

Original article

Plasma macrophage-derived chemokine (CCL22) and its receptor CCR4 on peripheral blood T lymphocytes of asthmatic children

Background: The macrophage-derived chemokine (MDC/CCL22) acts on CC chemokine receptor-4 (CCR4) to direct trafficking and recruitment of T helper-2 (TH2) cells into sites of allergic inflammation. It was previously found overexpressed in lesional samples from adult asthmatics.

Objective: This study is aimed to investigate the participation of CCR4/MDC axis in the development of TH2-dominated allergen-induced childhood asthma in relation to disease activity, attack severity, and response to therapy, and to outline its value in differentiating atopic asthma from infection-associated airway reactivity.

Methods: Proportion of CCR4-expressing peripheral blood T lymphocytes (CCR4⁺PBTL%) were purified and quantitated by negative selection from peripheral blood mononuclear cells by flow cytometry, and the concentration of MDC in plasma was measured by ELISA in 32 children with atopic asthma (during exacerbation and remission), as well as in 12 children with acute lower respiratory tract infections (ALRTI), and 20 healthy children serving as controls.

Results: The mean plasma MDC level (925±471.5 pg/ml) and CCR4⁺PBTL% (55.3±23.6%) were significantly higher in asthmatic children during acute attacks in comparison to children with ALRTI (109±27.3 pg/ml and 27.6±7.5%) and healthy controls (99.6±25.6 pg/ml and 24.2±4.1%). Both values decreased significantly after subsidence of attacks (502±284.3 pg/ml and 32.5±10.5%) although remained higher than the other 2 groups which were actually comparable in terms of MDC and CCR4%. MDC and CCR4% values were higher among children with acute severe than mild or moderate asthma exacerbations, and in persistent than intermittent cases during stability. Positive correlations could be elicited between both markers during exacerbation or stability, and between the exacerbation level of each and its corresponding value during remission. Corticosteroid-treated patients had the highest expression of both markers when relation to therapy was studied.

Conclusion: Our findings reinforce the concept that up regulation of CCR4/MDC axis is implicated in the pathogenesis of pediatric atopic asthma and may represent a useful biomarker of monitoring allergic inflammation and response to therapy. Neutralization and manipulation of CCR4-expressing T cells, as well as MDC antagonism, may represent an adjuvant in the treatment of severe allergic disorders.

Key words: MDC; CCR4; T-lymphocytes; flowcytometry; asthma; children

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INTRODUCTION

Chemokines and chemokine receptors provide directional cues for leukocyte migration and concomitantly induce the recruitment of T cells into the sites of inflammation. This process is particularly important in atopic diseases such as bronchial asthma. Initial studies in this area have emphasized that chemokine receptor expression is tightly regulated on TH cells, and that TH cell

subsets express restricted chemokine receptors. For example, TH1 cells express the chemokine receptors CXCR3 and CCR5, while CCR4, CCR8, and CCR9 are found on TH2 cells.^{1,2}

CCR4 is a receptor that binds 2 chemokines; CCL17/TARC (Thymus and Activation-Regulated Chemokine), and CCL22/MDC (Macrophage-Derived Chemokine). The receptor was originally isolated from a basophilic cell line and shown to be

expressed on a subpopulation of peripheral blood lymphocytes, thymocytes, mature dendritic cells and recently on blood platelets.³ Current evidence suggests that CCR4 expression is associated with TH-2 responses. Activation of T cells through their T-cell receptor can induce elevated CCR4 levels, which can last up to two days.⁴

Chemokines basically have been divided into two main families, CXC and CC chemokines, based on the sequence homology and the position of the first two cysteine residues. CXC chemokines are typically chemotactic for neutrophils, whereas CC chemokines attract and activate monocytes and lymphocytes.⁵ MDC or CCL22 is a novel human 8 kDa CC chemokine. It was originally cloned from human monocyte-derived macrophage cDNA clones. It is composed of 69 amino acid residues and has been termed MDC because it appears to be synthesized specifically by cells of macrophage lineage.⁶ Unlike most other CC chemokines, MDC is clustered on chromosome 16q13. It was previously known as STCP-1 (stimulated T cell chemotactic protein-1). It has the 4 cysteine motif and other highly conserved residues characteristic of CC chemokines, but it shares less than 35% sequence identity with other human chemokines, especially TARC being its closest known human relative.^{7,8}

CCR4/MDC axis plays an important role in the long-term recruitment of TH2 cells into the inflammatory sites and the regulation of TH2-related immune responses. CCR4/MDC axis has been directly associated with an asthma diagnosis and found to be inversely related to lung function as measured by FEV1.⁹ The established in vivo significance of distinct CCR4 expression on TH2 cells, and chemotactic and activating effects of MDC on TH2 cells and the association of TH2 cells with asthma activity and severity suggest that this axis is pivotal in asthma.¹⁰ The current study is therefore aimed to elucidate the role of CCR4/MDC axis in pediatric asthma by tracing and following up their expression during activity and in-between attacks and to outline their relation to other parameters denoting disease activity, degree of severity, and response to therapy. Also, to evaluate their discriminative value between allergic asthma and infection-associated bronchospasm. This may have an implication for new non-conventional lines of therapy.

METHODS

This follow-up, case-control study was conducted over a period of 1 year from March 2004 to the end of February 2005. It comprised 64 children divided

into 3 main groups: 32 children with allergic bronchial asthma (group I), 12 children with acute lower respiratory tract infections (group II), and 20 healthy children not expressing airway disease (group III) serving as controls. An informed consent was obtained from the parents or caregivers of each child before enrollment.

Group (I): It comprised 32 children enrolled from the Pediatric Allergy and Immunology Unit of Ain Shams University Children's Hospital while presenting with an acute asthmatic exacerbation. They were followed-up until complete clinical subsidence of the attack and then re-evaluated. Their ages ranged between 3 and 12 years (mean \pm SD = 7.6 ± 2.3 years), and they comprised 20 boys (62.5%) and 12 (37.5%) girls. The asthma exacerbations were triggered by exposure to allergens (food, animal allergens or both) in 20 children and upper respiratory tract infections in 3 children. In 9 children, the triggering agent was not clear. Thirteen children (41%) presented with acute severe asthma, 9 (28%) with moderate severe asthma, and 10 (31%) had exacerbations of mild severity based on clinical data and peak expiratory flowmetry (PEFR).^{11,12} Sixteen patients (50%) had radiological evidence of hyperinflation and increased bronchovascular markings. Based on the criteria of asthma grading, the clinical severity of the recruited asthmatics during stability were classified into intermittent asthma (IA) in 17 children (53%) and persistent asthma (PA) in 15 children (47%) according to the Global Initiative for Asthma (GINA) guidelines.¹³ Asthmatic children were further subdivided according to intake of inhaled corticosteroids into those on therapy (n=14; 44%) (mean dose = $250 \pm 50\mu\text{g}$ and a mean duration of 8.2 ± 6.4 months and those who were not (n=18; 56%). The latter group was on one or more of the following medications: inhaled sodium cromoglycate, oral/inhaled β_2 agonists, or theophylline.

Group (II): It comprised 6 boys and 6 girls whose ages ranged from 4 to 11 years with a mean \pm SD of 7 ± 1.4 years. They were enrolled after exclusion of a past, current or family history of allergic disorders, peripheral blood eosinophilia or elevated serum total IgE for age.¹⁴ Detailed history taking, clinical examination, and chest auscultation findings as well as chest radiographs were consistent with the diagnosis of bronchopneumonia in 4 patients, lobar pneumonia in 3 patients, bronchiectasis in 3 patients (secondary to cystic fibrosis), and chronic bronchitis in 2 patients. Children with bronchiectasis and chronic bronchitis

were sampled during an acute exacerbation of infection.

Group (III): It comprised 11 boys (55%) and 9 girls (45%) whose ages ranged from 5 to 12 years with a mean \pm SD of 8 ± 2.2 years. They were enrolled after exclusion of a personal and family history of allergic disorders, peripheral blood eosinophilia or elevated serum total IgE for age.¹⁴ Patients or control subjects with clinical or laboratory evidence of any parasitic or other concomitant illness were excluded from the study. We also excluded children with weights and/or heights below the 5th percentiles for age.¹⁵

Study Design

Blood samples from group (I) were collected within 24 hours of the start of an asthma exacerbation defined by increasing cough, wheezing, dyspnea and nocturnal symptoms. Patients received a reliever medication in the form of a β_2 agonist by nebulizer, and if necessary, oral or intravenous steroids and oxygen. After subsidence of the episode, the patients were returned to the controller medications. Follow up was carried out for 2 to 3 weeks until stabilization of the patients' condition and quiescence of symptoms (remission or steady state), then a follow up blood samples were collected for re-assessment. By that time, none of the asthmatics had evidence of pulmonary infections (clinical or radiological and confirmed by a normal total leukocyte count).

Analytical Methods

Blood sampling

Six ml of venous blood were collected under complete aseptic precautions and were divided into 3 equal parts. Two of them were mixed with EDTA as an anticoagulant; one for blood counting and flow cytometric analysis, and the other for plasma MDC assay, and a third was not added to any anticoagulant. After coagulation, the latter sample was centrifugated for 15 minutes at 1500 x g. The separated serum was stored at -70°C for the subsequent assay of total IgE. Repeated thawing and freezing was avoided. Hemolyzed and lipemic samples were excluded.

MDC/CCL22 assay

MDC was determined by a quantitative sandwich enzyme immunoassay technique using reagents supplied by *Quantikine (R&D Systems, Inc. McKinley Place N.E., Minneapolis, Minnesota, MN 55413 U.S.A.)*. A monoclonal antibody specific for MDC had been pre-coated onto a microplate. Standards and samples were pipetted into the wells

and any MDC present was bound by the immobilized antibody. After washing away any unbound substances, a horseradish peroxidase-linked monoclonal antibody specific for MDC was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution of hydrogen peroxide-tetramethylbenzidine was added to the wells and color developed in proportion to the amount of MDC bound in the initial step. The color development was stopped by 2N sulfuric acid and the intensity of the color was measured at 450 nm and 540 nm. Readings at 540 nm were subtracted. For calculation of results, a standard curve was constructed on a log/log scale by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and drawing a best fit curve through the points on the graph.

Flow cytometric assay of CCR4 on peripheral blood T lymphocytes

The assay was performed on peripheral blood cells. The total leukocytic count in EDTA anticoagulated whole blood samples was adjusted to $5-10 \times 10^3$ cell/ μl and 100 μl of each sample were added to a tube containing 10 μl of the monoclonal antibodies under study. Contaminating serum components were removed by washing the cells three times in an isotonic phosphate buffer (supplemented with 0.5% BSA) by centrifugation at 500 x g for 5 minutes. 50 μl of packed cells were then transferred to a 5 ml tube for staining with monoclonal antibodies. Labeled antibodies with fluorescein isothiocyanate (FITC), and phycoerythrin 5 (PE5) in optimal dilutions were used (*Becton-Dickinson, CA, USA*). The studied antibodies included CD3-PE5, and CCR4-FITC. Negative isotype matched controls (FITC and PE5 labeled) were used to determine the nonspecific binding of monoclonal antibodies. CCR4 assay was performed using Coulter EPICS XL flow cytometer (*Coulter Electronics, USA*). CCR4 monoclonal antibodies (*monoclonal anti-human CCR4-Fluorescein FITC, Catalog Number: FAB1567F*), was purchased from *R&D Systems, Inc. McKinley Place N.E., Minneapolis Minnesota, MN, USA*. Assays were performed to quantitatively determine the percentage of T cells bearing CCR4 and qualitatively determine the density of CCR4 on cell surfaces by flow cytometry. Washed cells were incubated with the fluorescein-labeled monoclonal antibody that binds to the cells expressing human CCR4. Unbound fluorescein-conjugated antibody was then washed from the cells. Cells expressing CCR4 were fluorescently stained, with the intensity

of staining directly proportional to the density of CCR4. Cell surface expression of CCR4 was determined by flow cytometric analysis using 488nm wavelength laser excitation. T lymphocytes were electronically gated using CD3-PC5. Results were expressed as percentage of cells co-expressing the predetermined markers in comparison to isotype matched controls.

Serum total IgE

Serum total IgE was assayed in all subjects by quantitative enzyme immunoassay (*Medix Biotech, San Carlos, CA, USA*). Results were expressed in IU/ml. Owing to the variability in serum total IgE levels with age in childhood, we calculated the percentage values from the reference ranges by dividing the subject's actual level by the highest normal for age multiplied by 100.¹⁴ IgE levels used in the correlations were both the measured and the calculated percentage values. The serum total IgE level that exceeded the highest normal for age was considered elevated.

Complete blood count

The blood count was performed in every subject with the Coulter counter (*Coulter MicroDiff 18, Fullerton CA, USA*). The differential leukocytic counts were estimated manually from the blood film and expressed in absolute count values. Infants and children whose absolute eosinophil counts (AEC) exceeded the normal reference values for age¹⁴ were considered to have peripheral blood eosinophilia. Blood sampling of all subjects was performed at the same time (10 am) daily to avoid diurnal variations in eosinophil counts.

Statistical Analysis

All statistical analyses were carried out using SPSS (Statistical Package for the Social Science) version 9.02 for Windows system. Data were expressed as mean, standard deviation (SD), median, and interquartile range (IQR) (ranges between 25th-75th percentiles). Student's "t" test was used for comparing parametric data between two groups. For non-parametric comparison of plasma MDC and PBTL cells % bearing CCR4 among different groups, we used Mann-Whitney U test. The relation between the various numerical parameters was studied by the Pearson correlation coefficient (r) test with graphic representation using linear regression line, r value was considered weak if <0.5, moderate if ranged between 0.5-0.75 and strong if >0.75. p values below 0.05 were considered significant.

RESULTS

Plasma MDC levels and CCR4⁺PBTL % in the studied groups (table1):

Plasma MDC levels during acute attacks of bronchial asthma ranged between 420 and 1850 pg/ml (median = 1050, mean \pm SD = 925 \pm 471.5 pg/ml). After subsidence of acute attacks, there was a significant decrease in MDC levels which ranged from 200 to 890 pg/ml and the median and mean \pm SD values were 506 and 502 \pm 284.3 pg/ml respectively (Z = 2.8; p<0.0001). The healthy children had much lower plasma levels (range = 70-182, median = 101, mean \pm SD = 99.6 \pm 25.6 pg/ml) as compared to the patients' data whether during asthma exacerbation (Z = 4.3; p<0.0001) or quiescence (Z = 3.6; p<0.0001).

CCR4⁺PBLT % values of asthmatic patients during acute exacerbations ranged between 50 and 90% (median = 65, mean \pm SD = 55.3 \pm 23.6%). After subsidence of acute attacks, there was a significant decrease in CCR4% ranging from 22 to 60% and the median and mean \pm SD levels were 35 and 32.5 \pm 10.5% respectively (Z = 2.1; p<0.0001). Healthy controls had significantly lower % (range = 20-30, median = 25, mean \pm SD = 24.2 \pm 4.1%) as compared to the patients' data both during exacerbations (Z = 2.4; p<0.0001), and quiescence (Z = 1.6; p<0.05).

Plasma MDC levels of children with acute lower respiratory tract infections ranged from 80 up to 209 pg/ml (median = 115, mean \pm SD = 109 \pm 27.3 pg/ml). Their CCR4⁺PBTL% ranged between 20-35% (median = 27, mean \pm SD = 27.6 \pm 7.5%). These values were significantly lower as compared to the values of children with atopic asthma whether during exacerbations (Z = 3.8; p<0.0001 for MDC; and Z = 1.9; p<0.05 for CCR4⁺PBTL%), or during quiescence (Z = 3.2; for MDC; and 1.1 for CCR4⁺PBTL%, p<0.05 for both). On the other hand, they were statistically comparable to the values of controls (p>0.05 for both markers).

Plasma MDC levels and CCR4⁺PBTL% in relation to severity of acute asthma exacerbations, and asthma grading during stability (figure 1):

Plasma MDC levels and CCR4⁺PBTL% values were significantly higher in children enrolled with acute severe asthma as compared to those with mild attacks or those with moderate exacerbations. Also, children with moderate attacks had significantly higher levels than those with mild attacks (fig.1).

Concerning asthma grading, during stability, patients with persistent asthma (mild, moderate and severe persistent) had significantly higher plasma

MDC levels (median = 400; mean \pm SD = 325 \pm 252 pg/ml) and higher CCR4⁺PBTL% (median = 30; mean \pm SD = 30 \pm 27 %) as compared to intermittent asthma (median = 235; mean \pm SD = 223 \pm 265 pg/ml for MDC; median = 25; mean \pm SD = 22 \pm 21% for CCR4%). Again, higher levels were observed when data of patients with persistent asthma were compared to controls ($p < 0.05$ for both markers).

CCR4/MDC axis in relation to asthma clinical, laboratory and radiological variables and response to inhaled steroid therapy (table 2; figure 2, 3 & 4):

There were significant positive correlations between plasma MDC levels and CCR4⁺PBTL% both during exacerbations (fig.2A), and during quiescence (fig.2B). Plasma MDC levels during acute exacerbations were positively correlated with the corresponding values after remission meaning that the higher the levels got during attacks the higher they remained after remission (fig.3A). A similar correlation was found in terms of CCR4⁺PBTL% (fig.3B). Significant negative correlations were reported between PEFr during acute asthmatic attacks and plasma MDC levels ($r = -0.51$; $p < 0.05$) and CCR4⁺PBTL% ($r = -0.55$; $p < 0.05$). In quiescence, such correlations were absent ($p > 0.05$ for both markers). There were

significant positive correlations between the absolute eosinophil count during acute asthmatic attacks and plasma MDC levels and CCR4⁺PBTL% (fig.4). In quiescence, such correlations were absent ($p > 0.05$ for both markers). The plasma MDC levels and CCR4⁺PBTL% could not be related by correlation coefficient to age, weight centiles, height centiles, IgE concentrations or IgE percent values from normal during or after subsidence of asthma exacerbation. Both, markers were not influenced by age, gender, or family history.

The plasma MDC levels in-between asthmatic attacks were significantly higher in children who demonstrated X-ray findings of hyperinflation and increased bronchovascular markings than in patients with normal chest X-ray (median = 610; mean \pm SD = 559 \pm 410 pg/ml versus 450 and 410 \pm 313 pg/ml; $p < 0.05$). However, the same did not apply when studied during acute exacerbations (median = 1000 versus 950 pg/ml, $p > 0.05$). Concerning CCR4⁺PBTL%, the results were not influenced by the presence of radiological findings whether in activity or remission.

Plasma MDC levels and CCR4⁺PBTL% expression did vary according to the corticosteroid inhalation therapy, being significantly higher in steroid-treated patients both during exacerbation or quiescence (table 2).

Table 1. Plasma macrophage-derived chemokine (MDC/CCL22) and the percentage of peripheral blood T lymphocytes expressing the CC chemokine receptor-4 (CCR4) in the studied groups

| | Bronchial Asthma (n=32) | | ALRTI (n=12) | Controls (n=20) |
|---------------------------------|----------------------------|-----------------|-----------------|--------------------|
| | Acute attacks | In-between | | |
| Plasma MDC/CCL22 (pg/ml) | | | | |
| Range | 420-1850 | 200-890 | 80-209 | 70-182 |
| Median | 1050 | 506 | 115 | 101 |
| Mean \pm SD | 925 \pm 471.5 | 502 \pm 284.3 | 109 \pm 27.3 | 99.6 \pm 25.6 |
| Z1 | | 2.8 ** | | |
| Z2 | 3.8 ** | 3.2 * | | |
| Z3 | 4.3 ** | 3.6 ** | 0.9 # | |
| CCR4⁺PBTL (%) | | | | |
| Range | 50-90 | 22-60 | 20-35 | 20-30 |
| Median | 65 | 35 | 27 | 25 |
| Mean \pm SD | 55.3 \pm 23.6 | 32.5 \pm 10.5 | 27.6 \pm 7.5 | 24.2 \pm 4.1 |
| Z1 | | 2.1 ** | | |
| Z2 | 1.9 * | 1.1 * | | |
| Z3 | 2.4 ** | 1.6 * | 0.5 # | |

ALRTI: acute lower respiratory tract infections; CCR4: CC receptor-4; MDC: macrophage-derived chemokine; PBTL: peripheral blood T lymphocytes
Mann-Whitney test was employed, Z1: acute asthmatic attacks versus stability; Z2: asthma versus ALRTI; Z3: patients (asthma or ALRTI) versus controls
*: Significant ($p < 0.05$), **: Highly significant ($p < 0.0001$), #: Non-significant ($p > 0.05$).

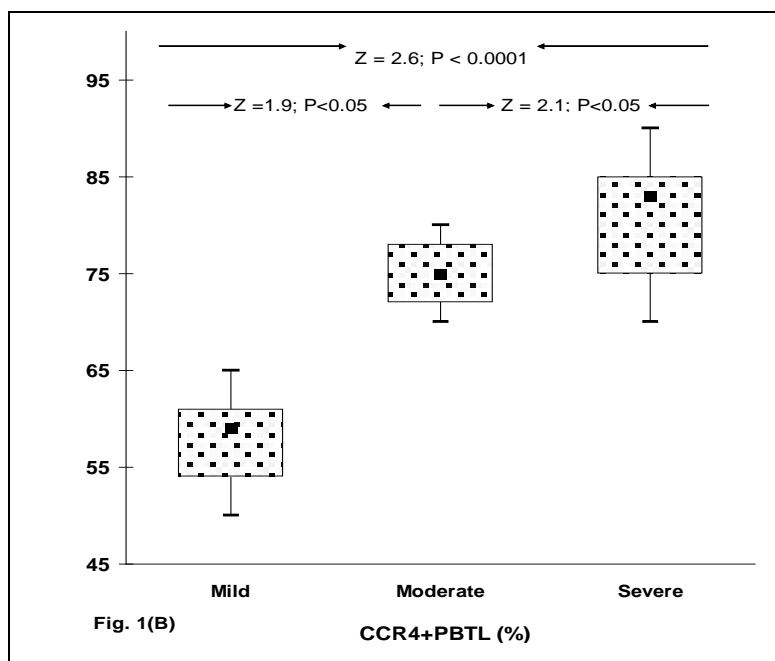
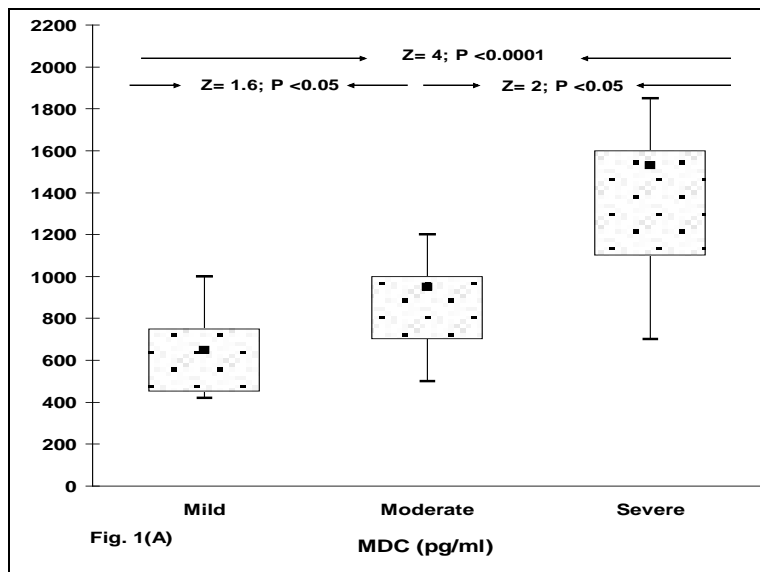


Figure 1. Box-plot summary of plasma macrophage-derived chemokine (MDC) levels (fig.1A) and the % of peripheral blood T lymphocytes expressing CCR4 (fig.1B) in relation to the degree of severity of acute asthmatic attacks. Mann–Whitney test was used in the comparisons. Black squares represent the median and the boxes encompass the interquartiles (25th and 75th percentiles). The ranges are marked as maximum and minimum.

Table 2. Variations of plasma macrophage-derived chemokine (MDC/CCL22) levels and the percentage of peripheral blood T lymphocytes expressing the CC chemokine receptor-4 (CCR4) according to corticosteroid inhalation therapy

| | +ve Steroid therapy (n = 14) | -ve Steroid therapy (n = 18) | Z value | p |
|---|---------------------------------|---------------------------------|---------|------------|
| Plasma MDC (pg/ml) (median; mean ± SD) | | | | |
| During exacerbation | 1320; 1200 ± 618 | 700; 650 ± 236 | 2.6 | p<0.0001** |
| After remission | 605; 555 ± 297 | 410; 449 ± 258 | 1.3 | p < 0.05* |
| CCR4⁺PBTL (%) (median; mean ± SD) | | | | |
| During exacerbation | 74; 63 ± 29.5 | 56; 50 ± 30.2 | 1.2 | p < 0.05* |
| After remission | 47; 32 ± 22.9 | 35; 33 ± 23.1 | 0.97 | p < 0.05* |

CCR4: CC receptor-4; MDC: macrophage-derived chemokine; PBTL: peripheral blood T lymphocytes
Mann-Whitney test was employed

*: Significant (P<0.05), **: Highly significant (P<0.0001)

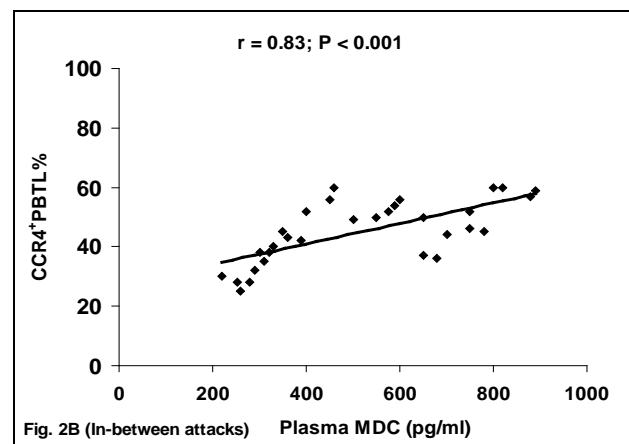
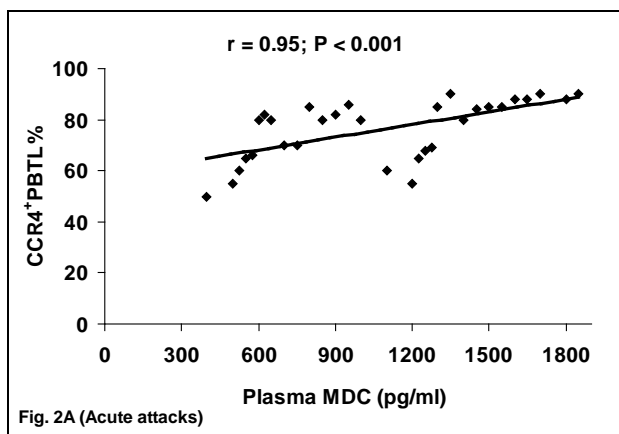


Figure 2. Positive correlations between the percentage of peripheral blood T lymphocytes expressing the chemokine CC receptor-4 (CCR4) and plasma levels of macrophage derived chemokine (MDC) among asthmatic children during acute attacks (Fig.2A) and after resolution (Fig.2B).

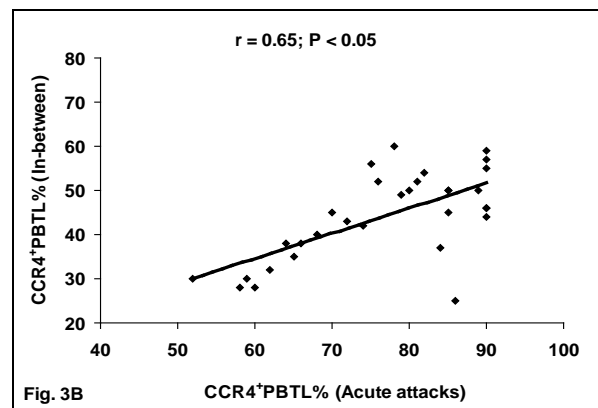
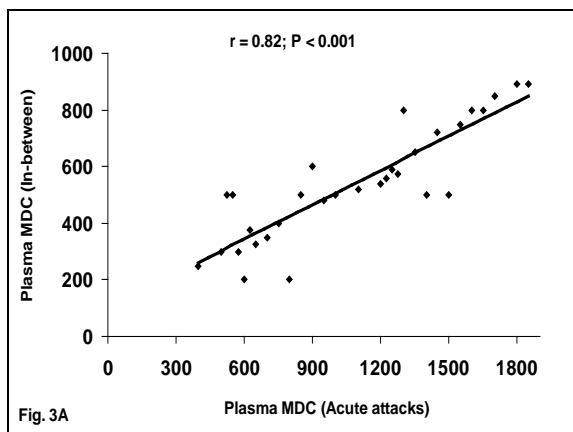


Figure 3. Positive correlations between the plasma levels of macrophage derived chemokine (MDC) (Fig.3A) and the percentage of CCR4 bearing T cells (Fig.3B) during and in-between asthma attacks.

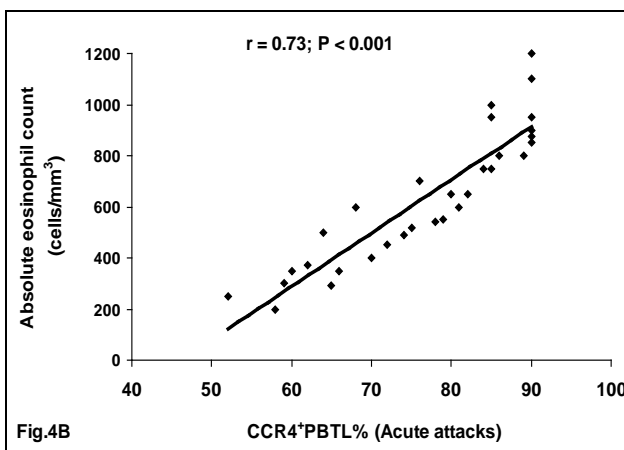
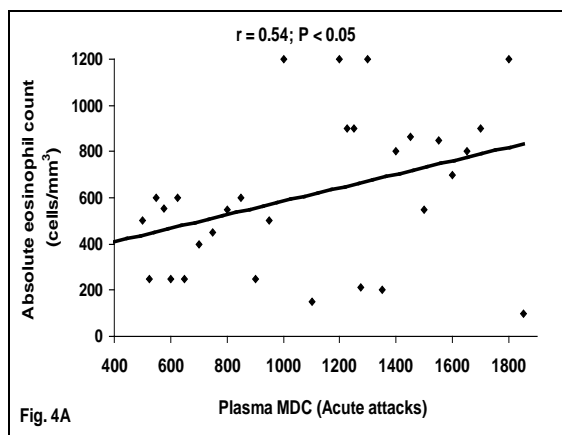


Figure 4. Positive correlations between the absolute eosinophil count (cells/mm³) and plasma levels of macrophage derived chemokine (MDC) (Fig.4A), and the percentage of CCR4 bearing T cells (Fig.4B) among asthmatic children during exacerbations.

DISCUSSION

The critical influence of chemokine biology on the outcome of asthma continues to be highlighted in recent reports describing novel mechanisms by which inflammatory cells are recruited into the lungs and the local TH2-cell dominance is maintained. Of considerable interest, is the increasing emphasis currently being realized for receptor mechanisms. In vitro, polarized human TH2 cells preferentially express the chemokine receptor CCR4, and migrate to its ligand MDC.¹⁶ Therefore; we were stimulated to elucidate the role of CCR4/MDC axis in a group of asthmatic children.

During acute asthma attacks, plasma MDC levels and CCR4⁺PBTL% were significantly higher in comparison to the corresponding values in the stable state and in comparison to controls. These data conform with several other reports.¹⁷⁻²¹ The results during exacerbations may reflect the upregulation of CCR4 and its ligand, MDC, in allergic inflammation, presenting potentially useful markers for the presence of atopic response. This was also observed in the sera and the lesional skin (keratinocytes) of atopic dermatitis and in animal models of allergy.^{4, 7, 21-23} When we followed-up our asthmatics to stability, we observed sustained elevation of both markers in comparison to control values. This might reflect the ongoing inflammatory process with persistent infiltration of airways by inflammatory cells. These cells release mediators that in turn recruit more and more inflammatory cells in a vicious circle manner. Such observations could be indicative of the continued exposure to triggering factors and/or the need for more intensive anti-inflammatory treatment. We, therefore, suggest

that MDC levels and CCR4% be used as markers of the ongoing airway inflammatory activity in terms of duration. Our results highlight the in vivo significance of distinct CCR4 expression on TH2 cells and CCR4/MDC axis in the setting of chronic inflammation and dendritic cells and lymphocytes homing in asthma.

Our data, however, contradict the report of Sekiya et al²⁴. Also, the plasma MDC levels in acute and stable asthma in the study of Leung et al¹⁷. were somewhat lower than our levels. This may probably reflect the presence of cases of acute severe asthma among our studied sample. We found very few published data on normal or pathological MDC levels in plasma or other biological fluids of infants and children. Moreover, MDC studies previously identified in the available databases were conducted in the bronchoalveolar lavage fluid, and exhaled breath condensate among adult asthmatics which made the comparison of the results somewhat difficult, especially that some authors²⁵ reported that MDC studies in vitro and in biological fluids cannot simply be extrapolated to normal human airway cells.

We sought to measure MDC in plasma because it is easier, rapid and less invasive and would still partially reflect the extent of local production by the large bronchial epithelial cells that leads to systemic expression via pooling in the plasma. This is expected to operate in the absence of other allergic diseases that might alter MDC levels.

The cellular source of MDC production has been a subject of debate. A number of sources had been identified, including macrophages, monocyte-derived dendritic cells (almost exclusively in the thymus), natural killer (NK) cells, and bronchial

epithelial cells. MDC mRNA was highly identified and expressed particularly upon stimulation with microbial products, or anti-CD40 antibody. High expression was detected in normal thymus (medullary epithelial cells) compared to lesser expression in the spleen.^{26, 27}

Human airway epithelial cells were found to release MDC upon cytokine stimulation.²¹ Immunofluorescent analysis of endobronchial biopsies from asthmatics, taken 24 hours after allergen challenge, demonstrates that virtually all T cells express CCR4.²⁰ Expression of the CCR4-ligand, MDC, is strongly upregulated on airway epithelial cells upon allergen challenge, suggesting a major involvement of this receptor/ligand axis in TH2-dominated allergen-induced asthma by the regulation of lymphocyte recruitment into the asthmatic bronchi. Recently, it was found that MDC induces concentration-dependent platelet aggregation (dependent on cyclooxygenase metabolites of arachidonic acid). Flow cytometric analysis revealed the expression of CCR4 on platelets and a monoclonal antibody to CCR4 inhibited MDC-induced platelet aggregation, confirming that this effect is mediated through its receptor CCR4. MDC-production is also upregulated by prostaglandin and cyclic AMP-elevating agents.²⁸ Therefore, CCR4/MDC axis may contribute to platelets involvement in TH2-type inflammation, which certainly deserves intense studies.

MDC chemotactic activity is dose-dependent. At low concentrations (1 ng/mL), MDC is chemoattractant for monocyte-derived dendritic cells, IL-2-activated NK cells and activated T lymphocytes. Monocytes and a subpopulation of thymocytes migrate in response to much higher concentrations of MDC (peak response at 100 ng/mL). Thus, in vivo production of MDC may first attract dendritic cells or NK cells, and further accumulation of MDC may cause a subsequent influx of monocytes.²⁹

The positive correlation between plasma MDC levels and CCR4% during exacerbations probably reflect excessive MDC production from bronchial epithelial cells during bursts of bronchial asthma. The current finding of positive correlations between plasma MDC levels and CCR4% during exacerbation and their levels after remission means that the higher they get during attacks the higher they remain after remission. This may reflect the non-stop allergic inflammation inside the airways of children mediated by inflammatory cells particularly lymphocytes even after subsidence of exacerbations. Our conclusion can be ascertained

also by the presence of a positive correlation between the plasma MDC levels and CCR4% during stability.

The concurrent acute lower respiratory tract infections in asthma are sometimes impossible to distinguish from spontaneous acute exacerbations. The question of whether or not the presence of airway infection in asthma would increase the expression of both markers was one of our concerns. Also, cough as a frequent respiratory symptom in children, is sometimes difficult to be diagnosed as being cough variant asthma. Therefore, we investigated whether analysis of these markers could facilitate the diagnostic discrimination of atopic asthma from infection-associated airway reactivity and other causes of spasmodic cough in children. We therefore, included 12 patients with acute lower respiratory tract infections in whom the diagnosis of atopic asthma was ruled out by medical history, chest auscultation and radiographic findings. Among these patients, the levels of both markers were statistically comparable to healthy controls and lower than levels of asthmatic children whether during exacerbation or stability. Our findings were concordant with some other relevant studies.^{30, 31} In view of these findings, it seems that CCR4/MDC axis activation is an inherent feature of atopy and that pathogen-induced inflammation in respiratory infections is independent on such axis. Both markers are thus expected to differentiate asthmatic children from those with spasmodic cough due to non-allergic causes. Our results are limited by the small number of children with infections, thus we recommend wider scale studies to verify the results.

MDC has been suggested as a useful objective marker for disease severity in asthma. We have demonstrated that MDC plasma levels and CCR4⁺PBTL% correlated with severity of acute asthma exacerbations (both parameters were significantly higher in children with acute severe asthma than in subjects with acute attacks of mild or moderate severity). Also, higher levels were reported in moderate attacks as compared to mild attacks. Similarly, CCR4% and MDC levels were reported to be inversely correlated with PEF. Similar results were reported by Leung et al¹⁷. and Lezcano-Meza et al²⁰. Our results may probably reflect excessive influx of inflammatory cells in acute severe attacks producing injury to bronchial mucosa mediating bronchospasm, bronchial obstruction and irritability. It seems that CCR4/MDC axis over expression is in favor of an asthma diagnosis and impaired lung function and is related to asthma severity.

Concerning asthma grading in-between attacks, CCR4% and MDC levels were significantly higher in persistent than intermittent asthma and control values. Our data conform with the results of van den Toorn et al³². This could be due to the ongoing process of sub-clinical allergic inflammation in the lungs of persistent asthmatics during clinical remission meaning that airway inflammation is still present in patients with seemingly stable conditions. Our results signify the role of CCR4/MDC axis in the persistence and long-term recruitment of TH2 cells, into the inflammatory sites, setting chronic inflammation, and lymphocyte homing. Thus, CCR4% and MDC levels might be useful indices in determining the magnitude of persistent airway inflammation.

Significant positive correlations could be elicited between CCR4% and MDC concentrations and the absolute eosinophil counts during acute attacks. It was previously mentioned that MDC may have a role in regulating the constitutive numbers of eosinophils in peripheral blood by inducing eosinophil chemotaxis in a CCR3 and CCR4-independent manner.^{6, 33} This conforms with our data and may signify the role of CCR4/MDC axis in the late allergic response. Moreover, such correlations highlight two important questions that deserve further studies; first: the possibility of eosinophil chemotaxis in a CCR4-dependant manner, second: whether the effect of MDC on eosinophils could be mediated at the level of bone marrow where eosinophil growth and development has been found predominantly dependent on specific chemokines e.g. eotaxin. This may pave way for targeting eosinophils in allergic diseases by manipulating CCR4/MDC axis.

In the current study, MDC levels and CCR4% could not be correlated to the serum total IgE concentrations or % values from normal either during or after subsidence of exacerbations. However, the number of cases in the normal IgE category was too small to come out with a conclusion. It may also reflect varying mechanisms regulating their actions.

Plasma MDC levels during stability were significantly higher in children who demonstrated X-ray findings of hyperinflation and increased bronchovascular markings than in patients with normal X-rays. This may enforce the relation between MDC expression and asthma chronicity and severity as reflected by the radiological findings. The same, however, did not apply during asthma exacerbations. Also, the absence of such correlation in terms of CCR4% values would limit the significance of the aforementioned conclusion.

Otherwise, such observation would have been better outlined with a larger sample size.

The effect of inhaled corticosteroid therapy on CCR4/MDC axis was highly impressive. We had observed significant higher levels of both markers in steroid-treated patients during and in-between attacks. This was contradictory to the reports of Leung et al¹⁷, who found that MDC concentrations in exhaled breath condensate were lower in patients with persistent asthma who received high-dose inhaled corticosteroids. The studied material is different in our series and this may account for the difference. Our finding might also be explained by the fact that severe cases received higher steroid doses and that their overexpression of the studied markers actually reflected the effect of severity rather than therapy. It is possible that in some asthmatic subjects, steroids do not inhibit the robust expression of MDC or its receptor CCR4. Although it was previously noted that glucocorticoids could inhibit the *in vitro* expression of many chemokines including MDC, the results of studies that used bronchial cell lines and mouse models may not apply to *in vivo* expression of this chemokine. This may lead to the speculation that therapeutic strategies aimed at blocking the effects of MDC by inhibiting its receptor (CCR4) might benefit some symptomatic, corticosteroid-dependent, asthmatic subjects.

In conclusion, plasma MDC levels and CCR4 expression on peripheral blood T lymphocytes were up regulated. They are expected to have biological significance in the regulation of lymphocyte recruitment into the asthmatic bronchi leading to development of the pathophysiological endpoint of asthma, the airway hyperreactivity. CCR4/MDC axis changes were not only related to asthma activity but to asthma severity as well. Also, the axis seems to be involved in the establishment and maintenance of chronic inflammation of the airways. CCR4 and its ligand MDC might be useful markers for monitoring allergic inflammation in asthma, and might be useful in differentiating atopic asthma from non allergic respiratory symptoms and infections-associated airway reactivity. Setting pediatric reference ranges for MDC in biological fluids, and exploring CCR4 expression on different immune cells involved in atopy are worthwhile. Moreover, the effect of MDC and CCR4 antagonists and other immunotherapeutics, like monoclonal antibodies on the MDC kinetics and CCR4 expression deserves intense evaluation. CCR4/MDC antagonism may prove value one day as a potential adjuvant therapeutic strategy in bronchial asthma.

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