



# Enhanced CCL26 production by IL-4 through IFN- $\gamma$ -induced upregulation of type 1 IL-4 receptor in keratinocytes

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## ABSTRACT

A Th2 cytokine, IL-4, induces various chemokines from epidermal keratinocytes which play crucial roles in the pathogenesis of skin disorders such as atopic dermatitis. In contrast, the role of IFN- $\gamma$ , a Th1 cytokine, on eosinophilic skin inflammation is unclear. This study investigated the effects of IFN- $\gamma$  on IL-4-induced production of eotaxin-3/CCL26, a potent chemoattractant to eosinophils, in normal human epidermal keratinocytes (NHEK). When the cells were stimulated with IL-4 and IFN- $\gamma$  simultaneously, IL-4-induced CCL26 production was attenuated. In contrast, prior stimulation with IFN- $\gamma$  enhanced IL-4-induced CCL26 production. NHEK constitutively expressed type 1 IL-4 receptor, and expression at the cell surface was upregulated by stimulation with IFN- $\gamma$ . This upregulation resulted in an enhanced IL-4-mediated cellular signal. These results indicate that IFN- $\gamma$  has opposite effects on IL-4-induced CCL26 production in NHEK depending on the time of exposure. Thus, changes in IL-4R expression by IFN- $\gamma$  might modulate eosinophilic skin inflammation.

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Epidermal keratinocytes represent the first line of defense against harmful effects such as heat wounds and virus skin infections [1,2]. Epidermal keratinocytes also play important roles in the pathogenesis of inflammatory skin diseases such as atopic dermatitis [3–5]. Inflammatory cells in the lesional skin of atopic dermatitis are mainly composed of lymphocytes, mast cells and eosinophils [6]. These cells are believed to infiltrate the skin by various cytokines and chemokines produced from epidermal keratinocytes. Overproduction of T helper type 2 (Th2)-specific chemokines has been observed in atopic-dermatitis-like lesions in mice [4]. Thymus- and activation-regulated chemokines (TARC/CCL17) produced from epidermal keratinocytes may be involved in recruiting Th2 lymphocytes to the skin of subjects with atopic dermatitis [7]. Human keratinocytes stimulated with IL-13, a pivotal mediator of the Th2 immune response, preferentially attract Th2 lymphocytes through production of macrophage-derived chemokines (MDC/CCL22) [8]. Thus, initiation of atopic dermatitis is thought to be mediated by means of early skin infiltration of Th2 lymphocytes. Epidermal keratinocytes also produce eosinophil-attracting chemokines such as eotaxin/CCL11, eotaxin-2/CCL24, and eotaxin-3/CCL26 [9,10]. Eosinophilic inflammation in lesional skin of patients with atopic dermatitis can be explained by the production of these chemokines from epidermal keratinocytes and the Th2 cytokines, IL-4, and IL-5.

In the chronic phase of lesional skin due to atopic dermatitis, however, the accumulation of activated monocytes, dendritic cells, and eosinophils causes a rise in IL-12 expression and the appearance of a mixed Th1/Th2 cytokine pattern. Concomitant with this rise are reduced concentrations of Th2 cytokines and the presence of a Th1 cytokine, IFN- $\gamma$  [5,11]. Although IFN- $\gamma$ -induced CCL17 production from epidermal keratinocytes indicates the infiltration of Th2 lymphocytes and the production of Th2 cytokines [12], IFN- $\gamma$  was found to attenuate IL-4-induced CCL26 production in a human keratinocyte cell line [10]. These results indicate that IFN- $\gamma$  has ambiguous roles in eosinophilic skin inflammation arising from atopic dermatitis. Interestingly, it had been demonstrated that IFN- $\gamma$  has dual effects on IL-4-induced CCL26 production in airway epithelial cells, depending on the length of exposure [13].

We therefore investigated the effect of IFN- $\gamma$  on IL-4-induced CCL26 production in primary human epidermal keratinocytes (NHEK). NHEK showed dual opposite responses against IFN- $\gamma$  stimulation regarding IL-4-induced CCL26 production. The mechanism behind this response involved type 1 IL-4 receptor (IL-4R) upregulation. The results of the current study suggest that enhanced IL-4-dependent inflammation might occur in the chronic phase of atopic dermatitis.

## Materials and methods

**Cell culture.** NHEK cells were purchased from Cambrex Bio Science (Walkersville, MD). The cells were cultured using a KGM-2 Bullet kit (Cambrex Bio Science) at 37 °C in a humidified

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atmosphere containing 5% CO<sub>2</sub>. NHEK were passed by trypsinization, and cells at 70–80% confluence at the third passage were used for each experiment. Cells were seeded 1 day prior to the experiments at  $2 \times 10^5$  cells per well for six-well plates and  $4 \times 10^5$  cells per well for 6-cm plates. Prior to stimulation, the culture medium was replaced with Dulbecco's modified Eagle's medium without fetal bovine serum (FBS). Various concentrations of IL-4 and/or IFN- $\gamma$  (Peprotec, London, UK) were added to the medium and the cells were incubated as indicated. In some experiments, the cells were pretreated with IFN- $\gamma$  before IL-4 stimulation.

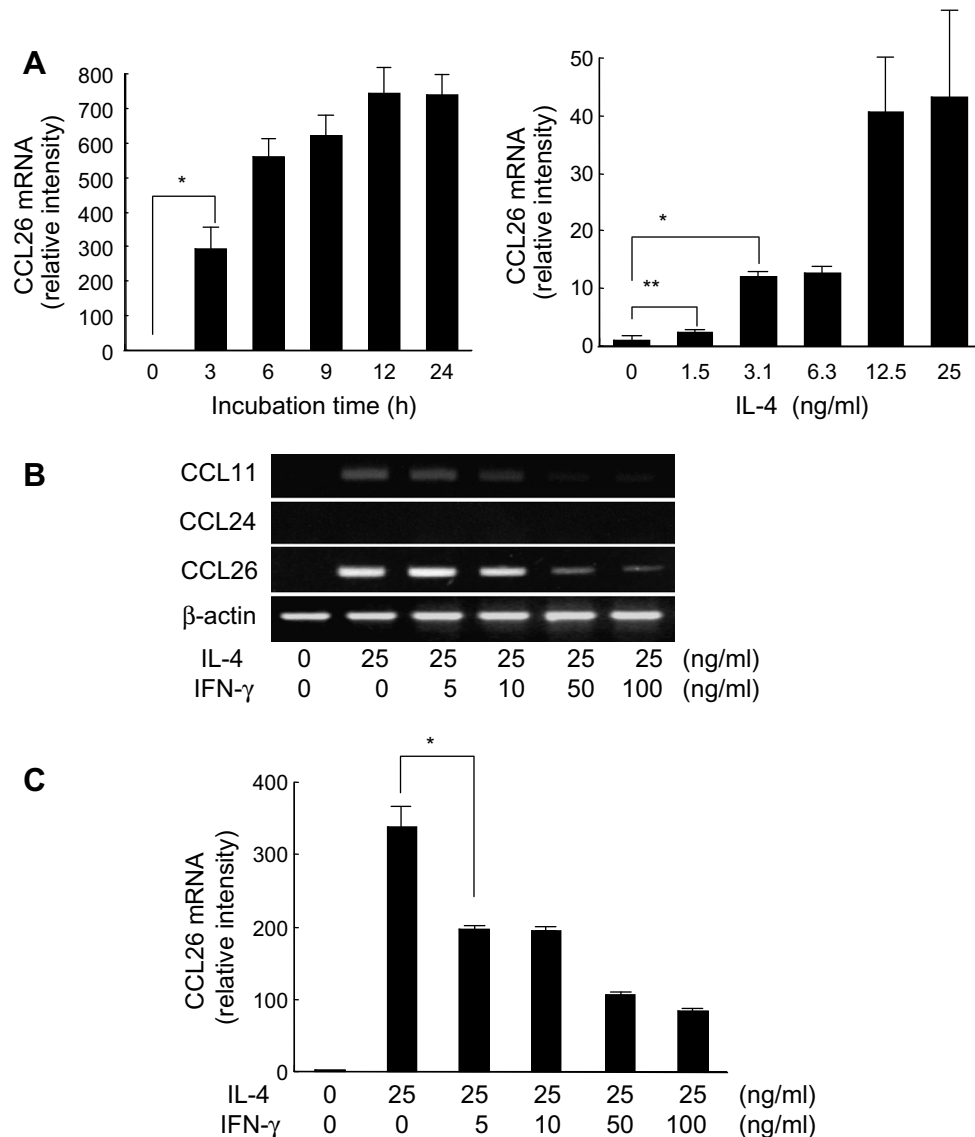
**Reverse transcription-polymerase chain reaction (RT-PCR).** Extraction of total RNA, reverse transcription and PCRs for CCL11, CCL24, CCL26, and  $\beta$ -actin were carried out as previously described [13]. PCR products were electrophoresed on a 1.5% agarose gel (Conda, Madrid, Spain) and photographed.

**Quantitative RT-PCR.** Messenger RNA expression of CCL26, IL-4R $\alpha$ ,  $\gamma$ C, IL-13R $\alpha$ 1, and IL-13R $\alpha$ 2 were also examined by quantitative

real-time PCR. All probes (CCL26: Hs 00171146\_m1; IL-4R $\alpha$ 1: Hs00166237\_m1;  $\gamma$ C: Hs00173950\_m1; IL-13R $\alpha$ 1: Hs00609817\_m1; IL-13R $\alpha$ 2: Hs00152924\_m1) were purchased from Applied Biosystems (Foster City, CA). GAPDH (Hs99999905\_m1) was used as an internal control for all PCR. Relative mRNA expression was quantified using the comparative C<sub>t</sub> ( $\Delta$ C<sub>t</sub>) method and expressed as means  $\pm$  SD.

**Enzyme-linked immunosorbent assay (ELISA).** The presence of CCL11, CCL24, and CCL26 in the culture supernatants was detected by a sandwich ELISA from R&D systems (Minneapolis, MN), performed according to the manufacturer's instructions.

**Western blotting.** Cellular protein was extracted using a lysis buffer as previously described [13]. Ten micrograms of protein were electrophoresed on 8–10% SDS polyacrylamide gel and transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA). In order to detect signal transducer and activator of transcription 6 (STAT6), phosphorylated STAT6 (pSTAT6) and IL-4R $\alpha$ , the following antibodies were used: rabbit anti-STAT6



**Fig. 1.** (A) Quantitative analysis of CCL26 mRNA expression from NHEK. Left panel: Cells were incubated with IL-4 (25 ng/ml). Right panel: Cells were incubated for 24 h. (B) Effect of IFN- $\gamma$  on CCL11, CCL24, and CCL26 mRNA expression. Cells were incubated for 24 h. Representative RT-PCR data from three separate experiments is shown. (C) Effect of IFN- $\gamma$  on IL-4-induced CCL26 mRNA expression. Results shown are means  $\pm$  SD of values from a representative experiment performed with triplicate samples (\* $P < 0.01$ ; \*\* $P < 0.05$ ).

(Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-pSTAT6 (Santa Cruz) and rabbit anti-IL-4R $\alpha$  (Santa Cruz). The membranes were developed using a West Dura detection kit (Pierce, Rockford, IL) and analyzed using a chemiluminescence analyzing system (Las-1000 plus; Fujifilm, Tokyo, Japan).

**Flow cytometry.** Flow cytometry analysis was performed as previously described [14]. The cells were labeled with phycoerythrin-conjugated mouse anti-human IL-4R $\alpha$  (Immunotech, Marseille, France) or rat anti-human  $\gamma$ C (Becton Dickinson, Mountain View, CA). The labeled cells were analyzed on a FACS Caliber (Becton Dickinson) and 10,000 events were collected.

**Statistical analysis.** Data are presented as means  $\pm$  SD. Significant differences were assessed using the paired Student's *t* test. *P* values less than 0.05 were considered statistically significant.

## Results

### Inhibitory effect of IFN- $\gamma$ on IL-4-induced CCL26 mRNA expression

CCL26 mRNA expression in NHEK was investigated first. Although no constitutive mRNA expression of CCL26 was observed, we confirmed that NHEK expressed CCL26 mRNA upon stimulation with IL-4 in a time- and dose-dependent manner (Fig. 1A). When IL-4 and IFN- $\gamma$  were present simultaneously in the culture medium, IL-4-induced CCL26 mRNA expression was significantly attenuated by IFN- $\gamma$  (Fig. 1B and C). No constitutive CCL11 mRNA was expressed

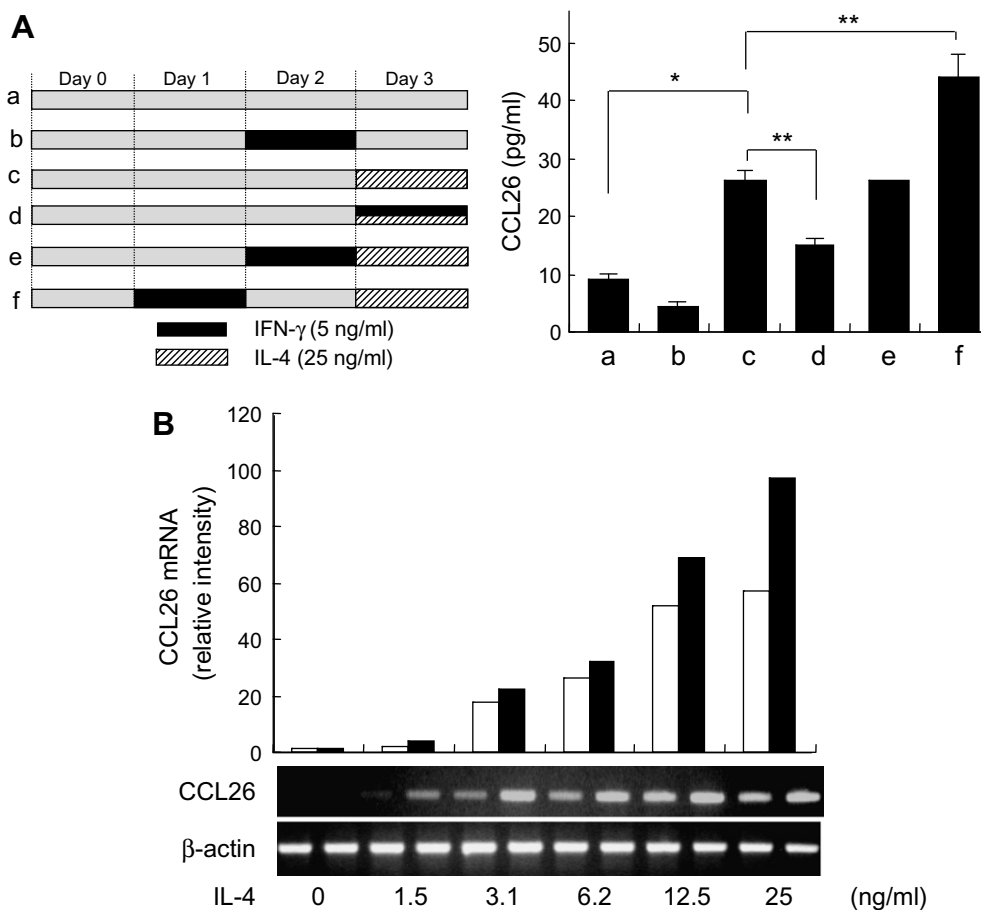
in NHEK. It was observed that a small amount of CCL11 mRNA expression was induced by IL-4, and was inhibited by IFN- $\gamma$  (Fig. 1B). No CCL24 mRNA was detected in this study (Fig. 1B).

### IFN- $\gamma$ pretreatment enhanced IL-4-induced CCL26 production

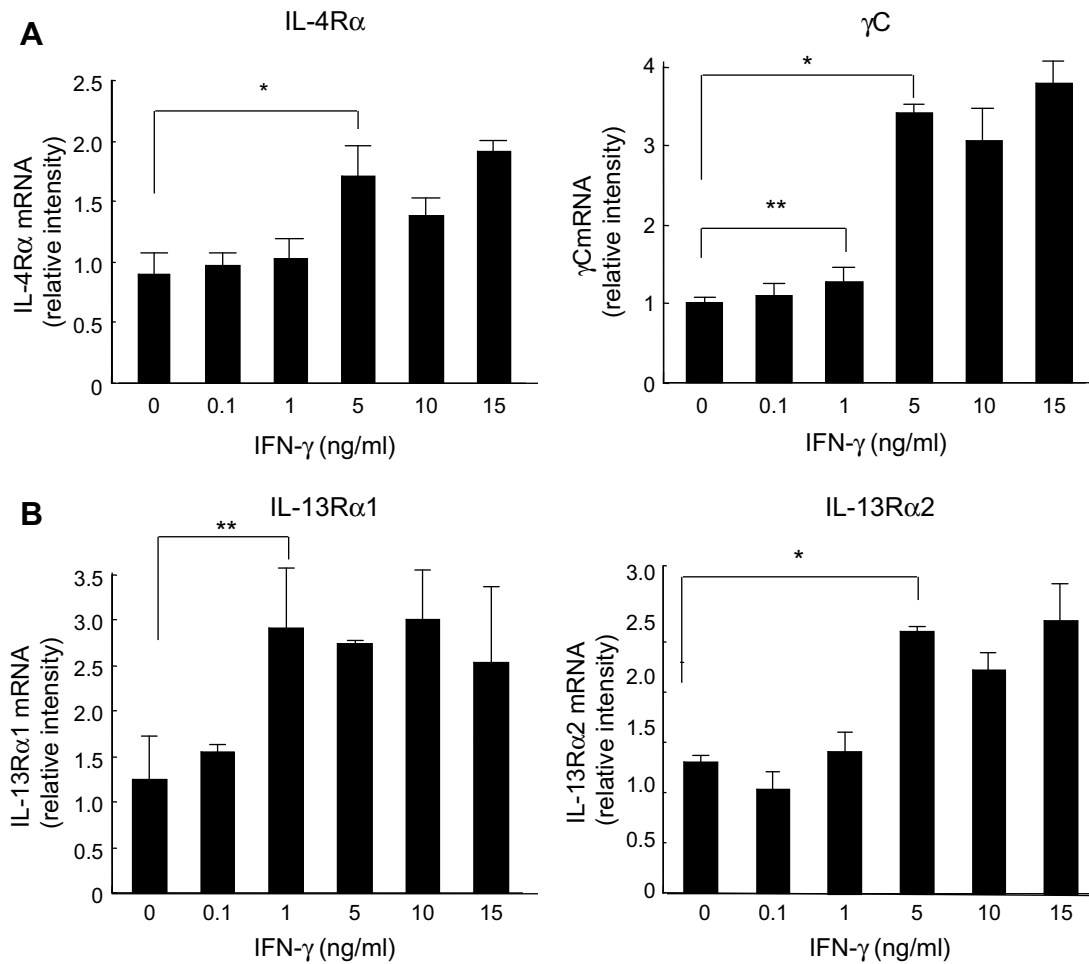
We previously observed that IFN- $\gamma$  had opposite dual effects on IL-4-induced CCL26 production in airway epithelial cells. Pretreatment with IFN- $\gamma$  enhanced IL-4-induced CCL26 production in the cells [13]. We therefore investigated the effect of IFN- $\gamma$  pretreatment on IL-4-induced CCL26 production in NHEK. NHEK were incubated with IL-4 and/or IFN- $\gamma$  as indicated in Fig. 2A. IL-4-induced CCL26 release into the culture medium was significantly enhanced in IFN- $\gamma$ -pretreated cells (Fig. 2A, column f). In contrast, CCL26 production was significantly attenuated when IL-4 and IFN- $\gamma$  were present simultaneously in the culture medium (Fig. 2A, column d). When NHEK were stimulated with IL-4 soon after IFN- $\gamma$  pretreatment, no enhanced IL-4-induced CCL26 production was observed (Fig. 2A, column e). IL-4-induced CCL26 mRNA expression was augmented in cells pretreated with IFN- $\gamma$  (Fig. 2B), consistent with the results from the CCL26 production experiments.

### IFN- $\gamma$ enhanced IL-4 receptor expression

We next investigated the mechanism by which IFN- $\gamma$  pretreatment enhances IL-4-induced CCL26 production. It was observed



**Fig. 2.** Effect of IFN- $\gamma$  pretreatment on IL-4-induced CCL26 production. (A) Left panel: schematic representation demonstrating how cells were incubated in the experiment. Gray bar, incubation only with culture medium; hatched bar, incubation with IL-4 (25 ng/ml); black bar, incubation with IFN- $\gamma$  (5 ng/ml). Right panel: CCL26 released into culture medium was determined by ELISA. Results shown are means  $\pm$  SD of values from a representative experiment performed with triplicate samples ( $P < 0.01$ ;  $P < 0.05$ ). (B) Quantitative analysis of IL-4-induced CCL26 mRNA expression. White bar indicates CCL26 mRNA expression in cells incubated as indicated in (A, column c); black bar, cells were incubated as indicated in (A, column f). Representative RT-PCR data from two separate experiments is shown.



**Fig. 3.** Quantitative mRNA expression analyses of components of IL-4Rs. (A) IL-4Rα and γC mRNA expression. (B) IL-13Rα1 and IL-13Rα2 mRNA expression. Cells were incubated with varying doses of IFN-γ for 24 h. Results shown are means ± SD of values from a representative experiment performed with triplicate samples. (\* $P < 0.01$ ; \*\* $P < 0.05$ ).

that NHEK constitutively expressed mRNA of IL-4Rα and γC, which are components of type 1 IL-4R. IFN-γ dose-dependently enhanced mRNA expression of IL-4Rα and γC (Fig. 3A). Constitutive mRNA expression of IL-13Rα1, a component of type 2 IL-4R, was observed, and its expression was upregulated by IFN-γ (Fig. 3B). These results indicate that NHEK express both type 1 and 2 IL-4R. Interestingly, mRNA expression of IL-13Rα2, a decoy receptor for IL-13, was also enhanced by IFN-γ (Fig. 3B). Western blotting showed constitutive IL-4Rα expression, which was upregulated by IFN-γ (data now shown). Flow cytometry analysis also revealed that NHEK express both components of type 1 IL-4R at the cell surface when expression is upregulated by IFN-γ (Fig. 4A). Dose-dependent upregulation of IL-4Rα and γC were also observed (Fig. 4B). Finally, we investigated the effect of IFN-γ pretreatment on IL-4-induced phosphorylation of STAT6, and found that pretreatment with IFN-γ for 24 h enhanced pSTAT6 generation induced by IL-4 (Fig. 4C).

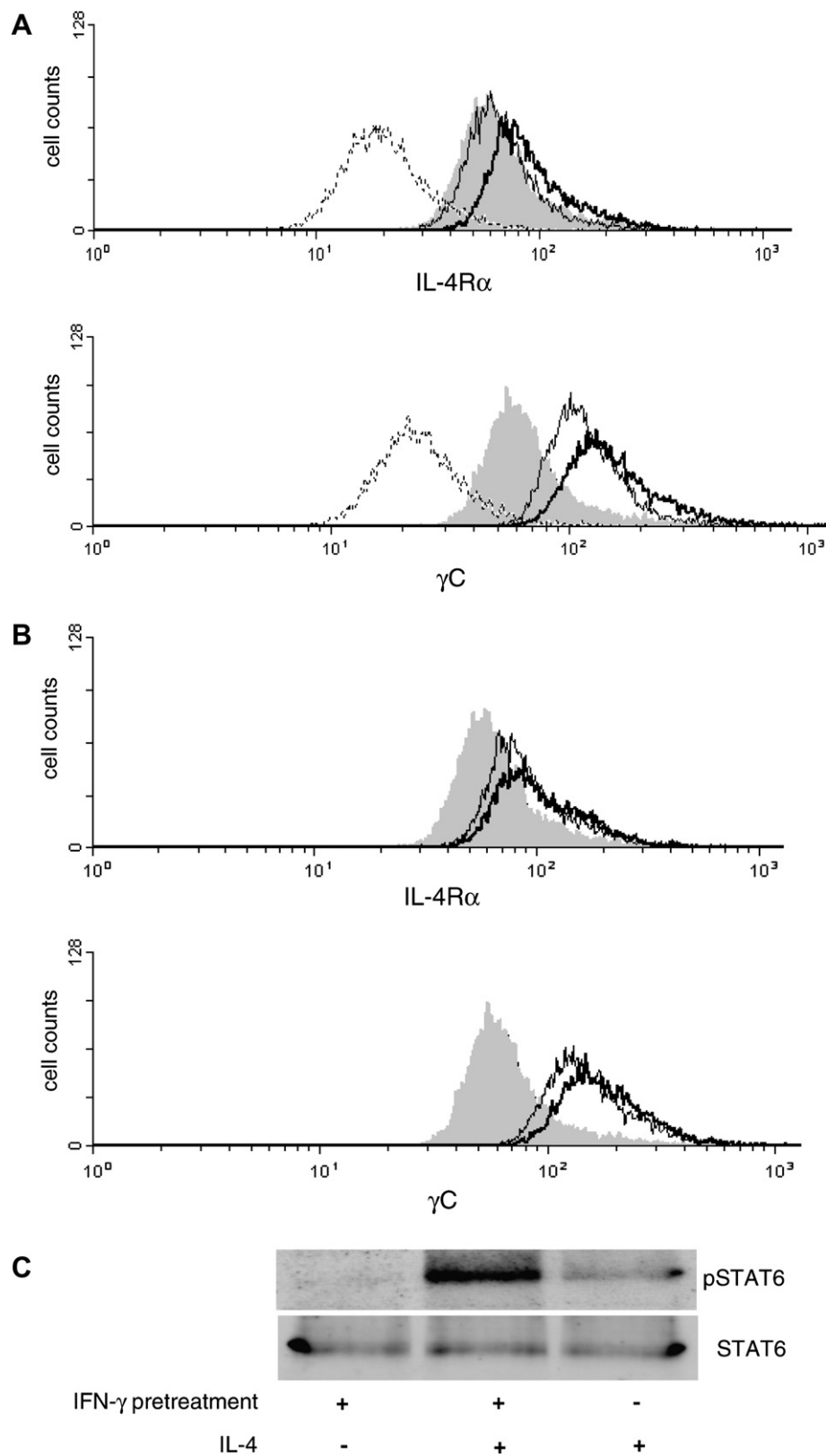
## Discussion

This study demonstrated that NHEK produce CCL26 upon stimulation with IL-4, which is consistent with results from previous studies [9,10]. Although IL-4 induced a small amount of CCL11 mRNA expression in the present study, no CCL11 protein was detected. However, neither CCL24 mRNA nor protein was detected. It can be speculated that CCL26 might be an eotaxin

mainly produced from epidermal keratinocytes upon stimulation with IL-4.

The inhibitory effect of IFN-γ on IL-4-induced CCL26 mRNA expression and protein production was observed in this study, which is in line with previous findings in HacaT cells [10]. Suppressor of cytokine signaling 1 (SOCS1)-dependent inhibition of IL-4-induced gene expression was demonstrated in keratinocytes [15]. In addition, IFN-γ-induced SOCS1 regulates STAT6-dependent CCL11 expression triggered by IL-4 and TNF-α in mouse embryonic fibroblasts [16]. In the IFN-γ pretreatment experiments in this study, an additional 24-h incubation without IFN-γ prior to IL-4 stimulation was required to enhance IL-4-induced CCL26 production (Fig. 2A, column f). No enhanced CCL26 production was observed in cells stimulated with IL-4 soon after completion of the IFN-γ pretreatment (Fig. 2A, column e). This additional incubation without IFN-γ might be necessary to counteract the effect of IFN-γ-induced SOCS1 in NHEK.

Several studies have shown that the cytokine profile in the skin changes during the course of atopic dermatitis, resulting in a Th1/Th2 mixed cytokine profile [11,17]. Given that IFN-γ inhibits CCL26 production from epidermal keratinocytes, a change in cytokine profile from Th2 to Th1 might indicate attenuation of eosinophilic inflammation in the chronic phase of atopic dermatitis; however, eosinophils were still observed in this phase [18]. Eosinophil infiltration in the chronic phase of atopic dermatitis may be explained at least in part by the observation that IFN-γ induces CCL17



**Fig. 4.** Protein expression of components of type 1 IL-4R. (A) Kinetics of IL-4R $\alpha$  and  $\gamma$ C expression at the cell surface over time. IL-4R $\alpha$  and  $\gamma$ C expressions were investigated by FACS as described in materials and methods section. Cells were treated with 5 ng/ml of IFN- $\gamma$  for 0 h (shaded area), 24 h (thin line) and 48 h (bold line). Dotted histogram: control cells labeled with isotype matched mouse (IL-4R $\alpha$ ) or rat ( $\gamma$ C) IgG. (B) Dose-dependent induction of IL-4R $\alpha$  and  $\gamma$ C. Cells were treated with 0 ng/ml (shaded area), 5 ng/ml (thin line), and 50 ng/ml (bold line) of IFN- $\gamma$  for 48 h. (C) Enhanced IL-4-induced pSTAT6 generation in cells pretreated with IFN- $\gamma$ . NHEK were pretreated with 5 ng/ml of IFN- $\gamma$  for 24 h. After washing with PBS, the cells were incubated with culture medium for 24 h. Cells were then stimulated with 25 ng/ml of IL-4 for 5 min. Representative Western blot data from three separate experiments is shown.

production from keratinocytes, perhaps similar to the way CCL17 preferentially stimulates the infiltration of Th2 lymphocytes [19]. The observation in this study that pretreatment with IFN- $\gamma$  enhanced IL-4-induced CCL26 production might reveal a novel mechanism by which eosinophil infiltration is maintained in the chronic phase of atopic dermatitis.

Receptor regulation is an important mechanism by which several mediators, including IL-4 and IL-13, work. IL-4 and IL-13 signal through IL-4R complexes [20], of which two types have been demonstrated. Type 1 IL-4R is composed of IL-4R $\alpha$  and  $\gamma$ C subunits and is primarily expressed in hematopoietic cells [20], whereas type 2 IL-4R is composed of IL-4R $\alpha$  and IL-13R $\alpha$ 1 subunits and is primarily expressed in non-hematopoietic cells [20,21]. Although IL-4 binds to both types of IL-4R, IL-13 signals only through type 2 IL-4R [20]. Important roles for IL-13 in skin inflammation have been demonstrated [22], and upregulation of IL-13R $\alpha$ 1 in human keratinocytes in the skin of atopic dermatitis and psoriasis patients has been described [23,24]. Recently, Purwar and colleagues showed modulation of type 2 IL-4R expression by several cytokines including IFN- $\gamma$  in keratinocytes [25]. In a keratinocyte cell line, however, no  $\gamma$ C mRNA expression was demonstrated, indicating that the cell line only expressed type 2 IL-4R [26]. Wongpiyabovorn and colleagues observed a small amount of  $\gamma$ C mRNA, and expression was upregulated by IFN- $\gamma$ ; however, no  $\gamma$ C protein was observed by FACS analysis of primary human keratinocytes [23]. Conversely, several reports have noted differential effects of IL-4 and IL-13, which suggests expression of both types of IL-4R in keratinocytes [23,27,28]. FACS analyses in the present study clearly demonstrate constitutive protein expression in NHEK of the type 1 IL-4R components, IL-4R $\alpha$ 1 and  $\gamma$ C. It is interesting that IFN- $\gamma$  enhanced protein expression of both IL-4R $\alpha$ 1 and  $\gamma$ C, suggesting upregulation of type 1 IL-4R in NHEK. In addition, IFN- $\gamma$ -induced type 1 IL-4R upregulation resulted in enhanced IL-4-induced CCL26 production through enhancement of the IL-4-induced cellular signal, STAT6. These results suggest that IL-4 might play an important role in skin inflammation, specifically in conditions associated with enhanced levels of IFN- $\gamma$  such as the chronic phase of atopic dermatitis.

We also observed IFN- $\gamma$ -enhanced expression of both IL-13R $\alpha$ 1 and IL-13R $\alpha$ 2 mRNA, which is in line with the findings of a recent study [25]. IL-13R $\alpha$ 2 has been suggested to function as a decoy receptor because the cytoplasmic region of IL-13R $\alpha$ 2 does not have a signaling motif or JAK/STAT binding sequence [29]. Since IL-13R $\alpha$ 2 shows high affinity towards IL-13 compared to IL-13R $\alpha$ 1, enhanced expression of IL-13R $\alpha$ 2 may reduce the extent of IL-13 in skin inflammation [29,30]. Although the expression of IL-13R $\alpha$ 1 and IL-13R $\alpha$ 2 protein was not examined in this study, it is likely that these receptors may be upregulated by IFN- $\gamma$  in NHEK. Taken together, results to date indicate that as the importance of IL-13 decreases, the important role of IL-4 might increase in IFN- $\gamma$ -dominant chronic skin lesions such as atopic dermatitis. Further studies will be needed to clarify the role of IL-4, IL-13, and IL-13R $\alpha$ 2 on keratinocytes in skin inflammation.

Atopic dermatitis is exacerbated by various causes, including bacterial skin infection [31] and viral infection [32]. The role of IFN- $\gamma$  in such conditions remains unclear. A Toll-like receptor-9 ligand, CpG oligodeoxynucleotide, is a known potent inducer of Th1 cytokines including IFN- $\gamma$ , and has been demonstrated to exacerbate atopic dermatitis-like skin lesion in NC/Nga mice [33]. In these mice, hyperproduction of IFN- $\gamma$  may play a role in exacerbating dermatitis in spite of the reduced production of Th2 cytokines. If such skin lesions are exposed to allergen, IL-4 induced from Th2 lymphocytes might result in enhanced CCL26 production from keratinocytes through upregulation of type 1 IL-4R by IFN- $\gamma$ .

In conclusion, this study demonstrated upregulation of type 1 IL-4R by IFN- $\gamma$ , which resulted in enhanced IL-4-induced

production of CCL26 from keratinocytes. Although the importance of IL-13 and its receptor (type 2 IL-4R) in lesional skin have been emphasized, the present results might also indicate the specific importance of IL-4 and type 1 IL-4R in chronic skin inflammation such as atopic dermatitis.

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