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ORIGINAL RESEARCH

Evaluation of Leukocytes, B and T Lymphocytes, and expression of CD200 and CD23 on B lymphocytes in Patients with Atopic Dermatitis on Dupilumab Therapy—Pilot Study

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ABSTRACT

Background: There are a lot of studies that describe the change in quantity of T cells in patients with atopic dermatitis (AD) compared with healthy subjects. Other components of lymphocytes such as B cells are not examined as well as T cells.

Objective: We focus on immunophenotyping of B cells with their subsets (memory, naïve, switched, non-switched) and the expression of CD23 and CD200 markers in patients with AD with and without dupilumab therapy. We also evaluate the count of leukocytes and their subsets, T lymphocytes ($CD4^+$, $CD8^+$), natural killer (NK) cells, and T regulatory cells.

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. We compared the absolute and relative count of leukocytes and their subsets, T lymphocytes ($CD4^+$, $CD8^+$), NK cells, T regulatory cells, absolute and relative count of B lymphocytes (memory, naïve, non-switched, switched, transient), and expression of CD23 and CD200 activation markers on B cells and on their subsets in patients with AD and control group. For statistical analysis we used nonparametric Kruskal–Wallis one-factor analysis of variance with post hoc by Dunn's test with Bonferroni modification of significance level.

Results: In patients with AD with and without dupilumab therapy we confirmed the significantly higher count of neutrophils, monocytes, and eosinophils; there was no difference in absolute count of B cells, NK cells and transitional B cells compared with control subjects. We confirmed higher expression of activation marker CD23 on total, memory, naïve, non-switched, and switched B lymphocytes and higher expression of CD200 on total B lymphocytes in both groups of patients with AD

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compared with controls. In patients without dupilumab therapy we confirmed significantly higher count of relative monocytes, relative eosinophils, and higher expression of CD200 on memory, naïve, and non-switched B lymphocytes compared with controls. In patients with dupilumab therapy we confirmed significantly higher expression of CD200 on switched B lymphocytes, higher count of relative $CD4^+$ T lymphocytes, and lower count of absolute $CD8^+$ T lymphocytes compared with controls.

Conclusion: This pilot study shows higher expression of CD23 on B lymphocytes and on their subsets in patients with AD with and without dupilumab therapy. The higher expression of CD200 on switched B lymphocytes is confirmed only in patients with AD with dupilumab therapy.

Keywords: Atopic dermatitis expression of activation marker CD23; CD200 on B cells dupilumab

Key Summary Points

Why carry out this study?
B cells are not examined as well as T cells in patients with AD.
We focus on immunophenotyping of B cells with their subsets (memory, naïve, switched, non-switched) and the expression of CD23 and CD200 activation markers patients with AD on dupilumab therapy compared with patients without dupilumab therapy and with control subjects. We compare also the absolute and relative count of leukocytes and their subsets, T lymphocytes ($CD4^+$, $CD8^+$), NK cells, and T regulatory cells in patients with AD and in controls.

What was learned from the study?
There was no difference in absolute count of B cells in patients with AD with and without dupilumab therapy compared with controls. Furthermore, the expression of CD23 on total B lymphocytes and on their subsets is significantly higher in both groups of AD patients compared with controls. Although the expression of CD200 on total B lymphocytes is also significantly higher in both groups of patients with AD compared with controls, it differs in subsets of B cells. In patients without dupilumab therapy, we confirmed significantly higher expression of CD200 on memory, naïve, and non-switched B cells in comparison with control. Furthermore, in patients with dupilumab therapy we confirmed higher expression of CD200 on switched B lymphocytes compared with control.
In patients with dupilumab therapy we confirmed significantly higher count of $CD4^+$ T lymphocytes and lower count of absolute $CD8^+$ T lymphocytes compared with controls.
Results of our study suggest that dupilumab may control the generation of aberrant immune responses. For a more accurate evaluation of the effect of biological treatment on the immunological profile in patients with AD, it is necessary to evaluate these data in one group of patients before starting the biological treatment and monitor these parameters during treatment. The following study should clarify the relationships between the expression of CD23, CD200, and the level of eosinophils and immunoglobulin E in patients with AD with and without dupilumab therapy.

INTRODUCTION

Atopic dermatitis (AD) disease is characterized by a biphasic inflammation, evolving from an initial, acute, and Th2- and Th22-dominated phase to a chronic phase characterized by the concomitant presence of T helper Th1 cells, Th2 cells, and Th17 cells [1–5]. Excessive production of Th2 lymphocytes leads to increased production of cytokines such as interleukins (ILs), including IL-4, IL-5, and IL-13. IL-4 has been shown to induce the differentiation of naïve $CD4^+$ T cells into Th2 effector cells, while IL-13 plays an important role in goblet cell metaplasia, mucus hypersecretion, and smooth muscle contractility. Both cytokines also promote class switching to immunoglobulin E (IgE) and the chemotaxis of eosinophils [1–5]. Factors influencing the destruction of the epidermis, such as damage, infections, or ongoing inflammation, stimulate keratinocytes to produce proinflammatory cytokines such as thymic stromal lymphopoietin (TSLP), IL-25, and IL-33. They also activate the Th2-mediated immune response. TSLP, through its receptor, activates immature dendritic cells and enhances the maturation of antigen-presenting cells (APCs) [6, 7]. The role of the innate immune system in the early phase of AD has been demonstrated in experimental animal models and is likely of clinical relevance in infancy [6, 7]. A key player of innate immunity is the epidermal barrier and the loss-of-function *FLG* gene variants that constitute a major predisposing factor for AD [6, 7]. There are a lot of studies that describe the change in quantity of T cells in patients with atopic dermatitis compared with healthy subjects [8–10]. Other components of lymphocytes such as B cells are not examined as well as T cells. Although T cells are considered the central component in immune-mediated diseases, supportive evidence has demonstrated that B cells also contribute to the progression of these diseases. B cells are divided into various subsets according to their secreted cytokines. Different B-cell subsets play diverse roles in immune-mediated dermatoses [11, 12]. Upon appropriate cytokine stimulation with or without T-cell-mediated CD40 ligation, B cells undergo class switching and/or enter germinal centers within secondary lymphoid organs to undergo affinity maturation. This maturation process produces both long-lived plasma cells and memory B cells capable of responding to secondary challenge with homotypic or heterotypic antigenic challenge. Class-switched memory B cells express high levels of the co-receptors required for T-cell interaction compared with naïve B cells. Furthermore, memory B cells have a potent antigen-presenting cell (APC) activity as compared with naïve B cells, which provides an effective activation of cognate helper T cells, resulting in increased efficacy of memory B-cell activation [11, 12].

The examination of the CD23 and CD200 molecules on B cells and on their subsets may lead to a better understanding of immune reactions in patients with AD. A low-affinity receptor FcεRII (CD23) is expressed by activated B cells, monocytes, follicular dendritic cells, and subsets of eosinophils and platelets [13–17]. The functions of CD23 include regulation of IgE synthesis, antigen capture and presentation, B-cell growth and differentiation, and activation of monocytes. However, the exact mechanism of IgE downregulation is a matter of debate. CD23 binds to IgE to regulate its activity, and can have either stimulatory or inhibitory functions [13–17]. CD200, a transmembrane glycoprotein type Ia belonging to the immunoglobulin protein superfamily, is broadly expressed on a wide variety of cell types, such as B lymphocytes, a subset of T lymphocytes, dendritic cells, endothelial cells, neuronal cells, and many cancer cells [18]. CD200 fulfill multiple functions in regulating inflammation interaction by promoting inhibitory activities of the immune system. This molecule is encoded by *CD200R1* gene. The interaction between CD200/CD200R results in activation of the intracellular inhibitory pathway and thus contributes to effector cell inhibition. CD200 activation stimulates the differentiation of T cells to the Treg subset and modulates cytokine environment from a Th1 to a Th2 pattern [19–28]. CD200 is also an immunoregulatory cell surface ligand with proven pro-tumorigenic credentials via its

ability to suppress CD200 receptor (CD200R)-expressing antitumor immune function [27].

AD can manifest with different clinical phenotypes, causing diagnostic difficulties. Long term is often required and systemic drugs are needed for moderate-to-severe forms. Limited data exist regarding the treatment in relation to individual clinical phenotypes. Dupilumab was shown to be effective in the treatment of atopic dermatitis, although in trials and real-life experiences the different phenotypes treated are usually not reported [29]. Dupilumab is a fully human monoclonal IgG4 antibody directed against the alpha subunit of the IL-4 receptor and blocks the signaling of IL-4 and IL-13. IL-4 and IL-13 are key drivers of the type 2 inflammatory response and are integral to the pathogenesis of atopic diseases including atopic dermatitis, asthma, and chronic sinusitis with nasal polyposis [30]. Clinical trials have shown that adults with moderate-to-severe AD who receive weekly or biweekly dupilumab injections have significantly improved clinical and patient-reported outcomes, including Eczema Area Severity Index, SCORing Atopic Dermatitis, Dermatology Life Quality Index, and Itch Numeric Rating Scale scores [31]. Biomarker analyses show that dupilumab modulates the AD molecular signature and other Th2-associated biomarkers. Common adverse events reported in the clinical trials were nasopharyngitis, upper respiratory tract infection, injection site reactions, skin infections, and conjunctivitis. These were mild to moderate in nature, and overall rates of adverse events occurred with similar frequency between the treatment and placebo groups. There were no significant serious safety concerns identified in phase III clinical trials. Dupilumab, as monotherapy or with concomitant use of topical corticosteroids, can significantly improve clinical outcomes and quality of life in patients suffering from moderate-to-severe AD [31–33].

Our study focuses particularly on expression of CD23 and CD200 molecules on B cells and their subsets in patients with AD with and without dupilumab therapy. The aim is also to measure the absolute and relative count of leukocytes, neutrophils, monocytes, eosinophils, basophils, T lymphocytes, natural killer cells, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, T regulatory cells, and B lymphocytes with their subsets (memory, naïve, non-switched, switched, transitional).

Methods

Dermatological Examination

Complete dermatological examination was performed in 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. We also evaluate the occurrence of bronchial asthma, allergic rhinitis, and onset of AD. This study was approved by the ethics committee of the Faculty Hospital Hradec Králové, Charles University of Prague, Czech Republic. Reference number is 2021 10 P 03. The study was approved by the Institutional Review Board—Ethics Committee of the Faculty Hospital Hradec Králové, Charles University of Prague, Czech Republic. Date of approval was 4 September 2021. The study was conducted in accordance with the Helsinki Declaration of 1964 and all subsequent amendments, and all patients provided written informed consent.

Severity of Atopic Dermatitis

Severity of AD is scored in agreement with Scoring of 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 quality of life are assessed every 3 months during 1 year in patients without dupilumab. In patients under dupilumab, the severity of atopic dermatitis and quality of life are assessed every 3 months during 1 year before dupilumab treatment and during 18 months with dupilumab treatment.

Bronchial Asthma

The diagnosis of bronchial asthma (AB) was determined according to the guidelines of the Global Initiative for Asthma (GINA) at the allergy outpatient clinic of the Institute of

Clinical Immunology and Allergology, Faculty Hospital Hradec Kralove, Czech Republic (Global Initiative for Asthma. Global Strategy for asthma management and prevention—Update 2015. www.ginasthma.com).

Allergic Rhinitis

The evaluation of allergic rhinitis (AR) was made according to the allergy testing and personal history of the Institute of Clinical Immunology and Allergology, Faculty Hospital Hradec Kralove, Czech Republic. AR was defined as a process that included three cardinal symptoms: sneezing, nasal obstruction, and mucus discharge. Symptoms occur with allergen exposure in the allergic patient.

Onset of Atopic Dermatitis

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

Inclusion Criteria

(1) Age 14 years and over and (2) AD as defined by the criteria of Hanifin and Rajka. We include patients with moderate and severe forms of AD and with dupilumab treatment lasting at least 18 months.

Exclusion Criteria

Pregnancy, breastfeeding, and systemic therapy (cyclosporin, systemic corticoids).

As a control group, we examined 30 healthy individuals, blood donors at Faculty Hospital Hradec Králové, Charles University, Czech Republic.

Evaluation of the Immunological Profile (Flow Cytometry)

Blood samples from the antecubital vein were collected in tubes precoated with ethylenediaminetetraacetic acid (EDTA) anticoagulant. The blood count was examined with a Sysmex XN 3000, Sysmex SP10, microscope DI60 for digital morphology evaluating cell division and microscope Olympus BX40.

Surface molecules expressed on immune cells were examined by flow cytometry using monoclonal antibodies labeled with fluorochromes purchased from Beckman Coulter. A total of 5 µl of each fluorochrome-labeled monoclonal antibody and 50 µl of peripheral blood was added to cytometric tube. Blood samples were incubated for 15 min with antibodies at room temperature in the dark. Then a lysis solution (OptiLyse C, Beckman Coulter) was added and samples were incubated for 10 min. The samples were measured with a Navios Flow Cytometer (Beckman Coulter). A minimum of 60,000 events were obtained for each stain and were supplied in list mode (LMD). Multiple peripheral blood parameters were assessed as absolute and relative count.

The gating strategies for the different leukocytes and lymphocytes subsets assessed were as follows:

- Leukocytes ($CD45^+$), eosinophils (high SSC, $CD49d^+$, $CD15^+$), monocytes ($CD45^+$, $CD14^+$), neutrophils ($CD15^+$, $CD16^+$)
- Lymphocytes (low SSC, $CD45^{++}$), T cells ($CD3^+$), helper T cells ($CD3^+$, $CD4^+$), cytotoxic T cells ($CD3^+$, $CD8^+$), natural killer (NK) cells ($CD3^-$, $CD56^+$, and/or $CD16^+$), B cells ($CD19^+$), and transitional B cells ($CD38^+$, $CD24^+$, $CD27^-$)
- B cells' regulatory surface molecules CD23 and CD200

Monoclonal antibodies CD23 and CD200 were incorporated into immunophenotyping of B cells. We examined samples of peripheral blood in the period from October 2021 to February 2022 (out of pollen season).

Examination using flow cytometry was performed according to recommended procedures [34–37].

STATISTICAL ANALYSIS

We compared the count of leukocytes (neutrophils, monocytes, eosinophils) and lymphocytes ($CD4^+$ T cells, $CD8^+$ T cells, NK cells and B lymphocytes), relative count of transitional B lymphocytes and expression of CD23 and CD200 on B cells and on their subsets in patients with AD (with or without dupilumab treatment) and in control group.

For statistical analysis we used nonparametric Kruskal–Wallis one-factor analysis of

variance with post hoc (follow-up multiple comparison) and Dunn's test with Bonferroni modification of significance level. We used statistical software: NCSS 2021 Statistical Software (2021). NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/ncss.

RESULTS

Characteristics of Patients

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. The characteristics of patients are recorded in Table 1. The representation of patients with atopic dermatitis was consistent with regard to gender, age, onset of AD, and the occurrence of other atopic diseases such as bronchial asthma and allergic rhinitis. Likewise, the representation of the control group matched with regard to gender and age to patients with AD.

The severity of atopic dermatitis and quality of life were assessed every 3 months during 1 year in patients without dupilumab. In patients under dupilumab, the severity of atopic dermatitis 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. The severity of AD was consistent in both groups of patients with AD before starting the dupilumab therapy. Patients on dupilumab therapy had suffered from moderate and severe forms of AD before starting the biological treatment; under dupilumab treatment the skin finding improved significantly 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 every 2 weeks.

The results of laboratory examinations (leukocytes, T lymphocytes, B lymphocytes, expression of CD23 and CD200) are presented in tables and graphs to these tables. We show the median value with the first and third quartiles and the results of statistical analysis in patients with AD (with and without dupilumab therapy) and in controls.

LEUKOCYTES

Our study shows the significantly higher amount of absolute leukocytes in patients with dupilumab in comparison with control group and the significantly higher amount of relative and absolute neutrophils and higher amount of absolute monocytes in patients with AD (with and without dupilumab) in comparison with control group.

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

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, graph to Table 2).

T LYMPHOCYTES

The significantly higher count of relative $CD4^+$ T lymphocytes and significantly lower count of absolute and relative $CD8^+$ T lymphocytes in patients with dupilumab in comparison with control group was confirmed. In patients with AD without dupilumab there is no difference in the count of T lymphocytes in comparison with control group (Table 3, graph to Table 3).

B LYMPHOCYTES

Our study did not confirm any statistically significant difference in count of B lymphocytes (relative, absolute, total memory, naïve, non-switched, switched, transitional) in patients with AD (with and without dupilumab treatment) in comparison with control group (Table 4, graph to Table 4).

Table 1 Characteristics of patients

Characteristics of patients	Patients with AD without dupilumab	Patients with AD with dupilumab	
Age, years	**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
Bronchial asthma	14 patients (43.8%)	6 patients (46.2%)	
Allergic rhinitis	22 patients (68.8%)	9 patients (69.2%)	
Onset of AD before 5 years of age	25 patients (78.1%)	11 patients (84.6%)	
Control group: 30 healthy subjects (10 men, 20 women), age 44.7 years (36.8–51.4)			

SCORAD, EASI, POEM, and DLQI–the mean values in bold (minimal, maximal) are recorded
SCORAD Scoring of Atopic Dermatitis, *EASI* Eczema Area and Severity Index, *POEM* Patient Oriented Eczema Measure, *DLQI* Dermatology Life Quality Index

EXPRESSION OF ACTIVATION MARKER CD23

In both groups of patients with AD, the significantly higher expression of activation marker CD23 was confirmed on memory, naïve, non-switched, switched, and total B cells in comparison with control group (Table 5, graph to Table 5).

Table 2 Leukocytes

Leukocytes	Median (first quartile, third quartile)			Statistical analysis (*p*-value)			
	DUP−	DUP+	Control	KW test *p*-value	DUP+ versus DUP−	DUP− versus control	DUP+ versus control
Absolute leukocytes 10^9/l	6.64	7.3	5.64	0.040			< 0.05
	(5.4, 7.8)	(5.9, 8.6)	(5.25, 7.01)				
Absolute lymphocytes 10^9/l	1.77	1.72	1.72	0.872			
	(1.02, 2.15)	(1.31, 2.55)	(1.43, 1.92)				
Relative neutrophils, %	67	69	54	0.010	0.062	< 0.05	< 0.05
	(52, 74.4)	(55, 71.5)	(48.25, 61)				
Absolute neutrophils 10^9/l	4.2	4.94	3.19	0.002		< 0.05	< 0.01
	(3.48, 5.23)	(3.6, 5.95)	(2.45, 4.07)				
Relative monocytes, %	7.5	7.0	6.0	0.075		< 0.05	
	(6, 9.75)	(5.5, 9)	5, 8				
Absolute monocytes 10^9/l	0.5	0.55	0.39	0.004		< 0.01	< 0.05
	(0.39, 0.65)	(0.34, 0.73)	(0.28, 0.47)				
Relative eosinophils, %	6.0	4.8	2.5	0.022		< 0.05	
	(3.35, 8.35)	(2.35, 13.5)	(1.45, 4.72)				
Absolute eosinophils 10^9/l	0.36	0.41	0.17	0.006		< 0.05	< 0.05
	(0.16, 0.57)	(0.2, 0.78)	(0.08, 0.25)				

Table 2 continued

Leukocytes	Median(first quartile, third quartile)			Statistical analysis (*p*-value)			
	DUP−	DUP+	Control	KW test *p*-value	DUP+ versus DUP−	DUP− versus control	DUP+ versus control
Relative basophils, %	0.45	0.4	0.5	0.241			
	(0.3, 0.67)	(0.25, 0.6)	(0.37, 1)				
Absolute basophils 10^9/l	0.025	0.03	0.03	0.523			
	(0.02, 0.05)	(0.02, 0.04)	(0.02, 0,06)				

DUP− patients without dupilumab treatment, *DUP+* patients with dupilumab treatment, *KW test* results of Kruskal–Wallis test. We show the significant difference in statistical analysis, *p*-value < 0.05

EXPRESSION OF ACTIVATION MARKER CD200

In patients with AD without dupilumab, the significantly higher expression of activation marker CD200 was confirmed on memory, naïve, and non-switched B cells in comparison with control group. Furthermore, the significantly higher expression of CD200 marker was confirmed on switched B cells in patients with AD with dupilumab treatment in comparison with control group (Table 6, graph to Table 6).

The review of followed parameters with significant difference in comparison with control group is recorded in Table 7.

DISCUSSION

Our study is focused on immunophenotyping of B cells and their subsets in patients with AD. We included patients with moderate-to-severe forms both with and without biological therapy, and we focused in particular on evaluation of expression of activation markers such as CD23 and CD200 molecules on B lymphocytes and on their subsets. The reason for investigating these molecules is the fact that the molecule CD200 serves as a naturally occurring immunomodulatory agent and is considered an immune checkpoint molecule capable of regulating inflammation; CD23 plays a central role in regulating IgE synthesis. To have a comprehensive overview, we also evaluated the count of leukocytes (neutrophils, monocytes, eosinophils), lymphocytes (T cells, B cells, NK cells), and relative count of transitional B cells. Historically, B cells have been considered to mediate humoral immune responses by differentiating into antibody secreting plasma cells [38]. Studies have revealed that B cells also serve as antigen-presenting cells, [39] secrete a variety of cytokines, [40] provide co-stimulatory signals, and promote T-cell activation [41]. Moreover, IL-10-producing B-cell subsets can inhibit innate and adaptive immune responses, inflammation, and autoimmunity, demonstrating the existence of regulatory B cells [38]. B cells differentiate into switched and non-switched B cell subsets. The differentiation is caused by activation of B cells, which are activated by T cell cytokines [11]. Class switching allows memory B cells to secrete different types of antibodies in future immune responses. The B cells then differentiate into either plasma cells, germinal center B cells, or

Table 3 T lymphocytes

T 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
Relative T lymphocytes, %	75.5 (68.5, 79.5)	72.0 (62.5, 80.0)	71.0 (65.75, 77)	0.332			
Absolute T lymphocytes 10^9/l	1.30 (0.82, 1.56)	1.41 (0.83, 1.73)	1.21 (1.03, 1.63)	0.944			
Relative natural killers (NK), %	9.0 (6.25, 13)	9.0 (6.5, 14)	9.5 (5.75, 14)	0.925			
Absolute NK 10^9/l	0.18 (0.06, 0.28)	0.2 (0.09, 0.28)	0.17 (0.07, 0.29)	0.983			
Relative CD4 T lymphocytes, %	65.0 (56.25, 73.0)	74.0 (68.5–80.0)	62.5 (54.0, 68.0)	0.002	< 0.05		< 0.01
Absolute CD4 T lymphocytes 10^9/l	1.1 (0.66, 1.45)	1.32 (0.95, 1.91)	1.07 (0.86, 1.38)	0.406			
Relative CD8 T lymphocytes, %	29.0 (21.25, 36.0)	22.0 (17.5, 25.0)	31.0 (26.5, 38.25)	0.001			< 0.001
Absolute CD8 T lymphocytes 10^9/l	0.48 (0.26, 0.65)	0.36 (0.28, 0.56)	0.59 (0.43, 0.73)	0.045	< 0.05		< 0.05
Relative T regulatory, %	1.0 (0.7, 1.25)	1.0 (6.8, 1.6)	0.8 (0.7, 1.2)	0.225			
Absolute T regulatory 10^9/l	0.01 (0.01, 0.03)	0.02 (0.01, 0.03)	0.015 (0.01, 0.02)	0.637			

DUP− patients without dupilumab treatment, *DUP+* patients with dupilumab treatment, *KW test* results of Kruskal–Wallis test, *NK* natural killer cells. We show the significant difference in statistical analysis, p-value < 0.05
% is stated from all leukocytes
% of relative T regulatory lymphocytes is stated from CD4 T lymphocytes
% of CD8 and CD4 T lymphocytes is stated from all T lymphocytes

memory B cells depending on the expressed transcription factors [38–41].

In our study we included patients with AD both with and without biological treatment. Before starting dupilumab therapy, patients suffered from moderate and severe forms of AD. During the course of dupilumab treatment lasting at least 18 months, the skin finding improved significantly. Patients without dupilumab suffer from a moderate-to-severe form of

Table 4 B lymphocytes

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
Relative B lymphocytes, %	11.5 (9.0, 13.75)	12.0 (9.0, 20.5)	12.5 (10.0, 14.25)	0.546			
Absolute B lymphocytes 10^9/l	0.17 (0.12, 0.26)	0.21 (0.13, 0.36)	0.2 (0.16, 0.32)	0.430			
Total memory B lymphocytes, %	50.0 (42.0, 62.0)	45.0 (37.0, 63.5)	43.5 (37.0, 57.25)	0.353			
B lymphocytes naïve, %	41.0 (34.0, 49.0)	44.0 (32.5, 55.0)	50.0 (36.25, 57.0)	0.193			
B lymphocytes Non-switched marginal, %	33.0 (22.0, 40.0)	30.0 (25.2, 43.0)	30.0 (24.5, 37.25)	0.764			
B lymphocytes switched, %	19.0 (12.0, 26.0)	18.0 (12,5, 23.5)	14.5 (10.75, 21.0)	0.502			
Transitional B lymphocytes, %	1.0 (0.7, 1.7)	1.0 (0.6, 1.25)	0.8 (0.57, 1.65)	0.750			

DUP− patients without dupilumab treatment, *DUP+* patients with dupilumab treatment, *KW test* results of Kruskal–Wallis test. We show the significant difference in statistical analysis, p-value < 0.05

The statistically significant difference (p-value < 0.05) in count of B lymphocytes (relative, absolute, total memory naïve, non-switched, switched, transitional) in patients with AD with and without dupilumab treatment in comparison with control group was not confirmed

% of relative B lymphocytes is stated from all lymphocytes

% of total, naïve, non-switched, and switched B lymphocytes is stated from all B lymphocytes

AD. In all patients we also evaluated the onset of AD and the occurrence of bronchial asthma and allergic rhinitis. These patients' characteristics were consistent. In both groups of patients with AD, we did not confirm any statistically significant difference in count of B lymphocytes (relative, absolute, total memory, naïve, non-switched, switched, transitional) in comparison with control group. However, our study reveals differences in expression of activation markers

Table 5 Expression of activation marker CD23 on memory, naïve, non-switched, switched, and total B lymphocytes

	Median count (first quartile, third quartile)			Statistical analysis (*p*-value)			
Expression of CD23 on B lymphocytes	**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, %	59.0 (45.0, 69.0)	61.0 (38.0, 70.5)	56.0 (45.75, 60.5)	0.525			
CD23 naïve MFI	11.02 (7.63, 20.72)	9.66 (8.77, 21.63)	6.7 (5.63, 8.27)	0.000		< 0.001	< 0.01
CD23 naïve, %	85.0 (82.0, 93.0)	91.0 (79.5, 92.0)	87.5 (82.25, 91.0)	0.925			
CD23 non-switched MFI	11.15 (6.67, 27.05)	12.2 (9.05, 27.22)	6.91 (5.19, 7.85)	0.000		< 0.001	< 0.001
CD23 non-switched, %	62.0 (37.0, 74.0)	64.0 (47.5, 72.5)	55.5 (47.0, 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, %	19.0 (11.0, 33.0)	14.0 (11.0, 25.5)	23.5 (17.0, 25.0)	0.249			
CD23 total B lymphocytes MFI	10.5 (7.66, 33.31)	9.54 (8.82, 21.7)	6.45 (5.35, 7.77)	0.000		< 0.001	< 0.001
CD23 total B lymphocytes, %	70.0 (59.0, 77.0)	73.0 (63.5, 78,0)	69.5 (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. We show the significant difference in statistical analysis, *p*-value < 0.05

CD23 and CD200 on B-cell subsets in patients with and without dupilumab therapy compared with control group. Although the CD200 expression on total B lymphocytes is significantly higher in both groups of patients with AD compared with controls, CD200 expression

Table 6 Expression of activation marker CD200 on memory, naïve, non-switched, switched, and total B lymphocytes

Expression of CD200 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
CD200 memory MFI	4.53 (3.69, 5.61)	4.13 (3.87, 4.24)	3.90 (3.43, 4.41)	0.055		< 0.05	
CD200 memory, %	39.0 (23.0, 50.0)	44.0 (27.5, 51.0)	34.0 (24.0, 46.75)	0.755			
CD200 naïve MFI	4.37 (3.75, 5.78)	4.35 (3. 90, 5.27)	3.89 (3.52, 4.38)	0.032		< 0.05	
CD200 naïve, %	67.0 (56.0, 80.0)	69.0 (62.0, 76.5)	70.0 (55.5, 79.0)	0.955			
CD200 non-switched MFI	4.72 (3.89, 7.34)	4.04 (3.89, 5.51)	3.78 (3.50, 4.38)	0.019		< 0.05	
CD200 non-switched, %	35.0 (19.0, 54.0)	42.0 (32.5, 50.5)	34.5 (23.5, 50.5)	0.725			
CD200 switched MFI	3.62 (2.79, 4.68)	4.05 (3.45, 5.05)	3.21 (3.02, 4.01)	0.076			< 0.05
CD200 switched, %	5.0 (2.0, 7.0)	4.0 (2.5, 5.5)	4.0 (2.75, 5.0)	0.482			
CD200 total B lymphocytes MFI	4.42 (3.73, 5.73)	4.31 (3.88, 5.34)	3.86 (3.52, 4.34)	0.020		< 0.05	< 0.05
CD200 total B lymphocytes, %	53.0	53.0	54.0	0.881			

Table 6 continued

Expression of CD200 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
	(38.0, 62.0)	(43.5, 57.0)	(35.5–62.75)				

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

is different in subsets of B lymphocytes in patients with and without dupilumab therapy. In patients without dupilumab therapy, we confirmed significantly higher CD200 expression on memory, naïve, and non-switched B lymphocytes in comparison with control. Furthermore, in patients with dupilumab therapy we confirmed higher CD200 expression on switched B lymphocytes compared with control. Non-switched B cells play the role in primary immune response; these cells could produce IgM antibodies in the first contact with antigens and are antigen dependent. Switched B cells are the most mature population of B cells; as they undergo immunoglobulin class switching from IgM, they can produce other types of antibodies than IgM and are antigen independent. Switched B cells are participating in the secondary immune response; this reaction is faster and more efficient [11]. CD200, by means of the interaction with its receptor, induces the suppression of T-cell-mediated responses [19–22]. Reduced Th1 cytokine (IL-2, IFNγ) production, increased IL-10 and IL-4 production, induction of T regulatory cells, inhibition of mast cell degranulation, downregulation of basophilic function, and suppression of NK cell function have been all experimentally demonstrated [42–46]. Higher expression of CD200 on switched B cells in patients with dupilumab therapy suggests that CD200 plays a role in regulating immune response, and we can hypothesize regarding the promotion of tolerance.

Regarding the CD23 molecule, we confirmed significantly higher expression of CD23 on total, memory, naïve, non-switched, and switched B lymphocytes in both groups of patients with AD compared with control group. The higher expression of CD23 on B lymphocytes and on their subsets could be caused by increased level of IL-4, but the level of IL-4 was not examined in our study. CD23 production is stimulated by IL-4, and the main function attributed to CD23 is the regulation of IgE synthesis [17, 47–55]. IL-4 also stimulates production of IgE. IFN-gamma also stimulates production of CD23, but suppresses production of IgE and inhibits IL-4-mediated production of CD23 [14]. IFN-alpha also suppresses these IL-4 mediated activities, and in addition, suppresses IFN-gamma-mediated stimulation of CD23 production. Changes in this coordinated regulation are involved in the development of AD [14].

A recent paper [16] has shown that CD23 can also negatively regulate B-cell receptor (BCR) activation on B cells by promoting B cell contraction. This explains the downregulation of CD23 on memory B cells that mount the high response of memory B cells to antigenic stimulation [15]. In contrast, upregulation of CD23 on switched memory B cells correlates with antigen-specific IgE levels and may be involved in some pathologies such as allergic rhinitis [16]. A further mechanism by which CD23 may regulate IgE levels is by acting as a direct decoy receptor for FcεRI. It was shown in mice that

Table 7 Parameters with significant difference in comparison with control group

Both groups of patients with AD, with and without dupilumab therapy	Patients without dupilumab	Patients with dupilumab
Relative neutrophils ↑	Relative monocytes ↑	$CD4^+$ T lymphocytes ↑
Absolute neutrophils ↑	Relative eosinophils ↑	$CD200^+$ switched B lymphocytes ↑
Absolute monocytes ↑	$CD200^+$ memory B lymphocytes ↑	Absolute $CD8^+$ T lymphocytes ↓
Absolute eosinophils ↑	$CD200^+$ naïve B lymphocytes ↑	Absolute leukocytes ↑
$CD23^+$ memory B lymphocytes ↑	$CD200^+$ non-switched B lymphocytes ↑	
$CD23^+$ naïve B lymphocytes ↑		
$CD23^+$ switched B lymphocytes ↑		
$CD23^+$ non-switched B lymphocytes ↑		
$CD23^+$ total B lymphocytes ↑		
$CD200^+$ total B lymphocytes ↑		

↓ significantly lower count in comparison with control group, ↑ significantly higher count in comparison with control group

B cells regulate serum IgE levels directly by absorbing free IgE molecules, thus preventing FcεRI loading and allergic sensitization. This more novel model of IgE regulation fits well with the generally higher IgE levels in CD23-deficient mice. CD23 cleavage could then be a mechanism to suppress this serum clearance and thereby enhance IgE levels [52–55]. Engeroff was engaged in this work with the role of CD23 in the regulation of allergic responses; he summarizes the importance of CD23: (1) it can absorb and clear IgE from the serum in a non-inflammatory fashion, (2) it reduces the synthesis of IgE from B cells, and (3) it regulates antigen-specific IgG and T-cell responses [17]. Oligomerization of CD23 on the surface of B cells could enhance IgE binding through an avidity effect. The higher expression of CD23 on B cells could be caused by activation of B cells with effect of allergens and could lead to elevated IgE levels [17]. This opinion of Engeroff et al. [17] could correlate with results of our study.

Czarnowicki sought to quantify B-cell populations and antibody-secreting cells in the blood of patients with AD, patients with psoriasis, and control subjects. They studied 34 adults with moderate-to-severe AD, 24 patients with psoriasis, and 27 healthy subjects using an 11-color flow cytometric antibody panel. IgD/CD27 and CD24/CD38 core gating systems were used to determine frequencies of plasmablasts and naïve, memory, transitional, and activated B cells. CD23 expression was highest in patients with AD and correlated with IgE levels and disease severity. The conclusion is that AD is accompanied by systemic expansion of transitional and chronically activated CD27(+) memory, plasmablast, and IgE-expressing memory subsets [56]. In another study, Czarnowicki et al. sought to define the frequency of B-cell subsets associated with progressive B-cell maturation and IgE class-switching [57]. They studied 27 children and 34 adults with moderate-to-severe AD and control subjects. Compared with adults, children showed T-cell predominance in the skin.

Circulating $CD19^+CD20^+$ B-cell counts were lower in patients with pediatric AD than in control subjects, whereas $CD3^+$ T-cell counts were higher. A decreased B-cell/T-cell lymphocyte ratio with age was observed only in pediatric control subjects. In pediatric patients with AD, a positive correlation was observed between B-cell/T-cell ratio and non-switched memory B-cell counts; positive correlations were observed between activated B-cell and memory T-cell counts. In patients with AD, IgE sensitization to most allergens clustered with age, T_H1, T_H2, total IgE levels, and B-cell memory subsets. Peripheral B and T cells are altered in pediatric patients with early AD, but T cells predominate in skin lesions [57].

Regarding the evaluation of other parameters, we confirmed in both groups of patients with AD higher count of inflammatory markers, such as neutrophils, monocytes, and eosinophils. Eosinophilia has been shown to be present in majority of patients with AD and their presence is correlated with the disease activity. Yamauchi et al. retrospectively reviewed data from 40 patients with AD who were treated with dupilumab. The number of eosinophils in the peripheral blood decreased at 4, 16, and 32 weeks after treatment initiation [58]. 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 [59]. According to our results, absolute eosinophils were significantly higher in patients in both groups of patients with AD in comparison with control group and there was no statistical difference in eosinophils count between patients with and without dupilumab treatment. Relative count of eosinophils was significantly higher in patients without dupilumab treatment compared with controls.

In patients with dupilumab therapy, we confirmed higher count of relative $CD4^+$ T lymphocytes and lower count of absolute and relative $CD8^+$ T lymphocytes compared with controls. This result is in accord with results of Szymanski et al. [60], who confirmed that IL-13 is also produced by $CD8^+$ T cells. The absolute $CD8^+$ T cell count in our study was reduced only in patients with dupilumab. It could be the consequence of this therapy because dupilumab blocks the subunit shared by receptors for IL-4/IL-13 [60]. Bakker et al.

Table 8 Changes in T and B lymphocytes in patients with atopic dermatitis, summary from other studies

Authors	Summary
Czarnowicki et al. [56]	Systemic expansion of transitional and chronically activated $CD27(^+)$ memory, plasmablast, and IgE-expressing memory subsets in children
Czarnowicki et al. [57]	↓ circulating $CD19^+CD20^+$ B cell in children ↑ $CD3^+$ T cell counts in children
Looman et al. [62]	Higher Th2, Treg, Treg-memory, $CD27^+$ IgA^+ memory B-cell numbers in children
Heeringa et al. [63]	Maritime and alpine climates normalizes memory B cells, $CD8^+$ T cells and Th2 cells
Czarnowicki et al. [65]	Immune activation and cytokine polarization with chronological changes in the blood of infants and children with AD through adolescence
Yanaba et al. [66]	CD19 expression in B cells plays a critical role in antigen-specific $CD4(^+)$ T-cell proliferation and T helper 2 and 17 responses in a murine model
Agrawall et al. [67]	↑ $CD4^+$ helper T cells T regulatory cells can convert to Th2 cells
Wang et al. [68]	↑ $CD4^+$ helper T cells (Th2) ↑ production of IL-4 and IL-13
Szegedi et al. [69]	↑ $CD4^+$ helper T cells (Th2) ↑ production of IL-31

studied the short- and long-term effects of dupilumab treatment on IL-4Rα expression and T-cell cytokine production within total and skin-homing (cutaneous lymphocyte antigen$^+$/CCR4$^+$) subpopulations in patients with moderate-to-severe AD. Dupilumab treatment rapidly and stably inhibited IL-4Rα, which was accompanied by a strong early functional immunological effect specifically on skin-homing T cells without affecting overall T helper type cell skewing in the long term [61].

We summarize other studies investigating the changes in T and B cells in Table 8. Looman confirmed that children with any atopic disease had higher Th2, Treg, Treg-memory, and CD27$^+$ IgA$^+$ memory B-cell numbers compared with children without atopic disease [62]. Heeringa et al. show that treatment in maritime and alpine climates normalizes B and T lymphocytes in children with moderate-to-severe AD [63]. Mizutani et al. revealed that CD8$^+$ T cells regulated by CD4$^+$CD25$^+$ Tregs in the early stage are key contributors to the development of Der f-induced skin lesions via increasing mast cell infiltration, indicating that CD8$^+$ T and Tregs could be potential therapeutic targets for AD [64]. Czarnowicki et al. sought to compare immune activation and cytokine polarization with chronological changes in the blood of infants and children with AD through adolescence. The adult AD phenotype is achieved only in adulthood. Unique cytokine signatures characterizing individual pediatric endotypes might require age-specific therapies [65]. The study of Yanaba et al. suggests that CD19 expression in B cells plays a critical role in antigen-specific CD4(+) T-cell proliferation and T helper 2 and 17 responses in a murine model of atopic dermatitis [66]. Agrawal et al. show that T(reg) cells can convert to Th2 cells and that this pathway is bidirectional [67]. Wang et al. focused on important discoveries of the contribution of CD4(+) T-cell cytokines to immunomodulation in AD, and particularly highlighted the multiple consequences of immune dysregulation on the barrier defect of the skin [68]. Szegedi et al. investigated the frequency of IL-31-producing T cells in AD lesions, as well as their cytokine profile. According to their results, many IL-31-producing T cells co-produced IL-13 and to a lesser extent IL-22, but rarely IFN-γ or IL-17 [69].

Limitations

In our study evaluating the immunological profile in patients with AD, we included patients both with and without biological treatment. For a more accurate evaluation of the effect of biological treatment on the immunological profile in patients with AD, it is necessary to evaluate these data in one group of patients before starting the biological treatment and monitor these parameters during treatment. However, our results suggest the effect of biological treatment. We selected the patients to match in terms of age, sex, and severity of AD before starting biological treatment, as well as with presence of other atopic diseases such as bronchial asthma and allergic rhinitis. The advantage of our study is that we show the immunological profile in two groups of patients with AD. All included patients are monitored long term at our department, they live in the same region, and their characteristics are comparable. The following study should clarify the relationships between the expression of CD23, CD200, and the level of eosinophils and IgE in patients with AD with and without dupilumab therapy.

CONCLUSIONS

The expression of CD23 is significantly higher on total B lymphocytes and on their subsets in both groups of patients with AD compared with controls. The expression of CD200 on total B lymphocytes is also significantly higher in both groups of patients with AD compared with controls, but it differs in subsets of B lymphocytes in patients with and without dupilumab therapy. In patients without dupilumab therapy we confirmed the significantly higher expression of CD200 on memory, naïve, and non-switched B cells in comparison with control. Furthermore, in patients with dupilumab therapy we confirmed the higher expression of CD200 on switched B lymphocytes compared with control subjects. Absolute number of

leukocytes, neutrophils, monocytes, and eosinophils are increased significantly in both groups of patients with AD compared with control subjects. There is no statistical difference in the absolute count of NK cells and relative count of transitional B cells in both groups of patients with AD compared with control subjects. In patients with dupilumab therapy, we confirmed the higher count of relative $CD4^+$ T lymphocytes and lower count of absolute and relative $CD8^+$ T lymphocytes compared with controls.

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Compliance with Ethics Guidelines. The study was conducted in accordance with the Helsinki Declaration of 1964 and all subsequent amendments, and all patients provided written informed consent. Patient-level data used for this analysis were de-identified. This study was approved by Ethics committee of the Faculty Hospital Hradec Králové, Charles University of Prague, Czech Republic. Reference number is: 2021 10 P 03. The study was 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.

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