Serum Level of CD48 and Its Expression on Blood Leukocytes in Persistent Asthmatic Patients

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CD48 is a surface receptor (mCD48) expressed on most hematopoietic cells, as well as in a soluble form (sCD48). It seems to play a major role in asthma through the interactions of mast cells with eosinophils via its ligand CD244. Hence, this study was done to evaluate the role of CD48, in its membrane and soluble forms, as a novel biomarker in asthma with various degrees of severity. One hundred participants were enrolled in this study and divided into 4 equal groups matched in age and sex; mild asthma, moderate asthma, severe asthma and apparently healthy controls. All were investigated for blood leukocytes mCD48 expression using flow cytometry and sCD48 in serum using ELISA. Our results revealed that the sCD48 was significantly elevated in patients with mild asthma compared with the controls (*P*<0.001) while significantly decreased in severe asthma than mild asthma (*P*<0.001) and moderate asthma (*P*=0.002) patient groups. Expression of mCD48 on eosinophils in moderate asthma group was significantly elevated compared with the control and the mild asthmatic groups (*P*<0.001). However, it was significantly decreased in severe asthma compared with moderate and mild asthma (*P*<0.001 and *P*=0.03, respectively). While it was significantly upregulated in severe asthmatic group compared to controls, patients with mild and moderate asthma on T-cell, B cells, Monocytes and NK cells (*P*<0.001 for all). In conclusion, CD48 may play a role in asthma and its level varies with severity of the disease being a useful marker in mild asthma.

Asthma is not considered a separate disease entity, but a "syndrome" with different phenotypes. There are various precipitating factors responsible for the clinical, physiological, and pathological manifestations [1].

The complex interaction between variable cells both resident and recruited and their mediators results in inflammation in the airways [2]. Eosinophilia in blood or sputum is a major predicting factor of risk of severe asthma exacerbations and the response to corticosteroid therapy [3]. However, measuring blood and/or sputum eosinophils is not a straightforward process and lacks specificity [4].

CD48 is a glycosylphosphatidylinositol-anchored receptor; a member of the CD2 subfamily of immunoglobulin-like receptors, expressed on most hematopoietic cells especially eosinophils and mast cells; the two major guiding cells in asthma. It exists as a surface receptor (mCD48) on cells and as a soluble form (sCD48) in serum [5, 6].

CD48 was previously found to be involved in various allergic disorders and infections and to have a pathophysiological role in inflammatory and autoimmune disorders. Being a costimulatory receptor of different immune responses and its involvement in the airway allergy, CD48 is strongly linked to asthma [7].

CD48 is implicated in B cell growth, differentiation, and immunoglobulin production. In addition, activation of neutrophils, proliferation of T-cells and stimulation of IL-2 synthesis and its receptor expression [8].

CD244, an activating receptor on eosinophils is the high-affinity ligand for

CD48. Crosslinking of CD48 on eosinophils triggers degranulation of eosinophils and subsequently cytokine release [9,10].

Various Studies in a mouse model of allergic airway inflammation ensures the regulatory role of CD48. In vivo administration of anti-CD48 mAb one day before antigen challenge dramatically reduce inflammation, lung cell infiltration, cytokines, and histological signs of inflammation [11].

These observations suggest a role for CD48 in asthma. Therefore, the current study was carried out to determine whether CD48 serum level and membrane expression, as a biomarker, would vary with severity of the disease.

Patients and Methods

Study design

This case-control study was conducted at the outpatient clinic of Chest Department of Zagazig University Hospitals, Microbiology & Immunology and Clinical pathology Departments, Faculty of Medicine, Zagazig University and started from November, 2019 to May, 2020. The study protocol was reviewed and approved by the Institutional Review Board (IRB), Faculty of Medicine, Zagazig University, Egypt (March 2020). All study participants agreed and signed informed consents.

Patient enrollment

A total of 100 participants were included in the study. These included 25 apparently healthy non-atopic, (negative skin prick tests) as controls, 25 patients with mild asthma receiving SABA (short-acting β2 adrenergic receptor agonist), 25 patients with moderate asthma receiving inhaled corticosteroid (ICS) ±LABA (long-acting β2 adrenergic receptor agonist) and 25 patients with severe asthma receiving high dose of ICS.

Study tools

Patients were diagnosed to have mild, moderate and severe persistent bronchial asthma according to referral of their clinical manifestations to the current Global Initiative for Asthma (GINA) criteria and confirmed by spirometry (Spirotube, PC Spirometer, THOR laboratories, Budapest, Hungary). The forced expiratory volume in the first second (FEV1) and the forced vital capacity (FVC) were measured in relation to reading of spirometry before and after bronchodilator (two puffs of inhaled salbutamol (each of 200 μg)). The reversibility is considered when the FEV1 increment by (≥12%) approaches the pre-bronchodilator value.

Skin prick test (SPT) to participants was performed at the Allergy and Immunology Unit, Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University. Allergen panel used (homemade extracts of pollen, house dust, smoke, wool, cotton, mixed fungi, hay rice dust, clover and maize). Histamine was used as a positive control and saline as a negative control. The test was read by measuring the size of the wheal after 20 min. A wheal diameter 3 mm or more, associated with erythema, was considered as a positive reaction [12].

Exclusion criteria

Patients beneath 19 years old, patients on medications as systemic steroids or immunosuppressant, Patients with intermittent asthma (not on regular treatment), having other complications like bronchiectasis or chest infections and unconsented patients, were excluded from the study.

Samples

Five ml of blood were obtained from each participant and divided into two tubes; EDTA containing tube for flowcytometric analysis and Wassermann's tube for serum collection for ELISA. Serum was obtained by allowing samples to clot for 30 minutes followed by centrifugation for 10 minutes at approximately 3000×g. and stored at -20 °C until used.

Flow cytometric analysis for immunophenotyping (FCI) of CD 48 on blood leucocytes

Immunophenotyping was done using flowcytometry (FACScan, Becton Dickinson, and San Jose, California, USA) acquisition and analysis was performed with Cell Quest software (BD Biosciences).

- Antibody panels design

Isotype control IgG1-FITC/IgG2a-PE was used at the start of each run. We used the following markers (BD Bioscience, USA): CD48 FITC, CD 45 E., CD19 PE; CD3 PE, CD15 PE, CD56 PE, CD14 PE, and CD 193 PE (CCR3).

- Staining for FCI analysis [13]

It was performed according to the manufacturer's instructions (Becton Dickinson, Bioscience, USA). In separate tubes, 20 µl of each of the monoclonal antibodies were added to 100 µl of EDTA blood sample and vortex mixed. Then incubated at room temperature in the dark for 30 min, then washed twice in 2 ml of PBS and centrifuged for 5 min at 300 × g. Finally, for fixation, 0.5 ml of 1% paraformaldehyde was added and samples were acquired on the Flowcytometer (FCM) after 30 min.

- Acquisition

Acquisition and analysis of stained suspension was performed by acquiring at least 10.000 cells at a high rate of 400–500 cells/second for each marker. Negative isotype control was run first to identify the position of the negative and the positive populations. At least two plots were drawn during the acquisition of each tube. The first plot; displayed forward scatter (FSc) on X axis versus side scatter (SSc) on Y axis to identify the size and granularity of cells and to exclude debris and dead cells. The second plot displayed the antibody marker on X axis versus FSc or the other marker in case of dual markers on Y axis.

- Sample analysis

Analysis of sample tubes was performed as follows: setting cursors for differentiating positive and negative populations (Isotype control plot analysis). The tube containing CD45 (gating reagent) was analyzed first to set a gate around lymphocyte clusters using FSc and SSc patterns and fluorescence staining based on low FSc and SSc patterns and bright stain of lymphocytes with CD45. Light-scattering patterns were examined on each sample tube and the remaining sample tubes were analyzed with the cursors previously set based on the isotype control. The data was reported as a percentage of the total lymphocytes and/or percentage of gated population. Absolute numbers of positive and negative populations were also reported.

- Interpretation of data

A negative (isotype) reagent control was used with each specimen to determine non-specific binding of the mouse monoclonal antibody to the cells and to allow setting of markers for distinguishing fluorescence-negative and fluorescence positive cell populations. The marker set on the negative control plot was copied on each analyzed plot of dual markers plots, so dividing it into 4 quadrants. Analysis of the population in each quadrant was as follows: the lower left quadrant; double negative to both markers, the lower right quadrant; positive for X axis marker only, the upper left quadrant; positive for Y axis marker only, and the upper right quadrant; positive to both markers.

Evaluation of sCD84 Levels in serum

Quantitative determination of sCD48 concentrations was done using Human CD 48 ELISA kit (Cloud clone, New Test Company, USA) according to the manufacturer's instructions:

All reagents, standard solutions and samples were allowed to reach the room temperature. The required strips were inserted in the frames. Fifty (50µl) of standard were added to standard well. Ten µl of each sample were added to sample wells and then 40µl sample diluent were added to sample wells but not the blank well, and then 100µl of HRP-conjugate reagent were added to sample wells and standard wells. Careful mixing was done, the plate was covered with a seal and was incubated at 37°C for 60 minutes. Then the sealer was removed, and the plate was washed with the washing buffer for 5 times; we used 400ul wash buffer for 1 minute for each wash. Fifty microliters of chromogen solution A were added to each well then 50µl of chromogen solution B added. The plate was covered with a new seal and was incubated in the dark for 15 minutes at 37°C. Finally, 50µl of Stop Solution were added to each well, the blue color was changed into yellow immediately. The optical density of each well was immediately determined using a micro plate reader (Palm city, USA) set to 450 nm.

The results were calculated by drawing the standard curve, the average O.D. obtained for each of the six standard concentrations on the vertical (Y) axis was plotted versus the corresponding concentration on the horizontal (X) axis.

Statistical Analysis

Statistical packages (EPI-info Version 6.04 and SPSS Version 20 inc. Chicago, USA) were used to analyze collected data. Quantitative data was represented as mean, median, standard deviation and range. Multigroup comparison was done using One Way Anova test (for parametric data) and Kruskal-Wallis (for nonparametric data). Parametric quantitative data was compared using Independent t-test and for the nonparametric data, Mann-Whitney test was used. Chi square test was used for comparing qualitative data. A P Value < 0.05 was considered statistically significant at 95% confidence interval.

Results

A total 100 participants were enrolled in this study and were divided into 4 groups matched in age and sex; group 1 included 25 patients with mild asthma with mean age of 36.8±9.3, range of (19-52) years and male/female =12/13, Group 2 included 25 patients with moderate asthma with mean age of 42.5±8.5, range of (24-57) years and male/female =10/15, group 3 included 25 patients with severe asthma with mean age of 46±9.3, range of (29-61) years and male/female =11/14 and finally group 4 included 25 controls with mean age of 39.8±10, range of (24-59) years and male/female =12/13.

There were no statistically significant differences between asthmatic patients (n=75) and controls (n=25) as regards gender and smoking (P=0.72 and P=0.46) as shown in table 1.

Table 1. Comparison of gender and smoking habit among asthma patients and controls.

	Asthma patients (n=75) N (%)	Controls (n=25) N (%)	*P value
Male	33 (44)	12 (48)	NS
Female	42 (56)	13 (52)	
Smoker	13 (17.4)	6 (24)	NS
Nonsmoker	62 (82.6)	19 (76)	

*Chi square test; NS (P>0.05 is non-significant)

Serum level of CD48 was assessed by ELISA in all our study groups and was found to be significantly elevated in patients with mild asthma compared with the controls (P<0.001). However, sCD48 in severe asthma group was observed to be significantly decreased than mild asthma (P<0.001) and moderate asthma patient groups (P=0.002). Although sCD48 was also decreased in patients with severe asthma compared to controls, but it did not reach the significant level (P=0.15) as shown in table 2.

Table 2. Serum level of CD48 (pg/ml) among the study groups.

	Mild Asthma	Moderate asthma	Severe asthma	Controls	P value
Mean ±SD	2962.4±389.2	1917.4±310.6	1590±379.8	1739.6±385.2	$^{*}P$<0.001
Median	2890	1780	1540	1780	$^{**}P^{1}$<0.001
Range	(2433-3760)	(1420-2655)	(1010-2290)	(1080-2244)	$^{**}P^{2}$=0.09
					$^{**}P^{3}$=0.15
					$^{**}P^{4}$<0.001
					$^{**}P^{5}$<0.001
					$^{**}P^{6}$=0.002

*One way Anova test, **Post Hoc test; P^{1}; control vs mild, P^{2}; control vs moderate, P^{3}; control vs severe, P^{4};mild vs moderate,P^{5}; mild vs severe and P^{6}; moderate vs severe, (P<0.05 is significant).

Membrane CD48 expression was evaluated by flowcytometry on different hematopoietic cells. Expression of mCD48 on eosinophils in moderate asthma patients was significantly elevated compared with mild asthma patients and controls (P<0.001) for both. However, its expression on eosinophils in severe asthma was significantly decreased compared with moderate and mild asthma (P<0.001 and P=0.03) respectively, table3.

No significant difference was observed in mCD48 expression on neutrophils and basophils (P=0.2 and P=0.5) respectively, table 3.

T-cell mCD48 expression in severe asthma patients was significantly increased compared with controls, mild and moderate asthma (P<0.001) for all, table 3.

Similarly, mCD48 expression was significantly increased on B cells, Monocytes and NK cells in patients with severe asthma (P<0.001) for all, table 3.

Table 3. Expression of membrane CD48 on different blood leukocytes among our study groups.

	Mild asthma	Moderate asthma	Severe asthma	Control	*P* value
Eosinophils(MFI)					$^{*}P$<0.001 $^{**}P^{1}$<0.001 $^{**}P^{2}$<0.001 $^{**}P^{3}$<0.001 $^{**}P^{4}$<0.001 $^{**}P^{5}$=0.03 $^{**}P^{6}$<0.001
Mean ±SD	39.2±11.5	84.1±23.7	30.1±12	13.5±7.2	
Median	36	90	28.9	10	
Range	17-58	37-113	15.3-53	5.3-29.4	
Neutrophils(MFI)					
Mean ±SD	23.8±6.3	25.8±7.6	24.8±5.4	23.5±7.1	$^{*}P$=0.565
Median	25	24	25	21.4	
Range	14-34	15-51	17-31	14.3-37.2	
Basophils (MFI)					
Mean ±SD	73.2±28.2	78.3±37.7	58.4±30.2	73.8±32.6	$^{*}P$=0.223
Median	67	87	56	83	
Range	37-138	23-170	15-92	19-117	
Monocytes (MFI)					$^{*}P$<0.001 $^{**}P^{1}$=0.25 $^{**}P^{2}$=0.2 $^{**}P^{3}$<0.001 $^{**}P^{4}$=0.01 $^{**}P^{5}$<0.001 $^{**}P^{6}$<0.001
Mean ±SD	147±16.9	103.3±60.6	334.8±82.4	126.5±73.9	
Median	149	76	351	111	
Range	113-171	33.5-198	201-441	15.4-211	
T Cells (MFI)					$^{*}P$<0.001 $^{**}P^{1}$<0.001 $^{**}P^{2}$=0.02 $^{**}P^{3}$<0.001 $^{**}P^{4}$<0.001 $^{**}P^{5}$<0.001 $^{**}P^{6}$<0.001
Mean ±SD	138.8±40.9	72.8±33.5	195.2±21.9	93.7±27.1	
Median	119	61	198	84	
Range	80-190	32-141	156-234	54-132	
B cells (MFI)					$^{*}P$<0.001 $^{**}P^{1}$<0.001 $^{**}P^{2}$<0.001 $^{**}P^{3}$<0.001 $^{**}P^{4}$=0.52 $^{**}P^{5}$<0.001 $^{**}P^{6}$<0.001
Mean ±SD	128.4±27.7	134.7±34.5	188.8±35.5	89.3±39.4	
Median	119	130	200	96	
Range	109-212	92-201	119-233	20.9-160	
NK cells (MFI)					$^{*}P$<0.001 $^{**}P^{1}$<0.001 $^{**}P^{2}$=0.1 $^{**}P^{3}$<0.001 $^{**}P^{4}$<0.001 $^{**}P^{5}$<0.001 $^{**}P^{6}$<0.001
Mean ±SD	145.7±51.7	90.7±51.31	196.1±22.8	71.1±33.1	
Median	167	102	200	76	
Range	48-201	21-189	158-229	19-151	

MFI: mean fluorescence intensity, $^{*}P$: Kruskal-Wallis test for nonparametric data and one way Anova for parametric data, $^{**}P$: Mann Whitney test for nonparametric data and Independent T-test for parametric data; P^{1}; control vs mild, P^{2};control vs moderate, P^{3}; control vs severe, P^{4};mild vs moderate,P^{5};mild vs severe and P^{6}; moderate vs severe, (P<0.05 is significant and p<0.001 is highly significant).

When we analyzed the association of sCD48 level with gender and smoking among all study participants, we found no significant differences of sCD48 level between males and females (P=0.59) or between smokers and nonsmokers (P=0.24) as demonstrated in table 4.

Table 4. Differences of sCD48 level according to gender and smoking among all participants.

sCD48	Groups		*P value
	Male N=45	Females N=55	
Mean ±SD	2014±696.7	2084±614.9	
Median	1890	1960	NS
Range	(1016-3650)	(1010-3760)	
sCD48	Smokers N=19	Nonsmokers N=81	*P value
Mean ±SD	2066.1±823	2049.3±609.3	
Median	1690	1980	NS
Range	(1080-3650)	(1010-3760)	

*Independent T- test and**Mann Whitney test; NS (P>0.05 is non-significant).

Discussion

We aimed in this study to determine the role of membrane CD48 as well as its soluble form (sCD48) as a novel biomarker in asthma with various degrees of severity to provide a new potential target for treatment.

A total 100 participants were enrolled in this case/control study and were categorized into 4 groups (25 patients each); mild asthma, moderate asthma, severe asthma and finally the apparently healthy controls. All groups were matched in age and sex.

Initially, we assessed the sCD48 by ELISA in all study groups and found it to be significantly elevated in patients with mild asthma compared with the controls. By contrast, sCD48 in severe asthma group was observed to be significantly decreased than mild and moderate asthma. However, it was decreased in patients with severe asthma but with no statistical difference from controls.

These results come in accordance with the results of Gangwar and his colleagues [10] who attributed the decreased serum level in severe asthma patients to the use of high dose of corticosteroids or to disease severity. Moreover, Minai-Fleminger and his co-workers [14] suggested that CD48 is only upregulated in tissues where the inflammatory reaction takes place and so not systemically increased in allergic inflammation.

Our flowcytometric results of the mCD48 showed decreased expression on eosinophils in patients with severe asthma compared with moderate and mild asthma. A possible explanation for this phenomenon was introduced by Johansson [15] and Munitz and his colleagues [16] who stated that there was a high degree of extravasation of the most activated eosinophils in severe or poorly controlled asthma than the less severe disease.

In our study, mCD48 expression was significantly upregulated on T cells, B cells, monocytes and NK cells in patients with severe asthma, but with no difference in mCD48 expression on neutrophils and

basophils. CD48 is involved in cell activation in response to infection and inflammation via its interactions with CD244 on eosinophils, mast cells, basophils and T-cells, and after activation sCD48 may be released from these cells and so it may have a role in the cellular response regulation [17].

Interestingly, our results did not show any significant relationship between the serum levels and some patient characteristics, such as gender and smoking which has been implicated in asthma due to underlying inflammatory reactions which may be due to small numbers of smokers in our study or the corticosteroid therapy. These results come in accordance with the results of Breuer *et al*. [17] who found no relationship with sex, smoking, asthma medications or any chronic medications.

In conclusion, CD48 may play a role in asthma and its level varies with severity of the disease suggesting that it may be considered a useful marker in mild asthma.

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