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ORIGINAL RESEARCH

The association between eosinophils (CD16+ eosinophils), basophils (CD203+ basophils), and CD23 B lymphocytes in patients with atopic dermatitis on dupilumab therapy: pilot study

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ABSTRACT

Background: Eosinophils, basophils, and the molecule CD23 on B cells are involved in the pathophysiology of atopic dermatitis (AD). The molecule CD23 is involved in the regulation of IgE synthesis and is expressed by activated B cells. The molecule CD16 is used to assess the activation of eosinophils and CD203 of basophils. The association between the count of eosinophils, basophils, CD16+ eosinophils, CD203+ basophils and the expression of the activation marker CD23 on B cells in patients with AD (with and without dupilumab therapy) is not described.

Objective: The aim of this pilot study is to evaluate the association between the blood count of eosinophils, basophils, relative CD16+ eosinophils, relative CD203+ basophils, and the expression of molecule CD23 on B cells and on their subsets (total, memory, naive, switched, non-switched) in patients suffering from AD (with and without dupilumab therapy) and in control group.

Methods: A total of 45 patients suffering from AD were examined; 32 patients without dupilumab treatment (10 men, 22 women, average age 35 years), 13 patients with dupilumab treatment (7 men, 6 women, average age 43.4 years), and 30 subjects as a control group (10 men, 20 women, average age 44.7 years). Immunophenotype was examined by flow cytometry in which monoclonal antibodies with fluorescent molecules were used. For statistical analysis we used non-parametric Kruskal–Wallis one-factor analysis of variance with post hoc by Dunn's test with Bonferroni modification and the Spearman's rank correlation coefficient; for coefficients higher than 0.41, we report R^2 (percent of variation explained).

Results: The absolute count of eosinophils was significantly higher in patients with AD (with and without dupilumab) in comparison to healthy subjects. The difference in the relative count of CD16+ eosinophils in patients with AD (with and without dupilumab therapy) compared with control is not statistically significant. In patients with dupilumab therapy the significantly lower count of relative CD203+ basophils was confirmed compared with control. The higher association between the count

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of eosinophils (absolute and relative) and the expression of CD23 marker on B cells was confirmed in patients with dupilumab therapy; in contrast, this association was low in patients with AD without dupilumab therapy and in healthy subjects.

Conclusion: The higher association between the count of eosinophils (absolute and relative) and the expression of CD23 marker on B cells was confirmed in patients with AD under dupilumab therapy. It suggests that IL-4 production by eosinophils may play a role in B lymphocyte activation. The significantly lower count of $CD203^+$ basophils has been demonstrated in patients with dupilumab therapy. This reduction of $CD203^+$ basophil count may contribute to the therapeutic effects of dupilumab by reducing the inflammatory response and allergic reactions in patients with AD.

Keywords: Eosinophils; Basophils; Activation markers CD16; CD203; Expression of CD23 on B cells; Atopic dermatitis patients; Dupilumab therapy

Key Summary Points

Why carry out this study?

Eosinophils, basophils, and the activation marker CD23 on B cells are involved in the pathophysiology of AD.

The association between the blood count of eosinophils, basophils, $CD16^+$ eosinophils, $CD203^+$ basophils, and the expression of CD23 molecule on B lymphocytes in patients with AD (with and without dupilumab therapy) is not described.

What was learned from the study?

The higher association between the count of eosinophils (absolute and relative) and the expression of CD23 marker on B cells was confirmed in patients with AD under dupilumab therapy; meanwhile, this association is low in patients with AD without dupilumab therapy and in healthy subjects. Our results suggest that B-lymphocyte activation (increased expression of molecule CD23 on B cells) in patients with dupilumab therapy may be mediated with IL-4 production by eosinophils.

In patients under dupilumab therapy, the significantly lower count of $CD203^+$ basophils was confirmed compared with patients without dupilumab and with healthy subjects. Dupilumab therapy further reduces the relative count of $CD203^+$ basophils by inhibiting the activity of IL-4 and IL-13, which are key drivers of basophil activation and function. This reduction of $CD203^+$ basophil count may contribute to the therapeutic effects of dupilumab by reducing the inflammatory response and allergic reactions in patients with AD.

For a more accurate evaluation of the effect of biological treatment on the immunological profile in patients with AD, it is necessary to measure the count of $CD203^+$ basophils, $CD16^+$ eosinophils, and the expression of CD23 marker on B cells before starting the biological treatment and to monitor these parameters during treatment in one group of patients. Further studies are needed to demonstrate that IL-4 produced by eosinophils in dupilumab patients is responsible for the activation of B cells with help of CD23 molecule.

INTRODUCTION

Atopic dermatitis (AD) is the most common chronic skin condition, affecting up to 20% of the population [1]. It is characterized by intense pruritus and epidermal barrier dysfunction [1]. The inflammation is biphasic, evolving from an initial, acute, Th2- and Th22-dominated phase to a chronic phase characterized by the concomitant presence of T helper Th1, Th2 cells, and Th17 cells [1]. The type 2 inflammatory pathway is driven by activation of type 2 CD4$^+$ helper cells and innate lymphoid type 2 cells (ILC2), resulting in tissue infiltration of inflammatory cells such as eosinophils, mast cells, basophils, and production of proinflammatory cytokines, including IL-4, IL-5, and IL-13 [2]. The IL-4Rα antibody, dupilumab, binds IL-4Rα with high affinity, directly blocking IL-4 and IL-13/IL-13Rα1 complex binding to IL-4Rα, and thereby prevents IL-4Rα-mediated signaling induced by both IL-4 and IL-13 [3, 4]. IL-4 and IL-13 can each stimulate effector cells, such as eosinophils, to migrate from the blood to sites of inflammation by inducing the production of eosinophil-promoting factors, including IL-5 and eotaxins from Th2 cells and epithelial cells [3, 4].

Basophils are rare circulating granulocytes that can secrete type 2 cytokines and histamine [5]. They exacerbate Th2 cell differentiation, and they infiltrate the skin to ILC2 expansion. In the context of allergic inflammation, the function of basophils is the rapid secretion of IL-4 and IL-13 at levels greater than any other cells capable of secreting these cytokines, including Th2 cells, mast cells, and eosinophils [6, 7]. By producing IL-4 and IL-13, basophils regulate infiltration of eosinophils [6–9]. Basophils have been considered both proinflammatory and antiinflammatory [10] and they infiltrate the dermis in diverse skin conditions to induce epidermal hyperplasia [11, 12] or support the resolution of inflammation [13]. Activation of human basophils results in the release of many different mediators and the expression of new cell surface proteins. The markers CD63 and CD203c have been used in recent years to assess basophil activation, but

there have been many studies that demonstrate that expression of these markers can be dissociated from histamine release [14]. Eosinophil numbers as well as eosinophil granule protein levels in peripheral blood are elevated in most patients with AD and appear to correlate with disease activity [15]. Moreover, eosinophil granule proteins, which possess cytotoxic activity, are deposited in the skin lesions. IL-5 specifically acts on eosinophils, resulting in accelerated eosinophilopoiesis, chemotaxis, cell activation, and delayed apoptosis [15]. Transient increases in blood eosinophil counts were reported in clinical trials with dupilumab in patients with asthma, chronic rhinosinusitis with nasal polyps, and in atopic dermatitis [16, 17]. Blood eosinophils have mRNA for FcγRIIIB (CD16) but no or minimal spontaneous CD16 expression, and they express CD16 on their surface when stimulated in vitro with platelet-activating factor or IFN gamma. Transient expression of CD16 is also observed in vivo following aeroallergen challenge of asthmatic subjects [18, 19]. In various experimental allergy models, basophils and eosinophils appear to be closely linked by directly or indirectly influencing each other since they are responsive to similar cytokines and chemokines [20–22]. However, the direct evidence that eosinophils and basophils interact is still rarely described.

CD23 is an integral membrane glycoprotein involved in the regulation of IgE synthesis and proinflammatory activities, such as triggering the release of regulatory cytokines tumor necrosis factor (TNF), IL-1, IL-6, and granulocyte–macrophage colony-stimulating factor by human monocytes. It is expressed by activated B cells, monocytes, follicular dendritic cells, and subsets of eosinophils and platelets. IL-4 produced by Th2 cells is thought to provide antigen-specific help to B cells, promoting CD23 induction and isotype switching [24]. CD23 binds to IgE to regulate its activity, and can have either stimulatory or inhibitory functions [23, 24]: (1) CD23 can absorb and clear IgE from the serum in a noninflammatory fashion, (2) CD23 reduces the synthesis of IgE from B cells, and (3) CD23 regulates antigen-specific IgG and T cell responses [24].

The expression of CD23 on total B lymphocytes and on their subsets was evaluated in our previous study [25]; the significantly higher expression of the activation marker CD23 was confirmed on total B lymphocytes and on their subsets in patients with AD both with and without dupilumab therapy compared with controls [25].

The aim of this pilot study is to compare the absolute and relative count of neutrophils, eosinophils, basophils, relative CD16+ eosinophils, relative CD203+ basophils in patients with AD (with and without dupilumab therapy) and in control group. Our aim is to evaluate the association between basophils (relative, absolute, relative CD203+ basophils) and eosinophils (relative, absolute, relative CD16+ eosinophils) in patients with AD with and without dupilumab therapy and in controls. We also evaluate the association between the expression of CD23 marker on B cells and on their subsets (memory, naive, switched, non-switched, total B lymphocytes) and basophils (relative, absolute, relative CD203+ basophils) and eosinophils (relative, absolute, relative CD16+ eosinophils) in patients with AD with and without dupilumab therapy and in controls.

METHODS

During the years 2021–2022 we examined 32 patients suffering from AD without dupilumab treatment, 13 patients with dupilumab treatment, and 30 subjects as a control group. Patient characteristics are recorded in Table 1.

Dermatological Examination

Complete dermatological examination was performed in all patients included in the study. All these patients were examined in the Department of Dermatology, Faculty Hospital Hradec Králové, Charles University, Czech Republic. The diagnosis of AD was determined according to Hanifin and Rajka diagnostic criteria.

Inclusion criteria: (1) age 14 years and over and (2) AD as defined by the criteria of Hanifin

and Rajka. Patients with moderate and severe forms of AD without dupilumab and patients with dupilumab therapy lasting at least 18 months were included. Exclusion criteria: pregnancy, breastfeeding, systemic therapy (cyclosporin, systemic corticoids).

Severity of Atopic Dermatitis

Severity of AD was scored in agreement with SCORing Atopic Dermatitis (SCORAD), with assessment of topography items (affected skin area), intensity criteria and subjective parameters, and with the Eczema Area and Severity Index (EASI) system. Quality of life is evaluated with Patient Oriented Eczema Measure (POEM) and with Dermatology Life Quality Index (DLQI). The severity of AD and the quality of life were assessed every 3 months during 1 year in patients without dupilumab. In patients under dupilumab, the severity of AD and the quality of life were assessed every 3 months during 1 year before dupilumab treatment and during 18 months with dupilumab treatment.

The Onset of Atopic Dermatitis

The onset of AD was evaluated according to the patient's history (the onset of atopic dermatitis under 5 years of age or later).

The representation of patients was consistent with regard to gender, age, and onset of AD. Likewise, the representation of the control group matched with regard to gender and age to patients with AD.

As a control group, we examined 30 healthy individuals, blood donors at Faculty Hospital Hradec Králové, Charles University, Czech Republic. Total IgE was examined in control group and only healthy subjects with negative total IgE were included.

Laboratory Examination

The count of neutrophils, eosinophils, basophils Blood samples from the antecubital vein were collected in tubes pre-coated with EDTA-anticoagulant. The blood count was examined with a Sysmex XN 3000, Sysmex SP10, microscope

Table 1 Patient characteristics

Patient characteristics	AD patients without dupilumab	AD patients with dupilumab	
Age	**35.0 years** (27.2–48.7)	**43.4 years** (38.6–48.3)	
Number of patients	**32** (10 men, 22 women)	**13** (7 men, 6 women)	
SCORAD	**33.2** (26.5–38.7)	**36.1** (30.5–45.2)	Before dupilumab therapy
		10.5 (7.1–18.2)	Average value after 1.5 years of treatment with dupilumab
EASI	**32.1** (26.8–38.5)	**35.2** (30.1–44.2)	Before dupilumab therapy
		10.1 (8.2–17.2)	Average value after 1.5 years of treatment with dupilumab
POEM	**14.3** (10–18)	**17.1** (13–21)	Before dupilumab therapy
		4.2 (2–6)	Average value after 1.5 years of treatment with dupilumab
DLQI	**13.8** (9–16)	**17.3** (12–20)	Before dupilumab therapy
		3.2 (1–5)	Average value after 1.5 years of treatment with dupilumab

Control group: 30 healthy subjects (10 men, 20 women), age 44.7 years (36.8–51.4), negative total IgE in all healthy subjects. The average values (minimal, maximal values) of SCORAD, EASI, POEM, DLQI are recorded in bold
SCORAD SCORing Atopic Dermatitis, *EASI* Eczema Area and Severity Index, *POEM* Patient Oriented Eczema Measure, *DLQI* Dermatology Life Quality Index, [25]

DI60 for digital morphology evaluating cell division and microscope Olympus BX40.

Evaluation of the Immunological Profile (Flow Cytometry)

The expression of CD23 molecule on B cells, $CD16^+$ eosinophils, and $CD203^+$ basophils were examined by immunophenotyping with the use of flow cytometry (using NAVIOS Flow Cytometer Beckman Coulter); we evaluated the expression of surface CD traits characterizing individual subpopulations and subsets of immune cells, including their relative numbers. Examination using flow cytometry was performed according to recommended procedures [26–28].

Individual monoclonal antibodies (anti-CD23, anti-CD16, anti-CD203, and anti-signs for B lymphocytes, basophils, and eosinophils) were dropped into a cytometric tube. Peripheral blood collected in EDTA reagent was added to this cocktail of antibodies. Incubation takes about 15 min when antibodies bind to individual cell structures. After this time, erythrocytes are lysed using OptiLyze, which are undesirable for analysis (approximately 10 min) and the sample was subsequently washed to stop the lysis reaction (the sample is spun for 6 min at 1100 rpm, the RCF value is 250). The sample was measured on a flow cytometer. Individual marker expressions and cell subpopulations are evaluated in the Kaluza program.

Compliance with Ethics Guidelines

The study was conducted according to the guidelines of the Declaration of Helsinki 1964. This study is approved by the Institutional Review Board-Ethics Committee of the Faculty Hospital Hradec Králové, Charles University of Prague, Czech Republic. Data of Approval 4 September 2021, reference number 2021 10 P 03.

Statistical Analysis

1. The absolute and relative count of neutrophils, eosinophils, basophils, relative CD16$^+$ eosinophils, relative CD203$^+$ basophils, and expression of CD23 marker on B cells and on their subsets (memory, naive, switched, non-switched) were compared in patients with AD with and without dupilumab and in control group. For statistical analysis we used non-parametric Kruskal–Wallis one-factor analysis of variance with post hoc by Dunn's test with Bonferroni modification of significance level. The median values with the first and third quartiles of the examined parameters are recorded in tables.
2. The association between the count of basophils (relative, absolute, relative CD203$^+$ basophils) and eosinophils (relative, absolute, relative CD16$^+$ eosinophils) was evaluated in patients with AD with and without dupilumab therapy and in controls.

The association between the expression of CD23 on B cells with their subsets (memory, naive, switched, non-switched) and the count of basophils (relative, absolute, relative CD203$^+$ basophils) and eosinophils (relative, absolute, relative CD16$^+$ eosinophils) was evaluated in patients with AD (with and without dupilumab therapy) and in controls. We used the non-parametric Spearman's rank correlation coefficient. It is a non-parametric method that uses the order of values of monitored variables in the calculation, so it does not require the normality of the data. The advantage is that this method can be used to describe any dependence, linear or non-linear. For coefficients higher than 0.41,

we report R^2 (%, percent of variation explained). R^2 of one quantity (dependent) can be explained by another quantity (independent). Several measures of the goodness-of-fit of the regression model to the data have been proposed, but by far the most popular is R^2. R^2 is the square of the correlation coefficient between Y and $\hat{Y}$. It is the proportion of the variation in Y that is accounted by the variation in the independent variables. R^2 varies between zero (no linear relationship) and one (perfect linear relationship). The level of statistical significance was set to $\alpha = 0.05$. We used statistical software: NCSS 2021 Statistical Software (2021). NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/ncss.

RESULTS

Characteristics of Patients and Control Group

During the years 2021–2022 we examined 32 patients suffering from AD without dupilumab treatment, 13 patients with dupilumab (treatment lasting 18 months), and 30 subjects as a control group. Patient characteristics are recorded in Table 1. The representation of patients with AD was consistent with regard to gender, age, and onset of AD. Likewise, the representation of the control group matched with regard to gender and age to AD patients with AD.

AD severity and quality of life were assessed every 3 months during 1 year in patients without dupilumab. In patients under dupilumab, AD severity and quality of life were assessed every 3 months during 1 year before dupilumab treatment and during 18 months with dupilumab treatment. The average values of SCORAD, EASI, POEM, and DLQI are presented in Table 1. AD severity was consistent in both groups of patients with AD before starting dupilumab therapy. Patients on dupilumab therapy had suffered from moderate and severe forms of AD before starting biological treatment; the skin finding improved significantly under dupilumab treatment and they suffered from a mild form of AD (Table 1). The treatment involves the use of moisturizers and application of

dupilumab 300 mg subcutaneously (s.c.) every 2 weeks.

Results of Laboratory Examination

In patients with AD and in control group we examined the absolute and relative count of neutrophils, eosinophils, basophils, relative CD16$^+$ eosinophils, relative CD203$^+$ basophils, and expression of CD23 molecule on B cells and on their subsets (memory, naive, switched, non-switched). The median value with the first and third quartiles of these examined parameters are demonstrated.

Count of Neutrophils

The absolute count of neutrophils is 3.19×10^9/l in control, 4.94×10^9/l in patients with dupilumab therapy and 4.20×10^9/l in patients without dupilumab therapy. The relative count of neutrophils is 54% in control, 69% in patients with dupilumab therapy, and 67% in patients without dupilumab therapy. The significantly higher count of relative and absolute neutrophils in patients with AD (with and without dupilumab therapy) compared with controls was confirmed (Table 2).

Count of Eosinophils

The absolute count of eosinophils is 0.17×10^9/l in control, 0.41×10^9/l in patients with dupilumab therapy and 0.36×10^9/l in patients without dupilumab therapy. The relative count of eosinophils is 2.5% in control, 4.8% in patients with dupilumab therapy, and 6% in patients without dupilumab therapy.

Absolute eosinophils were significantly higher in patients with AD (with and without dupilumab) in comparison with control group; there was no difference in absolute eosinophils count between patients with and without dupilumab treatment. Relative count of eosinophils was significantly higher in patients without dupilumab compared with controls (Table 2).

Relative CD16$^+$ Eosinophils

The relative count of CD16$^+$ eosinophils is 21% in control, 30% in patients with dupilumab therapy, and 15.5% in patients without dupilumab therapy. The significant difference in the relative count of CD16$^+$ eosinophils in patients with AD (with and without dupilumab) in comparison with control group was not confirmed (Table 2).

Basophils

The absolute count of basophils is 0.03×10^9/l in control, 0.03×10^9/l in patients with dupilumab therapy, and 0.02×10^9/l in patients without dupilumab therapy. The significant difference in count of relative and absolute basophils in patients with AD (with and without dupilumab) in comparison with control group was not confirmed (Table 2).

Relative CD203$^+$ Basophils

The count of relative CD203$^+$ basophils is 77% in control, 50% in patients with dupilumab, and 72% in patients without dupilumab. There is a difference at the limit of statistical significance between patients with and without dupilumab therapy (p-value = 0.054); the relative CD203$^+$ basophils are significantly lower in patients with dupilumab compared with controls (Table 2).

In Table 3 the association between basophils (relative, absolute, CD203$^+$ basophils) and eosinophils (relative, absolute, CD16$^+$ eosinophils) is recorded in patients with AD (with and without dupilumab therapy) and controls. The low association between eosinophils, basophils, relative CD203$^+$ basophils, and relative CD16$^+$ eosinophils in all subjects was confirmed (Spearman's rank correlation coefficient is lower than 0.41).

Table 2 The median count (with first and third quartiles) of neutrophils (absolute, relative), eosinophils (absolute, relative, CD16$^+$ eosinophils), and basophils (absolute, relative, CD203$^+$ basophils) in patients with dupilumab (DUP+), without dupilumab (DUP−) and in control group

	Median count (first quartile, third quartile)			Statistical analysis			
	DUP−	DUP+	Control	KW test p value	DUP+/ DUP−	DUP−/control	DUP+/control
Relative neutrophils, %	67.00 (52.00, 74.40)	69.00 (55.00, 71.50)	54.00 (48.25, 61.00)	0.010		< 0.05	< 0.05
Absolute neutrophils 10^9/l	4.20 (3.48, 5.23)	4.94 (3.60, 5.95)	3.19 (2.45, 4.08)	0.002		< 0.05	< 0.01
Relative eosinophils, %	6.00 (3.35, 8.35)	4.80 (2.35, 13.5)	2.50 (1.45, 4.72)	0.022		< 0.05	
Absolute eosinophils, 10^9/l	0.36 (0.16, 0.57)	0.41 (0.20, 0.78)	0.17 (0.08, 0.25)	0.006		< 0.05	< 0.05
Relative CD16$^+$ eosinophils, %	15.50 (1.50, 82.75)	30.00 (6.50, 41.0)	21.00 (2.00, 58.25)	0.916			
Relative basophils, %	0.45 (0.30, 0.67)	0.40 (0.25, 0.60)	0.50 (0.37, 1.0)	0.241			
Absolute basophils, 10^9/l	0.02 (0.02, 0.05)	0.03 (0.02, 0.04)	0.03 (0.02, 0,06)	0.524			
Relative CD203$^+$ basophils, %	72.00 (44.75, 90.00)	50.00 (37.00, 62.50)	77.00 (58.50, 91.25)	0.031	0.054		< 0.05

The significant difference in statistical analysis, p -value < 0.05, is recorded

KW test results of Kruskal–Wallis test

Table 3 The association between the count of basophils (absolute, relative, CD203$^+$ basophils) and eosinophils (absolute, relative, CD16$^+$ eosinophils) in patients with AD with dupilumab (DUP+) and without dupilumab (DUP−) and in controls

	Relative basophils, %		
	Controls	DUP−	DUP+
Relative eosinophils, %			
Spearman correlation	0.294	0.395	0.069
Spearman p-value	0.261	0.062	0.874
Relative CD16$^+$ eosinophils, %			
Spearman correlation	0.149	0.009	0.014
Spearman p-value	0.123	0.965	0.790
	Absolute basophils, 10^9/l		
	Controls	DUP−	DUP+
Absolute eosinophils, 10^9/l			
Spearman correlation	0.173	0.347	−0.114
Spearman p-value	0.471	0.009	0.943
	Relative CD203$^+$ basophils, %		
	Controls	DUP−	DUP+
Relative eosinophils, %			
Spearman correlation	0.122	0.109	0.061
Spearman p-value	0.727	0.449	0.253
Relative CD16$^+$ eosinophils, %			
Spearman correlation	−0.176	−0.352	−0.002
Spearman p-value	0.319	0.026	0.882

According to Spearman correlation, the association is low

Expression of Activation Marker CD23 on B Lymphocytes

In Table 4 the expression of CD23 on B lymphocytes and on their subsets (memory, naive, non-switched, switched) is recorded [25].

The significantly higher expression of activation marker CD23 on B cells and on their subsets (memory, naive, non-switched, switched) in patients with AD (with and without dupilumab treatment) in comparison with control group was confirmed [25].

The Association Between the Expression of CD23 Marker on B Cells with Basophils and Eosinophils

The association between the expression of CD23 molecule on B cells with the count of basophils (relative, absolute, relative CD203$^+$ basophils) and with the count of eosinophils (relative, absolute, relative CD16$^+$ eosinophils) was evaluated in patients with AD (with and without dupilumab therapy) and controls. Spearman correlation and Spearman p-values

Table 4 Expression of activation marker CD23 on B lymphocytes (memory, naive, non-switched, switched, total)

Expression of CD23 on B lymphocytes	Median count (first quartile, third quartile)			Statistical analysis (*p*-value)			
	DUP−	DUP+	Control	KW test *p*-value	DUP+ versus DUP−	DUP− versus control	DUP+ versus control
CD23 memory, MFI	10.97 (7.37, 23.69)	10.43 (9.34, 23.65)	6.48 (4.73, 7.41)	0.000		< 0.001	< 0.001
CD23 memory, %	59.00 (45.00, 69.0)	61.00 (38.00, 70.5)	56.00 (45.75, 60.5)	0.525			
CD23 naive, MFI	11.02 (7.63, 20.72)	9.66 (8.77, 21.63)	6.70 (5.63, 8.27)	0.000		< 0.001	< 0.01
CD23 naive, %	85.00 (82.00, 93.00)	91.00 (79.50, 92.00)	87.50 (82.25, 91.0)	0.925			
CD23 non-switched, MFI	11.15 (6.67, 27.05)	12.20 (9.05, 27.22)	6.91 (5.19, 7.85)	0.000		< 0.001	< 0.001
CD23 non-switched, %	62.00 (37.00, 74.00)	64.00 (47.50, 72.50)	55.50 (47.00, 66.25)	0.835			
CD23 switched, MFI	5.17 (3.54, 9.19)	4.22 (3.85, 9.81)	2.34 (1.84, 3.30)	0.000		< 0.001	< 0.001
CD23 switched, %	19.00 (11.00, 33.00)	14.00 (11.00, 25.50)	23.50 (17.00, 25.00)	0.249			
CD23 total B lymphocytes, MFI	10.50 (7.66, 33.31)	9.54 (8.82, 21.7)	6.45 (5.35, 7.77)	0.000		< 0.001	< 0.001

Table 4 continued

Expression of CD23 on B lymphocytes	Median count (first quartile, third quartile)			Statistical analysis (p-value)			
	DUP−	DUP+	Control	KW test p-value	DUP+ versus DUP−	DUP− versus control	DUP+ versus control
CD23 total B lymphocytes, %	70.00 (59.0, 77.0)	73.00 (63.5, 78,0)	69.50 (60.5, 75.5)	0.710			

DUP− patients without dupilumab treatment, *DUP+* patients with dupilumab treatment, *KW test* results of Kruskal–Wallis test, *MFI* laboratory unit in flow cytometry. The significant difference in statistical analysis, p -value < 0.05, is recorded

were calculated. We show associations with Spearman correlation higher than 0.41 (Table 5). Spearman correlation higher than 0.41 was recorded only in group of patients with AD with dupilumab therapy; the following associations were confirmed: between CD23 on memory B cells and relative and absolute eosinophils (Spearman correlation 0.555, resp.0.495), between CD23 on naive B cells and relative eosinophils and absolute eosinophils (Spearman correlation 0.560, resp.0.533), between CD23 on switched B cells and relative and absolute eosinophils (Spearman correlation 0.698, resp. 0.609), and between CD23 on total B lymphocytes and relative and absolute eosinophils (Spearman correlation 0.615, resp.0.593). In patients with AD without dupilumab and in controls these associations are not confirmed. For coefficients higher than 0.41 we report R^2 (%) (percent of variation explained). R^2 of one quantity (dependent) can be explained by another quantity (independent), (Table 5). The results in percentages explain the association between the monitored parameters. The highest association is confirmed between the relative count of eosinophils and expression of CD23 on total B cells ($R^2 = 37.9\%$) and on their subsets as switched ($R^2 = 48.7\%$), naive ($R^2 = 31.4\%$), and memory ($R^2 = 30.8\%$) B cells in patients with dupilumab therapy. The association is also confirmed between the absolute count of eosinophils and expression of CD23 on total B cells ($R^2 = 35.2\%$) and on their subsets as switched ($R^2 = 37.2\%$), naive ($R^2 = 28.4\%$), and memory ($R^2 = 24.5\%$) in patients with dupilumab therapy. We show for comparison R^2 in patients with AD without dupilumab and in controls.

The association between the expression of CD23 marker on B cells with the count of basophils (relative, absolute, relative CD203$^+$ basophils) in patients with AD (with and without dupilumab therapy) and in controls is low (Spearman's rank correlation coefficient is lower than 0.41).

DISCUSSION

Eosinophils, basophils, and the activation marker CD23 on B cells are involved in the pathophysiology of AD, but their association has not been studied in the context of patients with AD and dupilumab therapy. Dupilumab is a monoclonal antibody that targets the IL-4 receptor alpha subunit, and it is approved for the therapy of moderate-to-severe forms of AD. IL-4 is a cytokine that is involved in both the activation of B cells and the promotion of IgE production. CD23 is a low-affinity receptor for IgE and its expression on B cell is upregulated by IL-4. By blocking IL-4 and IL-13, dupilumab affects multiple downstream effector cells and cytokines, such as both IgE and eosinophils, but also IL-5, IL-13, FcεRIα (a high-affinity IgE receptor), pathogenic Th2 cells, and alveolar macrophages

Table 5 The associations (Spearman correlation higher than 0.41) between the count of eosinophils (relative, absolute) and expression of activation marker CD23 on B lymphocytes and on their subsets in patients with dupilumab therapy

Expression of molecule CD23 on B lymphocytes (MFI)	Relative count of eosinophils, (%)			
		Dupilumab yes	Dupilumab no	Control
Switched B lymphocytes	Spearman correlation	0.698	0.137	0.414
	R^2	**48.7%**	**1.9%**	**17.1%**
B lymphocytes total	Spearman correlation	0.615	0.286	0.265
	R^2	**37.9%**	**8.2%**	**7.1%**
Naive B lymphocytes	Spearman correlation	0.560	0.314	0.225
	R^2	**31.4%**	**9.9%**	**5.1%**
Memory B lymphocytes	Spearman correlation	0.555	0.311	0.313
	R^2	**30.8%**	**9.7%**	**9.8%**
	Absolute count of eosinophils (10^9/l)			
		Dupilumab yes	Dupilumab no	Control
Switched B lymphocytes	Spearman correlation	0.609	0.122	0.479
	R^2	**37.2%**	**1.5%**	**22.9%**
B lymphocytes total	Spearman correlation	0.593	0.254	0.208
	R^2	**35.2%**	**6.5%**	**4.3%**
Naive B lymphocytes	Spearman correlation	0.533	0.282	0.208
	R^2	**28.4%**	**7.9%**	**4.4%**
Memory B lymphocytes	Spearman correlation	0.495	0.279	0.2489
	R^2	**24.5%**	**7.8%**	**6.2%**

We compare R^2 in patients with AD with and without dupilumab therapy and in controls. Dupilumab no, patients without dupilumab treatment; dupilumab yes, patients with dupilumab treatment. R^2 = percent of variation explained, R^2 of one quantity (dependent) can be explained by another quantity (independent); the results in percentages explain the association between the monitored parameters (bold)

[4]. Analysis comparing 11 studies indicates that dupilumab treatment resulted in a transient increase in mean blood eosinophil counts in patients with asthma or AD, which typically declined to baseline or below baseline over time and was not generally associated with clinical symptoms or an impact on efficacy [29]. Because IL-4 and IL-13 do not mediate eosinophil maturation and release into the blood, this reduced eosinophil migration into the tissue may lead to transient rises in blood eosinophil counts [29].

Dupilumab therapy reduces the levels of IL-4, which may result in a lower activation of B cells. Further, results of our study show the activation of B cells according to the high expression of CD23 molecule on B cells and on their subsets in patients with AD with and without dupilumab therapy compared with control. It suggests that other cytokines or factors may compensate the loss of IL-4 signaling,

leading to the upregulation of CD23 on B cells. For this reason, our pilot study evaluates the count of basophils, eosinophils, relative CD16$^+$ eosinophils, relative CD203$^+$ basophils, the expression of activation marker CD23 on B cells (memory, naive, non-switched, switched), and their associations in patients with AD (with and without dupilumab therapy) and in controls. Both CD16 and CD203 markers were selected as activating molecules for eosinophils and basophils. CD16 is an activation molecule that is usually increased in allergic diseases or asthma; the marker CD203 is commonly used to detect basophils, but its increased expression is related to their activation. In our study, although the difference in the relative count of CD16$^+$ eosinophils in patients with AD (with and without dupilumab therapy) compared with control is not statistically significant, the higher count of CD16$^+$ eosinophils (30%) is recorded in patients with dupilumab and the lower count in patients without dupilumab (15.5%). In the control group, the count of CD16$^+$ eosinophils is higher than in patients with AD without dupilumab therapy (21%). The explanation may be, in fact, that CD16 activation marker may be altered in patients with AD without dupilumab with displacement of eosinophils to the skin lesions. The explanation for the higher count of CD16$^+$ eosinophils in patients with AD with dupilumab therapy may be, in fact, that these patients have higher absolute count of eosinophils. Another explanation may be to maintain IL-4 production, because this also occurs through eosinophils. Dupilumab blocks the production of IL-4, which is important for the activation of B lymphocytes. To maintain B cell activation with IL-4, we can hypothesize that this occurs through IL-4 production by eosinophils, while the IL-4 produced by basophils could be blocked by dupilumab. This corresponds to an increased relative count of CD16$^+$ eosinophils (30.0%) in patients with AD with dupilumab. This finding may also explain the significant association between count of eosinophils (relative and absolute) and expression of the activation marker CD23 on B lymphocytes and their subsets. Although the expression of CD23 is significantly higher on B lymphocytes and their subsets in both groups of patients with AD (with and without dupilumab therapy) compared with controls, the high association between eosinophils (absolute and relative) is confirmed only in patients with dupilumab therapy. Our results suggest that B-lymphocyte activation (increased expression of molecule CD23 on B cells) in dupilumab patients may be mediated with IL-4 production by eosinophils. According to the literature, eosinophils produce significant amounts of IL-4 [30]. Piehler et al. demonstrated an immunoregulatory role of eosinophils that contribute to IL-4-dependent immunopathological features during murine pulmonary *C. neoformans* infection, and provided evidence for previously unrecognized features of eosinophils during bronchopulmonary infection [30]. Further studies are needed to elucidate the mechanisms underlying this association and its implications.

As for the activation marker for basophils, a significantly lower count of CD203$^+$ basophils has been demonstrated in patients with dupilumab therapy. The explanation for this finding can be in fact that the main function of basophils is the rapid secretion of IL-4 and IL-13 at levels greater than any other cells capable of secreting these cytokines, including Th2 cells, mast cells, and eosinophils. Dupilumab therapy probably further reduces the relative count of CD203$^+$ basophils by inhibiting the activity of IL-4 and IL-13, which are key drivers of basophil activation and function. This reduction of CD203$^+$ basophil count may contribute to the therapeutic effects of dupilumab by reducing the inflammatory response and allergic reactions in patients with AD. This also corresponds to our other finding, that the association between basophils (relative, absolute, CD203$^+$ basophils) and CD23 activation on B cells has not been proven in our study. The marker CD203 on cell surface has been identified as specific for basophils and mast cells among cells of hematopoietic lineage [14]. CD203c is expressed on resting cells at low levels and its expression is rapidly upregulated following activation. The activation is transient and more rapid than expression of CD63 (CD63 is a good marker for flow cytometric quantification of in vitro activated basophils for diagnosis of IgE-

mediated allergy). Moreover, the CD203 antigen is upregulated after activation of basophils by anti-IgE antibodies and allergens [14]. Several lines of evidence from murine models suggest basophils' direct role in antigen presentation for Th2 responses through MHC-II expression [31–33], initiation, and maintenance of IgE-mediated Th2 responses as possibly independent of T cells and mast cells [34]. Despite this evidence from mouse models of the multifaceted roles of basophils in Th2 allergic inflammation, the crucial function of basophils in human allergic diseases remains undefined because of inadequate supporting evidence [5].

We speculate that blood eosinophils and basophils interact in vivo, but according to our results, the association was low between the blood count of basophils (relative, absolute, CD203$^+$ basophils) and eosinophils (relative, absolute, CD16$^+$ eosinophils) in patients with AD with and without dupilumab therapy and in controls.

Mountz et al. report a case of AD in a patient who received biweekly doses of dupilumab. Dupilumab treatment resulted in upregulation of genes associated with apoptosis and inhibition of B cell receptor signaling and downregulation of class-switch and memory B cell development genes. Their data suggest that intact and persistent IL-4 signaling is necessary for maintaining robust survival and development of naive B cells [35]. Hadebe et al. confirmed that IL-4Rα–responsive B cells play a critical role in house dust-mite-induced allergic asthma when the load of house dust mites is limited. IL-4Rα signaling on B cells is required at both sensitization and effector stages of allergic disease [36]. IL-4 can be secreted by several cell types involved in allergic inflammatory reactions, and therefore can affect eosinophil function similarly. Dubois et al. demonstrated that both IL-4 and IL-13 were capable of inducing PI-3 kinase activity (enzyme involved in cellular functions such as cell growth, proliferation, differentiation, motility, survival, and intracellular trafficking) in human eosinophils. Dubois et al. concluded that both cytokines can activate human eosinophils through binding to a receptor complex that makes up the IL-4Ralpha and yet-to-be-identified-associated proteins.

According to Dubois, IL-4 appeared to "prime" eosinophils to respond chemotactically toward regulated on activation and normal T cells expressed and secreted, but did not affect platelet-activating factor-induced chemotaxis; these data show the presence of a functional IL-4R on human eosinophils [37].

Regarding the activation marker CD23, some studies have shown that CD23 is expressed on B cells [38], monocytes [39], T cells [40], dendritic cells [41], platelets [42], and neutrophils [43]. CD23 has an important function in IgE-facilitated allergen presentation to T cells [44, 45]. IgE-facilitated antigen presentation strongly activates allergen-specific T cells and secretion of proinflammatory and T$_H$2-driving cytokines [44–47]. According to Selb, CD23 surface density on B cells of allergic patients is correlated with allergen-specific IgE levels and determines allergen uptake and subsequent activation of T cells [48]. In the context of a T helper cell–B cell interaction, IL-4 produced by Th2 cells is thought to provide antigen-specific help to B cells, promoting CD23 induction and isotype switching [45, 46]. Floch et al. show that only the dual ligand blocker (IL-4Rα antibody) prevents B-cell activation, as measured by increased cell surface of the low affinity receptor for IgE, CD23. Importantly, it has been shown that IgE-CD23 interaction contributes to allergic inflammation through IgE-facilitated allergen presentation by B cells to T cells [4].

The explanation for the association between eosinophils and expression of CD23 on B cells may also be, in fact, that it is described as participation of CD23 or a related molecule in IgE-dependent eosinophil functions [49]. Another study confirms that the receptor for IgE (Fc epsilon RII) on human eosinophils presents some common characteristics with CD23 [50].

Limitations

For a more accurate evaluation of the effect of biological treatment on the immunological profile in patients with AD, it is necessary to measure the count of CD203$^+$ basophils, CD16$^+$ eosinophils, and the expression of CD23 marker on B cells before starting biological treatment,

and to monitor these parameters during treatment in one group of patients. Further studies are needed to demonstrate that IL-4 produced by eosinophils in dupilumab patients is responsible for the activation of B cells with help of CD23 molecule.

CONCLUSIONS

The high association between the count of eosinophils (absolute and relative) and the expression of CD23 marker on B cells is confirmed only in patients with dupilumab therapy. Our results suggest that B-lymphocyte activation in dupilumab patients may be mediated with IL-4 production by eosinophils.

The significantly lower count of CD203$^+$ basophils has been demonstrated in patients with dupilumab therapy. This reduction of CD203$^+$ basophil count may contribute to the therapeutic effects of dupilumab by reducing the inflammatory response and allergic reactions in patients with AD.

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