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Title: Th2 cell differentiation from naive CD4⁺ T cells is enhanced by autocrine CC chemokines in atopic diseases

A short running title: CC chemokines production by T cells in atopy

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Abstract

Background:

Chemokines are involved not only in regulating leukocyte recruitment, but also in other activities. However, functions other than cell recruitment remain poorly understood. We have already shown that the production of CC chemokine ligand (CCL)17 and CCL22 by antigen-stimulated naïve CD4⁺ T cells was higher in asthmatic patients than in healthy

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controls. However, the role of these chemokines in stimulated naïve CD4⁺ T cells remains unclear.

Objective:

To clarify the biological function of CCL17 and CCL22 on naïve CD4⁺ T, we examined effects of these two chemokines on naïve CD4⁺ T cells expressing CC chemokine receptor (CCR)4 (a receptor for CCL17 and CCL22) during differentiation of Th2 cells in asthmatic patients as allergic subjects.

Methods:

Naïve CD4⁺ T cells were prepared from healthy controls and patients with asthma. We analyzed effect of CCL17 and CCL22, and blocking their receptor on differentiation of Th2 cells.

Results:

Production of CCL17 and CCL22 by activated naïve CD4⁺ T cells under Th2 condition was much more in asthmatic patients than in healthy controls. Proliferation and survival of the Th2 differentiating cells and restimulation-induced IL-4 production were much greater in asthmatic patients than in healthy controls. These cell biological phenomena were inhibited by blockade of CCR4. The biological effects of exogenous CCL17 and CCL22 were apparently observed in both healthy controls and asthmatic patients. The effectiveness of these chemokines on naïve CD4⁺ T cells from healthy controls was stronger than those from asthmatic patients. We found

that thymic stromal lymphopoietin (TSLP), a Th2 promoting chemokine, is involved in the activation of CD4⁺ naïve T cells via production of CCL17 and CCL22.

Conclusions & Clinical Relevance:

These data suggest that CCL17 and CCL22 produced by TSLP-primed naïve CD4⁺ T cells in asthma might contribute to an increase in Th2 cells via autocrine loops.

Key words: Allergy; Asthma; Chemokines; Human; Th1/Th2 Cells

Abbreviations:

BALF: bronchoalveolar lavage fluid

CCR: CC-chemokine receptor

CCL: CC chemokine ligand

CXCR: CXC chemokine receptor

CXCL: CXC chemokine ligand

DC: dendritic cell

FEV1: forced expiratory volume in one second

MDC: macrophage-derived chemokine

MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MFI: mean fluorescence intensity

MAPK: mitogen-activated protein kinase

PI: propidium iodide

TARC: thymus and activation-regulated chemokine

TCR: T cell receptor

TSLP: thymic stromal lymphopoietin

INTRODUCTION

Th2 cells are considered to have a critical role in the pathogenesis of allergic diseases such as bronchial asthma. Th2 cells are recruited into the peripheral tissues in response to allergic inflammation. Because chemokines influence inflammatory-cell trafficking and regulate immune responses, recent efforts have focused on the roles of chemokines in allergic inflammation [1- 3].

Chemokine receptor expression can be modulated either temporally or by the tissue environment. The chemokine receptor expression of T cells is also exquisitely regulated depending on the stages of activation and differentiation. Such expression coordinates tissue localization and encounters with antigen-presenting cells [4]. Many chemokine receptors are differentially expressed among Th1 and Th2 cells. Th2 cells preferentially express CCR3, CCR4, and CCR8. CC-chemokines, CCL17 and CCL22 are CCR4 ligands that attract Th2 cells [5]. CCL1 and CCL17 are ligands for CCR8 that is also chemotactic for Th2 cells. CCL17 appears to bind CCR8, in contrast to CCL22 [6], but this remains controversial [7]. Thus, CCR4⁺ Th2 cells are thought to prefer to migrate principally to inflammatory sites, where CCL17 and CCL22 are produced [11-13].

Mouse models of allergic airway disease have been extensively used to investigate the roles of chemokines in inflammatory response. The functional roles of chemokines in attracting effector Th2 cells have been investigated in mouse models of allergic asthma. Neutralization with specific antibodies (Abs) against CCL22 or CCL17 reduces airway hyperreactivity as well as lung eosinophilia [11-14].

A number of cellular sources of CCL17 and CCL22 have been identified, including not only macrophages, dendritic cells (DCs), and bronchial epithelial cells, but also T cells [12,15]. Galli et al. reported that CCL22 was produced by activated human T cells in vitro and in vivo, particularly in association with Th2 cytokines [16]. Previously, we demonstrated that naïve CD4⁺ T cells produced CCL17 and CCL22, with higher levels in asthmatic patients than in healthy controls [17]. These results suggest that naïve CD4⁺ T cells from asthmatic subjects fundamentally differ in their ability to release CCL17 and CCL22 before their development into effector/memory cells. However, the roles of CCL17 and CCL22 produced by naïve T cells remain unclear.

In the current study, we propose that CCL17 and CCL22 produced by naïve CD4⁺ T cells in allergic disease such as asthma might contribute to an increase in Th2 cells by means of autocrine loops.

METHODS

Subjects

All patients were treated at the Department of Medicine and Clinical Immunology of Dokkyo University School of Medicine. The study was approved by the Dokkyo Medical University Research Ethics Committee (Approval No. 2070), and written informed consent was obtained from each participant prior to enrollment. Eleven patients with asthma were enrolled in this study as an allergic

group (mean age, 38.3±5.4 years; 4 women and 5 men; mean forced expiratory volume in 1 s [FEV1], 97.0±2.1% of predicted value; PEF variability <20%, geometric mean provocation concentration of histamine required to decrease the FEV1 by 20 percent [PC20], 3.0 mg/ml histamine; geometric mean IgE, 312.4 kU/l), along with 11 healthy controls (mean age, 37.1±3.7 years; 4 women and 5 men; mean FEV1, 106.2±5.8% of predicted value; geometric mean PC20, greater than 16 mg/ml histamine; geometric mean IgE, 14.3 kU/l). All patients had mild allergic asthma as defined according to Classification of Asthma Severity according to the NAEPP/NHLBI Guidelines [18]. They had a history of intermittent wheeze with reversible airflow obstruction, and asthma had been diagnosed previously by an independent physician. Levels of specific IgE to *Dermatophagoides farinae* (Df) and *D pteronyssinus* (Dp) were measured using the CAP-RAST (Pharmacia). Atopy was determined by the positive response (score ≥ 3) of specific IgE to Df or Dp. Each patient had elevated Df-specific IgE levels (geometric mean, 64.9 U/ml) in sera. All patients had chronic asthma but were asymptomatic for asthma. The patients were not given oral corticosteroids for at least 2 weeks before vein puncture. To avoid analytical bias from various factors, the patients in this study were recruited such that they all had clinical backgrounds that were as similar as possible. Also, the patient group did not include those with a past history or complications of other obvious allergic diseases such as atopic dermatitis, rhinitis, or conjunctivitis. The 11 healthy controls had normal lung function test results, no bronchial hyperresponsiveness, and no history of allergic or other diseases. All subjects were nonsmokers, and none had had an episode of acute bronchitis during the 4 weeks before the study. Informed consent was obtained from all subjects before enrollment.

Abs for flow cytometry

The human Abs used for staining and sorting (FITC-conjugated anti-CD45RA, PE-conjugated anti-CD45RO, APC-conjugated anti-CD4) were purchased from Becton Dickinson. FITC-conjugated anti-CCR4, anti-CCR8, PE-conjugated CD25, and FITC-conjugated CD69 were purchased from Pharmingen. Flow cytometric analysis and cell sorting were performed with a FACScan flow

cytometer (Becton Dickinson) equipped with Cell Quest software, and a FACS Aria cell sorter (Becton Dickinson), respectively.

Cell preparation

Peripheral blood was obtained from the subjects by vein puncture, with heparin as an anticoagulant. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque density gradient centrifugation (Pharmacia). Cells were washed three times with PBS supplemented with 2% FCS (Gibco Life Technologies) and were resuspended at a concentration of 1×10^6 /ml. CD45RA⁺RO⁻ (naïve) CCR7⁺ CD4⁺ T cells and CD45RA⁻RO⁺ (memory) CD4⁺ T cells were sorted from PBMCs with a FACS Aria cell sorter (Becton Dickinson). T cells were phenotypically rested by confirming no significant expression of CD69 or CD25. The purity of each cell preparation was >99%.

Generation of Th1 and Th2 cells

Short-term Th1 and Th2 cell lines were derived from PBMCs from patients with asthma and healthy controls as previously described. Naïve CD4⁺ T cells (2×10^5 /well in 96-well plates) were stimulated with immobilized anti-CD3 monoclonal Abs ($10 \mu\text{g/ml}$) (R&D Systems) and anti-CD28 monoclonal Abs ($1 \mu\text{g/ml}$) (eBioscience) in polarizing conditions or a default condition as follows: human rIL-12 (R&D Systems; 2 ng/ml) and neutralizing anti-IL-4 (R&D Systems; 200 ng/ml) for Th1 skewing; human rIL-4 (R&D Systems; 10 ng/ml) and neutralizing anti-IFN- γ (BioSource International; 5 $\mu\text{g/ml}$) for Th2 skewing; and no cytokine and no Ab for the default condition. The culture medium used in all experiments was RPMI 1640 supplemented with 10% FCS at 37°C in a 5% CO₂ environment. Cells were removed from activation signaling after 2 days and expanded in medium containing 100 U/ml rIL-2 (Boehringer Mannheim).

Measurement of CCL17 and CCL22 produced by activated naïve CD4⁺ T cells

To analyze the production of CCL17 and CCL22 by activated naïve T cells in each skewing condition or the default condition, the supernatants of cells incubated in 96-well plates were collected 48 h after stimulation. The chemokines were measured using ELISA kits for CCL1, CCL17, and CCL22 (R&D Systems). The assay sensitivities were higher than 4.12, 31.2 and 125 pg/ml, respectively.

Effects of CCL17 and CCL22 on differentiation into Th2 cells

To elucidate the effects of CCL17 and CCL22 on the generation of Th2 cells, differentiating cells were cultured in the presence of rCCL17 (10 ng/ml) and/or rCCL22 (10 ng/ml) (Pharmingen) or of anti-CCR4 Abs (5 µg/ml) and/or anti-CCR8Ab (5 µg/ml) (BD Biosciences) [19] as neutralizing Abs. Subsequently, after 7 days of culture, the obtained Th2 cells were washed three times with PBS supplemented with 2% FCS. Each cell s (3×10^5 /well in 98-well plates) was then plated again and restimulated with immobilized anti-CD3 mAbs and anti-CD28 mAbs for 48 h. IL-4 in the culture supernatants were measured using ELISA kits (BioSource). The assay sensitivities were higher than 15.6 pg/ml, respectively.

Effects of TSLP on cellular biological function of activated naïve CD4⁺ T cells.

Naive CD4⁺ T cells were stimulated with immobilized anti-CD3 mAbs and anti-CD28 mAbs under a default condition. Amounts of CCL17 and CCL22 in the culture supernatants were measured using ELISA at 2 days and cell numbers activated naïve CD4⁺ T cells were counted at 6 days following stimulation in the presence or absence of rTSLP (10 ng/ml) (Pepro Tech). In some experimental settings, culture was carried out with anti-CCR4 Abs.

Cells counts and proliferation/viability

The numbers of viable and non-viable differentiated Th2 cells and memory CD4⁺ T cells were directly counted with a hemocytometer in the presence of 0.5% trypan blue. To analyze cell proliferation and viability of Th2 cells, MTT assay was performed on day 2 (48h) after activation of naïve CD4⁺ T cells and memory T cells, using a Premix WST-1 Cell Proliferation Assay System (TaKaRa). At designated intervals during the incubation, 10 µl of the premix WST-1 reagent was added to each well, and the plates were then reincubated under the prescribed conditions for an additional 2 h. The absorbance of the wells was measured with a microplate reader (Molecular Devices) at a wavelength of 570 nm.

Determination of surface marker expression and survival/apoptosis by flow cytometry

Cell apoptosis was measured with the use of an annexin V-FITC/propidium iodide (PI) apoptosis assay kit (BD Biosciences). Cells were labeled with annexin V-FITC according to the manufacturer's instructions. Immediately before analysis, PI was added to single-cell suspensions (2 µg per ml final dilution). The cells were analyzed on a FACScan flow cytometer. The combined use of PI and FITC-annexin V allowed the simultaneous analysis of viable cells (PI⁻ annexin V⁻), cells in early apoptosis (PI⁻ annexin V⁺), and dead cells (PI⁺ annexin V⁺).

Statistics

The statistical significance of differences between groups was examined by analysis of variance with Student's t test. P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

Production of CCL17 and CCL22 by stimulated naïve CD4⁺ T cells

Naïve CD4⁺ T cells from PBMCs of asthmatic patients and healthy controls were sorted (Fig 1, A). We measured amounts of CCL17 and CCL22, ligands for CCR4 and CCL1, a ligand for CCR8, in supernatants of the naïve CD4⁺ T cells activated in culture conditions of default, Th1, and Th2 (Fig 1 B). Amounts of CCL17 and CCL22 were significantly 3 to 4 folds higher in culture supernatants of naïve CD4⁺ T cells from asthmatic patients than from healthy controls after stimulation in a default condition for 2 days. The production of these chemokines was augmented by the addition of IL-4 (Th2 condition), with 2~3 folds greater increments in asthma than in controls. In contrast, IL-12 (Th1 condition) had no significant influence on production of these chemokines by each stimulated naïve CD4⁺ T cells. On the other hand, CCL1 was not detected in the supernatants under any culture conditions in asthmatic patients or healthy controls. The number of naïve CD4⁺ T cells was not significantly different between asthma and control groups in each condition for 2 day-culture (data not shown). These results indicate that the higher CCL17 and CCL22 in stimulated naïve CD4⁺ T cells is not associated with cell proliferation.

Effects of CCL17 and CCL22 on Th2 cell differentiation from naïve CD4⁺ T cells

Next, we investigated production of IL-4 by the restimulated CD4⁺ T cells which had been cultivated in the presence of CCL17, CCL22, blockade of CCR4 alone, or blockades of CCR4 and CCR8 during Th2 skewing (Fig 2). Although production of IL-4 by Th2 cells differentiated with exogenous CCL17 or CCL22 significantly increased after restimulation in asthma and healthy controls, the cytokine levels were 2-fold higher in asthma than in normal controls regardless of the presence of chemokines. However, the effectiveness of these chemokines on IL-4 production by the T cells was higher in healthy controls than in asthmatic patients. The treatment of CCR4 blockade apparently decreased the production of IL-4 by restimulated T cells of asthma, but not healthy controls. The presence of CCR8

blockade had no significant influence on the effect of CCR4 blockade on the production of IL-4 by restimulated T cells of asthma and healthy controls.

Effects of CCL17 and CCL22 on CCR4 expression on differentiating Th2 cells

We observed that the naïve CD4⁺ T cells from asthmatic patients as compared with healthy controls expressed CCR4 along with CD45RO as an activation marker, from approximately 3 days after stimulation with anti-CD3 and anti-CD28 Abs under the default condition (Fig 3, A). Thus, we assumed that naïve CD4⁺ T cells might activate themselves by producing CCL17 and CCL22. Because CCL17 and CCL22 were produced largely by stimulated naïve CD4⁺ T cells in Th2 condition to compare with Th1 condition in both healthy controls and asthmatic patients, we analyzed the ability of CCL17 and CCL22 to induce expression of CCR4 and CCR8 by stimulated naïve CD4⁺ T cells during differentiation into Th2 cells in 6-day cultures (Fig 3, B). The frequency of the CD4⁺ T cells co-expressing CCR4 and CD45RO in asthma ($44.1 \pm 10.0\%$) was significantly 3-fold greater than those of healthy controls ($15.2 \pm 8.2\%$) in the control group, respectively (Fig 3, C), while CCR8 expression was apparently not observed in asthmatic patients or healthy controls (Fig 3, B). The frequency of CD4⁺ T cells co-expressing CCR4 and CD45RO was significantly 1.5- and 2.5-folds increased in asthma and healthy controls, respectively by both exogenous CCL17 and CCL22 (Fig 3, C). The absolute number of Th2 cells co-expressing CCR4 and CD45RO was also increased in a manner similar to the results of flow cytometry analysis in the controls (Fig 3, D). The mean fluorescence intensity (MFI) of CCR4 expression was not significant different between asthma and healthy controls regardless of presence of CCL17 and CCL22 (Fig 3, E). These results suggest that CCL17 and CCL22 augment the proliferation of differentiating Th2 cells, independently of changes in CCR4 expression in particularly asthmatic subjects.

Effects of CCL17 and CCL22 on cell proliferation during Th2 differentiation

The increases in cell counts of the cultivated CD4⁺ T cells from asthma, stimulated under Th2 conditions, were strikingly attenuated by blockade of CCR4 as compared to treatment with control IgG, whereas no appreciable effect of the blockade on T cells from healthy controls was seen (Fig 4, A). Furthermore, to analyze the effects of exogenous CCL17 and CCL22 on cell proliferation in Th2 conditions, MTT assay was performed using CD4⁺ naïve T cells from asthmatic patients at 48h after stimulation (Fig 4, B). Cell proliferation was significantly inhibited by blockade of CCR4 and augmented in the simultaneous presence of CCL17 and CCL22 (Fig 4, B). It seems that the increases in IL-4 production might depend on cell proliferation, in which CCL17 and CCL22 might be involved. Therefore, naïve CD4⁺ T cells in asthma might reinforce their own proliferation during Th2 cell differentiation by intrinsic CCL17 and CCL22.

Roles of CCL17 and CCL22 in apoptosis of differentiating cells in asthma

To investigate the effects of CCL17 and CCL22 on increases in cell numbers after activation of naïve CD4⁺ T cells in asthma, we analyzed survival and apoptosis by flow cytometry (Fig 5, A). When cell survival was examined during Th2 differentiation for 6 days, the ratio of apoptotic cells (annexin V⁺) to differentiating Th2 cells in asthma was significantly less than in healthy controls in the presence control IgG, while the number of living cells decreased significantly with CCR4 blockade in both groups. The effects of exogenous CCL17 and CCL22 were demonstrated to rescue the cells from apoptosis in both groups (Fig 5, B). Therefore, these results indicate that CCR4-mediated signaling, and perhaps CCL17 and CCL22, are involved in cell survival during differentiation of Th2 cells.

Effects of CCL17 and CCL22 on proliferation and apoptosis of memory CD4⁺ T cells

We analyzed memory CD4⁺ T cells isolated from PBMCs and observed that CCR4 expression even in the absence of activation was much higher in asthmatic patients than in healthy controls (Fig 6, A).

However, we observed that CCL17 and CCL22 had no significant effect on apoptosis (Fig 6, B, C) and proliferation of memory CD4⁺T cells 48 h after activation (Fig 6, D).

Effects of TSLP on production of CCL17 and CCL22 by activated naïve CD4⁺ T cells

Naïve CD4⁺ T cells from asthmatic patients, as compared with healthy controls, were prone to differentiating toward Th2 cells. Because TSLP, a Th2 promoting cytokine can directly drive the Th2 differentiation of naïve CD4⁺ T cells [20,21] and is shown to have higher systemic levels in asthmatic patients rather than the other diseases groups and healthy controls [22,23], we focused on the effect of TSLP on the production of CCL17 and CCL22. We analyzed the production of these chemokines by the naïve CD4⁺ T cells activated with anti CD3 Abs plus anti-CD28 Abs as a default condition for 3 day-culture in the presence or absence of TSLP (Fig 7, A). For healthy controls' CD4⁺ T cells, TSLP significantly augmented CCL17 production, while demonstrating a tendency to increase CCL22 production. Regarding asthma, TSLP showed a tendency to hinder the production of these cytokines already at a high level. The cultivated cells were counted at 7days following stimulation under the default condition. Consistent with the results of cytokine production, TSLP significantly augmented an increment of the activated naïve CD4⁺ T cell of healthy controls, whereas in a case of asthma, a trend towards increase in cell number was observed to be enhanced by TSLP (Fig 7, B).

DISCUSSION

In our study, novel roles for CCL17 and CCL22 were suggested by the augmented production of these chemokines by naïve CD4⁺ T cells from patients with asthma. Surprisingly, we found that proliferation of activated naïve CD4⁺ T cells after stimulation in Th2 conditions was much greater in asthma than in healthy controls. Such proliferation was inhibited by blockade of CCR4. CCR4 is known to be preferentially expressed on Th2 cells [8-10]. We have confirmed that CCR4 is expressed on activated CD4⁺ T cells in a default condition, as well as during differentiation into Th2 cells in vitro. However, the present study showed that CCR8 was not apparently expressed during differentiation into Th2 cells in 6-day cultures, while CCR8 was reported to be up-regulated in Th2 cells [10]. Furthermore, there was no significant difference in the production of the CCR8-specific ligand CCL1 by naïve CD4⁺ T cells in Th2 skewing conditions between healthy controls and asthmatic patients in our study. In addition, there was no significant difference in the data between pre-treatment, treatment with CCR4 Abs alone, or with both CCR4 and CCR8 Abs. Therefore, CCR8, CCL1, or both most likely did not contribute to the auto-activation of naïve CD4⁺ T cells.

In this current study, we observed that exogenous CCL17 and CCL22 have proliferative effects on activated naïve CD4⁺ T cells. Mitogenic functions of some chemokines have been reported. For example, CXCL13 and CCL19 cooperatively induce resistance to apoptosis in B-cell-lineage acute and chronic lymphocytic leukemia B cells in humans [24, 25]. CXCL12 also enhances survival of chronic lymphocytic leukemia B cells [26]. In addition, the CCR8-ligand CCL1 has been reported to rescue thymic lymphoma cells from apoptosis [27, 28]. The mitogen-activated protein kinase (MAPK) pathway is involved in the antiapoptotic activity of CXCL12 and CCL1. Thus, the proliferative and antiapoptotic effects of CCL17 and CCL22 may be established through the MAPK pathway as well as other chemokines.

Although the molecular mechanisms underlying the mitogenic effects of CCL17 and CCL22 and the details of CCR4-mediated signaling remain unclear, the novel functions of CCR4, CCL17, and

CCL22 may contribute to an increment in helper T cells, particularly Th2 cells. Thus, further analysis to clarify the mechanism of chemokine-mediated Th2 cell differentiation is required in the future. For now, we speculate that CCL17 and CCL22 may reinforce Th2 cell differentiation by means of both antiapoptotic and proliferative functions, which may contribute to cell cycle dependent IL-4 gene activation [29], rather than by directly promoting differentiation. Because IL-4 could induce production of CCL17 and CCL22 [30] [31], augmented self-production of these chemokines by naïve CD4⁺ T cells in asthma might result from pre-activation by priming with an IL-4-rich environment in patients with asthma. Therefore, CCR4-mediated signaling in activated CD4⁺ T cells might be involved in the pathogenesis of allergic diseases through acceleration of Th2 cell differentiation by CCL17 and CCL22 autocrine loops in asthma. As another impact on production of CCL17 and CCL22, we identified an effect of TSLP to enhance production of these chemokines, which mediated auto-proliferation of naïve CD4⁺ T cells of both healthy controls and asthmatic patients. Notably, the effect of TSLP was remarkable in healthy controls as compared with asthmatic patients, although baseline chemokine production and cell proliferation without TSLP was less than in asthmatic patients. Therefore, in asthmatic patients, TSLP is a candidate for priming naïve CD4 T cells to produce substantial amounts of CCL17 and CCL22, which may be involved in formation of a dominant milieu of Th2 cell population. In contrast to naïve CD4⁺ T cells, CCL17 and CCL22 had no significant effect on proliferation and apoptosis of memory CD4⁺ T cell. Thus, such a novel role for these chemokines is adapted for activated naïve CD4⁺ T cells, particularly in Th2 conditions.

Current evidence suggests that CCL17 and CCL22 and their receptors maintain allergic immune responses such as asthmatic inflammation by supplying newly generated effector T cells, which may be related to disease chronicity. Moreover, naïve T cells as well as antigen-presenting cells such as DCs may serve as cellular sources of CCL17 and CCL22. Therefore, CCL17 and CCL22 production by each cell type may have an important role in not only the migration of memory Th2 cells into lymphoid tissue and peripheral tissues such as lung, but also in accelerating helper T cell

differentiation, particularly that of Th2 cells, which may potentiate Th2 immune response and contribute to chronic airway inflammation.

We have described a novel role of chemokines, apart from cell recruitment. We concluded that the differences in the effects of CCR4-mediated signaling between the asthmatic and control groups are specific to asthma. However, these differences may be related to atopy rather than asthma. Therefore, further study will be required with inclusion of an experimental group of subjects with atopy and no asthma. In addition, most functions of chemokines probably remain unknown. Clarification of the mechanisms responsible for the establishment and maintenance of allergic diseases such as asthma will require further analyses of the roles and functions of chemokines, potentially leading to a new generation of therapeutics for allergic diseases.

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Figure 1. Production of CCL17 and CCL22 by stimulated naïve CD4⁺ T cells.

(A) Representative results of flow cytometry analysis showing naïve CD4⁺ CD45RA⁺ CD45RO⁻ CCR7⁺ T cells sorted from PBMCs of a subject. **(B)** Naïve CD4⁺ T cells activated under default, Th1, or Th2 conditions. Following 2-day culture, amounts of CCL17, CCL22, and CCL1 in the supernatants were measured using ELISA. Individual data are indicated as open circles (healthy controls, n=11) and closed circles (patients with asthma, n=11). The horizontal bar in each group indicates the mean value. *P<0.05 between each group. NS: not significant

Figure 2. Effects of CCL17 and CCL22 on Th2 cell differentiation.

Naïve CD4⁺ T cells were prepared from healthy controls (n=11) and asthmatic patients (n=11). Naïve CD4⁺ T cells were activated under Th2 condition in the presence of blockade of CCR4, control IgG, CCL17, or CCL22. Following 6-day culture, Amount of IL-4 in culture supernatants was measured by ELISA 48 h after restimulation with plate-bound anti CD3 Abs. Data represent means ± SEM for each treatment group. *P<0.05 between each group.

[§]P<0.05 as compared with healthy controls. NS: not significant.

Figure 3. Effects of CCL17 and CCL22 on induction of CCR4 and CCR8 in CD4⁺ CD45RO⁺ T cells.

Naïve CD4⁺ T cells were prepared from healthy controls (n=11) and asthmatic patients (n=11). Naïve CD4⁺ T cells were activated under default condition **(A)** or Th2 condition in the absence (control) or presence of CCL17, or CCL22 for 6 days **(B)**. **(A, B)** Representative data of flow cytometry analysis from a healthy control (upper) and a patient with asthma (lower). The numbers in the corners represent the percentages of gated T cells. **(C-E)** Individual data on frequencies of CCR4⁺CD45RO⁺ Th2 cells **(C)**, absolute cell number **(D)**, and mean fluorescence intensity (MFI) of CCR4 expression

(E). (C, E) Horizontal bars in each group indicate the mean value. (D) Data represent means $\pm$ SEM for each group. *P<0.05 between each group. [§]P<0.05 as compared with healthy controls.

Figure 4. Effects of both CCL17 and CCL22 on cell proliferation during Th2 cell differentiation.

Naïve CD4⁺ T cells were prepared from healthy controls (n=11) and asthmatic patients (n=11). Naïve CD4⁺ T cells were activated under Th2 conditions in the presence of blockade of CCR4 or control IgG, or CCL17 and CCL22 for 6 days. (A) Total cell numbers were counted. (B) MTT assay for cell proliferation was carried out. Individual data are indicated. The horizontal bar in each group indicates the mean value. *P<0.05 between each group. [§]P<0.05 as compared with healthy controls

Figure 5. Involvement of CCL17 and CCL22 in apoptosis of CD4⁺ T cells during differentiation in Th2 condition.

Naïve CD4⁺ T cells were prepared from healthy controls (n=11) and asthmatic patients (n=11). Naïve CD4⁺ T cells were activated under Th2 conditions in the presence of blockade of CCR4 or control IgG, or CCL17 and CCL22 for 6 days.

(A) Representative results of apoptosis analysis by flow cytometry. The numbers in the upper right corners represent the percentages of total annexin V positive T cells. PI: propidium iodide. (B) Individual results for the frequencies of live cells and apoptotic cells (annexin V⁺). The horizontal bar in each group indicates the mean value. *P<0.05 between group. [§]P<0.05 as compared with healthy controls.

Figure 6. Effects of CCL17 and CCL22 on proliferation and apoptosis of CD4⁺ CD45RO⁺ (memory) T cells. (A) Representative results of flow cytometry analysis for CCR4 expression in unstimulated memory CD4⁺ T cells isolated from PBMCs of healthy controls (n=11) and asthmatic patients (n=11).

The numbers in the corners represent the percentages of gated T cells. **(B)** Representative results of apoptosis analysis with flow cytometry after stimulation with immobilized anti-CD3 mAbs and anti-CD28 mAbs in the absence (control) or presence of CCL17 and CCL22 for 48 h. The numbers in the upper right corners represent the percentages of total annexin V positive T cells. **(C)** Individual results for the frequencies of live cells and apoptotic cells (annexin V⁺). **(D)** MTT assay for cell proliferation of memory CD4⁺ T cells after activation. Data represent means ± SEM for each group. NS: not significant.

Figure 7. Effects of TSLP on production of CCL17 and CCL22 by activated Naïve CD4⁺ T cells.

Naïve CD4⁺ T cells were prepared from healthy controls (n=11) and asthmatic patients (n=11). Naïve CD4⁺ T cells were activated under a default condition in a presence of combination of TSLP and blockade of CCR4, or control IgG. Following 6-day culture, amounts of CCL17 (upper) and CCL22 (lower) in the supernatants were measured using ELISA **(A)** and total cell numbers were counted **(B)** for each group. **(A, B)** Individual results for the data are indicated. The horizontal bar in each group indicates the mean value. *P<0.05, **P<0.01, between group. [§]P<0.05, ^{§§}P<0.01, as compared with healthy controls.

Fig 1

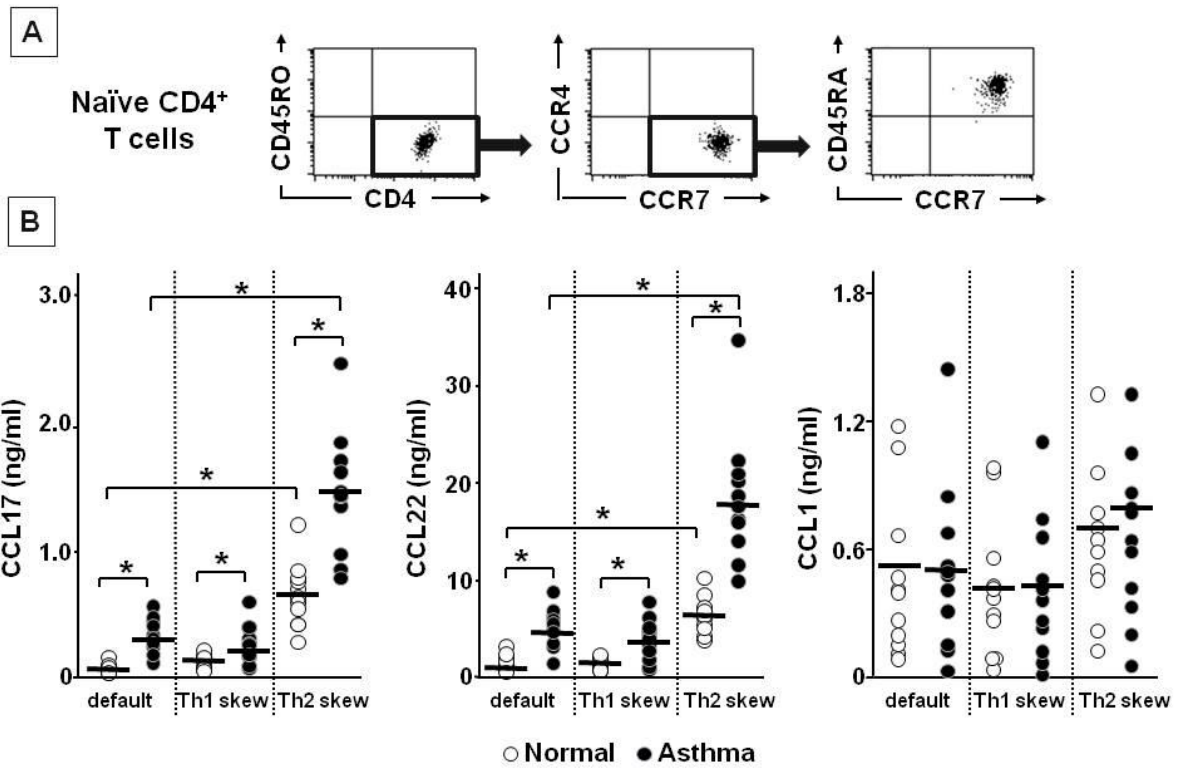


Fig 2

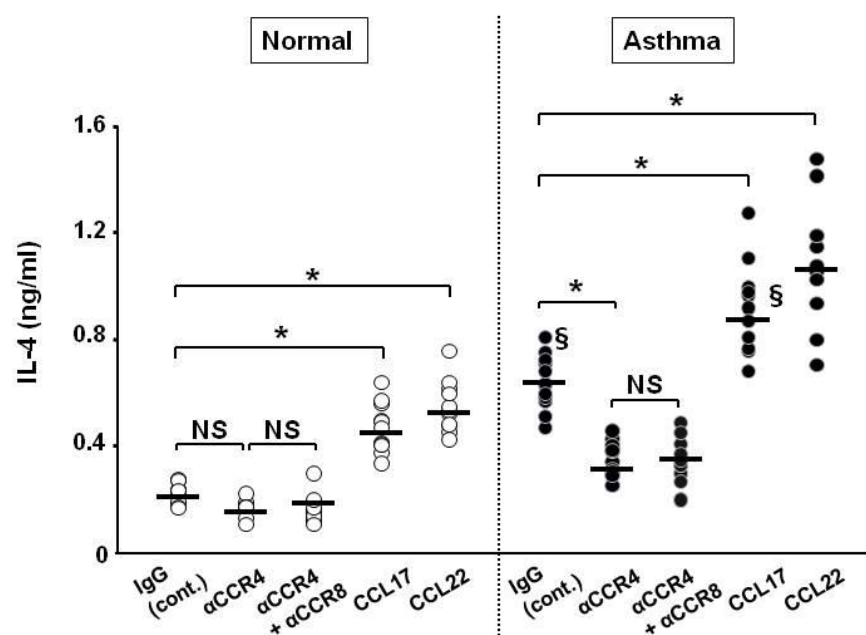


Fig 3

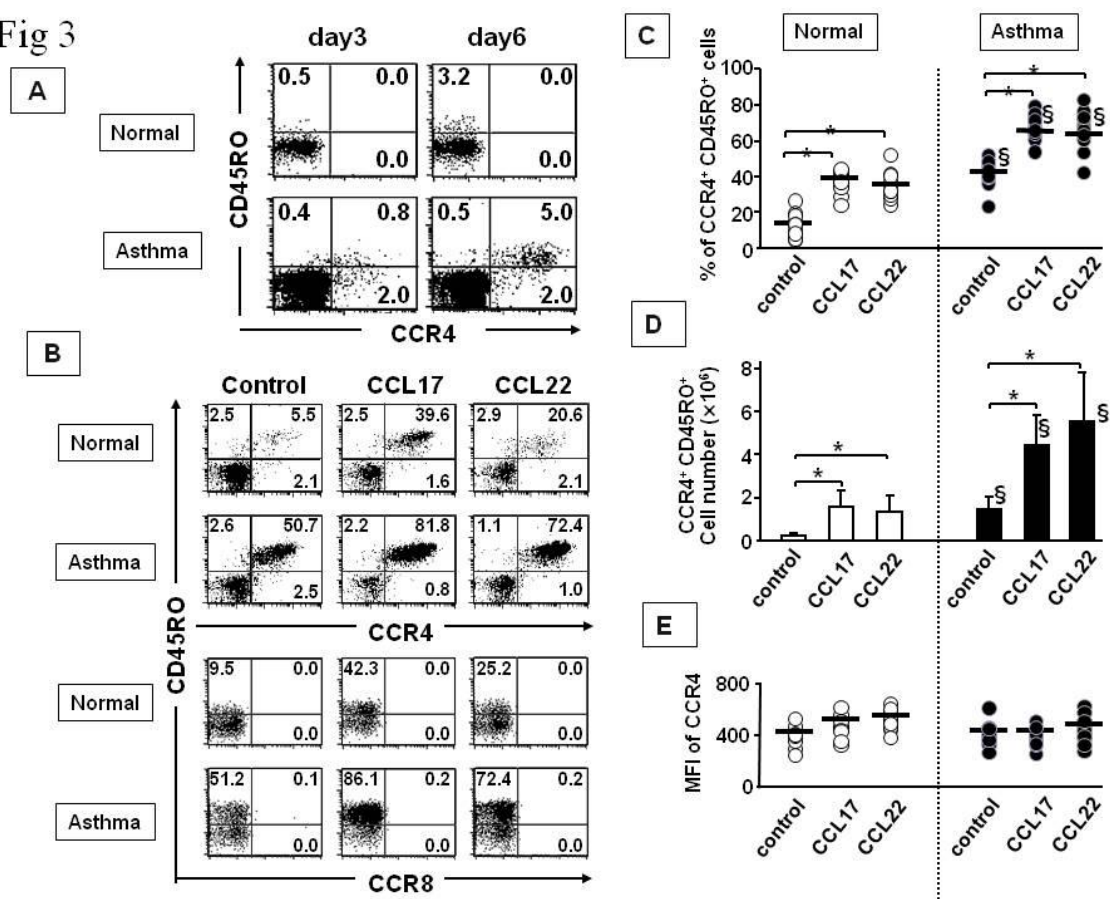


Fig 4

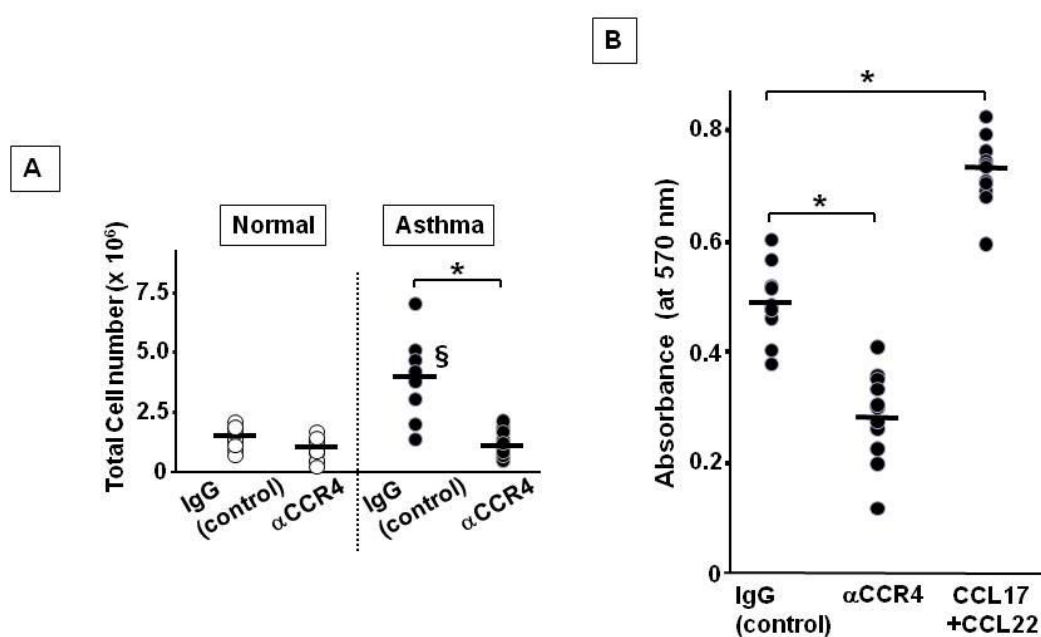


Fig 5

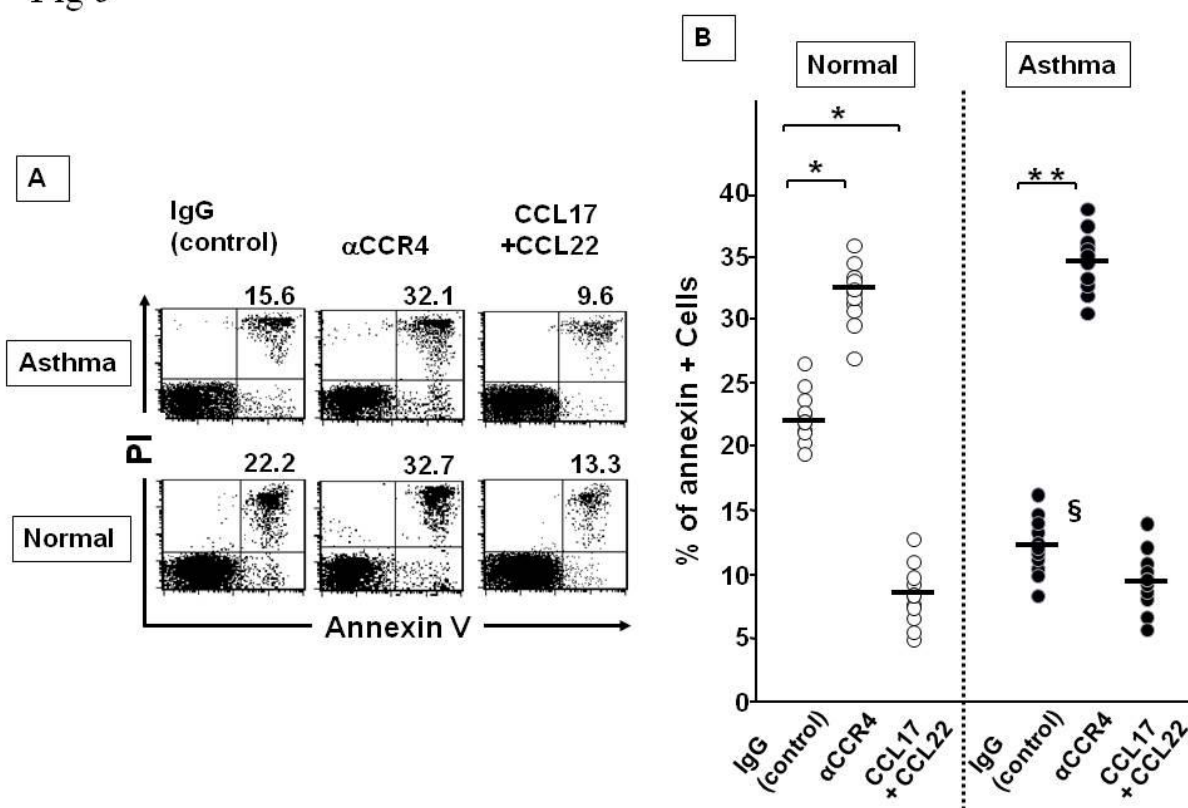


Fig 6

