Immune Signature of CCR7^{+} Central Memory T Cells Associates with Disease Severity and Immunoglobulin E in Bronchial Asthma

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Abstract

Objectives: CD4+T cell subtypes are the central orchestrators of airway inflammation in bronchial asthma (BA); however, the mechanisms that regulate their accumulation in asthmatic airways are still a challenging subject. In addition, neutrophils play a significant role in the development of airway remodeling and their presence may influence clinical presentation of BA being linked to the development of severe BA. Neutrophils have also been found to acquire antigen presenting functions, enabling them to directly activate T cells. The study aimed to evaluate the possible association of chemokine receptor 7 (CCR7)+ memory CD4+ T cells and CCR4+ effector T cells with disease severity and immunoglobulin E (IgE) production as well as to explore the relationship between these cells and neutrophil function in both allergic and non-allergic asthmatic patients.

Methods: Flow cytometry was used to determine the expression of different T cell subset phenotypes (CCR7 memory CD4+ and CCR4+ T cells using anti-human CD3, CD4, CD45RO, CCR4 and CCR7 monoclonal antibodies) utilizing peripheral blood mononuclear cells (PBMCs) isolated from 78 allergic asthmatic patients, 41 non-allergic asthmatic patients, and 40 healthy individuals. Moreover, neutrophils' phagocytic activity was assessed by ingestion of candida particles.

Results: We demonstrated increased percentages of CCR7+ memory CD4+ T cells and CCR4+ CD4+ T cells in patients compared to control, where this upregulation was significantly higher in allergic than non-allergic asthmatic patients. Additionally, these cells were negatively correlated with improved pulmonary tests and significantly associated with disease severity scores and IgE levels. The neutrophil phagocytic activity was markedly increased in patients compared to control, showing a significant positive correlation with disease severity.

Conclusion: These findings suggest that increased CCR4+ CD4+ T cells and CCR7+ memory CD4+ T cells (Tcm) may be associated with BA severity, especially in allergic BA patients and can potentially contribute to the rational design of new therapeutic approaches for asthma in the future.

Keywords: asthma, allergic, CCR4, CCR7, phagocytic activity, IgE.

1. Introduction

Bronchial asthma (BA) is considered as a globally major public health issue that has a negative impact on quality of life, and is associated with high levels of co-morbid diseases [1]. It is estimated that numbers of BA patients worldwide may be as high as 334 million with a suggested steady increase [2]. The prevalence among adults was estimated to be 6.7% of the general population in Egypt [3] and about 8.2% in children aged 3–15 years [4].

BA is a heterogeneous disease with different phenotypes, being one of the main obstacles to successful management [5]. The clinical phenotype of allergic BA is the most recognizable one, since it is associated with history of allergic diseases and reversible lung obstruction. It is characterized by eosinophilic airway inflammation, which is associated with immunoglobulin E (IgE) antibodies to various allergens, as evidenced by serology or skin prick test [6].

It has been characterized that the pathogenesis of asthma is classically defined as a T helper (Th2) -type inflammatory response. These elevated Th2-type lymphocytes have been characterized in the blood of BA patients, indicating that these immune cells responsible for chronic inflammation in the lung circulate in the blood [7]. The accumulation of Th2 cells in lungs is essential for both the initiation and persistence of airway inflammation being attributed to a number of candidates, including the chemokine receptor CCR4 because of its preferential expression on this type of cells [8]. Mucosal CD14+ mononuclear phagocytes are major producers of four chemokines (Chemokine C-C motif ligand 13 (CCL13), CCL17, CCL18, and CCL24), which are recognized as ligands for chemokine receptors that are typically expressed on differentiated Th2 cells including CCR4 that is involved in Th2 responses [9]. Though, roles of CCR4+CD4+T cells in the pathogenesis of asthma are still controversial in both humans and murine model of asthma.

However, it has been suggested that the pathogenesis of asthma must not be solely driven by Th2-type immune responses, owing to the high level of clinical heterogeneity of asthma [10]. Memory T cells have been previously reported to be associated with chronic inflammatory conditions and autoimmune diseases [11, 12]. They can be categorized into central memory T cells (TCM) that circulate among secondary lymphoid organs, and effector memory T cells (TEM) that search for their cognate antigen in the non-lymphoid organs. These two subsets also show differential chemokine receptor expression, where TCMs express high levels of CCR7, can migrate from peripheral tissues to the lymph nodes via the afferent lymph, and can quickly proliferate in response to infiltrating antigen-presenting cells (APCs) [13]. In response to this, memory CD4+ T cells can

acquire an effector-like phenotype with the secretion of cytokines and chemokines being considered as reactive memory cells [14]. The exit of cytokine-producing CCR7+ cells from peripheral tissues and entry into the draining lymph node might amplify and polarize the developing lymph node immune response and may contribute to the maintenance and distribution of the T cell memory pool [15], linking the lymphoid and peripheral T cell compartments with an important implication for the generation and maintenance of immune responses.

In addition, it has been demonstrated that neutrophils may play an important role in the development of airway remodeling and fibrosis in severe asthmatic airways being an important source of transforming growth factor beta (TGF-β1) and inducer of Epithelial-Mesenchymal transition [16]. In addition, freshly isolated human neutrophils can function as APCs to memory CD4+ T cells [17] with an evidence of the antigen-presenting capacity of human neutrophils for local allergen specific effector T cells in patients with allergic late phase reactions [18, 19].

Alternatively, reports have classified granulocytes as the main effector cells in inflammation, which migrate to inflammatory sites along the chemotactic gradient of inflammatory mediators. The migration of neutrophils to lymphoid organs has been linked to upregulation of the chemokine receptor CCR7 [20]. In addition, it has been shown that the severity of asthma affects the functioning of peripheral blood cells where in severe forms, the numbers of neutrophils and eosinophils are significantly increased in the blood [21] with altered expression profile of proinflammatory cytokines [22].

Although memory T cells have been intensively characterized in response to infections and autoimmunity, the importance of these cells in allergic diseases remains to be elucidated. Herein, we investigated the interactions of CCR7 expressing memory CD4+ cells and CCR4+ T cells in mediating severity and clinical outcomes of BA as well as the production of IgE, which is considered as a characteristic feature of allergic bronchial asthma and is thought to be critical for pathology. In addition, we explored the relation between these cells and neutrophil function in asthmatic patients.

2. Subjects and methods

Study population

The current study was conducted on 119 patients with bronchial asthma who were recruited from Chest Department, Main Alexandria University Hospital, Egypt. The diagnosis was based on the criteria of the Global

Initiative on Asthma (GINA; http://www.ginaasthma.org) [23]. Forty age and sex matched healthy controls with no history of asthma or any allergic disease, currently non-smokers and not receiving any drug at their inclusion in this study were included too.

Patients were further categorized into 78 allergic asthmatic patients and 41 non-allergic asthmatics. The allergic status of patients was determined by patient history, clinical examination, a positive specific IgE (ImmunoCAP test: ≥ 0.7 kUA/L; ThermoFisher) correlated with the clinical history or the allergen challenge, and a positive skin prick allergen test (wheal—a raised white bump surrounded by a small circle of itchy red skin to allergens ≥3 mm diameter above background) [24]. A positive family history of asthma and/or other allergic diseases, particularly allergic rhinitis, was also recorded in 100% of allergic BA patients.

All subjects had no change of asthma medications 4 weeks prior to recruitment to the study. All subjects were non-smokers and free from upper respiratory tract infection for at least 4 weeks preceding the study. Pulmonary flow rates were measured using DATOSPIR 120 spirometer with automatic dosimeter (FG0304-Datospir 120; Spain) (The DATOSPIR-120 spirometer). Interpretation of common test values: the forced vital capacity (FVC), forced expiratory volume in one second (FEV1), FEV-1/FVC was done [25]. Bronchial hyperresponsiveness was assessed with methacholine challenge test. The dose of methacoline that results in 20% reduction of FEV-1 was determined [26]. At entry to the study, patients were taking inhaled glucocorticoids at dosages up to 400 µg/day. All patients were taking inhaled β2-agonists "as required". Patients receiving increased inhaled glucocorticoid therapy were followed longitudinally for the purpose of the present study.

Disease severity was measured by different ways: All patients were assessed for their control of asthma by using asthma control test (ACT) scoring (total-score ranging from 5 to 25) and GINA guideline, Medical Research Council (MRC) dyspnea scale [27]; symptoms of asthma including: cough and wheezing which were scored from 0 to 3 according to GINA [28, 29]; and clinical severity score GINA (1995). C-reactive protein (CRP) was determined using (BN ProSpecNephlometry) (Siemenes, USA) [30] and erythrocyte sedimentation rate (ESR) was determined using (Westergren tube) [31]. The collection of blood samples and the related assays were approved by Ethical guidelines of Medical Research Institute, Alexandria University.

Total IgE assay

Total serum IgE was measured in duplicates using an enzyme linked immunosorbent assay (ELISA) kit (RIDASCREEN; R-Biopharm, Darmstadt, Germany) (RIDASCREEN® Total IgE A0141 (R-Biopharm AG)).Venous peripheral blood samples were collected from all subjects. Serum was used for total ELISA IgE assay (RIDASCREEN; Total IgE, R- Biopharm, Darmstadt, Germany) according to the manufacturer's instructions (RIDASCREEN® Total IgE A0141 (R-Biopharm AG)). Using the mean absorbance value for each sample, the corresponding concentration of IgE in IU/ml was determined from the standard curve and patients were divided into three categories (<20 IU/mL; 20-100 IU/mL; >100 IU/ml).

Isolation of peripheral blood mononuclear cells and lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from sodium heparin-treated blood obtained from healthy donors or BA patients by Ficoll-Hypaque 1077 (Sigma-Aldrich) gradient centrifugation [32]. Erythrocytes were lysed using an ammonium chloride solution. The suspension was centrifuged at 524×g for 10 min at RT. The pellets were washed with PBS and then resuspended in complete RPMI 1640 medium (Invitrogen, Grand Island, NY, USA, cat. 11875093) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Grand Island, NY, USA), 100 U/mL penicillin (cat. 15071163), 100 mg/mL streptomycin (15071163), 2 mM L-glutamine (cat. 25030031), and 50 mM 2-mercaptoethanol (cat. 21985023; Invitrogen, Grand Island, NY, USA).

Freshly isolated peripheral blood mononuclear cells (PBMCs) were labeled with the selected combination of cell surface antibodies including: anti-CD4-Percep, anti-CCR4 (CD194)-PE and anti-CD45RO-FITC, anti-CCR7 (CD197)-PE respectively.

Phenotypic characterization

The pooled PBMCs from the healthy donors and the BA patients were stained for flow cytometry. The following panel of mouse anti-human mAbs, all purchased from BD Biosciences (San Jose, CA, USA) or eBioscience (San Diego, CA, USA), was used: anti-human CD3-APC.cy7 (BD, 557832, SK7), anti-human CD4-Percp.cy5.5 (BD, 560650, RPA-T4), anti-human CD45RO-FITC (eBioscience, 11-0458-42, HI100), anti-human CCR4-PE.cy7 (BD, 557864) and anti-human CCR7-PE.cy7 (BD, 557648, 150503). The cell data were acquired using a 10-laser Gallios (Beckman Coulter Inc., Brea, CA, USA) analytical flow cytometer. Unstained and single fluorochrome-stained cells were used as controls to provide accurate compensation and data analysis.

The results were analyzed with BD FACS Calibur flow cytometer using Cell Quest software (Becton-Dickinson).

Assessment of phagocytic activity

This test relies on the uptake of heat killed candida albicans (yeast) by neutrophils over a brief period of time where stained intracellular candida can be identified and counted [33]. Heat-killed candida suspended in phosphate-buffered saline was adjusted to 2×10^7cells/mL where 250 µL of pooled serum and 250 µL of heat-killed candida were added to 250 µL of buffy coat obtained by using polymorphprep and incubated at 37°c for 1 hr with occasional mixing. Number of candida-engulfed neutrophils was counted as positive cells and phagocytic activity was calculated as follows:

Number of positive phagocytic cells/ total number of cells x 100

Statistical Analysis

Data were expressed as mean ± standard deviations (SD) (standard deviation of mean) and were compared with the tabulated probability value (P value) that was considered significant if it was 0.05 or less using SPSS statistical package (SPSS Inc., Chicago, IL). Student t-test was used for normally distributed data while Mann-Whitney U test was applied for non-normally distributed data. A Pearson chi-square test was applied for categorical variables. Multiple comparisons were performed using one-way ANOVA and Kruskal–Wallis tests. The correlation between two quantitative variables was evaluated using Pearson correlation coefficient (r).

3. Results:

Subjects' demographic and laboratory data

There was no statistical significant difference as regards age between allergic (mean ± SD = 48.2 ± 10.3), non-allergic patients (52.2 ±8) and control subjects (48 ±9.4; P=0.063), as well as sex distribution between groups (P = 0.731). Females represented 64.1% of allergic asthmatic patients, 58.5% and 57.5% of non-allergic and control subjects, respectively. Disease duration (years) showed a significant difference between the two patients' groups (P <0.001).

CBC data showed that total lymphocyte and eosinophil counts were significantly increased in allergic asthma patients compared to non-allergic BA patients and control. On the other hand, non-allergic asthma

patients had significantly more circulating neutrophils and monocytes compared to allergic BA patients and control (Table I). There was also a notable marked elevation in ESR (mm/hr) [median=38 (14.3 –52)] and CRP (mg/L) [median=4.5 (2.2–10.8)] in non-allergic BA patients' group relative to allergic patients and control groups.

Clinical indicators of respiratory function:

Clinical indicators of pulmonary function were measured on the day of sample acquisition. As shown in Table I, FEV1, FVC, FVC% pred., FEV1%/FVC, forced expiratory flow between the 25% and 75% of the FVC (FEF25-75%) (L/min), and FEF25-75% pred. were significantly lower in allergic than in non-allergic patients' groups (P <0.001), whereas, PD20 of the methacholine challenge test was significantly decreased in non-allergic BA patients (P <0.001).

Patients starting inhaled glucocorticoid therapy were given fluticasone propionate Diskus at a starting mean dosage of 350 μg/day. Those patients with increasing inhaled glucocorticoid therapy were followed up for the purposes of the present study, for a mean of 6.8 months (allergic patients) or 7.2 months (non-allergic patients). During this period, dosages of inhaled glucocorticoid were increased by a mean of 418.7 μg/day (95% confidence interval: 278–533) in the allergic asthmatics and 298 μg/day (95% confidence interval: 139–473) in the non-allergic BA patients. This was associated with significant improvements in FEV1% pred., asthma control test, coughs and wheezing score and alone, with significant reduction in inhaled β2-agonist usage in both groups (Table II). Table III showed the comparison between allergic and non-allergic patients with regard to different disease severity scores.

Phagocytic activity

Comparing the phagocyte activity in the blood of BA patients and controls, there was a marked increase in BA patients compared to control (P <0.001). On the other hand, no significant difference was observed between allergic and non-allergic patients regarding phagocytic activity (Figure 1a). We found a significant difference in phagocytic activity among patients based on asthma control test (P =0.041) where well controlled patients had lower phagocytic activity (92.0 ± 3.2 %) compared to both poorly controlled (93.1 ± 2.9 %) and not well controlled patients (93.5 ± 2.7%) (Figure 1b), while no significant difference in phagocytic activity was observed among patients regarding their IgE levels (P =0.734) (Figure 1c)

Total serum IgE

As regards total IgE level among study population, there was high significant difference between allergic (mean ± SD = 336.8 ± 171.9 IU/ml), non-allergic patients (mean ± SD = 31.6 ± 9.6 IU/ml) and the control group (mean ± SD = 11.3 ± 5.8 IU/ml; P <0.001), as well as between allergic and non-allergic patients (P <0.001). All allergic BA patients had the IgE level higher than 100 IU/ml whereas the IgE level in non-allergic patients ranged between (11 – 49 IU/ml).

CCR4+CD4+ T cells

Allergic asthma patients had a significant higher percentage of CCR4+ CD4+ T cells (mean= 24.2 ± 5.9) than non-allergic BA patients (mean± SD = 17.8 ± 6.4) in comparison with control (mean± SD =12.9 ± 2.5; P <0.001) (Figure 2a). To elucidate the clinical implication of increased CCR4+CD4+ T cells in asthma, our results revealed that percentages of CCR4+CD4+T cells were positively correlated with disease duration, lymphocyte count, phagocytic activity, total IgE level and disease severity scores, whereas a negative correlation was observed with improved pulmonary tests (Table IV, Figures 2b, 2c).

CCR7+ memory CD4+ T cells

We used the receptor CCR7 to define the subsets of CD45RO+ T cells. We gated the CD3+CD4+ CDRO+CDRA-CCR7+ T cells (TCM) in the healthy donors and BA patients. We found that the percentage of TCM cells was increased in patients compared to the control group. Interestingly, CCR7- cells (TEM) were also higher in patients than control. Moreover, our results showed that the percentage of CCR7+ memory CD4+ T cells was markedly increased in allergic (mean ± SD = 23.7 ± 5.4) than in non-allergic BA patients (mean ± SD = 13.8 ± 2.7; P<0.001) (Figures 3, 4a).

Due to the heterogeneity of asthma phenotypes and clinical variation, we next investigated whether the increase of CCR7+ memory CD4+ T cells is a common feature of different asthma subtypes. Allergic asthma patients were divided into 3 subgroups, based on their asthma control test. We found that poorly controlled and not well controlled patients had nearly similar percentages of circulating CCR7+ memory CD4+ T cells (mean ± SD = 24.0 ± 7.8; 21.2 ± 6.2, respectively; P=0.199), but both subgroups of patients had a significant higher percentage of CCR7+ memory CD4+ T cells than well controlled patients (mean± SD =17.8 ± 5.6) (Figure 4b). Regarding the relation between dyspnea scale categories and CCR7+ memory CD4+ T cells, our results revealed that the percentage of CCR7+ memory CD4+ T cells was upregulated with increasing score.

We further investigated whether percentage of the CCR7+ memory CD4+ T cells could impact the % predicted FEV1, where a negative correlation was observed between CCR7+ memory CD4+ T cells and FEV1% pred as well as other pulmonary functions (P <0.001). On the other hand, metacholine challenge test had a positive correlation with the percentage of CCR7+ memory cells CD4+ T cells (P =0.015), Table V.

Moreover, we found that the percentage of CCR7+ memory CD4+ T cells was significantly increased in patients with IgE level >100 IU/ml compared to those with IgE level <100 IU/ml (P <0.001) (Figure 4c). Of interest the percentage of CCR7- cells showed no difference between the studied subgroups (P =0.828) (Figure 5). Above all, we found that the percentage of CCR7+ CD45RO+ CD4+ T memory cells was positively correlated with CCR4+ CD4+ T cell (r=0.555, P <0.001) (Table IV) and negatively correlated with % of CCR7- CD45RO+ CD4+ T cell (r=-0.470, P <0.001) (Table V). However, no correlation was observed with phagocytic activity (r=0.073, P =0.429) (Table V).

4. Discussion:

Despite the improved understanding of the role of airway inflammation in asthma pathogenesis, the sequence of events that lead to persisting airway inflammatory cells and airway hyperresponsiveness in asthma remains to be clarified. A decline in apoptosis in peripheral blood lymphocytes might explain the extensive exacerbations but not the persistent inflammatory reactions seen exclusively in severe BA [34].

It has been shown that sustained allergic inflammation in the lower airway may require an abundant presence of readily primed memory T cells in peripheral blood that can respond to allergens [35]. CD4+ memory T cells were shown to be involved in recurrent episodes of inflammation in both murine models of BA and BA patients [36, 37]. We hypothesized that memory T cells in BA patients display distinctive phenotypes that can sustain chronic inflammation in the lung; and that the expression of certain chemokine receptors on T cells is associated with disease severity and worsening of symptoms.

Over the last few decades, chemokine family and their receptors attracted so much attention for their numerous roles in regulating leukocyte functions throughout inflammation and immune reactivity. A number of studies have speculated that the CCR7 plays essential roles in immune-cell trafficking in various tissue compartments during inflammation and in immunosurveillance [38]. Therefore, we analyzed memory (CD45RO+) CD4+ T cells based on their chemokine receptor (CCR7) expression and the results showed that the percentage of CCR7+ CD45RO+ CD4+ T memory cells was elevated significantly in allergic BA patients

compared to both non-allergic BA patients and controls, with an obvious non-significant difference between the latter two groups (P=0.956). This may be explained by the fact that the immunoregulation through CCR7 expression in T cells plays a role in allergen-specific sensitization in the airway where natural allergen exposure in patients with allergic respiratory syndrome affects T cell activation and their memory status [39].

More importantly, we found that the percentage of CCR7+ T memory cells was inversely correlated to improved pulmonary function tests, and positively correlated to disease severity scores, suggesting a central role of CCR7+ memory T cells in persistence of chronic inflammatory reactions in allergic BA patients' lungs with or without the existence of a specific allergen, and that the memory compartment of severe asthmatic patients expressing CCR7 is significantly expanded.

CCR7+ memory T cells (TCM) were also directly correlated to total IgE level being a critical factor for the development of bronchial hyperresponsiveness in asthmatics [40]. In concordance, it has been suggested that CCR7 may promote immune inflammation and that the role of cytokines and IgE in allergic asthma may be associated with the expression level of CCR7 where its downregulation was associated with reduced inflammatory cell infiltration and IL-4 levels [41]. They were also directly correlated to disease duration. TCMs are thought to have long-lived behavior and show superior engraftment capacities compared with other memory T cell subsets [42].

On the other hand, our results revealed an elevated percentage of CCR7−effector memory T cells (TEM) in BA patients as well, with no difference between allergic and non-allergic BA patients, endorsing that the increase of CCR7+ CD45RO+ CD4+ T cells (TCM) in BA patients was not due to a decrease of CCR7-CD45RO+ CD4+ T cells (TEM) in their blood. TEM cells did not show a significant correlation with any clinical variables, including ACT and % predicted FEV1 scores. In fact an imbalance in memory CD45RO+ T cells in peripheral blood of patients with allergic disease have been reported; however, results are inconsistent [43-45].

Parallel to their cytokine expression, subsets of effector T cell express distinct chemokine receptor patterns with an evidence of constant recirculation through the lungs and an immunosurveillance role. The subsequent variation in the local cytokine milieu might induce a change in chemokine receptor expression to allow correct migration within the surrounding airways. Th2 cells have been delineated by expression of CCR4 and CCR8 [35]. CCR4 has been long thought to take part in the recruitment of Th2 cells following allergen exposure, owing to its high expression on Th2 cells [46]. However, the role of CCR4+ T cells in the BA

pathogenesis is still controversial [8, 47]. In addition, resident pulmonary APCs can present allergen long after cessation of allergen exposure and have been shown to promote Th2 cell differentiation in situ [48].

An increase in the percentage of CCR4 expressing CD4+ T cells in BA patients has been previously described [8] which was also reported in our study; though, a correlation between proportion of CCR4+ CD4+ T cells in peripheral blood or in the lungs and the severity of asthma has been declined [49]. We described an inverse correlation of CCR4+ CD4+ cells and pulmonary functions. This could be explained by upregulated CCR4 specific ligands on airway epithelial cells upon allergen challenge suggesting an involvement of this receptor/ligand axis in the regulation of CD4+ T lymphocyte recruitment into the BA patients' bronchi. These findings were in line with the above results raising the possibility that the increased expression of CCR4 can be attributed to the expansion of Th2 cells, which could contribute to both chronic disease and allergen induced exacerbations. A direct correlation to IgE level was shown in our study as T cell help is a crucial factor for plasma cell differentiation and immunoglobulins production. Moreover, CCR4+ cells showed a direct correlation to TCM cells and an inverse correlation to TEMs.

Furthermore, our results revealed no significant association between neutrophil phagocytic activity and either of studied T cell subsets; however, a correlation to asthma control test was observed. Instead, it has been reported that neutrophils' phagocytic activity was most pronounced in BA patients irrespective of disease severity [50].

Conclusions

In this study, we report an increase of circulating long-lived TCM cells along with CCR4+ CD4+ effector cells in adult patients with allergic BA. This study also describes evidence of a clinical relevance of the existence of these cells as well as an association with increased disease severity, decreased lung functions, and increased production of immunoglobulin-E. Hence, these results might open a new horizon for proper understanding of the pathogenesis and progression of allergic BA in human, and further direct our efforts toward the rational design of new modalities of proper treatment candidates.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Table I: Comparison between the studied groups according to demographic and clinical characteristics

	Allergic asthmatic patients (n = 78)	Non-allergic asthmatic patients (n = 41)	Control (n = 40)	P
Age	48.2 ± 10.3	52.2 ± 8	48 ± 9.1	0.063
Sex				
Male	28 (35.9%)	17(41.5)	17 (42.5%)	0.731
Female	50 (64.1%)	24 (58.5%)	23 (57.5%)	
Family history of an allergic disease	78(100.0%)	5(12.2%)		<0.001*
Disease duration (years)	22.3 ± 2.2	15.7 ± 4.6	-	<0.001*
WBCs (x10^3 /mm^3)	10.9(4.6 – 25.6)	10.7(8.1 – 17.8)	5.1(4.2 – 7.4)	<0.001*
Sig. bet. groups		P_1=0.861, P_2<0.001*, P_3<0.001*		
Lymphocytes	2.8(1.7 – 5.3)	1.8(1.3 – 3.1)	2.1(1.8 – 2.4)	<0.001*
Sig. bet. groups		P_1<0.001*, P_2<0.001*, P_3=0.005		
Basophils	0.03(0.01 – 0.2)	0.03(0.01 – 0.07)	0.03(0.01 – 3.0)	0.850
Monocytes	0.8(0.3 – 1.9)	0.97(0.5 – 7.9)	0.4(0.2 – 0.6)	<0.001*
Sig. bet. groups		P_1=0.001*, P_2<0.001*, P_3<0.001*		
Eosinophil's	0.2 (0 – 1.1)	0.1 (0 – 0.2)	0.1 (0 – 0.2)	<0.001*
Sig. bet. groups		P_1<0.001*, P_2=0.003*, P_3=0.029*		
Neutrophils	6.8 (1.8 – 21.1)	7.4 (5.3 – 15.5)	2.3 (1.3 – 4.8)	<0.001*
Sig. bet. groups		P_1=0.041*, P_2<0.001*, P_3<0.001*		
ESR 1st hr. (mm/hr.)	17.0 (4 – 53)	36 (14.3 –52)	9 (1 – 19)	<0.001*
Sig. bet. groups		P_1<0.001*, P_2<0.001*, P_3<0.001*		
CRP	3.6 (1.7 – 6)	4.5 (2.2 – 10.8)	0.8 (0.2 – 2.6)	<0.001*
Sig. bet. groups		P_1<0.001*, P_2<0.001*, P_3<0.001*		
FVC(L)	2.7 ± 0.7	3.5 ± 0.3	-	<0.001*
FVC% pred.	78.9 ± 11.1	87.9 ± 7.2	-	<0.001*
FEV1(L)	2.4 ± 0.7	2.8 ± 0.6	-	<0.001*
FEV1%/FVC	84.7 ± 7.1	89.2 ± 4.6	-	<0.001*
FEF25-75%(L/min)	2.5 ± 0.9	3.7 ± 0.7	-	<0.001*
PD20 of Methacholine challenge (mg/ml)	0.025(0.002–0.16)	0.014(0.002–0.032)	-	<0.001*

Data were assessed using: Chi square test (χ^2), student t test (t), Mann Whitney test (U), ANOVA test (F), and Kruskal Wallis test (H). Family history of an allergic disease including: allergic asthma or/ and allergic rhinitis; Sig. bet. groups: significance between groups; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; FVC: forced vital capacity; pred. : predicted; FEV1: forced expiratory volume in one second; FEF25-75%: mean forced expiratory flow between the 25% and 75% of the FVC

P: P value for comparing between the studied groups

P_1: P value for comparing between **allergic asthmatic patients** and **non-allergic asthmatic patients**

P_2: P value for comparing between **allergic asthmatic patients** and **control**

P_3: P value for comparing between **non-allergic asthmatic patients** and **control**

*: statistically significant at $p \leq 0.05$

Table II: Comparison between allergic and non-allergic asthmatic patients according to clinical measurements and symptoms before and after IGC

	Allergic asthmatic patients		Test of Sig. (P)	Non-allergic asthmatic patients		Test of Sig. (P)
	Before IGC increase	After IGC increase"		Before IGC increase	After IGC increase"	
FEV1% pred.	74.7 ± 11.1	107.4 ± 14.3	<0.001*	83.5 ± 6.7	124.5 ± 16	<0.001*
Asthma control test	17 ± 4	19.5 ± 3.7	<0.001*	18.6 ± 3	21.5 ± 3	<0.001*
Cough score	2 (0 – 3)	1 (0 – 1)	<0.001*	1 (0 – 3)	0 (0 - 1)	<0.001*
Wheezing score	2 (0 – 3)	0 (0 – 1)	<0.001*	1 (0 – 3)	0 (0 – 1)	<0.001*
Inhaled β2 agonists doses/day	3 (2 – 5)	2 (1 – 3)	<0.001*	2 (1 – 3)	0 (0 – 2)	<0.001*

IGC indicates inhaled glucocorticoid therapy; FEV1: forced expiratory volume in one second
Cough score and Wheezing score (GINA 2020): 0 is well controlled, 1-2 is partly controlled, and 3-4 is uncontrolled.
P: P value for comparing between before IGC and after IGC using: Paired t-test or Wilcoxon signed ranks test.
*: statistically significant at $P \leq 0.05$

Table III: Comparison between allergic and non-allergic asthmatic patients according to disease severity scores

	Allergic asthmatic patients (n = 78)	Non-allergic asthmatic patients (n = 41)	P
Asthma control test	17.0 ± 4.0	18.6 ± 3.0	0.025*
FEV1% pred.	74.7 ± 11.1	83.5 ± 6.7	<0.001*
Dyspnea scale	2 (0 – 4)	1 (0 – 2)	<0.001*
Cough score	2.0(0.0 – 3.0)	1.0(0.0 – 3.0)	0.040*
Wheezing score	2.0(0.0 – 3.0)	1.0(0.0 – 3.0)	0.026*

The data were assessed using Mann Whitney test (U) and student t-test (t). Dyspnea scale: modified Medical Research Council scale [24]. Data where relevant are expressed as the mean (range) and standard deviation values. P: P value for comparing between the studied groups

*: statistically significant at $P \leq 0.05$

Table IV: Correlation between percentages of CCR4+ in CD4+ T cell with different studied parameters

	% of CCR4+ in CD4+ T cell	
	r	**P**
Disease duration	0.333*	<0.001
Lymphocytes	0.242*	0.008
FVC(L)	-0.500*	<0.001
FVC% pred	-0.196*	0.032
FEV1(L)	-0.228*	0.013
FEV1%/FVC	-0.131	0.154
FEF25-75%(L/min)	-0.428*	<0.001
FEF25-75%pred.	-0.358*	<0.001
Methacoline challenge test	0.084	0.362
Asthma control test before IGC increase	-0.206*	0.025
Asthma control test after IGC increase	-0.247*	0.007
Cough score before IGC increase	0.196*	0.033
Cough score after IGC increase	0.225*	0.014
Wheezing score before IGC increase	0.186	0.043
Wheezing score after IGC increase	0.093	0.313
Inhaled β2 agonists doses/day before IGC	0.473*	<0.001
Inhaled β2 agonists doses/day after IGC	0.350*	<0.001
FEV1% pred Before IGC increase	-0.468*	<0.001
FEV1% pred after IGC increase	-0.273*	0.003
% of CCR7- CD45RO+ in CD4+ T cell	-0.344*	<0.001
% of CCR7+ CD45RO+ in CD4+ T cell	0.555*	<0.001
Total serum IgE	0.622*	<0.001
Phagocytic activity	0.143	0.121

r: Pearson coefficient

*: statistically significant at $P \leqslant 0.05$

Table V: Correlation between percentages of CCR7+ CD45RO+ in CD4+ T cell with different studied parameters

	% of CCR7+ CD45RO+ in CD4+ T cell	
	r	**P**
Disease duration	0.435*	<0.001
Lymphocytes	0.481*	<0.001
FVC(L)	-0.622*	<0.001
FVC% pred	-0.417*	<0.001
FEV1(L)	-0.431*	<0.001
FEV1%/FVC	-0.353*	<0.001
FEF25-75%(L/min)	-0.613*	<0.001
FEF25-75%pred.	-0.314*	<0.001
Methacoline challenge test	0.222*	0.015
Asthma control test before IGC increase	-0.469*	<0.001
Asthma control test after IGC increase	-0.514*	<0.001
Cough score before IGC increase	0.097	0.296
Cough score after IGC increase	0.014	0.882
Wheezing score before IGC increase	0.079	0.391
Wheezing score after IGC increase	0.067	0.468
Inhaled β2 agonists doses/day before IGC	0.526*	<0.001
Inhaled β2 agonists doses/day after IGC	0.310*	0.001
FEV1% pred Before IGC increase	-0.618*	<0.001
FEV1% pred after IGC increase	-0.393*	<0.001
Total serum IgE	0.692*	<0.001
Phagocytic activity	0.073	0.429
% of CCR7- CD45RO+ in CD4+ T cell	-0.470*	<0.001

r: Pearson coefficient

*: statistically significant at $P \leqslant 0.05$

Figure Legends

Fig. 1: The percentages of phagocytic activity in BA patients (a) Representative dot plots were shown from allergic asthmatic patients, non-allergic asthmatic patients and healthy individuals, (b) Representative dot plots were shown from BA patients who were classified into well controlled, not well controlled and poorly controlled patients based on asthma control test, (c) Representative dot plots were shown from BA patients who were classified according to their IgE serum levels into categories (<20, 20-100, >100 IU/ml)

Fig. 2: The percentages of CCR4+ CD4+ T cells in BA patients (a) Representative dot plots were shown from allergic asthmatic patients, non-allergic asthmatic patients and healthy individuals, (b) Representative dot plots were shown from BA patients who were classified into well controlled, not well controlled and poorly controlled patients based on asthma control test, (c) Representative dot plots were shown from BA patients who were classified according to their IgE serum levels into categories (<20, 20-100, >100 IU/ml).

Fig. 3: The percentages of CCR7+ CD45RO+ CD4+ T cells in allergic BA patients and healthy control where CD3+ CD4+ T cells were stained with anti-human CD45RO and anti-human CCR7 antibodies.

Fig. 4: The percentages of CCR7+ CD45RO+ CD4+ T cells in BA patients (a) Representative dot plots were shown from allergic asthmatic patients, non-allergic asthmatic patients and healthy individuals, (b) Representative dot plots were shown from BA patients who were classified into well controlled, not well controlled and poorly controlled patients based on asthma control test, (c) Representative dot plots were shown from BA patients who were classified according to their IgE serum levels into categories (<20, 20-100, >100 IU/ml)

Fig. 5: The percentages of CCR7- CD45RO+ CD4+ T cells in BA patients (a) Representative dot plots were shown from allergic asthmatic patients, non-allergic asthmatic patients and healthy individuals, (b) Representative dot plots were shown from BA patients who were classified into well controlled, not well controlled and poorly controlled patients based on asthma control test, (c) Representative dot plots were shown from BA patients who were classified according to their IgE serum levels into categories (<20, 20-100, >100 IU/ml)

figure 1

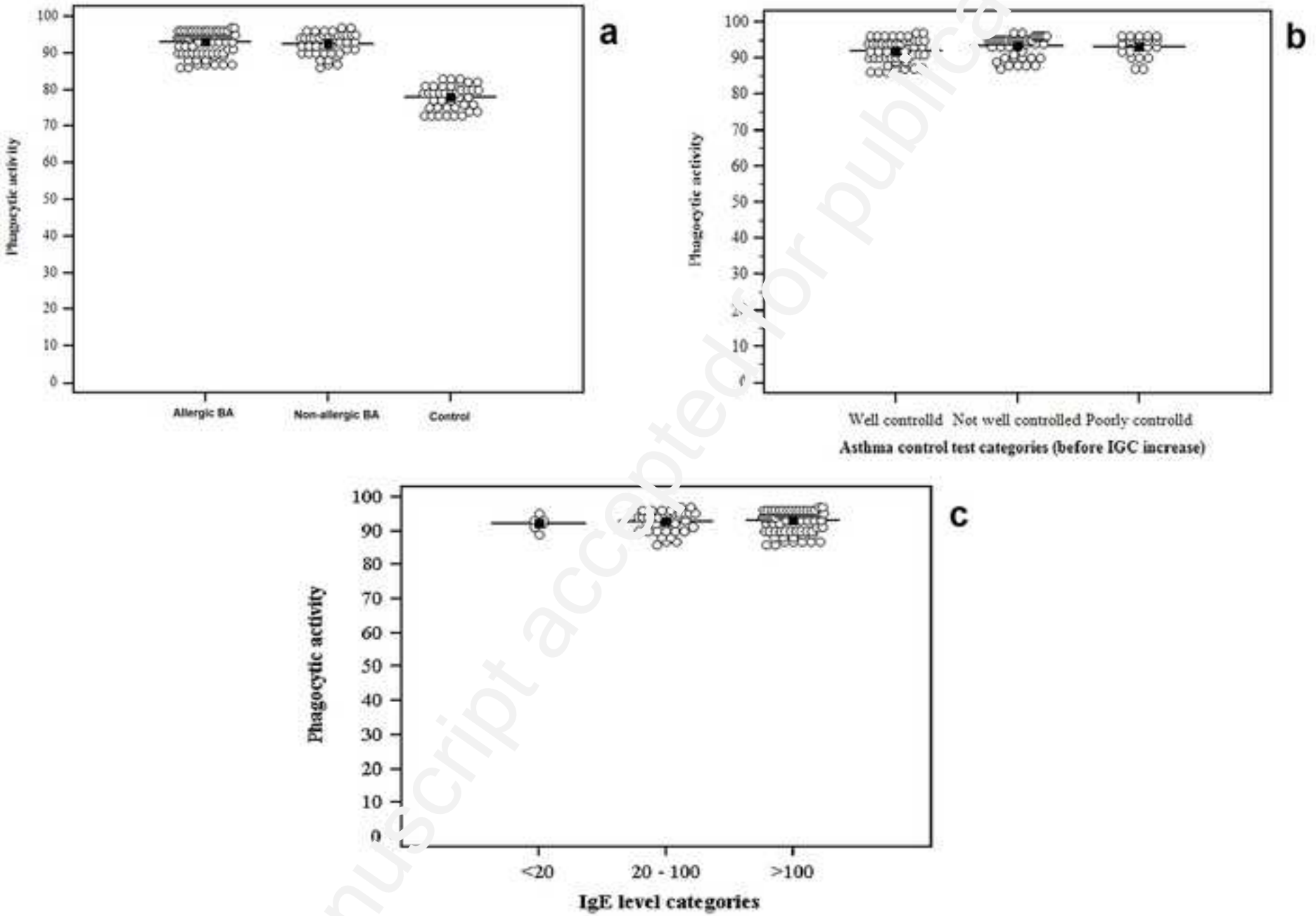
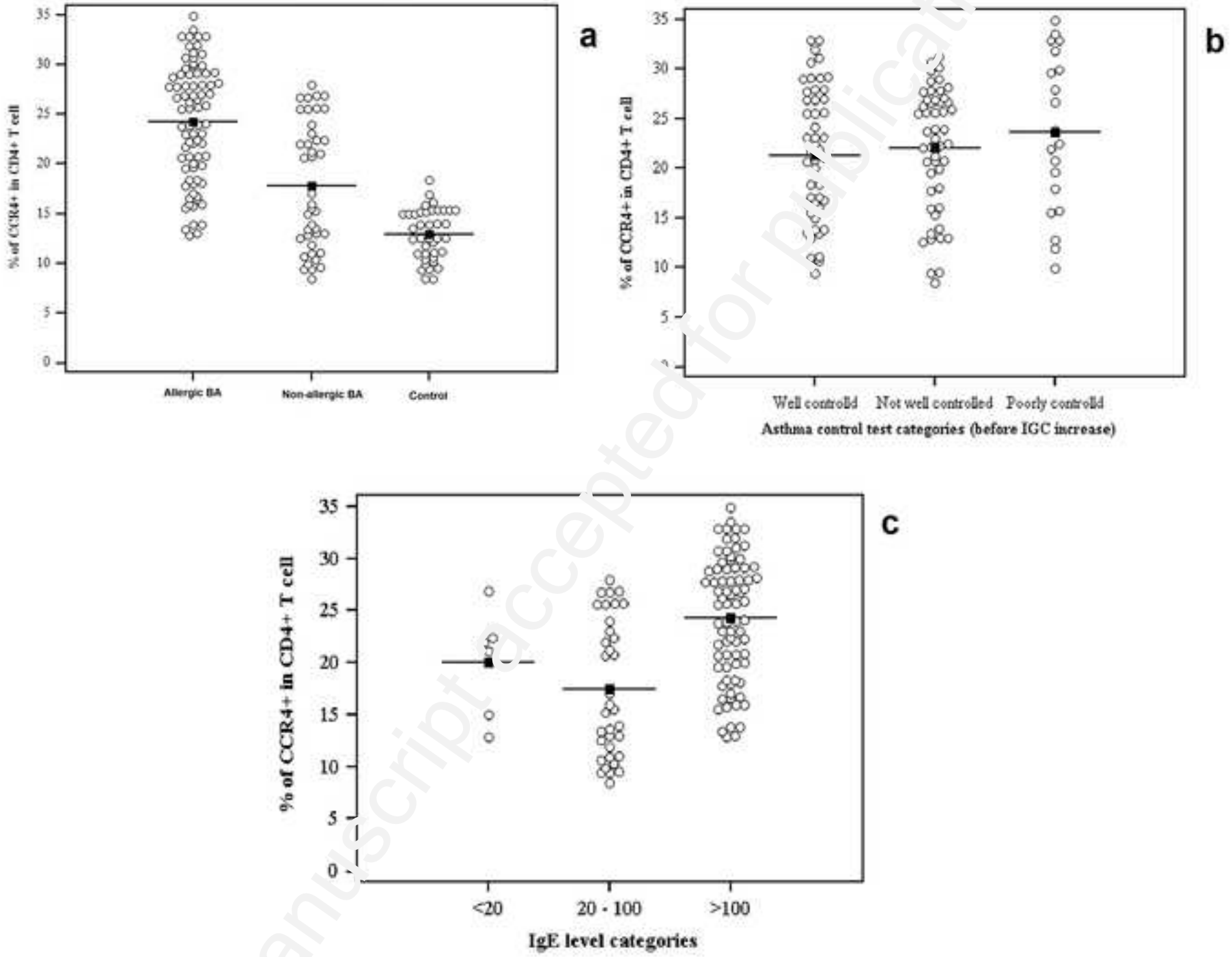


figure 2



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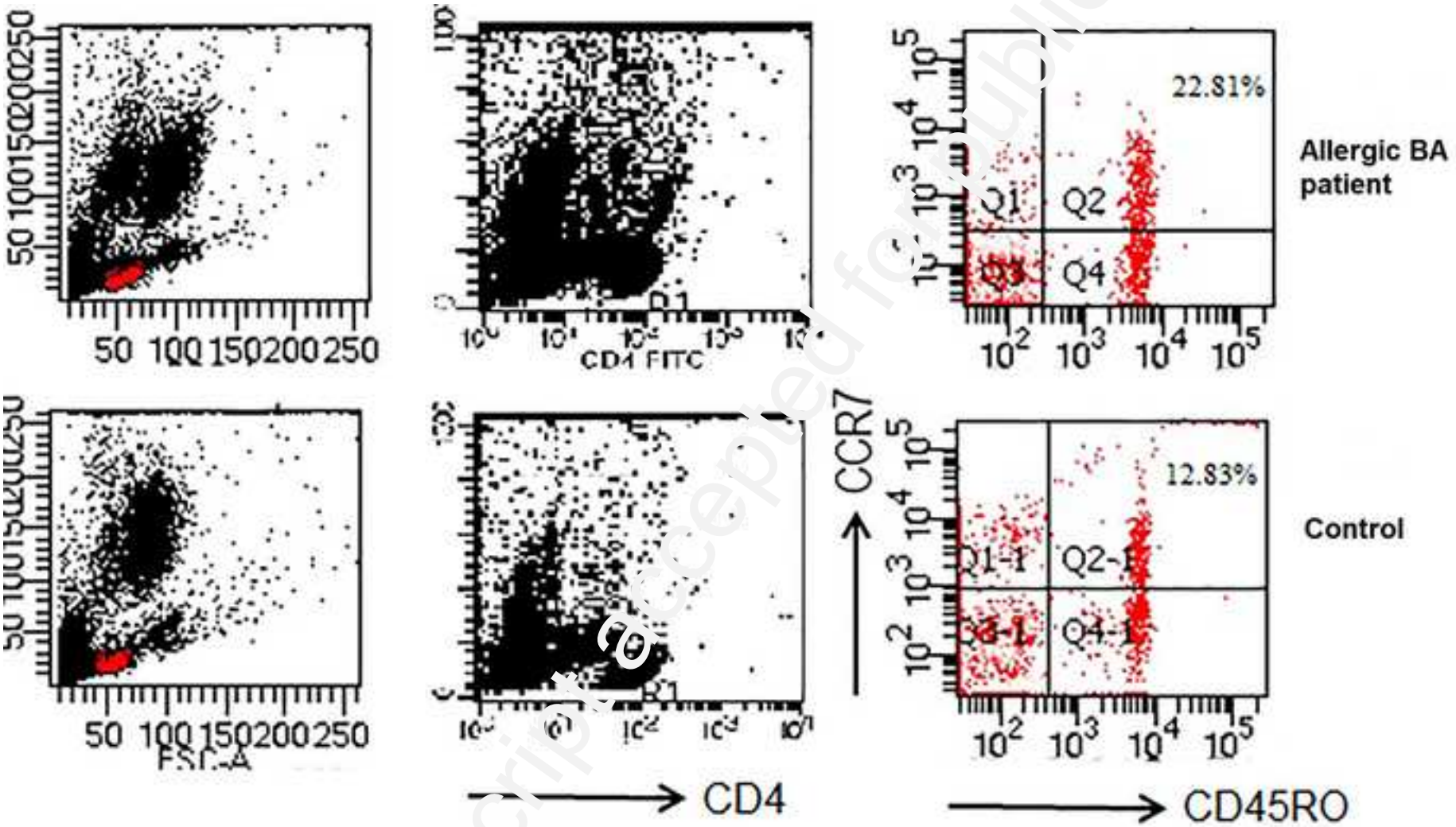

figure 4

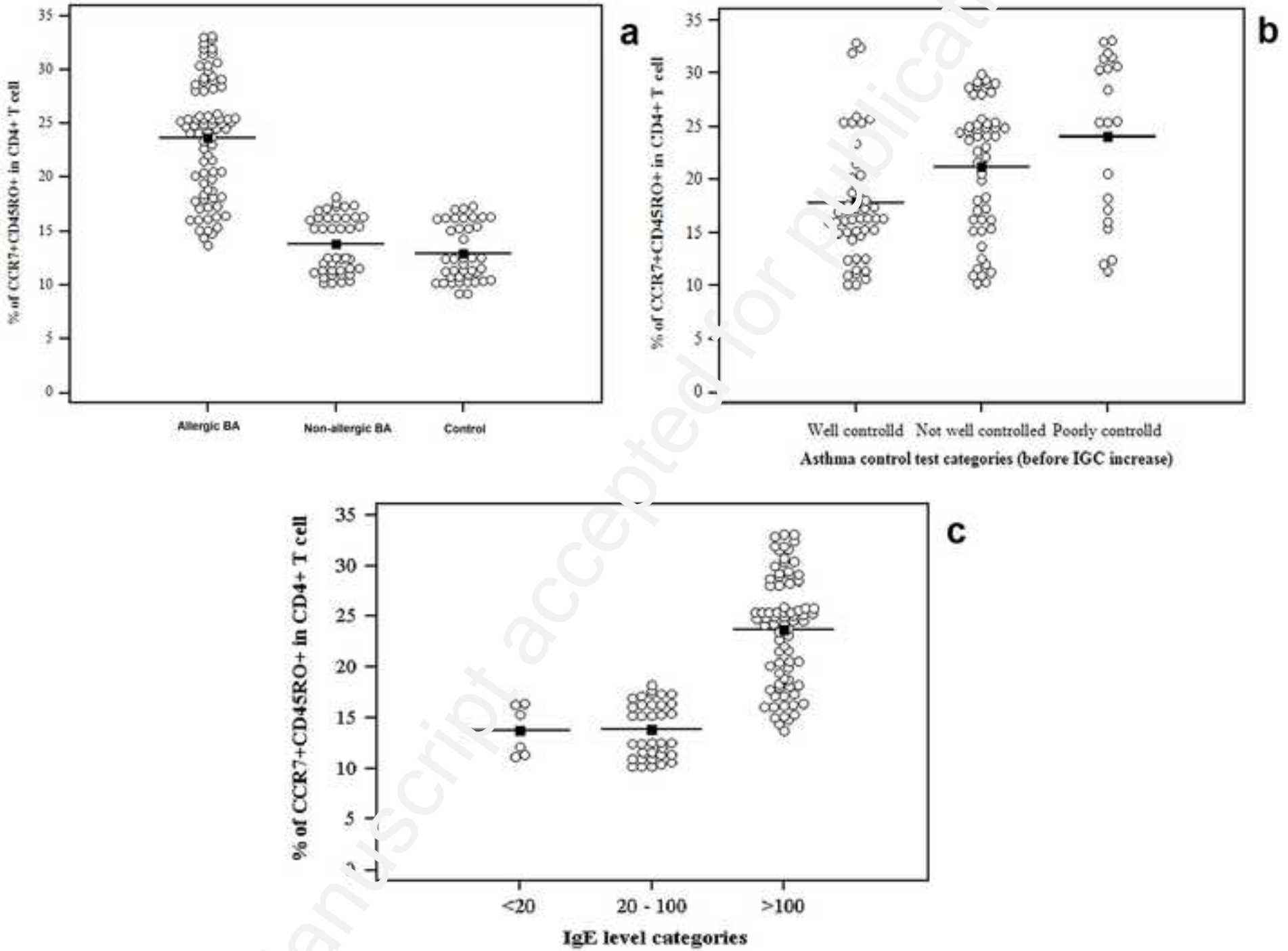

figure 5