchi *et al*, 1998). In HaCaT keratinocytes, the production of TARC and MDC are reciprocally regulated by Th1 and Th2 cytokines.

E-cadherin is a potent cell adhesion molecule and is indispensable in the maintenance of the structural and functional rigidity of the epithelium. The blockage of cadherins by antibodies induced tissue destruction and the dissociation of the cultured tissue (Vestweber and Kelmer, 1985). Also, misregulated E-cadherin expression or

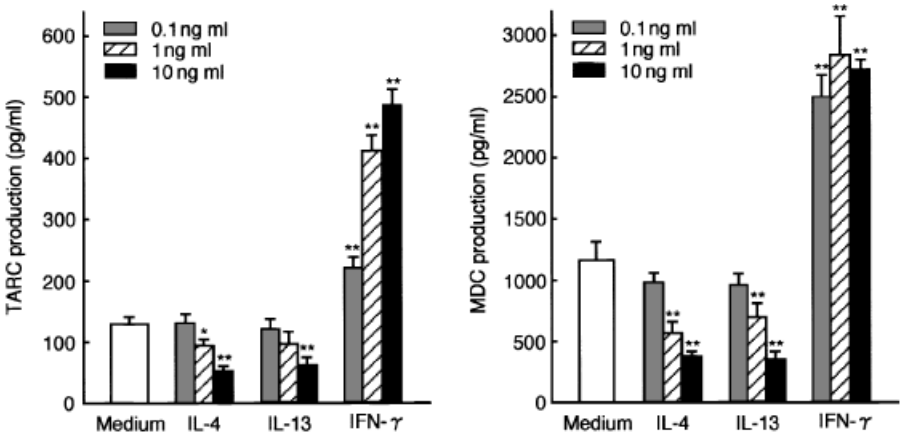



**Figure 2**
Effects of IL-4/IL-13 and IFN-γ on TARC/MDC production. Cells were incubated with medium alone or with IL-4, IL-13, or IFN-γ for 48 h. After incubation, the concentrations of TARC/MDC in the supernatants were quantified by ELISA procedures. Results represent the mean ± SEM of triplicate experiments. *$p < 0.05$ and **$p < 0.005$ indicate a statistically significant difference versus unstimulated control cells.

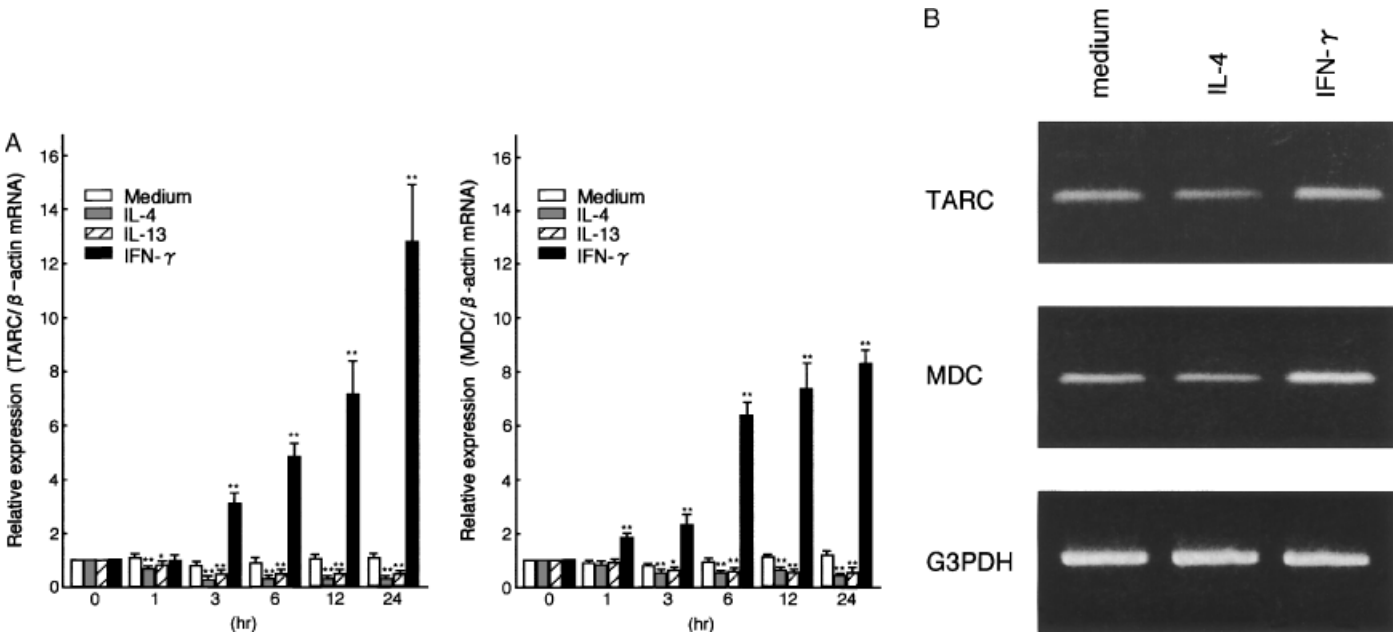



**Figure 3**
Effects of IL-4/IL-13 and IFN-γ on TARC/MDC mRNA expression. (*A*) HaCaT cells were incubated with medium alone or with 10 ng per mL each of IL-4, IL-13, or IFN-γ for the indicated times. The ratio of TARC/MDC mRNA to β-actin mRNA was determined by quanti-tative real-time RT-PCR. Results represent the mean ± SEM of triplicate experiments. *$p < 0.05$; **$p < 0.005$. (*B*) Normal human keratinocytes were incubated with medium alone or with 10 ng per mL each of IL-4 or IFN-γ for 24 h. mRNA for TARC/MDC was analyzed by RT-PCR. Like IL-4, IL-13 gave similar results (data not shown).

function can alter the pattern of epithelial cell growth, and differentiation (Gumbiner, 1996).

Several investigators have reported the regulation of E-cadherin expression by IL-4, IL-13, and IFN-γ (Fenyves *et al*, 1993; Kanai *et al*, 2000). In bovine microvascular endothelial cells, IFN-γ upregulated E-cadherin expression (Fenyves *et al*, 1993). In a human colon cancer cell line (colo205), IL-4 and IL-13 inhibit cell–cell adhesion by downregulating E-cadherin (Kanai *et al*, 2000). In this study, we demonstrated that the surface expression of E-cadherin in keratinocytes is suppressed by IL-4/IL-13, but is enhanced by IFN-γ. E-cadherin-mediated cell–cell adhesion is disrupted by Th2 cytokines, but enhanced by the Th1 cytokine. We demonstrated that the integrity of surface E-cadherin was closely linked to the production of TARC and MDC. Disruption of the homophilic adhesion of E-cadherins by anti-E-cadherin antibody blockade or by extracellular calcium chelating abolished the spontaneous production of TARC and MDC. This strongly suggests that E-cadherin is involved in the regulation of TARC/MDC production. In keratinocytes, E-cadherin forms an adherens junction complex with β-catenin or γ-catenin and α-catenin. β-Catenin or γ-catenin binds to the cytoplasmic tail of E-cadherin and to α-catenin. α-Catenin in turn binds to the actin cytoskeleton. This assembly is important for cell–cell adhesion (Kondo *et al*, 1998; Kobayashi *et al*, 2000; Pece and Gutkind, 2000; Hu *et al*, 2001). Interestingly, IL-4/IL-13 enhanced the internalization of E-cadherin/catenins complex into the cytoplasm and disrupted surface E-cadherin expression, resulting in the downregulation of TARC/MDC production. Conversely, IFN-γ augmented the surface membrane distribution of E-cadherin/catenins, resulting in the upregulation of TARC/MDC production.

Hu *et al* (2001) reported that in peroxovanadate-treated human keratinocytes, the distribution of adherens junction proteins is altered with the tyrosine phosphorylation of

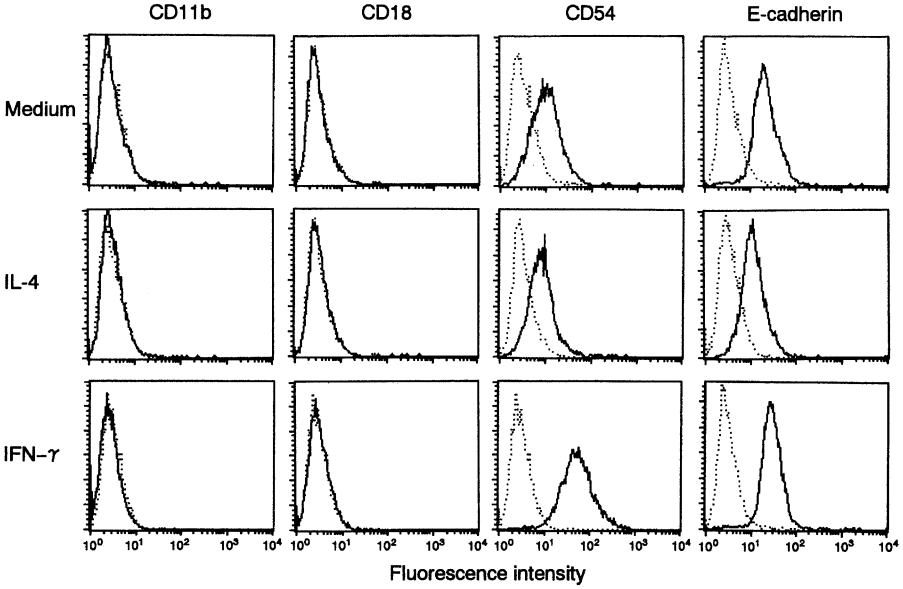



**Figure 4**
Effects of IL-4 and IFN-γ on CD11b, CD18, CD54, and E-cadherin expression. Cells were cultured with medium alone or with 10 ng per mL each of IL-4 or IFN-γ for 48 h. Expression of these adhesion molecules was analyzed by flow cytometry. Representative data are shown (n = 3). Like IL-4, IL-13 gave similar results (data not shown).

**Table I. Sequences of primers used for PCR**

| Amplified RNA | Primer sequence | Size of amplified product (bp) |
|---|---|---|
| IL-4Rα | 5′-GCTATGTCAGCATCACCAAGA-3′ | 830 |
| | 5′-TTCTACTTCCTCCAGGTGTCT-3′ | |
| IL-2Rγ | 5′-TTTCTTCCTGACCACTATGCC-3′ | 479 |
| | 5′-CAGTCCAGCTGTGGTCCCAG-3′ | |
| IL-13Rα1 | 5′-GGAGCCAGCTCAATTTGTAG-3′ | 510 |
| | 5′-CACACGGGAAGTTAAAGGCA-3′ | |
| IL-13Rα2 | 5′-ATGGCTTTCGTTTGCTTGGCTA-3′ | 620 |
| | 5′-TAGTCTGATGCCTCCAAATAGG-3′ | |
| TARC | 5′-ATGGCCCCACTGAAGATGCT-3′ | 351 |
| | 5′-TGAACACCAACGGTGGAGGT-3′ | |
| MDC | 5′-GCTCGCCTACAGACTGCACTC-CTG-3′ | 268 |
| | 5′-GCTTATTGAGAATCATCTTCACC-CAGG-3′ | |
| G3PDH | 5′-TGAAGGTCGGAGTCAACGGATTT-GGT-3′ | 983 |
| | 5’-CATCTGGGCCATGAGGTCCACC-AC-3′ | |

**Table II. Effects of IL-4, IL-13, and IFN-γ on E-cadherin expression in normal human keratinocytes[a]**

| | Mean fluorescence intensity | Significance versus medium |
|---|---|---|
| Medium | 7.01 ± 0.63 | |
| IL-4 | 5.69 ± 0.61 | $p < 0.2$ |
| IL-13 | 5.34 ± 0.83 | $p < 0.2$ |
| IFN-γ | 8.90 ± 0.87 | $p < 0.2$ |

[a]Cells were cultured with medium alone or with 10 ng per mL each of IL-4, IL-13, or IFN-γ for 48 h. Expression of E-cadherin was analyzed by flow cytometry. Results represent the mean fluorescence intensity ± SEM of triplicate experiments.

β-catenin and γ-catenin. Thus, we examined the tyrosine phosphorylation of adherens junction proteins in the presence of cytokines. No tyrosine phosphorylation was induced by any cytokine stimulation (data not shown). Nelms *et al* (1999) described that in the A431 keratinocyte cell line, IL-4 not only activated the JAK/STAT pathway, but also regulated the activity of various members of the MAPK family, which is in contrast to what has been described in most hematopoietic cells. Therefore, we investigated whether the activation of ERK1/2, p38 MAPK, and JNK were modulated by IL-4, IL-13, and IFN-γ. Our results show that the activation of p38 MAPK, JNK, or ERK1/2 was not modified by any cytokine.

The function of the keratinocyte is known to be regulated via intracellular signaling pathways triggered by growth factors (Hashimoto, 2000). We explored the role of some growth factors in the reciprocal regulation of TARC/MDC production by IL-4/IL-13 and IFN-γ. The growth factors examined, PDGF-AB, IL-6, amphiregulin, EGF, basic FGF, and PDGF-BB, had no effect on the regulation of TARC/MDC production by HaCaT cells (data not shown).

In conclusion, our results suggest that in HaCaT keratinocytes, IL-4/IL-13 and IFN-γ induce differing alterations in the distribution of adherens junctions and, thereby, contribute to the reciprocal regulation of TARC and MDC production. Although the molecular mechanisms triggered by these cytokines remain unclear, our findings that TARC and MDC production are regulated in parallel with E-cadherin expression in keratinocytes may provide a new clue for understanding the orchestrated regulation of TARC/MDC production.

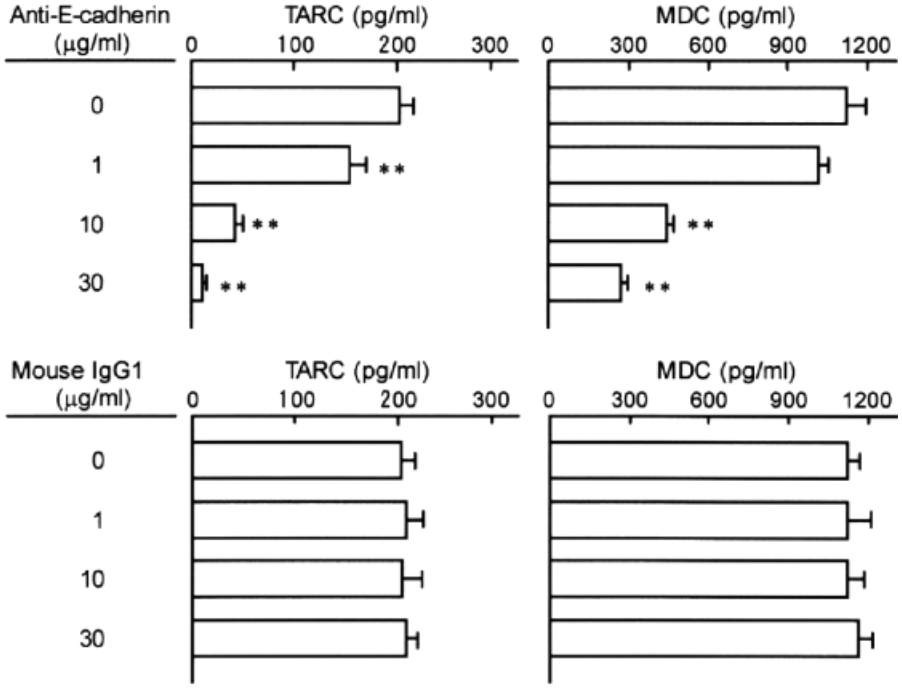



**Figure 5**
Inhibitory effect of anti-E-cadherin antibody on the spontaneous production of TARC/MDC. Cells were incubated with or without the anti-E-cadherin antibody or control antibody for 48 h. Levels of TARC/MDC in the supernatants were quantified by ELISAs. Results represent the mean ± SEM of triplicate experiments. *$p<0.05$; **$p<0.005$.

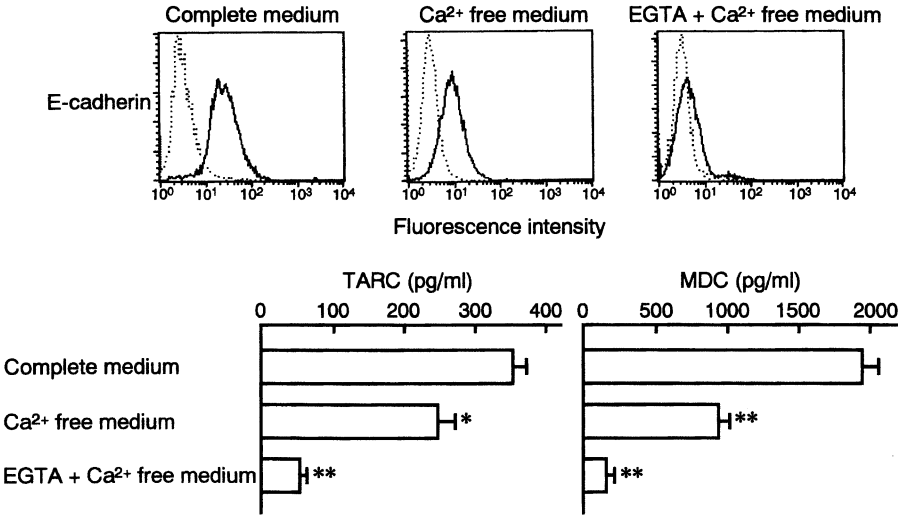



**Figure 6**
Effects of $Ca^{2+}$ depletion on E-cadherin expression and TARC/MDC production. Cells were cultured with ordinary $Ca^{2+}$-containing medium for 48 h, with $Ca^{2+}$-free medium for 48 h, or with $Ca^{2+}$-free medium for 47 h followed by EGTA (4 mM) treatment for 1 h. After incubation, E-cadherin expression and TARC/MDC production were determined using flow cytometry and ELISA procedures, respectively. Representative data (in top panels) and mean ± SEM (in bottom panels) are shown ($n=3$). *$p<0.05$; **$p<0.005$.

## Materials and Methods

**Reagents** Recombinant human IL-4, IL-13, and IFN-γ were purchased from Genzyme (Cambridge, MA). ELISA kits for TARC and MDC were obtained from R&D Systems (Minneapolis, MN). All reagents for quantitative real-time PCR were from Roche Molecular Biochemicals (Germany). For flow cytometry staining, polyclonal anti-IFN-γR1 antibody was obtained from R&D systems (Minneapolis, MN). Fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-CD11b antibody was purchased from Beckman Coulter (Fullerton, CA). FITC-conjugated monoclonal anti-CD18 antibody was purchased from Dako (Denmark). FITC-conjugated monoclonal anti-CD54 antibody was purchased from Serotec (UK). Monoclonal anti-E-cadherin antibody (Immunotech, France) was used for flow cytometry as well as for blocking experiments. Phycoerythrin-conjugated $F(ab')_2$ fragments of goat anti-mouse immunoglobulin antibody was from Biosource (Camarillo, CA). Streptavidin-phycoerythrin was purchased from Becton Dickinson (San Jose, CA). For immunoblotting and immunofluorescent staining, monoclonal anti-α-catenin, monoclonal anti-β-catenin, horseradish peroxidase-conjugated goat anti-mouse IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG were from Zymed (South San Francisco, CA). Monoclonal anti-E-cadherin and anti-γ-catenin antibodies were from Transduction Laboratories (Lexington, KY). Polyclonal anti-phospho (p)-ERK1/2 (Thr202, Tyr204), anti-p-p38 MAPK (Thr180, Tyr182), anti-p-JNK (Thr183, Tyr185), anti-ERK1/2, anti-p38 MAPK, and anti-JNK antibodies were obtained from Cell Signaling Technology (Beverly, MA). FITC-conjugated $F(ab')_2$ fragments of goat anti-mouse immunoglobulin antibody was from Biosource (Camarillo, CA).

**Cell culture** The HaCaT keratinocyte cell line was provided by N. E. Fusenig, DKFZ Heidelberg (Boukamp *et al*, 1988). HaCaT cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (Gibco-BRL), 100 U per mL penicillin, 100 μg per mL streptomycin (Gibco-BRL) at 37°C. In specific experiments described below, cells were serum-starved for 24 h before

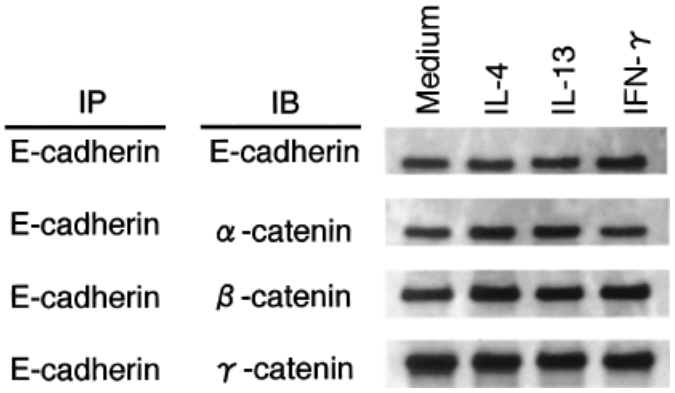



**Figure 7**
Analysis of adherens junction complexes composed of E-cadherin, α-catenin, β-catenin, and γ-catenin. Cells were stimulated with or without 10 ng per mL each of IL-4, IL-13, or IFN-γ for 48 h. The lysates were immunoprecipitated with anti-E-cadherin antibody and blotted with monoclonal antibodies to E-cadherin, α-catenin, β-catenin, or γ-catenin.

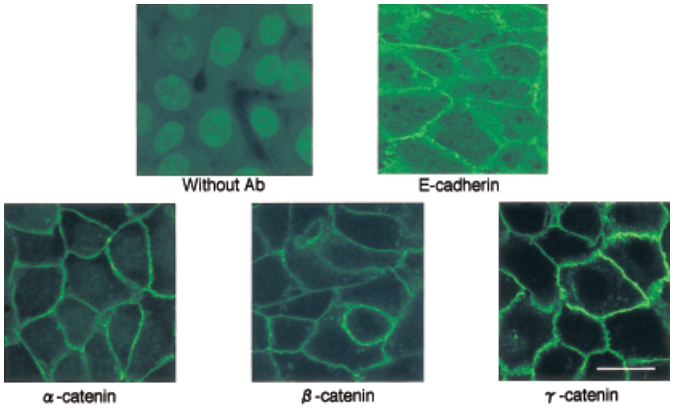



**Figure 8**
Distribution of adherens junction proteins. After fixation and permeabilization, cells were stained with monoclonal antibodies to E-cadherin, α-catenin, β-catenin, or γ-catenin and detected with FITC-conjugated anti-mouse IgG. Stained cells were analyzed by confocal laser microscopy. *Bar,* 10 μm.

treatment with or without IL-4, IL-13, or IFN-γ. Normal human keratinocytes from neonatal foreskin (Kurabou, Osaka, Japan) were grown initially in keratinocyte basal medium (HuMedia-KB2, Kurabou) containing 0.15 mM calcium supplemented with 0.1 ng per mL epidermal growth factor, 0.4% bovine pituitary extract, 50 μg per mL gentamycin, and 50 ng per mL amphotericin B.

**ELISA** Concentrations of TARC and MDC in culture supernatants were measured by commercially available ELISA kits according to the manufacturer's instructions. These ELISA procedures enabled detection of TARC concentrations of greater than 15.6 pg per mL and MDC concentrations of greater than 62.5 pg per mL.

**RT-PCR** Total RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden Germany). RT was performed using oligo(dT)$_{12\text{-}18}$ primer (Invitrogen Corp., Carlsbad, CA) and a Superscript II RNase reverse transcriptase kit (Invitrogen Corp.) according to the manufacturer's protocol. To analyze the expression of IL-4/IL-13 receptor components and TARC/MDC in unstimulated HaCaT cells, semiquantitative PCR was performed on a thermal cycler (TaKaRa, Tokyo, Japan) using a Pyrobest DNA polymerase kit (TaKaRa, Tokyo, Japan). The sequences of the PCR primers are shown in Table I. G3PDH was used as a reference gene. The thermal cycling conditions were set to 98°C for 5 min, followed by 28 cycles of 98°C for 10 s, 62°C for 30 s, 72°C for 1 min, and 72°C for 4 min. The amplified products were subjected to electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. We investigated whether 10 ng per mL each of IL-4, IL-13, or IFN-γ had any effect on the expression of TARC/MDC mRNA. Quantitative real-time PCR was performed on a LightCycler (Roche Molecular Biochemicals, Germany) in LightCycler capillaries using the LightCycler FastStart DNA master SYBR green I kit (Roche Molecular Biochemicals) and LightCycler primer sets for TARC (CCL17), MDC (CCL22), and β-actin (Roche Molecular Biochemicals) were used. Thermal cycler conditions were set to 95°C for 10 min, followed by 35 cycles of 95°C for 10 s, 68°C for 10 s, and 72°C for 16 s. To control for the specificity of the amplification products, a melting curve analysis was performed. No amplification of unspecific products was observed. The copy number was calculated from a standard curve.

**Flow cytometry** The surface expression of IFN-γR1 or adhesion molecules was determined by flow cytometry after 0 or 48 h of incubation with or without 10 ng per mL each of IL-4, IL-13, or IFN-γ. A total of $5 \times 10^5$ cells were suspended in 50 μL of phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and 0.05% $NaN_3$ (FACS buffer). To analyze IFN-γR1 expression, the cells were sequentially incubated with biotinylated anti-IFN-γR1 antibody for 30 min at 4°C and streptavidin-phycoerythrin for 30 min at 4°C. Expression of CD11b, CD18, and CD54 was assessed by staining with the appropriate antibodies labeled with FITC for 30 min at 4°C. To analyze E-cadherin expression, the cells were first incubated with monoclonal anti-E-cadherin antibody for 30 min at 4°C and detected with phycoerythrin-conjugated goat anti-mouse immunoglobulins. In control samples, staining was performed with isotype-matched control antibodies. After being washed and fixed with 0.5% paraformaldehyde, the cells were

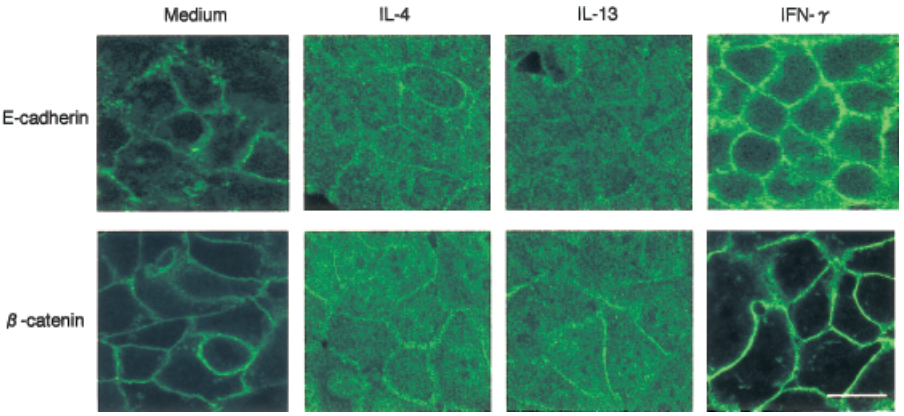



**Figure 9**
Effects of IL-4/IL-13 and IFN-γ on the distribution of E-cadherin and β-catenin. Cells were treated with medium alone or with 10 ng per mL each of IL-4, IL-13, or IFN-γ for 48 h. After fixation and permeabilization, immunostaining, and microscopic analyses were performed. Similar results were obtained for α-catenin and γ-catenin (data not shown). *Bar,* 10 μm.

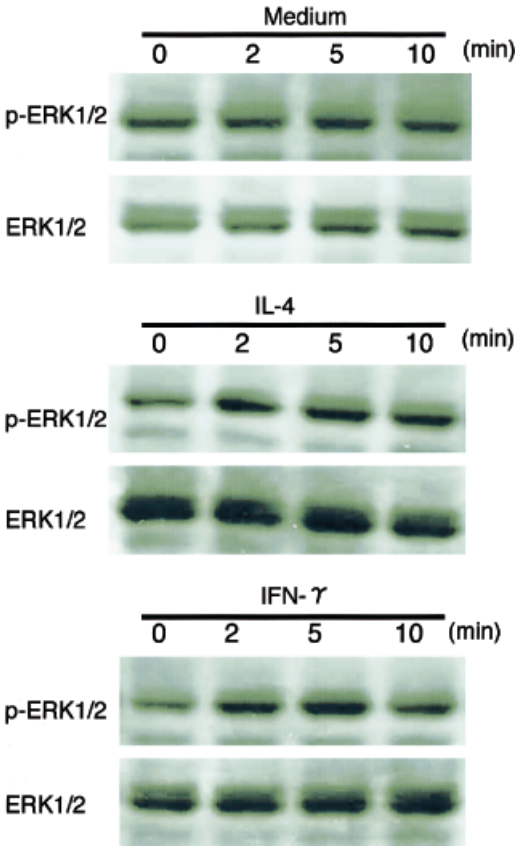



**Figure 10**
Effects of IL-4 and IFN-γ on ERK1/2 activation. Cells were treated with medium alone or with 10 ng per mL each of IL-4 or IFN-γ for the indicated times. The lysates were blotted with anti-p-ERK1/2 antibody. To evaluate the total amount of ERK present on the blot, the blot was stripped and reprobed with anti-ERK antibody. Like IL-4, IL-13 gave similar results (data not shown).

analyzed by FACSCalibur using CELLQUEST software (Becton Dickinson). Forward- and side-scatter gates were set to identify living cells and 10,000 events were acquired.

**Effect of anti-E-cadherin antibody on the spontaneous production of TARC/MDC** To assess the involvement of E-cadherin in the regulation of TARC/MDC production, an anti-E-cadherin antibody (1, 10, and 30 μg/ml) and isotype-matched control antibody were added to the cultures for 48 h. After incubation, the supernatant was collected for ELISA procedures for TARC/MDC. In addition, because E-cadherin is a calcium-dependent adhesion molecule, we examined the effect of $Ca^{2+}$ depletion on TARC/MDC production. Cells were cultured with ordinary $Ca^{2+}$-containing medium (1.8 mM) for 48 h, with $Ca^{2+}$-free medium for 48 h, or with $Ca^{2+}$-free medium for 47 h followed by EGTA (4 mM) treatment for 1 h. After incubation, E-cadherin expression and TARC/MDC production were determined using flow cytometry and ELISAs, respectively.

**Immunoprecipitation and western blotting** The effect of cytokines on the amount of adherens junction complex composed of E-cadherin, α-catenin, β-catenin, and γ-catenin was analyzed by western blotting. After stimulation with or without 10 ng per mL each of IL-4, IL-13, or IFN-γ for 48 h, the cells were lysed on ice with lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM β-glycerol phosphate, 1 mM phenylmethylsufonyl fluoride, 10 μg per mL aprotinin, and 10 μg per mL leupeptin. Protein concentration in extracts was determined by the Bradford assay (Bio-Rad, Hercules, CA). The lysates were precleared with protein A-Sepharose beads (Pharmacia, Sweden) for 1 h and incubated with anti-E-cadherin antibody (3 μg/ml) for 1 h at 4°C. Immunocomplexes were precipitated overnight with protein A-Sepharose beads at 4°C, washed with lysis buffer, eluted by boiling in SDS-PAGE sample buffer containing 2-mercaptoethanol (Wako, Japan), resolved on SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were then blocked with 2% BSA in PBS for 1 h and analyzed by western blotting with sequential probing of the same membrane using monoclonal antibodies to E-cadherin, α-catenin, β-catenin, and γ-catenin diluted to the manufacturer's recommendation in antibody dilution buffer (2% BSA, 0.1% Tween 20 in PBS) (1:250 E-cadherin, 1:1000 α-catenin, 1:1000 β-catenin, 1:2000 γ-catenin) for 1 h at room temperature. Blots were then washed in PBS with 0.1% Tween 20 and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:5000 in antibody dilution buffer. After being washed in PBS with 0.1% Tween 20, the blots were visualized by SuperSignal West pico chemiluminescent substrate (Pierce, Rockford, IL). When necessary, blots were stripped with Restore western blot stripping buffer (Pierce) for 15 min at 50°C, washed in PBS with 0.1% Tween 20, and sequentially reprobed with the appropriate antibodies.

The effects of cytokines on ERK1/2, p38 MAPK, and JNK activation were also examined. Cells were treated with or without 10 ng per mL each of IL-4, IL-13, or IFN-γ for 0 to 10 min. The lysates were blotted with polyclonal antibodies to p-ERK1/2, p-p38 MAPK, or p-JNK. To evaluate the total amount of ERK1/2, p38 MAPK, or JNK present on the blot, the same blot was stripped and reprobed with appropriate antibodies, respectively.

**Confocal immunofluorescent microscopy** For immunofluorescent staining, HaCaT cells were grown on ethanol-sterilized glass coverslips. Cells were incubated with or without 10 ng per mL each of IL-4, IL-13, or IFN-γ for 48 h before fixation in 3.7% paraformaldehyde in PBS for 5 min at room temperature, permeabilized with 0.25% Triton X-100 in PBS for 10 min at room temperature, and blocked with 2% BSA in PBS for 15 min at room temperature. Fixed cells were incubated overnight at 4°C in a humidity chamber with each primary antibody (E-cadherin, α-catenin, β-catenin, and γ-catenin) diluted 1:100 in antibody dilution buffer (2% BSA, 0.1% Tween 20 in PBS). After incubation, the coverslips of fixed cells were washed in PBS at 4°C, blocked with 2% BSA in PBS at 4°C for 20 min, incubated for 60 min at 4°C in the dark with FITC-conjugated anti-mouse immunoglobulins diluted 1:100 in antibody dilution buffer, washed in PBS at 4°C, and then mounted with FluoroGuard antifade reagent (Bio-Rad). Stained cells were analyzed by confocal laser microscopy at 600× magnification (Bio-Rad).

**Statistical analysis** Statistical analyses were performed using an unpaired Student's *t* test.

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