IL-13-Stimulated Human Keratinocytes Preferentially Attract $CD4^+CCR4^+$ T cells: Possible Role in Atopic Dermatitis

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Skin inflammation in atopic dermatitis (AD) is characterized by the predominant infiltration of T-helper (Th)2-cells in lesional skin. However, the mechanism of recruitment of these cells in lesional skin of AD is not yet fully elucidated. In this study, we investigated the role of IL-13-stimulated human primary keratinocytes (HPKs) in the recruitment of lymphocytes and further delineated the mechanism of enrichment of these cells. In the migration assays, we observed preferential enrichment of $CD4^+CCR4^+$ T cells towards IL-13-stimulated HPKs. Interestingly, $CD4^+CCR4^+$ T cells from AD showed a higher chemotactic response than those from healthy individuals. We observed a significant increase in the expression of CCL22 in IL-13-stimulated HPKs as compared to unstimulated cells. Blocking of CCL22 in IL-13-stimulated HPKs by a neutralizing antibody resulted in 70–90% inhibition in migration of $CD4^+CCR4^+$ T cells. Moreover, IL-13 upregulated IFN-γ-induced chemokines, CCL2 and CCL5, in HPKs. Taken together, our data suggest that IL-13-stimulated HPKs participate in a positive feedback loop by preferentially enriching Th2-cells in lesional skin of acute AD patients. However, in chronic phase, IL-13 may act in synergy with IFN-γ resulting in lymphocytes recruitment of a mixed phenotype at the site of inflammation, thus contributing to the chronification of eczema.

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INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease and characterized particularly by infiltration of lymphocytes into the skin compartment. Chemokines are potent chemoattractants and activating factors, which play a critical role in defining the nature of the inflammatory infiltrate in AD (Ono *et al.*, 2003). In acute skin lesions, accumulation of T-helper (Th)2-lymphocytes producing IL-4 and/or IL-13 and expressing CCR4 has been observed (Hamid *et al.*, 1996; Nakatani *et al.*, 2001; Wakugawa *et al.*, 2001). In contrast to a more Th2-like micromilieu in acute lesions, IFN-γ has been reported to be expressed in chronic skin lesions in AD (Werfel *et al.*, 1996).

IL-13 is known as a pivotal mediator of Th2 immune responses (Wynn, 2003). $CD4^+$ T cells and mast cells are major sources of IL-13 in acute lesions and in peripheral blood of AD (Obara *et al.*, 2002). Recently, a higher number of IL-13-expressing $CD4^+$ T cells in peripheral blood mononuclear cells have been observed in AD patients than in healthy individuals (La Grutta *et al.*, 2005). Additionally, it has also been described that, in AD skin lesions, there is a higher number of IL-13-positive cells in the acute phase than in the chronic phase (Hamid *et al.*, 1996).

IL-13 does not exert direct effects on T cells, which lack a functional IL-13 receptor (Zurawski and de Vries, 1994). Therefore, unlike IL-4, it does not support the proliferation and differentiation of naive T cells into Th2 phenotype. IL-13 has been described to upregulate the expression of vascular cell adhesion molecule-1 on endothelial cells (Sironi *et al.*, 1994) to enhance the expression of various adhesion molecules on monocytes, such as CD11b, CD11c, CD18, CD29, and CD49ε (VLA-4) (reviewed in de Vries, 1998), and to upregulate IL-6 (Derocq *et al.*, 1994) as well as IFN-γ-induced CCL5 secretion in keratinocytes (Wongpiyabovorn *et al.*, 2003).

Originally, Th1 and Th2 subsets have been functionally separated based on their profiles of cytokine production. However, it has now become obvious that certain types of chemokine receptors are selectively expressed on either Th1- (e.g. CXCR3, CCR5) or Th2-cells (e.g. CCR4, CCR8, CRTH2) (Bonecchi *et al.*, 1998a; Nagata *et al.*, 1999; Gombert *et al.*, 2005). *In vitro* studies have revealed that high-affinity ligands for CCR4, thymus, activation-regulated chemokine (CCL17), and macrophage-derived chemokine (CCL22) induce selective migration of Th2-cells (Imai *et al.*, 1998, 1999). However, expression of CCR4 has also been documented

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Abbreviations: AD, atopic dermatitis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPK, human primary keratinocyte; TNF-α, tumor necrosis factor-α

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on cutaneous lymphocyte antigen-positive memory T cells and Th0-cells (Campbell *et al.*, 1999). Recently, the chemoattractant receptor-homologous molecule that expressed Th2-cells (CRTH2) has been shown to be selectively expressed on Th2-cells (Cosmi *et al.*, 2000).

Expression of CCR4 has been speculated to be important for T-cell infiltration into skin lesions of AD (Wakugawa *et al.*, 2001). The frequency of $CD4^+CCR4^+$ T cells in peripheral blood of AD patients has been reported higher than that of healthy individuals and has been correlated with severity of the disease (Nakatani *et al.*, 2001). Additionally, in the epidermal layer of AD skin, keratinocytes have been shown to be positive for CCL17 and CCL22 (Vestergaard *et al.*, 2000; Horikawa *et al.*, 2002). Activated human primary keratinocytes (HPKs) are an important source of chemokines, hence capable of regulating the progression of the inflammatory response. Recently, interaction of T-lymphocytes and keratinocytes has been shown to promote Th1 cell accumulation in chronic inflammatory skin diseases (Albanesi *et al.*, 2001; Klunker *et al.*, 2003).

These observations led us to hypothesize that IL-13 plays an important role in the recruitment of inflammatory cells to the sites of AD skin lesions. Co-localization of IL-13-producing $CD4^+$ T cells and keratinocytes expressing IL-13 receptor in the skin of AD patients (Wongpiyabovorn *et al.*, 2003) prompted us to investigate if IL-13-stimulated HPKs can attract effector subsets of $CD4^+$ T cells. In this study, we report that IL-13-stimulated chemokine(s) released by HPKs preferentially enrich $CD4^+CCR4^+$ T cells. Higher chemotaxis indices of $CD4^+CCR4^+$ T cells from AD patients than from normal subjects could be observed. In addition, we have shown that IL-13 alone induces CCL22 and a little but significant amount of CCL2 in HPKs. Furthermore, IL-13 enhances IFN-γ-induced CCL2 and CCL5 in HPKs. Taken together, these findings suggest that IL-13-stimulated chemokine(s) released by HPKs may play a role in the initiation and persistence of skin inflammation in AD.

RESULTS

IL-13-stimulated HPKs preferentially attract $CD4^+CCR4^+$ T cells

To determine if IL-13-stimulated HPKs could contribute to the recruitment of $CD4^+$ T cells, we investigated the migratory capacity of $CD4^+$ T cells towards IL-13-stimulated HPKs in a transwell chemotaxis assay. In this direction, we added purified $CD4^+$ T cells, from healthy individuals, suspended in keratinocyte growth medium in the upper chamber of a transwell culture insert containing IL-13-treated or -untreated HPKs in the lower chamber. After 3 hours of transmigration, we observed a significant increase in the absolute number of migrated $CD4^+$ T cells in response to IL-13-stimulated HPKs as compared to unstimulated controls ($P=0.01$; Figure 1a). We further characterized whether chemokines produced by IL-13-stimulated HPKs could in turn promote the preferential migration of Th1-lymphocytes ($CD4^+CXCR3^+$) versus Th2-lymphocytes ($CD4^+CCR4^+$). We used the CXCR3 and CCR4 chemokine receptor to characterize Th1- and Th2-cells. As depicted in Figure 1a and b, a significantly higher number

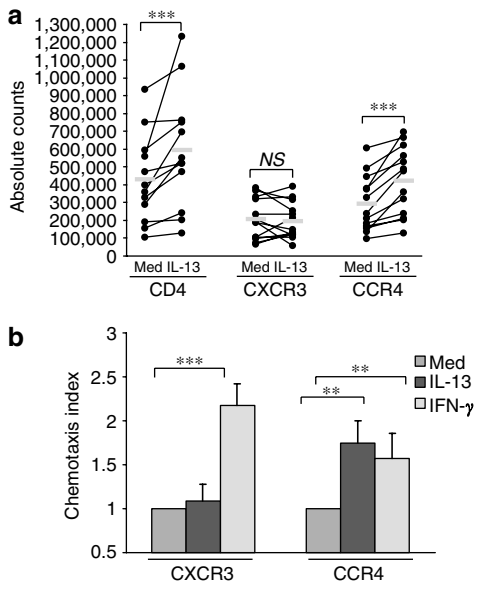

Figure 1. Chemotaxis of $CD4^+$ T cells and preferential recruitment of $CD4^+CCR4^+$ T cells in response to IL-13 stimulation of HPKs. HPKs were treated with IL-13 (50 ng/ml) or IFN-γ (10 ng/ml) for 24 hours. Purified $CD4^+$ T cells from healthy donors ($n=6$) were added in the upper chamber and IL-13-treated or -untreated HPKs in the lower chamber of the 24-well transwell plate. Migration was performed for 3 hours at 37°C in a humidified environment. (**a**, **b**) For phenotype analysis, transmigrated $CD4^+$ T cells were stained for CXCR3 (Th1) and CCR4 (Th2) by using respective mAbs and analyzed by flow cytometry. The numbers of cells transmigrated into the lower chamber were counted, and absolute numbers of each subtype and chemotaxis indices were calculated as described in Materials and Methods. The gray lines depict mean values. NS = nonsignificant. **P-value <0.02, and ***$P<0.01$.

of $CD4^+CCR4^+$ T cells migrated towards IL-13-stimulated HPKs than towards unstimulated cells ($P=0.002$). There was a basal migration of $CD4^+CXCR3^+$ T cells towards unstimulated and IL-13-stimulated HPKs; however, there was no significant difference in migration of these cells towards IL-13-stimulated HPKs than to unstimulated cells (Figure 1a). In contrast to this, as depicted in Figure 1b, significantly higher migration of $CD4^+CXCR3^+$ T cells and $CD4^+CCR4^+$ T cells was observed in response to IFN-γ-stimulated HPKs as compared to unstimulated cells ($P=0.01$). Chemotaxis index of $CD4^+CXCR3^+$ T cells was higher as than that of $CD4^+CCR4^+$T cells in IFN-γ-stimulated HPKs (Figure 1b).

IL-13-stimulated HPKs attract more $CD4^+CCR4^+$ T cells from AD patients than from healthy individuals

In the next series of experiments, we compared migration of $CD4^+CCR4^+$ T cells and $CD4^+CXCR3^+$ T cells from AD patients with those from healthy individuals (Figure 2a). We observed a significantly higher chemotaxis index of $CD4^+CCR4^+$ T cells in response to IL-13-stimulated HPKs than to unstimulated cells with $CD4^+$ T cells derived from AD patients (chemotaxis index 1.70 ± 0.16, $P=0.01$) as well as with $CD4^+$ T cells freshly isolated from healthy

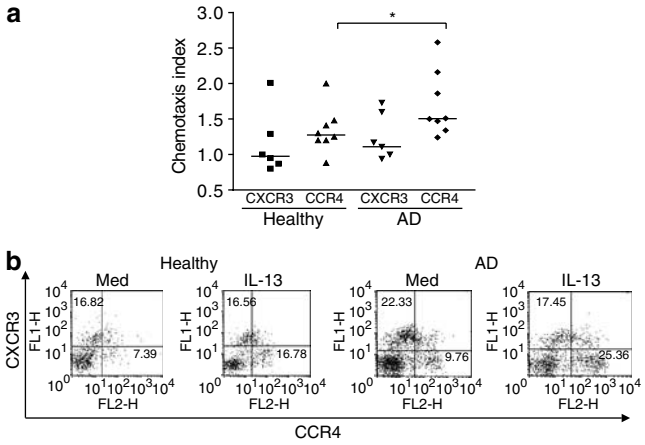

Figure 2. Higher migration of CD4$^+$CCR4$^+$ T cells derived from AD patients than from healthy individuals towards IL-13-stimulated HPKs. Purified CD4$^+$ T cells either from healthy donors or AD patients were added in the upper chamber and supernatants derived from IL-13-treated (50 ng/ml, 24 hours) or -untreated HPKs in the lower chamber of the 24-well transwell plate. Migrations were performed for 3 hours at 37°C in a humidified environment. For phenotype analysis, transmigrated CD4$^+$ T cells were stained for CXCR3 ($n=6$) and CCR4 ($n=8$) by using respective mAbs and analyzed by flow cytometry. The numbers of cells transmigrated into the lower chamber were counted, and absolute numbers of each subtype and chemotaxis indices were calculated as described in Materials and Methods (**a**). Horizontal line shows mean values. Representative dotplots of an AD patient and a healthy individual are shown ($n=6$ for CXCR3, $n=8$ for CCR4) (**b**). The numbers in each quadrant are indicated in percentages of respective subpopulations. *P-value <0.05.

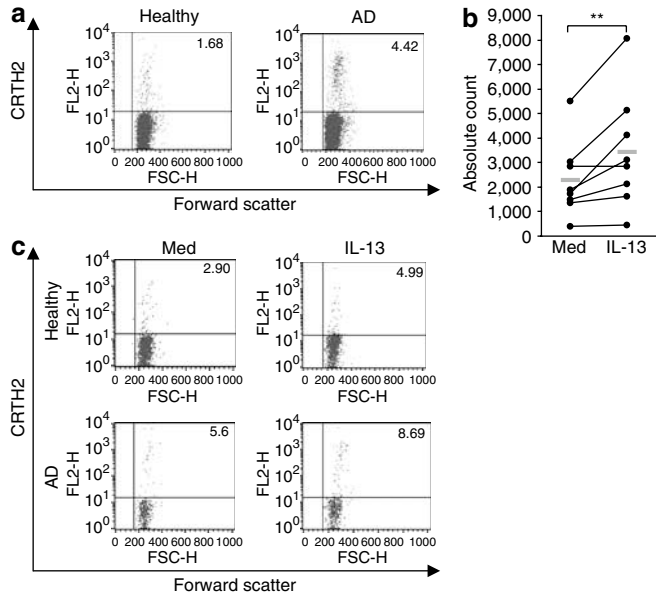

Figure 3. Migration of CD4$^+$CRTH2$^+$ T cells in response to IL-13-stimulated HPKs. (**a**) Freshly isolated CD4$^+$ T cells either from peripheral blood of healthy individuals or from AD patients were stained for CRTH2. Representative dotplots of a healthy individual ($n=6$) and an AD patient ($n=6$) are shown. (**b**) Purified CD4$^+$ T cells either from healthy donors ($n=5$) or from AD patients ($n=3$) were added in the upper chamber and IL-13-treated or -untreated HPKs (50 ng/ml) in the lower chamber of the 24-well transwell plate. Migration assays were performed for 3 hours at 37°C in a humidified environment. Transmigrated CD4$^+$ T cells were stained for CRTH2, the numbers of cells transmigrated into the lower chamber were counted, and the absolute numbers of migrated CD4$^+$CRTH2$^+$ T cells and chemotaxis indices were determined. (**c**) Representative dotplots of an AD ($n=3$) and a normal donor ($n=5$) are shown. The numbers in each quadrant are indicated in percentages of respective subpopulations. The gray lines depict mean values. **P-value <0.02.

individuals (chemotaxis index 1.34 ± 0.11, $P=0.02$). Interestingly, as shown in Figure 2a, chemotaxis indices of CD4$^+$CCR4$^+$ T cells from AD were significantly higher than those from healthy individuals ($P=0.044$). However, there was no observable effect in migration of CD4$^+$CXCR3$^+$ T cells derived from AD and healthy subjects towards IL-13-stimulated HPKs as compared to unstimulated cells (Figure 2a). Moreover, Figure 2b depicts the representative experiment of IL-13-induced transmigration of CD4$^+$CXCR3$^+$ and CD4$^+$CCR4$^+$ T cells derived from AD ($n=6$ for CXCR3, $n=8$ for CCR4) and healthy subjects ($n=6$ for CXCR3, $n=8$ for CCR4).

Recently, it has been shown that CCR4 could also be expressed on Th0- or Th1-cells (Campbell *et al.*, 1999). To confirm that migrated CD4$^+$ T cells are of Th2 origin, we studied another Th2 marker, CRTH2. Initially, we investigated the expression of CRTH2 in freshly isolated purified CD4$^+$ T cells and observed that AD patients have a higher number of CD4$^+$CRTH2$^+$ T cells than that of healthy individuals (Figure 3a), as previously observed by others (Cosmi *et al.*, 2000). Furthermore, we investigated the IL-13-induced transmigration of CD4$^+$CRTH2$^+$ T cells of AD and healthy individuals. Consistently, there was a significant increase in the migration of absolute numbers of CD4$^+$CRTH2$^+$ T cells in response to IL-13-stimulated HPKs as compared with negative controls ($P=0.01$, chemotaxis index 1.5 ± 0.15) in healthy individuals as well as in AD patients (Figure 3b and c). In addition, Figure 3c depicts the representative experiment of IL-13-induced transmigration of CRTH2 cells derived from AD ($n=4$) and healthy subjects ($n=3$).

Induction of CCL22 by IL-13 in HPKs attract CD4$^+$CCR4$^+$ T cells

To confirm that the observed effect on migration of Th2-cells was due to chemokine production by HPKs, we studied the ability of HPKs to synthesize Th2-type C–C chemokines such as CCL22 and CCL17 *in vitro*. HPKs were subjected to IL-13, tumor necrosis factor-α (TNF-α), IL-1β, or a combination of these cytokines for 4 hours. The mRNA levels of CCL22 and CCL17 were then determined by real-time quantitative RT-PCR. We used TNF-α and IL-1β stimulation, because HPKs respond well to these cytokines, which are known to be upregulated during inflammation. We showed that CCL22 mRNA was upregulated 2- to 8-fold by IL-13 stimulation (Figure 4a). However, TNF-α, IL-1β or its combination with IL-13 did not show synergistic effects within same time span (data not shown). In contrast to CCL22, CCL17 was not detected in IL-13-stimulated HPKs (Figure 4a).

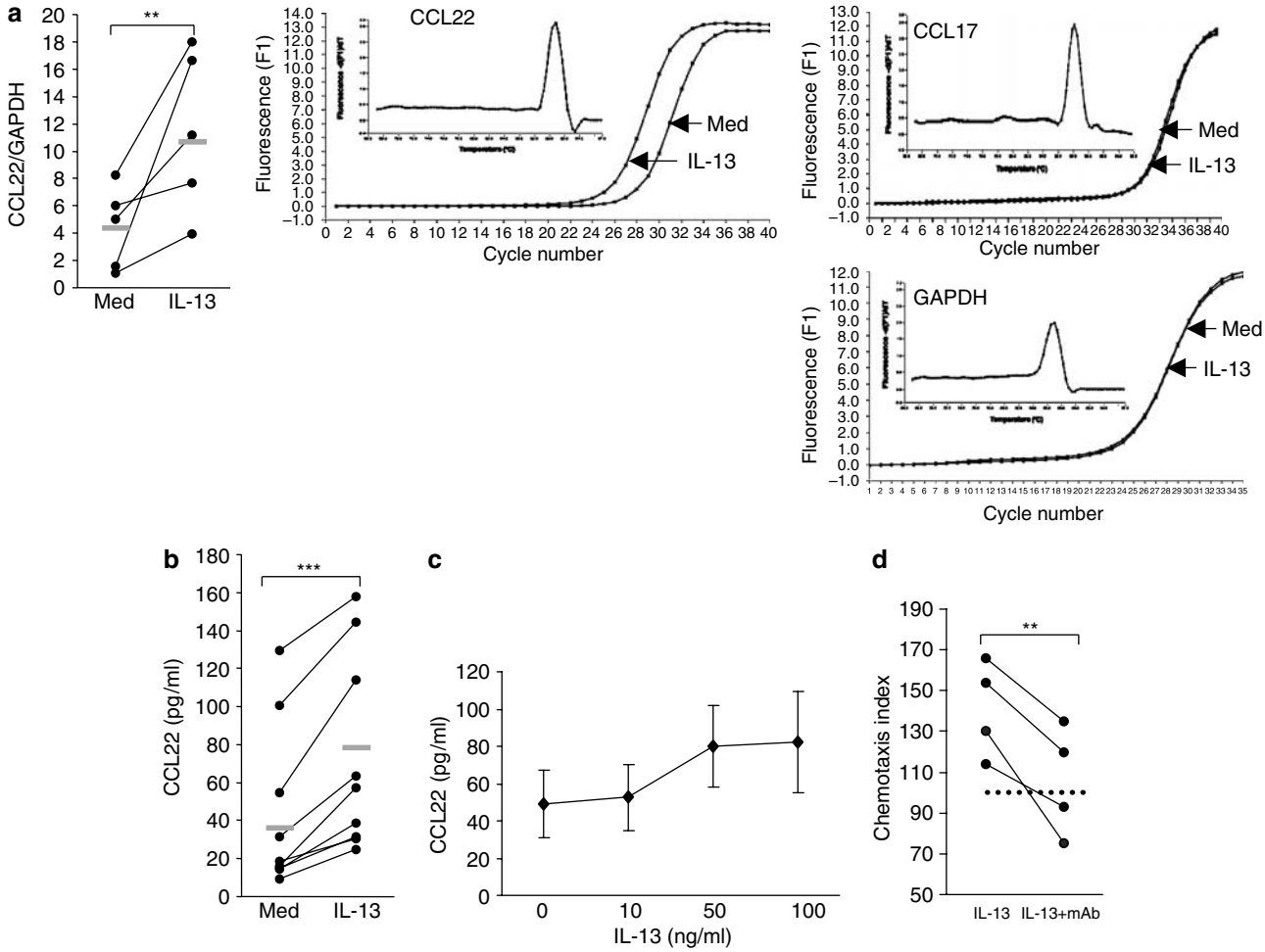

Figure 4. Induction of CCL22 by IL-13 in HPKs attract CD4$^+$CCR4$^+$ T cells. HPKs were stimulated with IL-13 (50 ng/ml) for 4 hours for RNA and with different doses of IL-13 for 24 hours for protein measurement. Total RNA was isolated and subjected to real-time quantitative LightCycler RT-PCR. (**a**) Data are shown as normalized ratio (CCL22/GAPDH), and a representative graph of the intensity of the fluorescence signal of the original data of CCL22 ($n=6$), CCL17 ($n=3$), and housekeeping gene GAPDH with their corresponding melting curves are shown ($n=6$) after IL-13 (50 ng/ml) stimulation as compared to un-stimulated cells ($n=6$). (**b** and **c**) In cell-free supernatants, CCL22 protein was measured by specific ELISA ($n=9$). The gray lines depict mean values. (**d**) Supernatant from IL-13-treated HPKs was preincubated for 2 hours with anti-CCL22 antibody before adding to the bottom chamber. Migration assays were performed with purified CD4$^+$ T cells derived from healthy donors for 3 hours at 37°C. Transmigrated CD4$^+$ T cells were stained for CCR4. Original data (un-processed) were normalized to basal migration (un-stimulated HPKs, dotted line) for each experiment and shown as chemotaxis index. $^{**}P$-value <0.02 and $^{***}P<0.01$.

The next question we addressed was whether CCL22 mRNA expression in unstimulated *versus* IL-13-stimulated HPKs could be confirmed at the protein level. We measured CCL22 in culture supernatants of HPKs collected 24 hours after IL-13 (50 ng/ml) stimulation by specific ELISA (Figure 4b). HPKs produced pg/ml quantities of CCL22 (mean 78.4 ± 17.26, range 24.5–157 pg/ml, $P=0.0004$) upon stimulation with IL-13 as compared to unstimulated HPKs. We further studied dose-dependent effects of IL-13 on CCL22 generation in HPKs. As depicted in Figure 4c, 10 ng/ml of IL-13 was insufficient to induce CCL22 production in HPKs, whereas 50 ng/ml of IL-13 could enhance the production of CCL22 in HPKs significantly. Induction of CCL22 by IL-13 in HPKs may be responsible for the accumulation of CD4$^+$CCR4$^+$ T cells; therefore, to prove this hypothesis, we performed blocking experiments with neutralizing anti-CCL22 antibody. As depicted in Figure 4d, CD4$^+$CCR4$^+$ T cells migrated in response to IL-13-treated HPKs, whereas pre-incubation with anti-CCL22 mAb significantly inhibited the migration of CD4$^+$CCR4$^+$ T cells (80–90%). With this set of experiments, we conclude that migration of these cells in response to IL-13-stimulated HPKs was due to CCL22.

As all migrated CD4$^+$ cells were not CD4$^+$CCR4$^+$ T cells, we studied other T-cell chemoattractants, CCL2, CCL5, and CCL20, after stimulation with IL-13 (50 ng/ml). At the mRNA level, CCL2 was upregulated about 2- to 4-fold by IL-13 stimulation (Figure 5a), but induction of CCL5 by IL-13 could not be detected in the same samples (Figure 5c). Furthermore, we observed a little upregulation of CCL2 production upon stimulation with IL-13 as compared with negative control (Figure 5b). In blocking experiments using neutralizing anti-CCL2 mAb, we could not detect a consistent

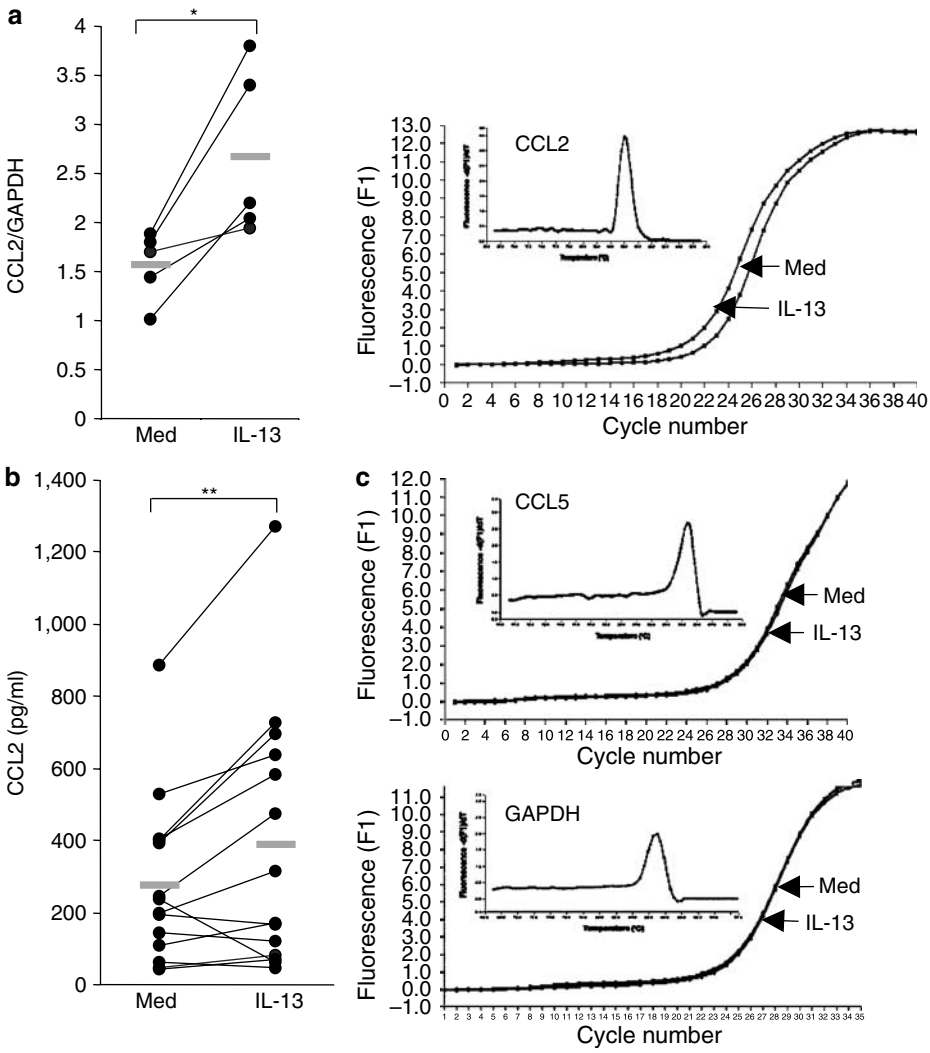

Figure 5. Induction of CCL2 mRNA and protein but not CCL5 by IL-13 in HPKs. Similar to CCL22, CCL2 and CCL5 were quantified in HPKs stimulated with or without IL-13 (50 ng/ml). (**a**) Data are shown as normalized ratio (CCL2/GAPDH), and a representative graph of the intensity of the fluorescence signal of the original data with the corresponding melting curves is shown ($n=5$). (**b**) In cell-free supernatants, CCL2 protein was measured by specific ELISA ($n=8$). The gray lines depict mean values. (**c**) Representative graph of the intensity of the fluorescence signal of the original data with the corresponding melting curves of CCL5 ($n=3$) and GAPDH ($n=5$) are shown. *P-value <0.05 and **$P<0.02$.

inhibition of $CD4^+$ T-cell migration. Furthermore, there was no induction of CCL20 in IL-13- or IL-4-stimulated HPKs alone or in combination with other pro-inflammatory cytokines (data not shown).

IL-13-positive cells have been shown to be present in the chronic phase of AD patients where IFN-γ, a Th-1 signature cytokine, dominates (Hamid *et al.*, 1996). Therefore, in the next series of experiments, we investigated the effect of IL-13 on IFN-γ-induced chemokines synthesis in HPKs. As shown in Figure 6, IL-13 and IL-4 markedly enhanced the IFN-γ-induced CCL5 and CCL2. However, neither IL-13 nor IL-4 could modulate the IFN-γ-induced CCL22 in HPKs.

DISCUSSION

HPKs play a pivotal role in skin inflammation through the regulated expression of chemokines attracting various leukocyte subsets, including monocytes, dendritic cells, and T-lymphocytes. Several evidences confirm a T-cell-mediated pathogenesis for inflammatory skin diseases such as AD, psoriasis, and allergic-contact dermatitis. Accumulation of T cells in the skin compartment from peripheral blood is highly dependent on chemokines and expression of chemokine receptors. Our study demonstrates that IL-13-stimulated HPKs express CCL22, which preferentially attract $CD4^+CCR4^+$ T cells. Furthermore, $CD4^+CCR4^+$ T cells from AD patients showed higher chemotactic indices towards IL-13-stimulated HPKs than those from healthy individuals.

We initially used transmigration assay to confirm preferential migration of Th2-cells toward IL-13-stimulated HPKs. Previously, IL-13 has been shown to be relevant in the recruitment of T cells and eosinophils to the inflammatory site by upregulating various adhesion molecules, and

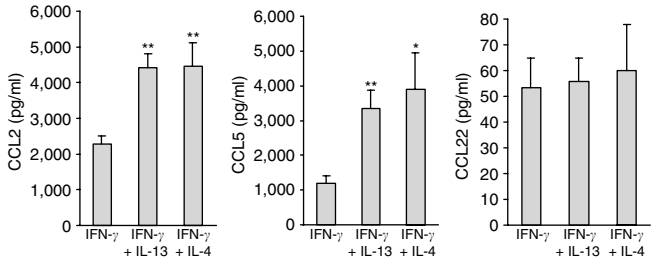

Figure 6. IL-13 enhances IFN-γ-induced chemokines CCL2 and CCL5 in HPKs. HPKs were stimulated with IFN-γ (10 ng/ml) alone and in combination with IL-13 (50 ng/ml) or IL-4 (50 ng/ml) for 24 hours. In cell-free supernatants, CCL2, CCL5, and CCL22 at protein levels were measured by specific ELISA ($n=4$). Data are expressed as mean pg/ml ± standard error of mean. *P-value <0.05, and **$P<0.02$ when compared with IFN-γ-stimulated cells.

chemokines (Li *et al.*, 2000; Lukacs, 2000). In AD patients, higher numbers of IL-13-positive cells appear to be present in acute lesions than in chronic lesions as shown on the mRNA level previously (Hamid *et al.*, 1996). Recently, it has been shown that IL-13 is a major inducer of Th2 milieu in the cutaneous microenvironment, being required independent of IL-4 (Herrick *et al.*, 2003). This fact, in combination with the relative abundance of IL-13 over IL-4 in subacute and chronic AD, emphasizes the potentially important role of IL-13 in the pathogenesis of AD (Tazawa *et al.*, 2004).

To investigate the phenotype of migrated $CD4^+$ T cells (Th1/Th2), we used CXCR3 and CCR4 surface markers as the inflammatory cells express different chemokine receptors depending on their differentiation and activation state. In particular, Th1-cells express high levels of CCR5 and CXCR3, whereas Th2-cells express high CCR4 and CCR8 levels (Yamamoto *et al.*, 2000; Gombert *et al.*, 2005). We observed higher chemotactic indices of $CD4^+CCR4^+$ T-cell migration in AD as compared to healthy individuals. It has been shown that CCR4 plays an important role in the migration of Th2-cells from peripheral blood into the inflamed skin (Reiss *et al.*, 2001). Our results point to the fact that the higher rate of migration of $CD4^+CCR4^+$ T cells in AD patients may not be only due to a higher frequency of $CCR4^+$ cells in AD patients (Nakatani *et al.*, 2001) but also because of higher migratory capacity of $CCR4^+$ cells from AD than healthy individuals.

To further confirm that migrated $CD4^+CCR4^+$ T cells are of Th2 phenotype, we used another Th2 marker, CRTH2, that has been shown to be expressed selectively on a subpopulation of Th2-cells but not on Th0- and Th1-cells (Cosmi *et al.*, 2000). Consistently, we observed higher frequency of CRTH2-positive cells in AD patients than in healthy individuals. Moreover, we observed higher migration of CRTH2 cells towards IL-13-stimulated HPKs in AD as well as in healthy individuals.

For $CD4^+CXCR3^+$ T cells, we observed almost the same or a marginal increase of migration towards IL-13-stimulated HPKs as compared to unstimulated cells. Moreover, no significant difference could be observed in AD *versus* healthy individuals. Recently, it has been shown that IL-4 upregulates the IFN-γ- or TNF-α-induced CXCR3 agonist expression (Albanesi *et al.*, 2000). As the cytokine micromilieu and timing of signal are important for the induction of several genes (Wittmann *et al.*, 1999), this may explain why we have observed preferential enrichment of $CD4^+CCR4^+$ T cells towards IL-13-stimulated HPKs (no pre-incubation with TNF-α or IFN-γ); however, no significant effect on migration of $CD4^+CXCR3^+$ T cells.

To understand the mechanism of migration of Th2-cells, we have evaluated the ability of HPKs to newly synthesize and release CCL22 and CCL17. Results of *in vitro* experiments demonstrated that HPKs were capable of generating significant quantities of CCL22 protein, but induction of CCL17 could not be observed in the same experimental settings. Recently, *in vivo* presence of CCL22-positive cells in skin lesions of AD in both the epidermis (keratinocytes) (Horikawa *et al.*, 2002) and the dermis (mature DCs) (Vulcano *et al.*, 2001) has been demonstrated. The *in vitro* secretion of CCL22 by IL-13-stimulated HPKs in this study strongly suggests that CCL22 expressed by HPKs may function to recruit $CCR4^+$ lymphocytes into inflamed epidermis. In monocytes, IL-4 and IL-13 have been reported to augment CCL22 expression (Bonecchi *et al.*, 1998b), whereas in HaCaT (spontaneously immortalized human keratinocytes) cells, IL-13 has been shown to downregulate the CCL22 release (Fujii-Maeda *et al.*, 2004). HaCaTs are a human keratinocytes cell line, which we have not used in this study. Here, we used HPKs, hence differences in the result could be because of differences between cell lines and primary cells. Recently, differences in the NF-κβ pathway have also been shown between HaCaTs and HPKs (Muller *et al.*, 2003). In our study, IL-13 itself exerted significant effects on HPKs, but TNF-α or IL-1β did not show synergy with IL-13 in the induction of CCL22, unlike in bronchial epithelial cells (Sekiya *et al.*, 2000).

Several recent studies demonstrated that infiltration of IFN-γ-positive cells into the epidermis leads to apoptosis of keratinocytes, and thus contributing to the skin inflammation in AD (Klunker *et al.*, 2003). Our study demonstrates that $CD4^+CXCR3^+$ and $CD4^+CCR4^+$ T cells migrate in response to IFN-γ-treated HPKs. IFN-γ induced CCL22 production in HPKs (our own observation) and in HaCaT keratinocytes (Fujii-Maeda *et al.*, 2004). These results were rather surprising, because CCL22 and CCL17 are usually considered to play a role in Th2-dominated disorders such as allergic responses, but not in Th1-mediated disorders. IFN-γ inhibits the development of Th2-cells, antagonizes Th2-dependent effects such as inhibition of signal transducer and activator of transcription 6 and its downstream genes, and downregulates CCL22 in monocytes (Bonecchi *et al.*, 1998b; Heller *et al.*, 2004). Therefore, it appears that IFN-γ may exert dichotomous effects on the pathogenesis of AD. This might be an important clue in understanding the pathogenesis of the chronic phase of AD where a mixed (Th1 and Th2) cytokine milieu has been observed.

Besides $CD4^+$-lymphocytes, CCL2 potently attracts monocytes and dendritic cells. In this study, we could observe a little induction of CCL2 in HPKs after IL-13 stimulation. The slight induction of CCL2 by IL-13 alone may

not account for biologically important effects; however, in concert with IFN-γ, we observed a marked induction of CCL2 as well as CCL5 in HPKs. This suggests that CCL2 and CCL5 may contribute to the mixed Th1/Th2 infiltrate that characterizes the chronic phase of AD. Basal keratinocytes strongly express CCL2 in patients with AD (Giustizieri *et al.*, 2001). IL-4 has also been shown to induce marginal increase in IFN-γ-induced CCL2 in oral keratinocytes (Li *et al.*, 2000). Therefore, it is another insight of the potential role of IL-13 in amplifying the inflammatory response at the target site by recruiting various leukocytes, thereby facilitating the chronification of inflammatory eczematous response.

Although it is well established that multiple C–C chemokines are produced by HPKs, there were remarkable variations within the HPKs isolated and cultured from different healthy individuals in the induction level of CCL22 and CCL2. These probably represent heterogeneity in the population with respect to genetic background. In agreement to our results, previous study also observed the donor-dependent variability in the expression of CCR-3 in HPKs (Petering *et al.*, 2001).

Taken together, our results indicate the mechanisms of regulation of epithelial chemokine production and thereby amplifying the inflammatory response in the skin. We have demonstrated that IL-13-activated HPKs represent an important source of the chemokine CCL22 and preferentially enrich Th2-cells as observed in the migration assays. Moreover, IL-13 in concert with IFN-γ induces high amounts of CCL2 and CCL5 in HPKs. With the recent evidences, we conclude that IL-13 plays a dichotomous role in the pathogenesis of AD by enriching Th2 cells in acute phase and a mixed lymphocytes population in chronic phases of allergic skin inflammation.

MATERIALS AND METHODS

Cytokines and reagents

All cytokines were used as purified recombinant human preparations. Human IL-13 was purchased from PromoCell GmbH (Heidelberg, Germany); IFN-γ from ImmunoTools (Friesoythe, Germany); and IL-4 from R&D Systems (Wiesbaden, Germany).

Cell isolation and culture

Peripheral blood mononuclear cells either from healthy individuals or AD patients were separated by Ficoll–Hypaque density-gradient centrifugation. AD patients suffered from the extrinsic form of the disease. SCORing Atopic Dermatitis were in the range of 12–35. At the time point blood samples were taken, none of the patients received systemic medication, but most of them used local steroids (class II–III). The study was approved by the ethics committee of the Hannover Medical School and was conducted according to the Declaration of Helsinki Principles. All participants gave their written informed consent. Monocytes were depleted by adherence and $CD4^+$ T cells were purified from nonadherent cells by using a negative selection kit (Miltenyi Biotech, Bergisch Gladbach, Germany). Purity of the resulting $CD4^+$ T cells was consistently $\geqslant$95%, as verified by flow cytometry analysis of $CD4^+$ T cells (mouse anti-human CD4 mAb; Beckman-Coulter, Krefeld, Germany). After separation, cells were resuspended in Iscove medium (Biochrom, Berlin, Germany) supplemented with 4% human AB serum.

Primary cultures of normal human keratinocytes were prepared from foreskin of children undergoing surgery as described previously (Wittmann *et al.*, 2005). In brief, the single-cell suspension of keratinocytes was cultured in serum-free keratinocytes growth medium (Keratinocyte Growth Medium 2 Kit; PromoCell GmbH, Heidelberg, Germany). All cell cultures were incubated in a humidified atmosphere containing 5% CO_2 at 37°C and were used at the passage 2–5. Hydrocortisone-free medium was used for all experiments. The purity of HPKs was verified by the expression of the epithelial marker cytokeratin (anti-human cytokeratin antibody, clone: MNF-116; DakoCytomation, Hamburg, Germany). All cells (more than 95%) were found to be uniformly positive for cytokeratin.

Flow cytometric analysis of intracellular and membrane molecules

Expression of surface antigens was assessed as described previously (Wittmann *et al.*, 2002). The following phycoerythrin- or FITC-labelled mAb were used: CXCR3 (clone 49801; R&D Systems, Wiesbaden, Heidelberg, Germany), CCR4, (clone 1G1; BD Biosciences), and CRTH2 (clone BM 16; Miltenyi Biotech, Bergisch Gladbach, Germany). Cytokeratin (DakoCytomation) was detected by intracellular staining using the Cytofix/Cytoperm kit (BD Biosciences). Stained cells were measured by flow cytometry (FACSCalibur) and analyzed using CELLQuestPro™ software (BD Biosciences).

Migration assay

Purified $CD4^+$ T cells (1×10^6/150 μl) from either healthy or AD donors were added to the top chamber of 5-μm pore size polycarbonate 24-well Transwell culture insert (Costar, NY) containing undiluted supernatants from either unstimulated or IL-13-stimulated HPKs (about 70% confluent) in the bottom chamber. Migration assays were carried out for 3 hours at 37°C in a CO_2 incubator. The numbers of migrated cells were counted by BD Truecount tubes (BD Biosciences) and absolute numbers were determined according to the manufacturer's instructions. To determine the Th1 or Th2 phenotype, migrated cells were stained with fluorochrome-conjugated CXCR3, CCR4, or CRTH2 mAbs, and the number of cells migrated were calculated with the following formula (total number of cells migrated $\times$ % age of subtype of cells/100). Chemotaxis indices were calculated as the ratio of the absolute numbers of cells migrated towards stimulated cells divided by the numbers of cells migrated towards unstimulated HPKs (negative control). For the blocking experiments, supernatants derived from IL-13-activated HPKs were pre-incubated with 10 μg/ml anti-human CCL22 antibody (clone 57226.11; R&D Systems) and anti-human CCL2 antibody (clone 24822; R&D Systems) for 2 hours, before the setup of chemotaxis assay.

mRNA isolation, reverse transcription, and LightCycler PCR

mRNA isolation and reverse transcription were performed as described previously (Wittmann *et al.*, 2004) by using the ''High Pure mRNA Isolation Kit'' (Roche Molecular Biochemicals, Mannheim, Germany) and ''First Strand cDNA Synthesis Kit'' (MBI Fermentas, St Leon-Rot, Germany). The quantitative real-time PCR

was performed on a LightCycler (Roche Molecular Biochemicals) by using ''LightCycler® FastStart DNA Master^{PLUS}SYBR Green I'' (Roche Molecular Biochemicals). CCL22, CCL17, CCL2, CCL5, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were run in a touchdown PCR program (target temperature, 68°C; secondary target temperature, 58°C; step size, 0.5; and step delay, 1). The following primers were used for PCR amplification: CCL22 sense – 5′-ACC TAT GTG TCG TGT CT-3′ and CCL22 antisense – 5′-CGC TAC GCT ACC ACA G-3′; CCL17 sense – 5′-ATG GCC CCA CTG AAG ATG CT-3′ and CCL17 antisense – 5′-TGA ACA CCA ACG GTG GAG GT-3′; CCL2 sense – 5′-AGA TGC AAT CAA TGC CC-3′ and CCL2 antisense – 5′-TGG AAT CCT GAA CCC AC-3′; CCL5 sense – 5′-CCA TGA AGG TCT CCG C-3′ and CCL5 antisense – 5′-CAT CCT AGC TCA TCT CCA A-3′; and GAPDH sense – 5′-CCA CAT CGC TCA GAC-3′ and GAPDH antisense – 5′-GGC AAC AAT ATC CAC TTT ACC AGA GT-3′. For quantitative analysis, standard curves for CCL22, CCL17, CCL2, and CCL5 were created covering a range of six orders of magnitude by dilution series (dilutions from these standard curves were used as calibrators in further experiments). These standard curves describing the PCR efficiencies of the target CCL22, CCL17, CCL2, or CCL5 and the reference gene (GAPDH) allow an efficiency-corrected quantification using the ''Relative Quantification Software'' (Roche Molecular Biochemicals). The calibrator-normalized relative quantification results in a target concentration expressed relative to the concentration of the reference gene in the same sample material.

Quantification of chemokines

In all experiments, HPKs were grown in a 24-well plate (Nunc Inc., Wiesbaden, Germany) in a 60–90% cell density in 0.5 ml of keratinocytes medium. After stimulation with appropriate cytokines for 24–48 hours, supernatants were collected. Concentrations of CCL22, CCL5, CCL20, and CCL2 in the cell-free supernatants of cytokine(s)-stimulated HPKs were measured by DuoSet ELISA of CCL22, CCL5, and CCL20 (R&D Systems, Wiesbaden, Germany) and CCL2 ELISA development kit (Peprotech, Germany) according to the manufacturer's instructions.

Statistical analysis

Comparative data were analyzed by using the Mann–Whitney rank-sum test (Figure 2), *t*-test (Figure 6), and paired *t*-test (paired data are depicted). The software used to perform the statistical analysis was Prism 3.03. Mean values ± standard error of mean are depicted. In the figures, *stands for a *P*-value <0.05, **$P<0.02$, and ***$P<0.01$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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