

LETTER

Innate lymphoid cell subsets in obese asthma patients: Difference in activated cells in peripheral blood and their relationship to disease severity

To the Editor,

The parallel increase in the prevalence of obesity and asthma has suggested a relationship between these two diseases in recent years. The identification of common or distinct pathophysiological mechanisms associated with these two chronic diseases has not yet been clarified. Although Type 2 asthma has classical characteristics of Th2 cells, group 2 innate lymphoid cells (ILC), their cytokines, IL-4, IL-5, and IL-13 and eosinophilia, the classical Type 2 immune response is more complex in obese patients, mainly due to the involvement of fat tissue in immune responses. In obese asthmatics, asthma is generally nonatopic, and ILC3s play a critical role in obesity-induced airway hyperreactivity. However, it has recently been shown that eosinophils are also predominant in the lung tissue of obese asthma patients. In addition, increased T2 response and ILC2s are observed in obese asthmatic patients.^{1,2}

Under homeostatic conditions, adipose tissue is infiltrated by ILC2, but ILC2 and ILC3 levels decrease, whereas ILC1 levels increase in response to the chronic ongoing inflammation in obesity.¹ In obese asthmatics, ILC2s and ILC3s are decreased in the adipose tissue and increased in lungs potentially secondary to the redistribution to lungs. Because of this trafficking, they can also be detected in peripheral blood.¹ It was reported that ILC3s mediate obesity-associated asthma through IL-1 β production by macrophages, leading to the release of IL-17.² Similarly, ILC2s are found to be activated in the blood or sputum of obese asthmatics.³ Particularly, total numbers of ILC2s were significantly increased in allergic and/or eosinophilic asthma and ILC2s were related to corticosteroid unresponsiveness in T2 asthma.^{3,4} While increased numbers of ILC2s may provide benefits for obesity by creating metabolic homeostasis, it is known to have detrimental effects in terms of asthma. These conflicting results call for further research. In the present study, we aimed to investigate the differences in the distributions of the main ILC groups (Lin⁻ CD45⁺CD127⁺CD161⁺total ILC, c-kit⁺CRTH2⁻ ILC1, c-kit⁺CRTH2⁺ ILC2, and c-kit⁺CRTH2⁻ ILC3) and the activated subgroups of the ILCs (CD25⁺ total ILC, ILC1, ILC2, ILC3; CD69⁺ total ILC, ILC1, ILC2, ILC3; Nkp44⁺ILC3) between obese asthma (OA) and normal-weight asthma (NWA) patients in comparison with obese control (OC) and normal-weight control (NWC). The c type lectin

receptor CD69 is the earliest activation and tissue residency marker. The most prominent cellular activation marker CD25 is the alpha chain of the trimeric IL2 receptor. We isolated peripheral blood mononuclear cells (PBMC) in total of 80 women (Figure S1) and counted main and activated subgroups of ILCs with flow cytometry (Figure S2). Demographic specificities of the patient groups were given at Table S1.

Obese asthma patients tended to have higher CD69⁺ILC subsets, while lower CD25⁺ILC subsets than OCs. However, significant results were found only in the percentages of CD69⁺total ILCs, CD69⁺ILC3, CD25⁺total ILCs, and CD25⁺ILC3s (Figure 1). On the contrary, in terms of cell counts in the viable CD45⁺lymphocytes, we showed that OA patients had lower CD161⁺total ILC, ILC1, CD69⁺ILC1 than NWCs. In addition, we found that both CD25⁺ and CD69⁺ innate lymphoid cell counts tended to be lower in NWA patients than NWCs. However, only significant results were detected in CD69⁺ILC1 and CD25⁺ILC3 groups (Figure 2A).

In terms of asthma severity, CD69⁺total ILCs, ILC2s, CD69⁺ILC2, CD69⁺ILC3 were higher in severe asthma patients than mild asthmatics in this study. In addition, we showed that CD161⁺total ILCs, CD69⁺ total ILCs, ILC2, CD69⁺ ILC2, ILC3, CD69⁺ ILC3 were higher in the circulation of moderate asthma patients compared to mild asthmatics (Figure 2B). Our results failed to show any significance between moderate and severe asthma, although there was a tendency.

Supporting our findings of low CD25 and high CD69 expression in total ILCs, ILC1, ILC2, and ILC3s in OA patients, it was found that under non-pathological conditions, the majority of ILCs expressed CD25 even in PBMCs, whereas CD69 was found to be upregulated in pathological tissues, such as omentum adipose tissue, lung tumor, and colorectal tumor.⁵ Later, it was reported that circulating ILCs did not express CD69, which is known as a marker of early activation and tissue residence, but the demonstration of CD69⁺ILCs in the peripheral blood may exhibit that these cells are redistributed from tissues after local tissue activation.⁶

Low peripheral ILC1 levels in OA may be secondary to the increased accumulation of ILC1s in the adipose tissue. In particular, ILCs accumulate and are activated in inflamed tissues. In NWA,

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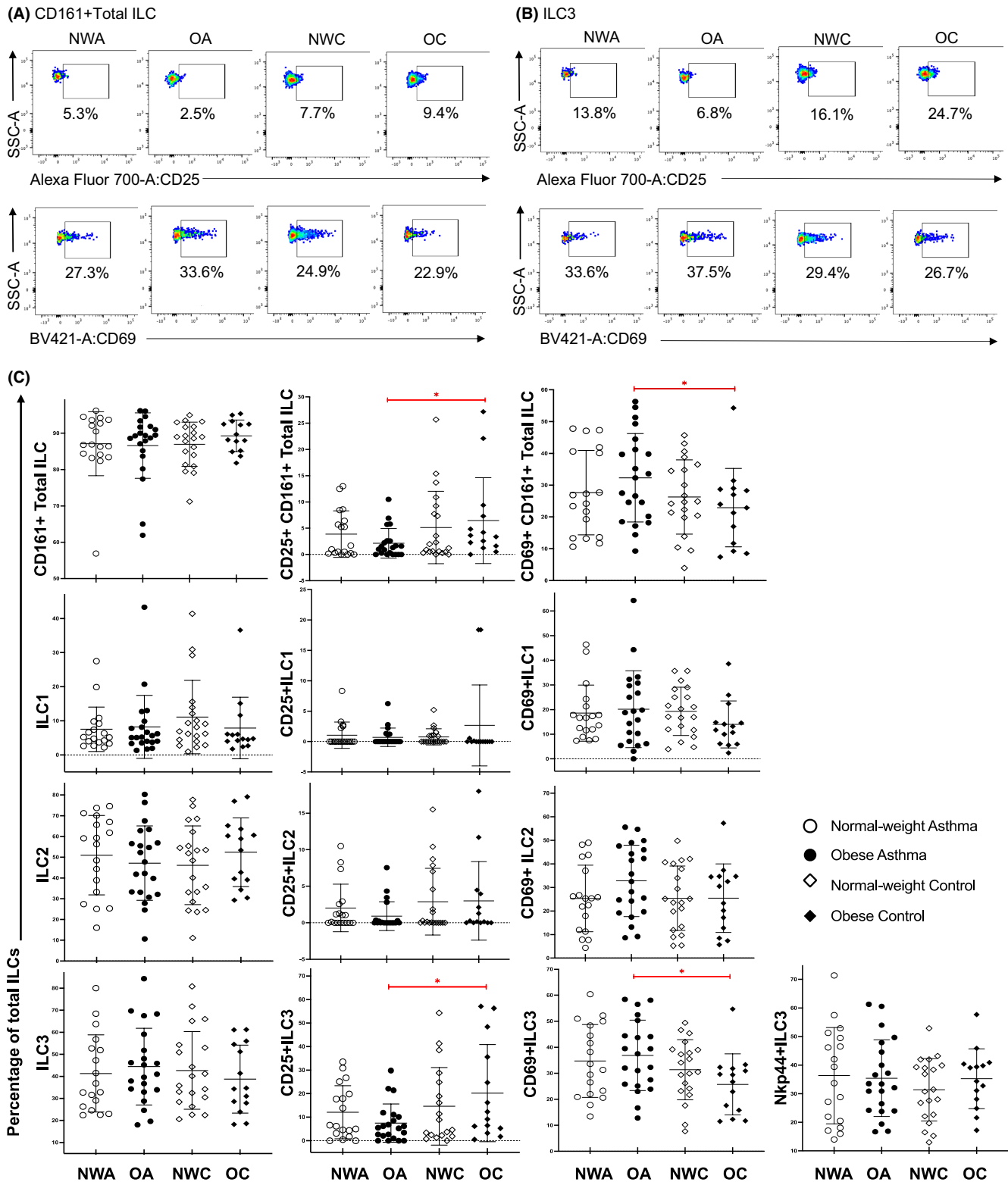


FIGURE 1 Innate lymphoid cell subsets in peripheral blood. (A) Representative data of CD25⁺CD161⁺total ILCs and CD69⁺CD161⁺total ILCs according to NWA, OA, NWC, OC study groups. (B) Representative data of CD25⁺ ILC3s and CD69⁺ ILC3s according to NWA, OA, NWC, OC study groups. (C) Cumulative data of the ILCs according to 4 main study groups. CD25⁺CD161⁺ total ILCs and CD25⁺ILC3s were lower in OA than OC ($p = .04$, $p = .04$, respectively). CD69⁺CD161⁺ total ILCs and CD69⁺ ILC3s were higher in OA than OC ($p = .02$, $p = .02$, respectively). (NWA, Normal-weight asthma; OA, Obese asthma; NWC, Normal-weight control; OC, Obese control)

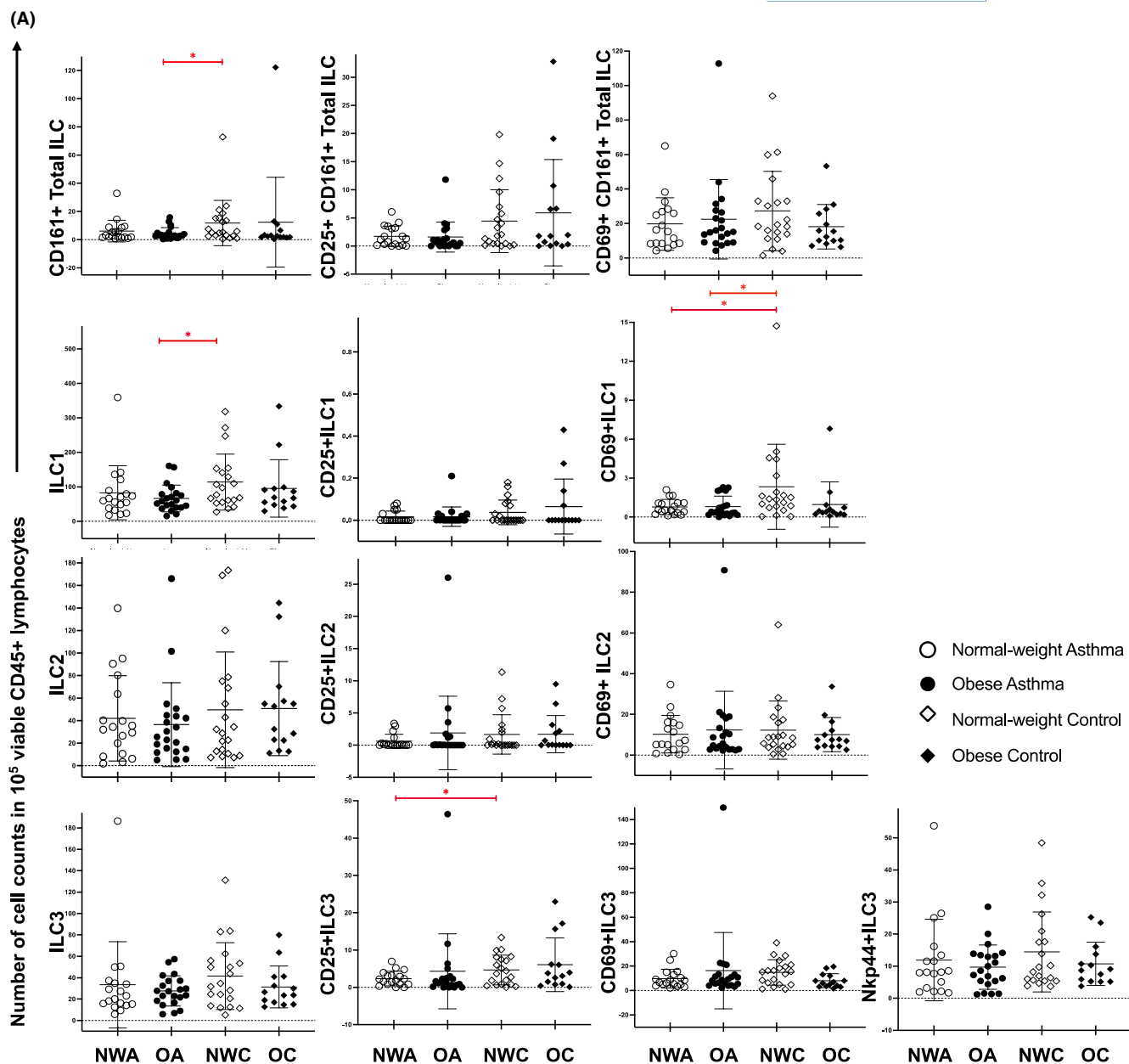


FIGURE 2 (Continued)

ILCs, particularly ILC2s and ILC3s, accumulate and become activated in lungs which may be the reason of the lower CD25⁺ and CD69⁺ILC subsets in the peripheral blood of NWA than in NWC.

ILCs and their relationship to asthma severity has been studied several times. It was shown that circulating ILC2s were higher in severe asthmatics compared with patients with mild asthma.⁷ Moreover, increased expression of IL-17 in lungs of severe asthma patients suggested ILC3s as well as ILC2s are involved in inflammation.² Consistent with these results, we found higher activated ⁺total ILCs, ILC2s, ILC3s in severe asthma patients than mild asthmatics.

Our results suggest that total ILCs and ILC3s are activated and migrated to the lungs in OA patients as they were low in the circulation. In addition, asthma severity was associated with an increase in ILCs, particularly in CD69⁺ activated CD161⁺total ILC, ILC2, and ILC3 subgroups. Indeed, altered epithelial barrier functions seem to play an essential role in the activation and upregulation of ILCs. Because of chronically persistent inflammation and impaired epithelial barriers in adipose and lung tissue in OA patients, it is not surprising that increased and activated ILCs are found in inflamed tissues. Altogether, the present study demonstrates that total ILCs and ILC3 and activated ILCs show major changes in chronic ongoing inflammation in OA patients.

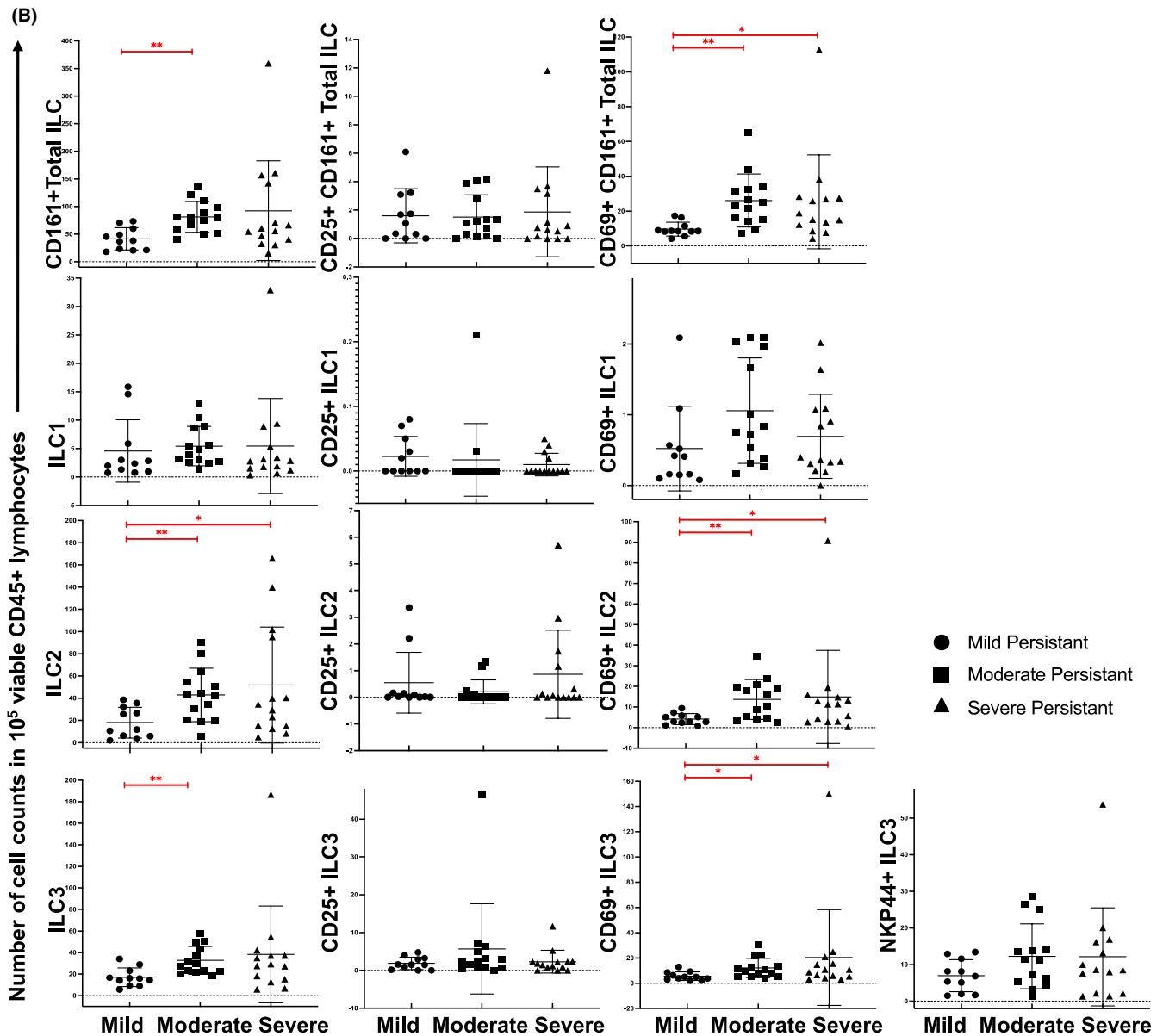


FIGURE 2 ILC subsets in peripheral blood show a relationship to asthma severity. Cell counts in 10^5 viable CD45⁺ lymphocytes. (A) CD161⁺total ILC, ILC1, CD69⁺ILC1 were lower in OA than NWC ($p = .04$, $p = .03$, $p = .03$, respectively). CD69⁺ ILC1 and CD25⁺ILC3 were lower in NWA than NWC ($p = .04$, $p = .03$, respectively). (B) CD161⁺total ILCs, CD69⁺ total ILCs, ILC2, CD69⁺ ILC3 were higher in moderate asthma patients than mild asthmatics ($p = .001$, $p = .001$, $p = .006$, $p = .003$, $p = .002$, and $p = .01$, respectively). CD69⁺total ILCs, ILC2s, CD69⁺ILC2, CD69⁺ILC3 were higher in severe asthma patients than mild asthmatics ($p = .01$, $p = .03$, $p = .03$, $p = .02$)

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Supplementary Material

Methods

Study design

This study was designed as a prospective study conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Ankara University (Approval no: 04-302-19). All patients signed the written informed consent forms and allowed their files to be evaluated.

Patient selection

Patients who were diagnosed with asthma according to the GINA criteria¹ and have been followed up for at least one year with complete file records, were evaluated according to the inclusion and exclusion criteria. Considering the fact that the female male ratio was approximately 4/5 in our outpatient clinic and high throughout the country² as well as the possible differential effects of sex-related hormones on adipose tissue, males were excluded from the study in order to avoid data confusion. Subjects who have had an asthma attack and/or a history of bacterial or viral infection in the past 1 month, who smoked more than 10 packs/year or are current smokers, who were pregnant or lactating, who were using oral corticosteroid therapy, who were receiving immunotherapy or biological agent treatment in the past 3 months, and those who were included in any diet and/or exercise program in the past 1 month were excluded from the study. In addition, subjects with comorbidities [cardiac (atherosclerotic), renal, hepatic, rheumatologic, malignancy] affecting ILC levels were excluded.

Study population and patient classification

Obese and normal-weight female asthmatic patients and a control group of obese and normal-weight healthy women were included in the study. Women with body mass index (BMI) values (kg/m²) of 18.5–24.9 were categorized as normal-weighted, 25.0–29.9 as overweighted and with BMI greater than or equal to 30 as obese.³ Phenotypic characteristics (allergic/nonallergic, early onset/adult onset/late onset, eosinophilic/non-eosinophilic) and disease severity (mild/moderate/severe) were determined by retrospectively examining the records of the asthmatic patients for the past 1 year.

Patients diagnosed under eighteen years of age were defined as early onset, between 18-40 years of age as adult onset and at 40 years and older as late onset asthma.^{4,5} According to skin

prick test results subjects demonstrating sensitivity to at least one inhalant allergen by using common aeroallergen extracts; *Dermatophagoides pteronyssinus*; *Dermatophagoides farinae*; mixtures of grass pollens, weed pollens, tree pollens, and cereal pollens; molds, and animal epithelia (cat and dog); (ALK, Abello, Spain) along with appropriate positive (histamine: 10 mg/mL) and negative controls (saline) with a wheal size ≥ 3 mm at 15th minute early readings and a compatible history were considered as allergic. Or sIgE measurement ≥ 3.51 kU/L with a compatible history was defined as allergic by CAP fluoroenzyme immunoassay according to the manufacturer's recommendation (Phadia, Uppsala, Sweden). Asthmatic patients were defined as eosinophilic if, at any time during follow up (between the diagnosis and the last visit), the absolute eosinophil count was measured at least once $\geq 300/\mu\text{L}$ during the oral corticosteroid free period or $\geq 150/\mu\text{L}$ under oral corticosteroids. They were classified as noneosinophilic if in at least three measurements, the absolute eosinophil count did not reach $300/\mu\text{L}$ during the oral corticosteroid free period or $150/\mu\text{L}$ under corticosteroid.¹ Asthma severity was determined according to the treatment step at the last visit. Patients receiving step 1 to 2 treatment were recorded as mild asthma and, those given step-3 treatment were classified as moderate. Patients were defined as severe asthma if they needed steps 4-5 to achieve control, or step 4-5 treatment was not sufficient to achieve control, although the correct inhaler technique was provided and disruptive factors such as treatment of comorbidities and reduction of exposure to triggers were managed.¹

Spirometric measurements

Spirometric measurements (forced expiratory volume in one second [FEV1], forced vital capacity [FVC], peak expiratory flow [PEF] and maximum mid expiratory flow [MMF]) were performed using a spirometry device (ZAN 100, Germany) and evaluated according to the American Thoracic Society/ European Respiratory Society (ATS/ERS) guidelines.⁶

Human blood samples & flow cytometry

Blood samples were taken from all subjects. Peripheral blood mononuclear cells (PBMC) were separated from the samples according to the density gradient method and stored in a liquid nitrogen tank. Frozen PBMCs were thawed according to the established procedure with minimal damage to cell viability in Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland. Number of cells and viability rates were evaluated in Neubauer chamber, 5 patients were excluded and not studied because of low cell count and/or low cell viability (Figure S1). Then, PBMCs were stained with the created ILC panel. This is a well-established method to stain ILCs.⁷

Accordingly, PBMC samples were stained first with Dump Stain Master Mix which is containing FITC-labelled surface antibodies to exclude dendritic cells, plasmacytoid dendritic cells, monocytes, macrophages, stem cells, NK cells, basophils, T and B lymphocytes. Then a Full Stain panel were used to isolate ILCs; containing CD45, CD161 and CD127 (IL-7R α) to isolate the total ILC population, and CRTH2 (PGD2 receptor), CD117(c-kit) to differentiate ILC1s, ILC2s and ILC3s. The Full Stain panel also includes Nkp44 for ILC3 subsets and CD69 (c-type lectin protein) and CD25 (IL2R α) to evaluate the activation of cells. Isotype control tubes containing the colours PE, PE-CF594, PerCPCy5.5, AF647, AF700, BV421 were prepared for each patient sample to precise the gates determined in the flow cytometric analysis (Table S2). Flow cytometric measurements were made according to the compensation matrix of the ILC panel prepared on the BD LSR FORTESA (16 colour analyzer) and were analyzed using the Flowjo LLC Software (Version 10, San Jose, CA, USA). 2 patients' measurements were excluded due to compensation error (Figure S1). The gating strategy was shown in Figure S2.

Statistical analysis

Statistical analyzes were performed using SPSS version 20 software, FlowJo and Graphpad prism 8 programs. The conformity of the variables to the normal distribution was examined using visual (histogram and probability graphs) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk tests). Descriptive statistics were calculated by using the mean and standard deviation for normally distributed variables, and using the median and interquartile range for non-normally distributed variables.

The data of the patients in the obese asthmatic (OA), normal-weight asthmatic (NWA), obese control (OC) and normal-weight control (NWC) groups, were compared with each other in terms of total ILCs, ILC1, ILC2, ILC3, ILC subgroups. In these comparisons, dependent groups were: CD127⁺ CD161⁺ total ILC; CD127⁺ CD161⁺ CD25⁺ total ILC; CD127⁺ CD161⁺ CD69⁺ total ILC; CD117⁺ CRTH2⁻ ILC1; CD117⁺ CRTH2⁻ CD25⁺ ILC1; CD117⁺ CRTH2⁻ CD69⁺ ILC1; CRTH2⁺ ILC2; CRTH2⁺ CD25⁺ ILC2; CRTH2⁺ CD69⁺ ILC2; CD117⁺ CRTH2⁻ ILC3; CD117⁺ CRTH2⁻ CD25⁺ ILC3; CD69⁺ CD117⁺ CRTH2⁻ ILC3; Nkp44⁺ CD117⁺ CRTH2⁻ ILC3. The independent variable consisted of OA, NWA, OC and NWC groups. Categorical data were obtained between these four independent groups with the Chi-square test. Significance of the difference between the means in the normally distributed groups calculated by the Student-T test or One-Way Anova Variance Analysis, and to find the significance of the difference between the median values in the non-normally distributed

groups Mann-Whitney-U or Kruskal Wallis tests were used. In similar studies in the literature, 15-20 patients were included in each four group. Accordingly, the sample size analysis, the minimum number of patients to be included in each group was calculated as 18 with 95% power and 0.05 error. (R.3.0.1 open source software).

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136 **Table S1: Demographic specificities of the study groups**

		NWA (n=18)	OA (n=21)	P value for asthma groups	NWC (n=20)	OC(n=14)	P value for all group
Age (mean ±SD)		41.11±12.01	55.76±9.38	<0.0001	35.95±12.64	42 ±14.02	<0.0001
BMI (mean±SD)		22.49±1.95	35.38±4.85	<0.0001	21.84±1.58	32.53±2.73	<0.0001
Allergy n, (%)	present	10, (55.6)	8, (38.1)	0.34	0	0	<0.0001
	absent	8, (44.4)	13, (61.9)		20(100)	14 (100)	
Eos. n, (%)	present	9, (50)	10, (47.6)	1.000	0	0	<0.0001
	absent	9, (50)	11 (52.4)		20(100)	14(100)	
Asthma duration (mean ±SD), year		9.39±8.79	14.48±9.88	0.100	-	-	-
Age of disease onset (mean ±SD), year		31.78±9.17	41.33±11.97	0.009	-	-	-
Asthma onset n, (%)	Early	3, (16.7)	1, (4.8)	0.040	-	-	-
	Adult	11 (61.1)	7, (33.3)		-	-	-
	Late	4, (22.2)	13, (61.9)		-	-	-
Asthma severity n, (%)	Mild	5, (27.8)	3, (14.3)	0.582	-	-	-
	Moderate	8, (44.4)	11, (52.4)		-	-	-
	Severe	5, (27.8)	7, (33.3)		-	-	-
Allergic Comor.* n, (%)	present	14, (77.8)	10, (47.6)	0.098	-	-	-
	absent	4, (22.2)	11, (52.4)		-	-	-
Systemic Comor.** n, (%)	present	3, (16.7)	10, (47.6)	0.05	2(14.3)	3(15)	0.04
	absent	15, (83.3)	11, (52.4)		12(85.7)	17(85)	
PFT (%)	FEV1	97.38±13.50	94.33±15.23	0.515	-	-	-
	FVC	103.11±10.56	100.95±14.45	0.603	-	-	-
	FEV1/ FVC	81.16±7.99	78.47±6.36	0.250	-	-	-
	MMF	74.22±20.83	65.09±23.96	0.216	-	-	-

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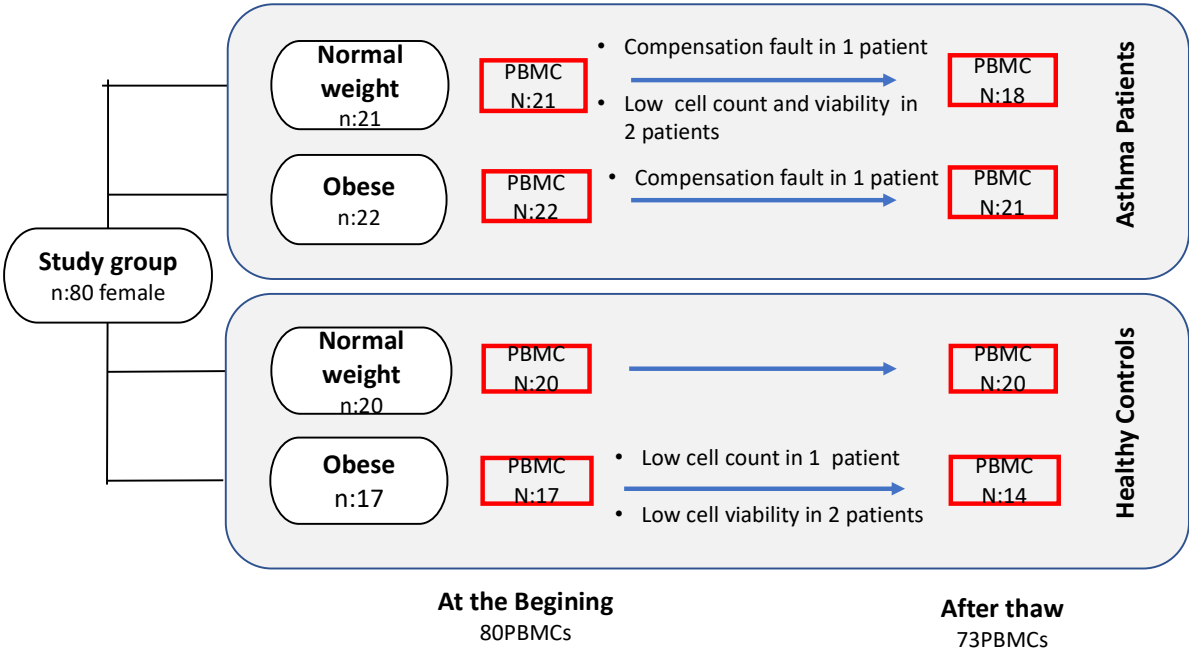
NWA: Normal-weight asthma, OA: Obese asthma, NWC: Normal-weight control, OC: Obese control, BMI: Body mass index, Eos: Eosinophilia, Comor: Comorbidity, PFT: pulmonary function test
 *Allergic rhinitis, food allergy, drug allergy, atopic dermatitis, venom allergy, latex allergy, urticaria
 **Gastroesophageal reflux disease, thyroid disorders, obstructive sleep apnoea syndrome, cardiac valve pathologies, cardiac rhythm problems, anxiety disorders, nasal polyposis.

Table S2: Antibodies used to discriminate ILC cell subsets

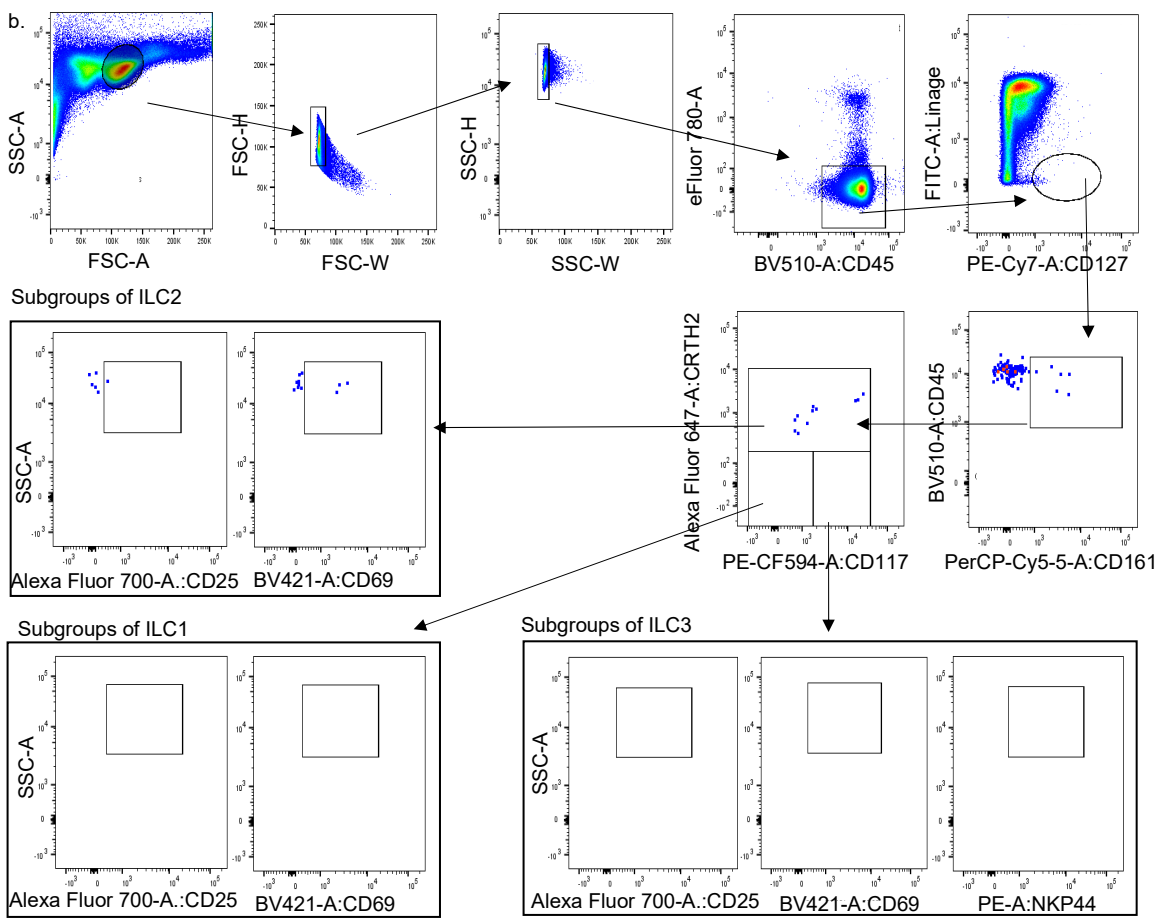
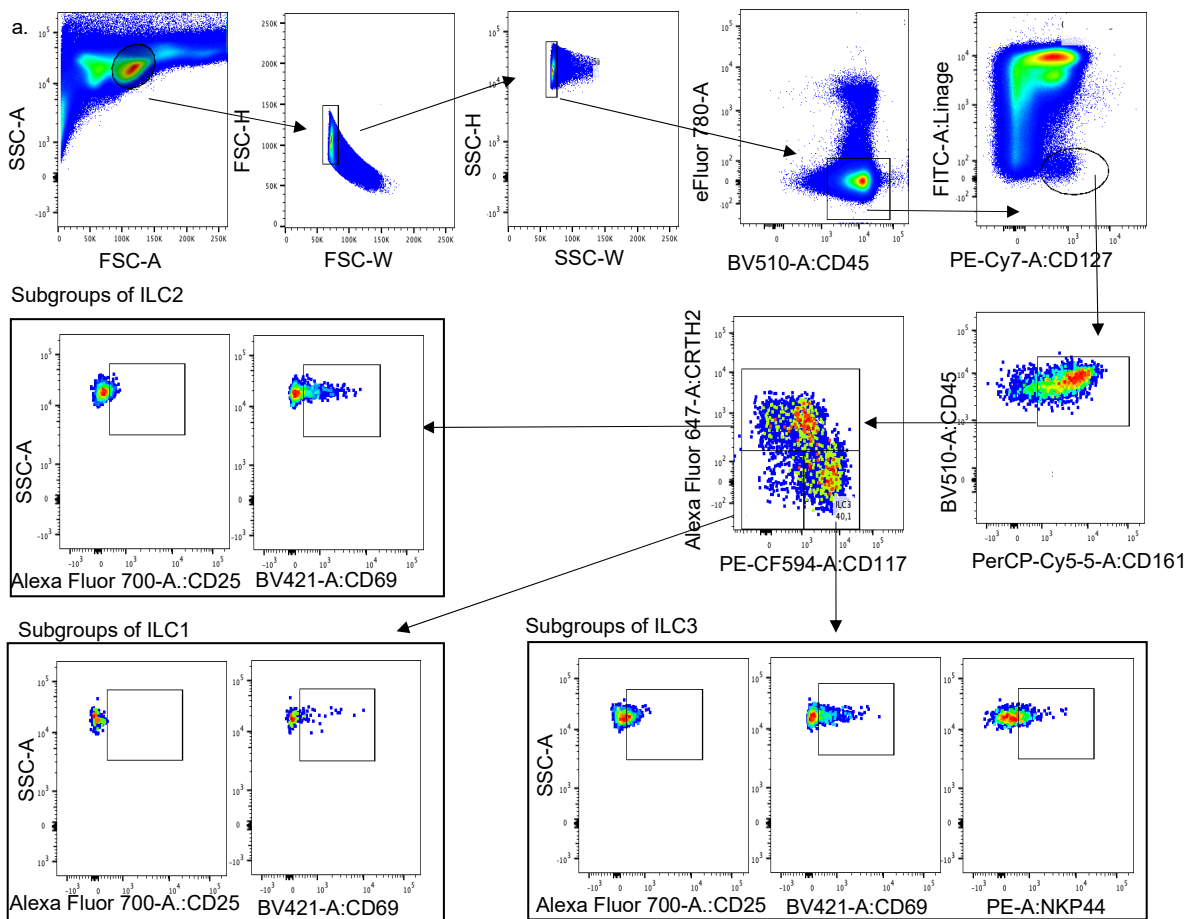
		Antibodies	Colour	Isotype	Clone
DUMP STAIN	Dendritic cell	CD11c	FITC	M IgG1k	3,9
	Hematopoietic stem cells	CD34	FITC	M IgG1k	581
	NK cell	CD94	FITC	M IgG1k	DX22
	Plasmacytoid dendritic cell	CD303	FITC	M IgG2ak	201A
		CD123	FITC	M IgG1k	6H6
	Dendritic cell, monocytes, mast cell, basophil	FceR1a	FITC	M IgG2bk	AER-37
	Monocytes	CD14	FITC	M IgG1k	HCD14
	NK cell, neutrophil, monocyte, macrophage	CD16	FITC	M IgG1k	B73.1
	T cell	CD1a	FITC	M IgG1k	HI149
		CD3	FITC	M IgG1k	UCHT1
		CD4	FITC	M IgG1k	RPA-T4
		CD8a	FITC	M IgG1k	RPA-T8
	B cell	CD19	FITC	M IgG1k	HIB19
	FULL STAIN	Total ILC	CD161	PerCPCy5.5	M IgG1k
CD127(IL-7Rα)			PE/Cy7	M IgG1k	HIL-712-M12
ILC2		CRTH2 (PGD2 receptor)	AF647	Rat, IgG2a, k	BM16
ILC3		CD117 (c-Kit)	PE-CF594	M IgG1k	YB5.B8
Activity		CD25 (IL2Rα)	AF700	M IgG1k	M-A251
		CD69 (c-type lectin protein)	BV421	M IgG1k	FN50
		NKp44 (CD 336)	PE	M IgG1k	P44-8
Lymphocyte		CD45	V510	M IgG1k	HI30
Viability		APC-Cy7			
AF780					
ISOTY PE			PE	M IgG1k Iso	MOPC-21
			PE-CF594	M IgG1k Iso	X40
			PerCPCy5.5	M IgG1k Iso	MOPC-21

	AF647	R IgG2ak Iso	
	AF700	M IgG1k Iso	MOPC-21
	BV421	M IgG1k Iso	MOPC-21
	PE	M IgG1k Iso	MOPC-21

NK: Natural killer, ILC:Innate lymphoid cell



Supplementary Figure 1: Study diagram. Initially we had 80 female patients; There were 21 normal weight, 22 obese asthma patients, 20 normal weight, 17 obese controls. We collected PBMCs from all patients. After thaw, we excluded 1 normal weight and 1 obese asthmatic patient due to compensation error during flow measurement and 2 normal weight asthmatic patients due to low cell count and viability. Among healthy controls, 1 patient was excluded due to low cell counts and 2 patients due to low cell viability.



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Supplementary Figure 2: Gating strategy for ILCs. **a.** Full Stain, **b.** Isotype control

Gating strategy for ILCs. Lymphocytes were first gated in the forward and side scatter plot. After doublet discrimination, live CD45⁺ lymphocytes were gated. CD127⁺CD161⁺ total ILCs were gated after lineage negative cells were gated. Among the CD127⁺CD161⁺ total ILCs, CD117⁻CRTH2⁻ ILC1, CRTH2⁺CD117⁺ ILC2 and CRTH2⁻CD117⁺ILC3 were then identified. Each ILC group were further gated separately from the side scatter plot to differentiate CD25⁺ and CD69⁺ cells. In addition, ILC3s were gated for Nkp44⁺ ILC3 cells.