Original article

Regulatory natural killer cell expression in atopic childhood asthma

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Introduction: *Different subsets of natural killer (NK) cells were found to play a role in pathogenesis of allergy. We sought to investigate the expression of regulatory NK cells (CD56+CD16+CD158+) in atopic children with bronchial asthma in order to outline the value of these cells as biomarkers of disease severity and/or control.* ***Methods:*** *A cross sectional controlled study was carried out in the Pediatric Allergy and Immunology Unit, Ain Shams University. The study included 45 atopic children [mean age(SD)= (2.9) years] with bronchial asthma (BA) and/or allergic rhinitis (AR)as well as 40 healthy matched controls. Enrolled subjects underwent complete blood counting and flow cytometric measurement of NK cell (CD16+ CD56+) and regulatory NK cells (CD16+CD56+CD158+).* ***Results:*** *Patients had significantly higher regulatory NK cell percentages [mean (SD)= 41 (52) %] than controls [mean (SD)=15 (7.1)]; p≤0.001. Regulatory NK cell counts and percentages did not vary with the concomitant presence of AR or the degree of asthma control. Regulatory NK cell counts tended to be higher in children with moderate/severe BA compared to those with mild asthma but the difference did not reach statistical significance (U= -1.8, p=0.06). NK cell counts [mean (SD)= 159 (164) cells/µl] and percentages [mean (SD)= 3.7 (3.2) %] were comparable among patients and controls and did not vary with the presence of AR (p= 0.51, 0.95) or with the degree of asthma control. NK cells absolute counts and percentages tended to be higher among patients with moderate/severe compared to mild asthma but the difference did not reach statistical significance.* ***Conclusions:*** *Regulatory NK cells seem to be increased in childhood asthma. We recommend wider scale prospective studies on steroid-naïve subjects involving measurement of cytokines that are secreted by different types of NK cells.*

Keywords: Natural killer, regulatory, asthma, children, allergy.

INTRODUCTION

Natural killer (NK) cells are large granular lymphocytes of the innate immune system that were originally defined by their capacity to lyse target cells and produce interferon-γ without prior activation.[1] Various subtypes of NK cells and specific NK-derived cytokines/chemokines contribute to the promotion/cessation of allergic sensitization.[2]

In 2008, Deniz et al characterized a regulatory subset of NK cell subsets that are characterized by IL-10 secretion. This specific subset of cells was found to suppress Ag-specific T cell proliferation in response to bee venom major allergen, phospholipase A_2 and purified protein derivative of *Mycobacterium bovis* (PPD), in addition to suppression of IgE production. Further characterization revealed that these cells express the inhibitory killer immunoglobulin-like receptors (KIR) (CD158a, CD158b). These cells are termed $CD56^{bright}$ and they are capable of killing, not only the immature dendritic cell (DC), but also mature DC in order to prevent DC over-activation.[3] By limiting the supply and recruitment of immature DC, activated NK cells exert the ability to control the subsequent innate and adaptive immune responses.[3,4] Little is known about the role of regulatory NK cells in atopy and asthma. We, therefore, sought to investigate the regulatory NK cell expression in atopic children with bronchial asthma in relation to disease severity and level of control. The ultimate objective is to outline the value of these cells as novel biomarkers of childhood asthma and may pave the way for using non-conventional therapeutic modalities.

METHODS

Study design

This study is a cross-sectional controlled study that was carried out in the Pediatric Allergy and Immunology Unit, Children's Hospital, Ain Shams University in the period from May 2014 to June 2015.

We enrolled 45 patients with ages ranging between two and 12 years, with physician diagnosed bronchial asthma with or without allergic rhinitis and atopy indicated by positive skin prick testing (SPT) to at least one of the common environmental aeroallergens). Children with any known chronic illness including autoimmune diseases or immune-deficiencies were excluded from the study. Forty age and sex-matched healthy children were enrolled as a control group.

Ethical considerations:

An informed consent was obtained from parents or care givers of all subjects prior to enrollment. The study protocol gained approval of the local Research Ethics' Committee of the Pediatric Department of Ain Shams University.

Study measurements:

The patients were subjected to detailed medical history taking and physical examination with special emphasis on the concomitant presence of allergic rhinitis, duration of allergic disease, severity of asthma in the last three months, degree of asthma control in the month prior to enrollment, and related therapeutic history.[5]

Asthmatic patients underwent SPT at the time of enrolment and only cases with positive results were included in the study. We used standardized allergen extracts of common environmental aeroallergen (house dust mites, alternaria, aspergillus, cockroach, cat epithelia, and pollens) as well as positive histamine (10 mg/mL) and negative saline controls (Omega Laboratories, Montréal, Canada).

Complete blood count (CBC) was performed by automated cell counter Coulter MicroDiff 18, Fullerton, CA, USA) with manual differential after staining with Leishman stain. Flow cytometric measurement of NK cells (CD16+ CD56+) and the regulatory NK cell subset **(**CD16+ CD56+ CD158+) using EPICS XL flow cytometer (Navios Beckman Coulter, USA) was done. Serum total IgE was measured in enrolled patients using enzyme linked immunosorbent assay (serum IgE ELISA, Affymetrix eBioscience, Inc., CA, USA). A serum IgE level was considered elevated if it exceeded the highest reference value for age.[6]

Statistical methods:

Data were collected, revised, coded and entered to the Statistical Package for Social Science (IBM SPSS) version 20. Numerical parametric data were described as mean ± standard deviation (SD) together with minimum and maximum range**.** Median and interquartile range (IQR) were used for non-parametric data. Categorical data were expressed as frequency and percentage. Comparison between qualitative data was done using Chi-square test and between independent groups with parametric continuous variables by Independent t-test. Non-parametric data were compared using Mann-Whitney U test. Spearman correlation coefficients were used to assess the relation between any two studied parameters. Confidence interval was set to 95% and the margin of error accepted was set to 5%. p-value was considered significant at values ≤ 0.05.

RESULTS

Patients' ages ranged between 2.4 and 11.8 years with mean±SD of 7.3± 2.9 years. Thirty- two (71.1%) of them were boys and 13 (28.9%) were girls. Their weight centiles ranged between the 5th and 95th centiles for age. Twelve of the asthmatic patients had concomitant allergic rhinitis (26.7%). The duration of allergic disease ranged from one to 9 years with a mean±SD of 5.9±2.7 years. The control group comprised 40 age- and gender-matched healthy children; their ages ranged from 4 to 12.2 years with mean±SD of 7.5±2.7 years. Thirty of the controls were boys (75%) and10 were girls (25%).

Ten children in our series had mild asthma (22.2%), 30 (66.7%) had moderate and 5 patients had severe asthma (11.1%). According to GINA guidelines[5] for asthma control, 24 patients (53.3%) had well controlled, 15 patients (33.3%) had partially controlled and 6 patients (13.3%) had uncontrolled asthma at the time of enrollment.

Differential leukocyte counts and flow-cytometric results for NK and regulatory NK cells among patients and controls are depicted in table 1. The asthmatic children had significantly lower weight percentiles for age and significantly higher regulatory NK cell percentages in comparison to the control group.

Patients with exclusive bronchial asthma (n=35) were comparable to those with concomitant AR (n=12) in terms of total leukocyte count (p=0.347), absolute lymphocyte count (p=0.257), absolute

eosinophil count (p=0.521), and relative and absolute counts of NK cells (p = 0.96 and 0.51, respectively) and regulatory NK cells (p = 0.7 and 0.65, respectively), as well as the total serum IgE levels (p= 0.173).

Patients with different grades of asthma severity were comparable in terms of their CBC counts, NK and regulatory NK cell counts or percentages and their total serum IgE levels. Although the absolute eosinophil and regulatory NK cell counts were slightly higher among patients with moderate/severe versus mild asthma, the differences did not reach statistical significance (table 2). Moreover, Patients with different degrees of asthma control were comparable in terms of their CBC counts, and NK and regulatory NK cell absolute counts and percentages, as well as total serum IgE levels (table 3).

Regulatory NK cell counts correlated positively with the total leucocyte counts of the asthmatic children (Figure 1). Neither absolute eosinophil count nor total IgE level showed any significant correlation with NK cell or its regulatory subset counts.

Table 1. Variation of blood cell counts among patients and controls.

Variables		Patients (n=45)	Controls (n=40)	Test value	*p*
TLC x10^3/µl	Min-Max	5.4-15.8	5.4-13.4	-0.819 #	0.413
	Median (IQR)	10 (4.6)	9.2 (3.3)		
ALC x10^3/µl	Min-Max	1.52-9.4	1.6-8.04	-1.3 #	0.164
	Median (IQR)	3.5 (2.6)	3.1 (2)		
Lymphocyte %	Min-Max	15.5-62	15.3-62.3	-0.814 #	0.415
	Median (IQR)	42.8 (15)	37 (23)		
ANC x10^3/µl	Min-Max	2-9	1.4-8.3	-0.225 #	0.822
	Median (IQR)	4 (2.4)	4.2 (2)		
AEC x10^3/µl	Min-Max	0.0125-0.7	0.017-0.84	-1.03 #	0.301
	Median (IQR)	0.2 (0.31)	0.231 (0.11)		
NK cell count /µl	Min-Max	15-623	10-984	-0.56 #	0.58
	Median (IQR)	96 (217)	118 (119)		
NK %	Min-Max	0.5-11.9	0.5-13.3	1.28 #	0.19
	Median (IQR)	2.4 (5.1)	3 (3.9)		
Regulatory NK cell count/µl	Min-Max	1-215	2-104	-0.269 #	0.788
	Median (IQR)	30 (40)	15 (25.2)		
Regulatory NK %	Min-Max	7.3-61.8	2.7-29.8	-3.896 #	**0.00 ***
	Median (IQR)	28.1 (23.7)	15 (11.1)		

ALC: absolute lymphocyte count; AEC: absolute eosinophil count; ANC: absolute neutrophil count; IQR: interquartile range; NK: natural killer cell; Max: maximum; TLC: total leucocyte count; SD: standard deviation; • Chi square test; # Mann-Whitney test; * statistically significant.

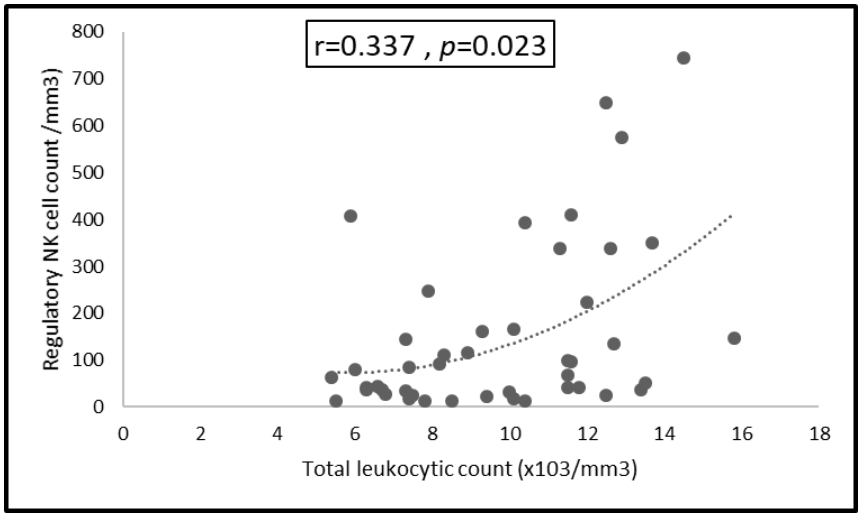

Figure 1. Positive correlation between total leukocyte and regulatory NK cell counts

Table 2. Variation of the laboratory data according to asthma severity.

Parameter		Mild (n=10)	Moderate/Severe (n=35)	U Test	*p* value
TLC (10^3/µl)	Min-Max Median (IQR)	6-13.4 9.4 (6.2)	5.4-15.8 9.2 (4)	-0.56	0.58
ALC (10^3/µl)	Min-Max Median (IQR)	1.8-5.7 3.4 (1.8)	1.5-9.4 3.4 (2.6)	-0.87	0.39
Lymphocyte (%)	Min-Max Median (IQR)	15.5-55.3 34 (13.1)	16.4-62.1 42 (16.4)	-0.13	0.904
ANC (10^3/µl)	Min-Max Median (IQR)	2-9 3.7 (3)	2-8 4.1 (2.3)	-0.08	0.94
AEC (10^3/µl)	Min-Max Median (IQR)	0.012-0.53 0.09 (0.19)	0.013-2 0.23 (0.39)	-1.8	0.06
IgE (IU/ml)	Min-Max Median (IQR)	110-275 227 (74)	35-800 220 (190)	-0.31	0.75
NK cell count (/µl)	Min-Max Median (IQR)	15-359 61 (43)	16-623 113 (266)	-1.6	0. 09
NK cell (%)	Min-Max Median (IQR)	0.5-8 1.3 (2.5)	0.5-11.9 3.2 (5.7)	-1.5	0.11
Reg NK cell count (/µl)	Min-Max Median (IQR)	4-39 12 (14)	1-215 34 (48)	-1.8	0.06
Reg NK cell (%)	Min-Max Median (IQR)	7.3-61.8 30.7 (23.8)	7.7-59.1 26 (24.9)	-0.12	0.9

AEC: absolute eosinophil count; ALC: absolute lymphocyte count; ANC: absolute neutrophil count; Min: minimum; Max: maximum; NK: natural killer; Reg NK: regulatory natural killer; TLC: total leucocyte count; SD standard deviation; U: Mann Whitney test.

Table 3. Variation of the laboratory data according to degree of asthma control

Parameters		Asthma control		U Test	*p*
		Good (n=24)	Partial or Non (n=21)		
TLC (10^3/µl)	Min-Max	5.4-14.5	5.5-15.8	-0.17	0.86
	Mean (SD)	9.6 (± 2.6)	9.8 (± 2.9)		
	Median (IQR)	9.7 (4.5)	10 (5)		
ALC (10^3/µl)	Min-Max	1.8-7.9	1.5-9.4	-0.12	0.228
	Median (IQR)	3.4 (1.9)	4.8 (2.9)		
Lymphocyte (%)	Min-Max	15.5-55.4	25.4-62	-1.4	0.14
	Median (IQR)	42.2 (15.6)	45 (13.6)		
ANC (10^3/µl)	Min-Max	2-9	2.1-8.2	-0.34	0.7
	Median (IQR)	4 (2.3)	3.9 (2.8)		
AEC (10^3/µl)	Min-Max	0.012-0.7	0.03-0.6	-0.216	0.82
	Median (IQR)	0.2 (0.33)	0.14 (0.28)		
IgE (IU/ml)	Min-Max	35-800	103-495	-0.49	0.62
	Median (IQR)	204 (144)	230 (102)		
NK count per µl)	Min-Max	15-623	16-563	-1.3	0.17
	Median (IQR)	57 (224)	115 (193)		
NK %	Min-Max	0.5-8.7	0.6-11.9	-0.71	0.47
	Median (IQR)	2.39 (4.8)	2.5 (5.5)		
Reg NK count per µl)	Min-Max	16-563	1-215	-1.3	0.18
	Median (IQR)	13 (35)	32 (43)		
Reg NK (%)	Min-Max	7.3-61.8	7.7-59.1	-0.94	0.34
	Median (IQR)	25.2 (18.9)	30 (26.8)		

AEC: absolute eosinophil count; ALC: absolute Lymphocyte count; ANC: absolute neutrophil count; Min: minimum; Max: maximum; NK: natural killer cell; Reg NK: regulatory natural killer cell; TLC: total leucocyte count; SD standard deviation; U: Mann Whitney test.

DISCUSSION

In the current study, regulatory NK cells, identified by the expression of CD16+CD56 +CD158+, were significantly higher in asthmatic patients compared to controls. Thus, although the total NK cell counts were not elevated in our series, there was relative over expression of the regulatory subset. Regulatory NK cells, however, did not vary significantly with the coexistence of allergic rhinitis. The small sample size and enrollment while under therapy might have affected our results. Regulatory NK cells, characterized by IL-10– and TGF-β– secretion, were previously reported to play major roles in immune regulation in the context of viral infection and they are thought to promote transplant and pregnancy tolerance.[16] IL-10 has been shown to suppress both cytokine production and antigen-specific proliferation of T-helper 1 (Th1) and Th2 cells.[17] In 2015, Deniz et al demonstrated an increase in percentage of both IL-10 secreting regulatory NK and IL-4 NK2 subsets in atopic patients with allergic rhinitis in comparison with healthy controls.[18]

Among our series, regulatory NK cell absolute counts tended to be higher among patients with moderate/severe asthma versus those with mild asthma but the difference did not reach statistical significance. This finding might be limited by the sample size. Regulatory NK cell relative and absolute counts did not vary significantly with the degree of asthma control in our subjects despite seemingly higher percentage values among the partially controlled/uncontrolled group. The elevation of regulatory NK cells among asthmatic patients might occur in the context of allergic reaction control attempts. The expected increase in IL-10 secretion may thereby decrease levels of Th2 cytokines and suppress proinflammatory Th1 cytokine secretion, thus decreasing host tissue damage.[19-21] Further studies linking the regulatory NK cells to viral replication and cytokine secretions may elucidate the actual role of NK regulatory cell subset in respiratory allergy.

The total counts and percentages of $CD16^{+}CD56^{+}$ NK cells were comparable among our atopic children and matched controls. Also, their expression did not vary significantly with the coexistence of allergic rhinitis or the degree of asthma control. However, NK cells absolute counts and percentages tended to be higher among patients with moderate/severe asthma as compared to mild asthma, but the difference did not reach statistical significance. Several studies have addressed the role of total NK cells in allergy.[7-11] A relevant study that included 12 adult atopic patients with history of allergic rhinitis (mean age 33 years) of whom four had concomitant asthma, reported that a NK cell subpopulation expressing CD56+CD16+ (INF-γ secreting) was significantly reduced in the patients as compared to 9 non-atopic healthy controls. Also, patients' NK cells showed decreased capability of inducing dendritic cell maturation and proliferation, proved by assessment of IL-12 secretion. The authors concluded that allergic diseases may be characterized by an impairment of peripheral blood NK cells to interact with dendritic cell and promote and maintain appropriate TH-1 responses.[12]

In another study, a significant decrease in NK ($CD16^{+}$ $CD56^{+}$) cell count was demonstrated in the peripheral blood of 20 children with atopic dermatitis (AD) as compared to 20 matched healthy children. The authors attributed this reduction in peripheral NK cell numbers to chemokine-dependent NK cell recruitment from peripheral blood to the inflamed skin.[13] Similar findings were also previously reported by *Katsuta et al. (2006).*[14] In animal models, increased NK cells were observed in the lung following antigen challenge.[11]In asthmatics, the NK cell phenotype was found to be altered and may thus contribute to the promotion of a proinflammatory Th2-type environment.[2] The ability of NK cells to produce Th2-type cytokines and their pivotal role in combating respiratory infections which cause airway dysfunction in asthmatics suggest that they may directly contribute to the immunopathogenesis of allergic airway disease.[15]

In our series, house dust mite was the most common aeroallergen causing sensitization among our patients (93%), followed by aspergillus (33%) and cockroach allergens (8%). Our results are supported by previous studies conducted on asthmatic children from our community.[22,23]

Our study has some limitations; it was conducted in a cross-sectional pattern on a small sample. Second, all patients enrolled were on treatment including inhaled and/or intranasal corticosteroids. Assessment of both total and regulatory NK cells in steroid naïve allergic patients might reveal the effect of treatment on NK cell. We also relied on detecting surface receptors for recognizing NK and regulatory NK cell subsets while measuring cytokine secretion by NK cell subsets could have provided more conclusive results. Finally, the age range was wide in respect to our sample size and this may have impeded the interpretation of data as inflammatory parameters do vary with age.

In conclusion, the results of this pilot study show that regulatory NK cell are increased in asthmatic children and may have a tendency to increase with severity. A possible protective role in the context of

asthma pathogenesis needs verification from wider scale studies. These should involve measurement of different pro- and anti- inflammatory cytokines and viral loads in correlation to regulatory NK cells. Studies investigating the impact of blocking the action of regulatory NK cells in respiratory allergy might also unmask their role whether protective or pathogenic and may have possible therapeutic potentials.

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