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# Immunological Parameters in Patients Suffering from Atopic Dermatitis and Either Treated or Non-Treated with Dupilumab

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## ABSTRACT

Objective: The aim of the study is to analyze the absolute count of leukocytes, neutrophils, monocytes, eosinophils, T cells, natural killer cells, B cells and to evaluate the expression of functionally important CD23 and CD200 molecules on B cells in patients suffering from atopic dermatitis (AD), (with and without dupilumab therapy).
Materials and Methods: We examined 45 patients suffering from AD – 32 patients without dupilumab treatment (10 men, 22 women, average age 35.0 years), 13 patients with dupilumab treatment (7 men, 6 women, average age 43.4 years) and 30 healthy control (10 men, 20 women, average age 44.7 years). Immunophenotype was examined by flow cytometry (Navios Flow Cytometer – Beckman Coulter). The blood count was examined with a Sysmex XN 3000, Sysmex SP10, microscope DI60 for digital morphology evaluating cell division and microscope Olympus BX40. We compared the absolute count of leukocytes and their subsets, T cells (CD4, CD8), natural killers cells, absolute and relative count of B lymphocytes and expression of surface molecules CD23 and CD200 on B cells in AD patients and in control group. Non-parametric Kruskal-Wallis one-factor analysis of variance with post-hoc (follow-up multiple comparison) and Dunn's test with Bonferroni modification of significance level were used for statistical analysis.
Results: We confirmed the significantly higher number of neutrophils, monocytes and eosinophils and higher expression of CD23 and CD200 on B cells in peripheral blood of AD patients (either with or without dupilumab) therapy. We demonstrated the lower number of CD8+ T cells.
Conclusion: We demonstrated the difference in the count of white blood cells populations in patients suffering from AD compared with healthy control. There were a differences in the expression of immunoregulatory molecules CD23 and CD200 on B cells in AD patients (either with or without dupilumab therapy) in comparison to healthy controls.

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## INTRODUCTION

Atopic dermatitis (AD) is a common, chronic inflammatory skin disease characterized by immune abnormalities and a disturbed epidermal barrier, resulting in increased transepidermal water loss and increased penetration of allergens, irritants, and microbes. The key role of filaggrin (FLG), a protein present in the granular layer of the epidermis regulating the aggregation of keratin filaments, was evidenced in AD because some loss-of-function mutations in *FLG* gene resulting in FLG deficiency contribute to epidermal barrier dysfunction and is strongly associated with AD (1–4).

AD disease is characterized by a biphasic inflammation, evolving from an initial, acute phase dominated by Th2– and Th22 functionaly polarized T helper cells to a chronic phase characterized by the concomitant presence of various subsets of CD4+ T helper Th1, Th2 cells, and Th17 cells (1, 2). Excessive polarization of Th2 T cells leads to increased production of selected interleukins (IL) such as IL-4, IL-5 and IL-13. IL-4 has been shown to participate on the differentiation of naive 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 IgE and the chemotaxis of eosinophils (1, 2). Factors influencing the destruction of the epidermis, such as damage, infections (*Staphylococcus aures, Streptococcus spp.* and viral infections) or ongoing inflammation, stimulate keratinocytes to produce proinflammatory cytokines such as Thymic stromal lymphopoietin (TSLP), IL-25 and IL-33. They also contribute to the Th2-mediated immune response and activate innate lymphoid cells (subtype 2). TSLP, through its receptor, activates immature dendritic cells, enhances their maturation to the effective antigen-presenting cells (APCs) (2, 5–7). The Th2 cytokines interleukin 4 (IL-4) and IL-13 and the heterodimeric IL-4 receptor (IL-4R) complexes that they interact with, play a key role in the pathogenesis of allergic disorders. The multifaceted roles of IL-4 and Il-13 is an attractive target for treatment strategies. IL-4 is multifunctional cytokine, which promotes mature B cells activation and differentiation, proliferation and secretion of antibodies. IL-4 plays the role in prologing the survival of transitional B cells and promoting their maturation. There are multistep approaches to treat patients suffering from AD. The most effective therapy seems to be biological therapy. Dupilumab is a humane IgG4 monoclonal antibody that targets the IL-4 receptor alpha chain (IL-4Rα), common to both IL-4R receptors: type 1 (IL-4Rα/γc; IL-4 specific) and type 2 (IL-4Rα/IL-13Rα1; IL-4 and IL-13 specific) (5, 8–11).

Clinical studies with non specific and targeted therapeutics have helped to elucidate the contribution of various immune mechanisms to the disease phenotype. Besides skin lesions in AD patients, the blood components display specific inflammatory changes. The suppression of inflammatory changes is demonstrated in the case of dupilumab treatment, which inhibits the formation of key IL-4 and IL-13 cytokines. B cells proliferation and diferentiation is stimulated by IL-4, followed by terminal B cells differentiation to plasma cells. This cytokine increases expression of CD23 and supports isotype switching in antibodies (5, 6, 12–14).

The role of B cells in innate and adaptive immunity is rapidly evolving with acknowledgement of their complex multifactorial role in innate immunity through functions including antigen presentation, non-specific antibody secretion and cytokine secretion. A lot of new therapeutics for AD and other inflammatory diseases were derived from our understanding of T cells contribution to allergic inflammation. The function of B cells and their surface markers provided additional layers of complexity to understand of B cell function in normal and damaged skin. AD demonstrate high number of skin mature B cells (15). The other subtype of B cells, such as transitional B cells, represent a link between immature B cells in the bone marrow and mature B cells in peripheral blood. Transitional B cells represent one of the B cells in healthy subjects. Their count could be altered in patients with autoimmune immunopathological diseases such as multiple sclerosis, neuromyelitis optica spectrum disorders, systemic lupus erythematosus, rheumatoid arthritis and others. Transitional B cells can also produce homeostatic IL-10 and regulate proliferation of CD4+ T cells (6, 8). Activated Th2 cells produce IL-13. IL-13 could be also produced by basophils, natural killer and innate lymphoid cells (subtype 2). Activated eosinophils also produce IL-13 and during this process factors essential for polarization of eosinophils are induced and B cells are diferenciated for production of IgE (6, 8). IL-13 is involved to reshaping of B cells through the transitional B cells. However the function of transitional B cells remain largely unclear. This may partially be due to their low frequency in circulation (8, 13).

Activated B cells express CD23 surface molecule which is also expressed on monocytes and subsets of eosinophils. CD23 is low affinity immunoglobulin E, participating in the regulation of IgE synthesis and numerous pro-inflammatory activities. This molecule could trigger the release of proinflammatory cytokines, for example tumor necrosis factor alfa (TNF alfa), IL-1 beta, IL-6 (16–18). Transmembrane glycoprotein CD200 belong to the immunoglobulin superfamily. This molecule is expressed on lymphocytes (B-cells and T-cells) and endothelial cells. CD200 induces the downregulation of T-cells by interaction with its receptor CD200R. CD200 molecules demonstrated the inhibition of macrophage function, induction of regulatory T cells and suppression of the function of natural killer cells (19–22).

For these above mentioned facts that IL-4 and IL-13 are playing an important role in immunopathogenesis of AD and biological effect of these cytokines is blocked in patients treated by dupilumab, we focus on immunophenotype of blood cells as neutrophils, eosinophils, monocytes and lymphocytes. IL-4 supports switching B cells and subsequent output of antibodies and increased the expression of CD23 molecule. This cytokine play the role in formation of antibodies, the CD23 expression was followed in immunophenotyping analysis of peripheral blood of patients either treated or not with dupilumab. Assumed that the CD200 molecule could regulate myeloid cell activity and delivers an inhibitory signals for the macrophage lineage, this marker determination was also included in our immunophenotyping analysis (8, 14, 23, 24).

The aim of our study is to analyze the absolute count of leukocytes (neutrophils, monocytes, eosinophils), lymphocytes (T cells, B cells and NK cells) and relative count of transitional B cells and to evaluate the relation to the expression of CD23 and CD200 molecules on B cells in patients suffering from AD (with and without dupilumab therapy).

The evaluation of the expression of CD23 and CD200 surface molecules on B cells compared with absolute count of phagocytic cells could help us to asses the severity of AD and can reflect response to biological treatment with dupilumab.

## MATERIAL AND METHODS

### DERMATOLOGICAL EXAMINATION
Complete dermatological examination was performed in all patients included in the study. The diagnosis of atopic dermatitis was determined according to Hanifin-Rajka's diagnostic criteria. Severity of AD was scored in agreement with SCORAD (Scoring of Atopic Dermatitis), with assessment of topography (affected skin area), intensity criteria and subjective parameters and with the EASI system (Eczema Area and Severity Index). We also examined 30 healthy volunteers – blood donors (matched to age and sex).

The severity of atopic dermatitis was evaluated with SCORAD as a mild form to 25 points, as moderate over 25 to 50 points, as a severe form over 50 points. This examination was performed during one year every two month and the average SCORAD index was calculated.

The biological treatment (dupilumab) was indicated in patients with moderate or severe form of AD (SCORAD index = from 25 to 50 points and over). This is systemic treatment; the dose of dupilumab is 300 mg s.c. every two weeks. During the biological treatment these AD patients showed improvement of clinical signs to the mild form of AD (SCORAD index = to 25 points).

Inclusion criteria: 1) age 14 years and over 2) atopic dermatitis as defined by the criteria of Hanifin and Rajka. The severity of atopic dermatitis evaluated with SCORAD index and EASI score.

Exclusion criteria: pregnancy, breastfeeding, systemic therapy (cyclosporine A, systemic corticoids).

### EVALUATION OF THE IMMUNOLOGICAL PROFILE
The blood samples were collected from antecubical fossa vein into sample tubes pre-coated with 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. 5 μl of each fluorochrome-labelled monoclonal antibodies and 50 μl of peripheral blood was added to cytometric tube. Blood samples were incubated for 15 minutes with antibodies at room temperature in the dark. Then a lysis solution (OptiLyse C, Beckman Coulter) was added

and samples were incubated for 10 minutes. The samples were measured with a Navios Flow Cytometer (Beckman Coulter). A minimum of 60,000 events (60,000 cells) were obtained for each stain and were supplied in list mode (LMD), which are necessarily for assessment. 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+), 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).

This study was approved by Ethics commitee of Faculty Hospital Hradec Králové, Charles University, Czech Republic and it have been performed according to the Declaration of Helsinki. The informed consent has been obtained from all participants.

### STATISTICAL ANALYSIS
We compared the absolute 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 in patients suffering from AD (with or without dupilumab treatment) and in control group. For statistical analysis we used non-parametric 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

### CHARACTERISTIC OF PATIENTS
During the period from October 2021 to February 2022 we examined 75 subjects: thirty-two patients suffering from AD without dupilumab treatment, thirteen patients with dupilumab treatment and thirty subjects as a heatlhy control. The characteristic of patients is recorded in Table 1.

The severity of atopic dermatitis was consistent in both group of AD patients before starting the dupilumab therapy. Patients on dupilumab therapy had suffered from a moderate and severe form of AD before starting the biological treatment. AD patients have been under dupilumab treatment at least 18 months; there was a significant improvement in the skin finding and we observe mild symptoms of AD in these patients (Table 1). The treatment

**Tab. 1** Characteristic of patients.

| | Number of patients | Age | SCORAD | | EASI | |
|---|---|---|---|---|---|---|
| AD patients with dupilumab treatment | **13** (7 males, 6 females) | **43.4** (38.6–48.3) | Before therapy with dupilumab | **36.1** (30.5–45.2) | Before therapy with dupilumab | **35.2** (30.1–44.2) |
| | | | after 1.5 years | **10.5** (7.1–18.2) | after 1.5 years | **10.1** (8.2–17.2) |
| AD patients without dupilumab treatment | **32** (10 men, 22 women) | **35.0** (27.2–48.7) | **33.2** (26.5–38.7) | | **32.1** (26.8–38.5) | |
| Control group | **30** (10 men, 20 women) | **44.7** (36.8–51.4) | 0 | | 0 | |

involves the use of moisturizers and application of dupilumab 300 mg s.c. every two weeks.

The changes in absolute counts of leukocytes (neutrophils, monocytes, eosinophils), lymphocytes (T cells, B cells and NK cells) and relative count of transitional B cells are shown in Table 2. In AD patients treated with dupilumab the absolute number of leukocytes was increased compared to control group. The absolute number of eosinophils, neutrophils and monocytes in both groups of AD patients was increased compared to healthy controls. Absolute counts of T cells, B cells and NK cells were not statistically significantly different in AD patients when compared to the control group. There was significantly decreased the absolute number of CD8+ T cells in patients with dupilumab treatment compared to control group.

The number of transitional B cells has not been changed for any analyzed group. However, the expression of CD23 and CD200 on B cells were increased. This change was apparent in patients with dupilumab treatment and in patients without dupilumab compared to controls. The expression of selected markers on B cells is shown in Table 3.

Absolute counts of leukocytes and expression of CD23 and CD200 markers are recorded in Figures 1–6.

## DISCUSSION

The Th2 pattern inflammatory pathway in AD atopic dermatitis is driven by activation of functionally polarised CD4+ helper T cells and innate lymphoid type 2 cells (ILC2). The tissue infiltration is characterized by inflammatory cells such as eosinophils, mast cells, basophils, and production of proinflammatory cytokines, including IL-4, IL-5, and IL-13 (1, 2). The aim of our study was to evaluate the absolute number of leukocytes, T cells, B cells and NK cells in atopic dermatitis patients with and without

**Tab. 2:** Characterization of changes in absolute and relative counts of leukocytes (median values are recorded). Explanation: "DUP–" patients without dupilumab treatment, "DUP+" patients with dupilumab treatment, "KW test" results of Kruskal Wallis test, "MFI" mean fluorescence intensity. *p-value < 0.05* is considered as statistically significant.

| | DUP– | DUP+ | control | DUP+/DUP– | DUP–/control | DUP+/control |
|---|---|---|---|---|---|---|
| abs. count of leukocytes ($10^9$/l) | 6.64 | 7.30 | 5.640 | | | <0.050 |
| abs. count of neutrophils ($10^9$/l) | 4.20 | 4.94 | 3.200 | | <0.050 | <0.001 |
| abs. count of monocytes ($10^9$/l) | 0.50 | 0.55 | 0.390 | | <0.001 | <0.050 |
| abs. count of eosinophils ($10^9$/l) | 0.36 | 0.41 | 0.170 | | <0.050 | <0.050 |
| abs. count of T cells ($10^9$/l) | 1.31 | 1.41 | 1.210 | | | |
| abs. count of CD4+ T cells ($10^9$/l) | 1.10 | 1.32 | 0.075 | | | |
| abs. count of CD8+ T cells ($10^9$/l) | 0.48 | 0.36 | 0.590 | | | <0.050 |
| abs. count of B cells ($10^9$/l) | 0.18 | 0.21 | 0.200 | | | |
| rel. count of transitional B cell (%) | 1.00 | 1.00 | 0.800 | | | |
| abs. count of NK cells ($10^9$/l) | 0.18 | 0.20 | 0.175 | | | |

**Tab. 3:** Expression of markers CD23 and CD200 on B cells (median values are recorded). Explanation: "DUP-" patients without dupilumab treatment, "DUP+" patients with dupilumab treatment, "KW test" results of Kruskal Wallis test, "MFI" mean fluorescence intensity. *p-value < 0.05* is considered as statistically significant.

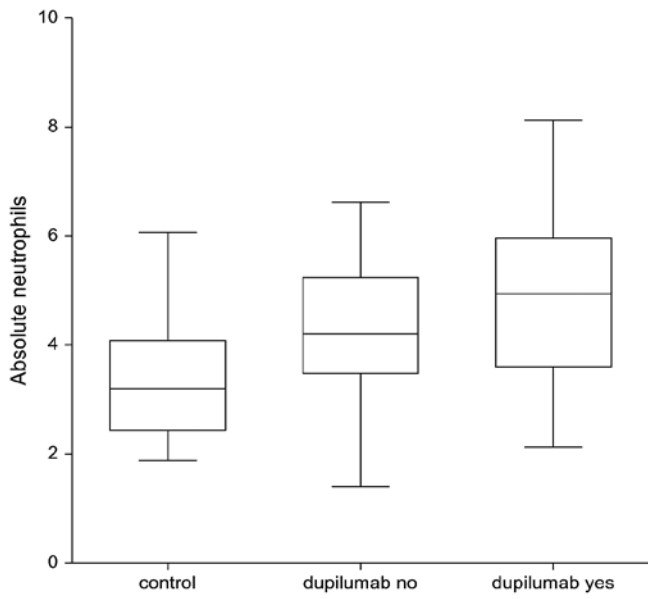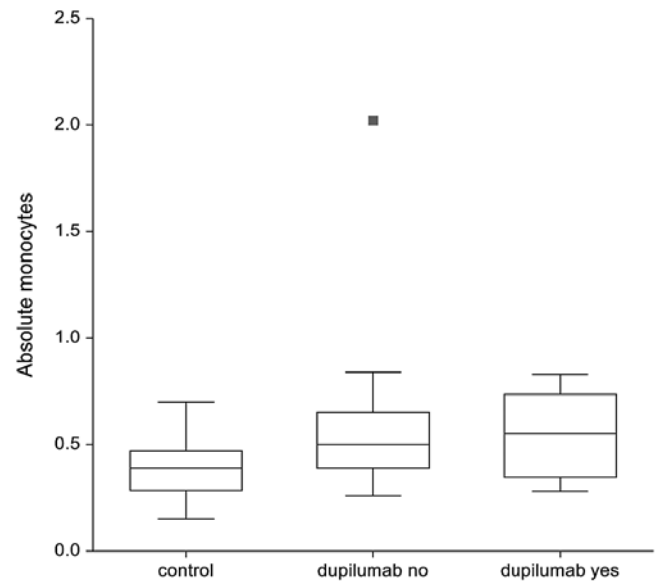| | DUP– | DUP+ | control | KW test | DUP+/DUP– | DUP–/control | DUP+/control |
|---|---|---|---|---|---|---|---|
| expression CD23 B cells (MFI) | 10.50 | 9.54 | 6.46 | 0.0000 | | <0.001 | <0.001 |
| expression CD200 B cells (MFI) | 4.42 | 4.31 | 3.86 | 0.0202 | | <0.050 | <0.050 |

Fig. 1 Absolute count of neutrophils (10⁹/l).
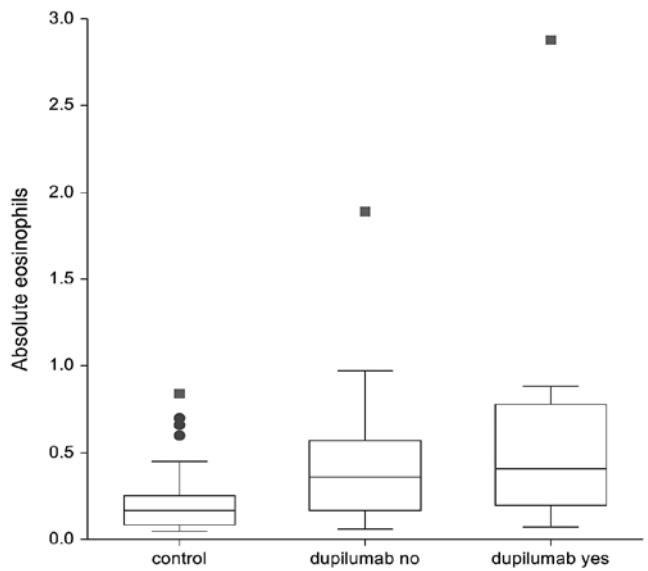


Fig. 2 Absolute count of monocytes (10⁹/l).



Fig. 3 Absolute count of eosinophils (10⁹/l).
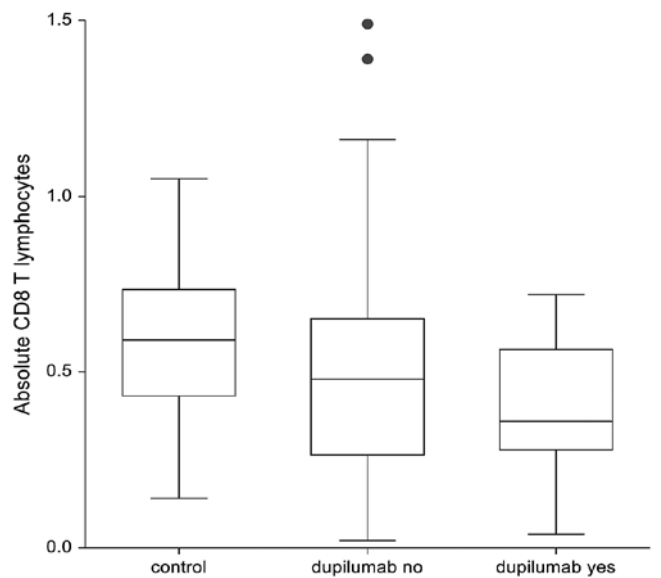


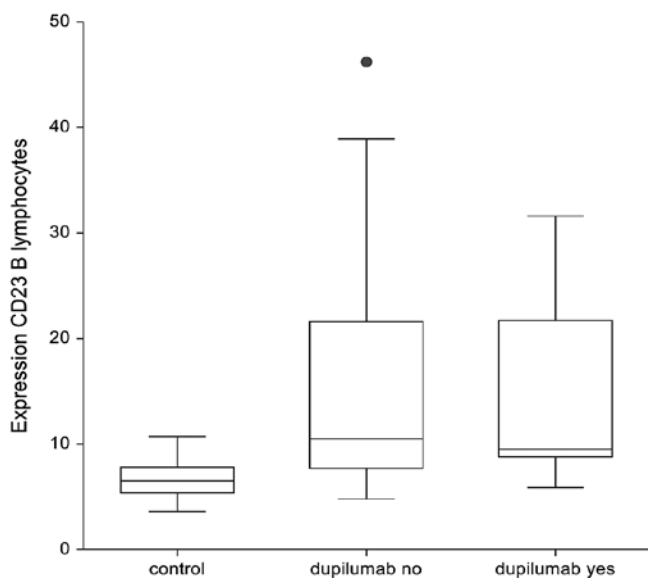Fig. 4 Absolute count of CD8 T lymphocytes (10⁹/l).



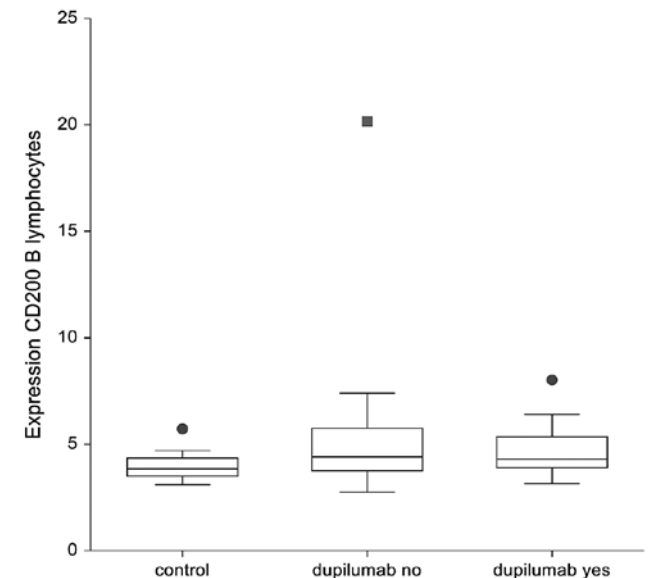Fig. 5 Expression of CD23 on B lymphocytes (MFI).



Fig. 6 Expression of CD200 on B lymphocytes (MFI).

dupilumab treatment in comparison to healthy control. We focused on detailed immunophenotyping of B cells and we determined the expression of CD23 and CD200 regulatory molecules.

The study of Jiang (25) showed higher count of white blood cells, neutrophils and lymphocytes, which is in accord with results of our study. The inflammatory markers as a count of neutrophils, monocytes and eosinophils are increased in patients who were either treated with dupilumab or not. Eosinophilia has been shown to be present in majority of patients with AD and it correlated with the disease activity (25–28). Yamauchi et al. (29) demonstrated reduction of eosinophil number in peripheral blood in patient treated by dupilumab. In contrary, increased eosinophil counts have been reported in some dupilumab clinical trials. This increase generally occurred in the first few weeks and returned to baseline or is lower in the end of the treatment period (30). In our study, we recorded the higher absolute count of eosinophils in both group of patients (with or without dupilumab treatment).

The monocytes are a significant component of skin immunopathology such as atopic dermatitis or *psoriasis vulgaris*. These cells could invade the inflamed skin and differentiate there into macrophages. Macrophages can act as antigen-presenting cells in the skin lesion directly in patients with atopic dermatitis (31–33). IL-13 is a cytokine which is produced not only by stimulated Th2 lymhocytes, but also by CD8+ T cells, NK cells and keratinocytes. This cytokine acts on monocytes, but also stimulates the B cells proliferation (28, 33). Our study showed the difference in absolute count of CD8+ T cells in patients with dupilumab treatment. This result is in accord with results of Szymanski et al. (28), who claimed the IL-13 is also produced by CD8+ T cells. The absolute CD8+ T cells count in our study was reduced in patients with dupilumab treatment only. It could be the consequence of dupilumab treatment, because dupilumab blocks the subunit shared by receptors for IL-4/IL-13 (10, 28). Vestergaard et al. (31) showed the higher level of monocytes, which expressed CCR2 in patients with AD compared to healthy control. We analyzed monocytes without expression of CCR2 chemokine receptor, but the absolute number of monocytes was increased in both group of patients with AD compared with the control group. This result correlates with the study of Vestergaard et al. (23) and is reflecting ongoing inflammation. Apparently, in patients with dupilumab treatment the relief of clinical signs of disease activity is seen. However, the inflammatory response is still active as the monocytes and neutrophils counts are increased in patients with AD either treated or not treated with dupilumab (10, 26, 28, 32).

The higher count of leukocytes such as neutrophils, monocytes and eosinophils correlated positively with a diagnosis of AD. The result is similar to the study of Jiang et al. (25) who also found higher number of neutrophils and eosinophils in patients with AD. The neutrophils represent the most abundant population of circulating leukocytes in peripheral blood. These cells are indispensable for antimicrobial immunity. The activity of neutrophils is controlled by immune mechanism including chemotaxis of neutrophils to tissue-draining lymph nodes, resulting in antimicrobial immunity and inflammation. For this reason better understanding of the role of neutrophils in AD immunopathogenesis and impact of biological therapy of AD is warranted (34, 35).

There is the difference in the abundance of NK cells in the skin lesions compared with nonlesional skin in AD patients. NK cells are apparently more abundant in lesional skin (36). Mobus et al. (36) found, that the number of NK cells in skin lesions was upregulated after dupilumab treatment. We observed that the absolute count of NK cells in blood is not statistically significantly different in both examined patients groups compared to healthy control in our study. It could be caused by increased migration of NK cells from blood to skin lesions (28, 36–38).

The number of activity of B cells is also correlated with ongoing inflammation. Simon et al. (39) in their study claimed that the loss of B cells and their function as antigen presenting cells will ultimate to a lower T cell activation and consequently to decreased cytokines and mediators release. This could be the mechanism responsible for the clinical improvement in patients with AD. This statement is in accordance with effect of dupilumab treatment. The cytokine IL-4 is responsible for promoting Th2 cell functional polarisation and consequently the secondary production of IL-4 and IL-13, potent stimulators of IgE production by B cells (40, 41). No statistical difference in the absolute count of B cells in AD patients compared to healthy control was found in our study. However, there were the statistically significant differences in the expression of CD23 and CD200 molecules on B cells. It could be interpreted that B cells are activated and participate in the ongoing inflammation. The marker CD23 is expressed on B cells and IL-4 is required for its expression as found by Getahun et al. (42). In our study expression of CD23 on B cells was increased in both AD group compared to healthy control. It could be probably caused by increased level of IL-4, but this was not examined by us (42).

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 it leads to elevated IgE levels (43). This opinion of Engeroff et al. (43) could correlate with results of our study.

Furthermore, our study confirmed the higher expression of CD200 molecule on B cells in the patients with and without dupilumab treatment compared to the control group. It could be in accord with work of Mucha et al. (44) who evidenced that the genes *DOK2* and *CD200R1* contribute to AD risk (44). Also CD200 is expressed on the cell surface and this protein is considered as an immune checkpoint molecule. CD200 is present on the membrane of macrophages and other immune cells and this marker is responsible for the process leading to secretion of high level of IL-10. IL-10 is recognized as homeostatic cytokine preventing immune activation. Higher expression of CD23 and CD200 molecules as activation markers is correlating with increase of absolute count of neutrophils, monocytes and eosinophils adverts to ongoing phagocytosis presumably and general dysregulation of immune response (45, 46).

The laboratory results in both group of patients with AD (DUP+/DUP–) are almost comparable despite

of difference in SCORAD and EASI score. Whereas the SCORAD and EASI score include assessment of skin lesions, results of these scores are different in both group of patients with AD. In our study the immunophenotype was investigated from peripheral blood. There could be difference in immunological process in peripheral blood and skin lesions. Concurrently, the skin lesions were improved after dupilumab treatment, but inflammatory process is ongoing in peripheral blood. From this reason there could be unevenness between laboratory results and SCORAD and EASI score.

Patients who suffer from severe or persistent form of AD experience significant impairment in their quality of life which is also associated with substantial economic burden on society as a whole (7, 28). For that reason, the better understanding of immunopathological mechanisms in AD patients with or without dupilumab treatment related with B cells could be a hopeful step in improving the long term quality of their lives.

## CONCLUSION

The expression of CD23 and CD200 on B cells is elevated in both group of AD patients compared with controls but there was not the difference in absolute count of B cells. The absolute count of CD8+ T cell is lower in AD patients treated with dupilumab. Absolute number of leukocytes, neutrophils, monocytes and eosinophils are increased significantly in both groups of AD patients compared to healthy control. There is no statistically difference in the absolute count of NK cells and relative count of transitional B cells in blood in both groups of AD patients compared to controls. It is feasable that these cells (NK cells and trasitional B cells) could be localized in skin lesions in patients without dupilumab.

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