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Evaluating the role of circulating dendritic cells in methimazole-treated pediatric Graves' disease patients.

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Abstract: Graves' disease (GD) is hyperthyroidism associated with organ-specific autoimmune inflammation. GD occurs more frequently in adults than in children, however, pediatric patients are a therapeutic challenge due to cycles of remissions and relapses requiring constant monitoring at every stage of treatment administered. Dendritic cells (DCs) are considered a link between innate and adaptive immunity. DCs as antigen-presenting cells (APCs) are involved in antigen presentation to T lymphocytes, thereby, initiate shift towards effector cells. In accordance, DCs participates also in the modulation of tolerance to specific antigens. To date, the data on DC role in Graves' pathological processes are scarce. Therefore, here we evaluated frequencies and role of circulating DCs in GD pediatric patients treated with methimazole. Flow cytometric analysis was implemented to evaluate mDC1, mDC2 and pDC cells and their correlation with clinical GD-related parameters. We found significantly higher levels of DC subsets in patients at admission. Furthermore, methimazole treatment seemed to effectively reduce subsets of DCs which, in addition, were found to differentialy correlate with thyroid function. Our study shed a new light on DCs role in pediatric GD pathomechanism. Further studies are required for mechanistic assessment of DCs exact role in disease progression and influence on thyroid function.

Keywords: Graves' disease; autoimmunity; dendritic cells; methimazole (3-10 key words)

1. Introduction

Graves' disease (GD) is the most common cause of hyperthyroidism, a condition associated with abnormal thyroid gland function resulting from an organ-specific autoimmune reaction. Despite local autoagression of the immune system, affected thyroid function contributes significantly to changes in systemic regulation leading to, inter alia, weight loss, palpitation, hyperactivity, orbitopathy [1,2]. In children, GD occurs less frequently compared to adults, however, it remains a severe therapeutic problem and requires constant monitoring due to alternate periods of remissions and relapses [3]. Although the etiology of GD is still not fully understood, the classical paradigm involves role of genetic, environmental, and immune-related factors. Abnormalities in immune system function leads to uncontrolled production of thyroid-stimulating hormone receptor (TSHR) autoantibodies (TRAb), thus, inducing excessive thyroid hormone synthesis and consequently resulting in hyperthyroidism [4]. Moreover, previous research indicates that increased activity of T

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lymphocytes, helper Th1 and Th2 predominantly, is responsible for the inflammation phenomenon occurring in the GD [5]. To date, participation of other populations of immune cells has been demonstrated by numerous papers, including effector Th17, regulatory T lymphocytes (Treg), and even tissue-resident fibroblasts [6-7]. However, to date there are no comprehensive data on the role of dendritic cells in the course of pediatric Graves' disease.

Dendritic cells (DCs) are a heterogeneous population of cells originating from the bone marrow, classified as highly specialized antigen-presenting cells (APCs). Their primary role is to induce and regulate the immune response through effector cells activation. Dendritic cells are also considered to be a link between the innate and acquired immune response, which is due to the fact they recognize pathogens by receptors like Toll-like receptors (TLRs) belonging to the non-specific immunity. Apart from pathogens presentation to T-lymphocytes and inducing effector phenotypes, DCs modulate response of T lymphocytes to specific antigens in the body, thus controlling tolerance mechanisms. [8-10]. Furthermore, dendritic cells are thought to be involved in T lymphocytes plasticity through controlling Th1 and Th2 differentiation [1,11-12].

Today, two major subpopulations of dendritic cells can be distinguished: plasmacytoid DCs (pDC) indicated by the presence of CD303 marker and myeloid/classical DCs (mDC/cDC). Additionally, myeloid DCs can be further divided into two subgroups: mDC1 marked by CD1c, and mDC2 recognized by the CD141 marker [8]. Plasmacytoid dendritic cells (pDCs), immediately after contact with an antigen, produce mainly type I interferons (IFNα) responsible for modulating activity of immune cells by increased TLRs expression induction. Effects caused by pDC also include an increase in production of proinflammatory cytokines and chemokines. On the contrary, myeloid dendritic cells are involved predominantly in presenting antigens to T cells, as they demonstrate higher levels of MHC class II molecules [8,13]. Dendritic cells can contribute to the initiation of tolerance mechanisms in various ways, but therefore, they can also play role in immune tolerance impairment that is one of the causes of autoimmune diseases onset. Accordingly, there is a gradually increasing number of the reports on alleged involvement of dendritic cells in autoimmune diseases by affecting tolerance mechanisms [14-16].

Considering scarce data on dendritic cells in pediatric Graves' disease, that in fact are involved in tolerance induction counteracting the development of autoimmune diseases, our research aimed to assess the changes in dendritic cells in children with the disease at the admission. Moreover, here we evaluated how DCs subsets correlate with thyroid function-related parameters and influence of methimazole (MMI) treatment on these dependencies.

2. Materials and Methods

Patients.

Study was performed on 22 pediatric patients with active Graves' disease (GD). The additional control group (HC) involved 31 healthy patients with autoimmune and inflammatory conditions excluded – euthyroid and no personal or family history of autoimmune thyroid disease (AITD). The clinical description of the patient groups was included in the supplementary table (Supp. Tab. 1). Informed consent was obtained from each of the patients (parent or legal guardian for underage subjects). The protocol of the study was approved by the Ethical Committee at the Medical University of Bialystok (R-I-002/422/2010).

The research material was EDTA-anticoagulated peripheral blood collected by venipuncture at three time-points: before treatment (T0), after 3 months (T1), and 1 year of treatment (T2) with methimazole from pediatric patients. Peripheral blood mononuclear cells (PBMC) were isolated through gradient centrifugation using Pancoll with a density of 1.077g/l (PAN Biotech). Cells were washed and suspended in cryoprotectant (10% DMSO (Sigma-Aldrich) in fetal bovine serum (FBS; PAN Biotech) and were stored as viable cells in liquid nitrogen until described experiments.

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Parameter	Graves' disease (GD)	Control group (HC)
Age [years]	14 (10.75; 16.00)	13.5 (9.50; 14.50)
Sex distribution [male to female]	18% to 82%	39% to 61%
TSH [mIU/l]	0.02 (0.01; 0.48)	2.39 (1.40; 2.80)
fT4 [ng/dl]	2.03 (1.04; 7.77)	
fT3 [ng/l]	6.47 (3.35; 28.56)	
TRAb [IU/l]	27.90 (15.20; 36.77)	

Supp. Tab. 1 Clinical description of the studied groups. Data presented as median values with 25th and 57th percentile in the brackets.

Flow cytometry.

Following thawing of PBMC from liquid nitrogen, cell counting, and viability verification, cells were subjected to flow cytometric analysis. PBMCs of Graves patients (GD) and control group(HC) were stained with monoclonal antibodies conjugated to fluorochromes aimed at selected cell surface markers allowing for dendritic cells analysis, including anti-Lineage2 (anti-CD3 FITC, clone SK7; anti-CD14 FITC, clone MφP9; anti-CD19 FITC, clone SJ25C1; anti-CD20 FITC, clone L27; anti-CD56 FITC, clone NCAM16.2), anti-CD1c AlexaFluor647 (clone F10/21A3), anti-CD141 PE (clone 1A4) (BD Bioscience), anti-CD303 PE-Cy7 (clone 201A) (Biolegend). Equal amounts of PBMC were stained in the same buffer volume for all of the patients to investigate absolute numbers of cells. Following staining of cells and washing unbound antibodies in phosphate-buffered saline (PBS; Corning), cells were fixed with the use of CellFix reagent (CellFix; BD Bioscience) and stored shortly until the acquisition in +4°C. Data were acquired on FACS Calibur flow cytometer (BD Bioscience) and subsequently analyzed using FlowJo software (Tree Star Inc.).

Dendritic cells were distinguished based on morphological properties (relative size – forward scatter (FSC), and relative shape/granularity – side scatter (SSC)) and lack of cell surface markers included in the Lineage2 reagents: CD3, CD14, CD19, CD20, CD56. Furthermore, stratification of dendritic cells was possible by evaluating the differential expression of CD1c, CD141, and CD303 cell surface markers. Accordingly, we were able to monitor changes in plasmacytoid DCs (CD303+, pDC) and two subtypes of myeloid/classical DCs (CD141+, cDC2; CD1c+, cDC1). In addition, various combinations of selected markers were included in the analysis of DCs: CD1c+CD141+, CD1c+CD303+, CD141+CD303+, and CD1c+CD141+, CD303+). The implemented gating strategy was included in the supplementary materials (Supp. Fig. 1).

Statistical analysis.

Biostatistical analysis of the obtained flow cytometric data was performed with the use of GraphPad Prism 9.0.0 statistical software (GraphPad Prism Inc.). Considering the lack of Gaussian distribution of the data within studied groups, two-way ANOVA tests were applied with Sidak correction. Test for paired data was used for assessment of changes in Graves patients in the course of treatment, and unpaired when comparing Graves patients to the control group. The evaluation of

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correlations between studied dendritic cells-related parameters and clinical results was performed using the Spearman test. The final results were presented on graphs as the frequency of studied markers within Lineage2-negative PBMC cells, frequency of Lineage2-negative cells with selected marker within total PBMC cells, and as an absolute number of cells.

3. Results

Graves' disease is associated with higher numbers of circulating dendritic cells compared to healthy control groups.

We found that untreated Graves patients demonstrated significantly higher levels of dendritic cells compared to the healthy control group. These differences were observed in all studied subtypes of dendritic cells, CD141- and CD1c-expressing myeloid mDCs and CD303+ plasmacytoid pDCs, especially when frequency within Lineage-negative PBMC or absolute numbers were analyzed. Although CD141+ subtype of dendritic cells was the dominant population among others in the context of absolute cell numbers, the ratio of changes between Graves' patients and the control group seemed to be similar among all monitored DC-related parameters (Fig. 1, a-c). Interestingly, we found a significant difference in ratio between mDC2 and pDC with a strong significance level. Such a phenomenon was not found in mDC2/mDC1, with the only slight tendency in mDC1/pDC ratio (Fig. 1d).

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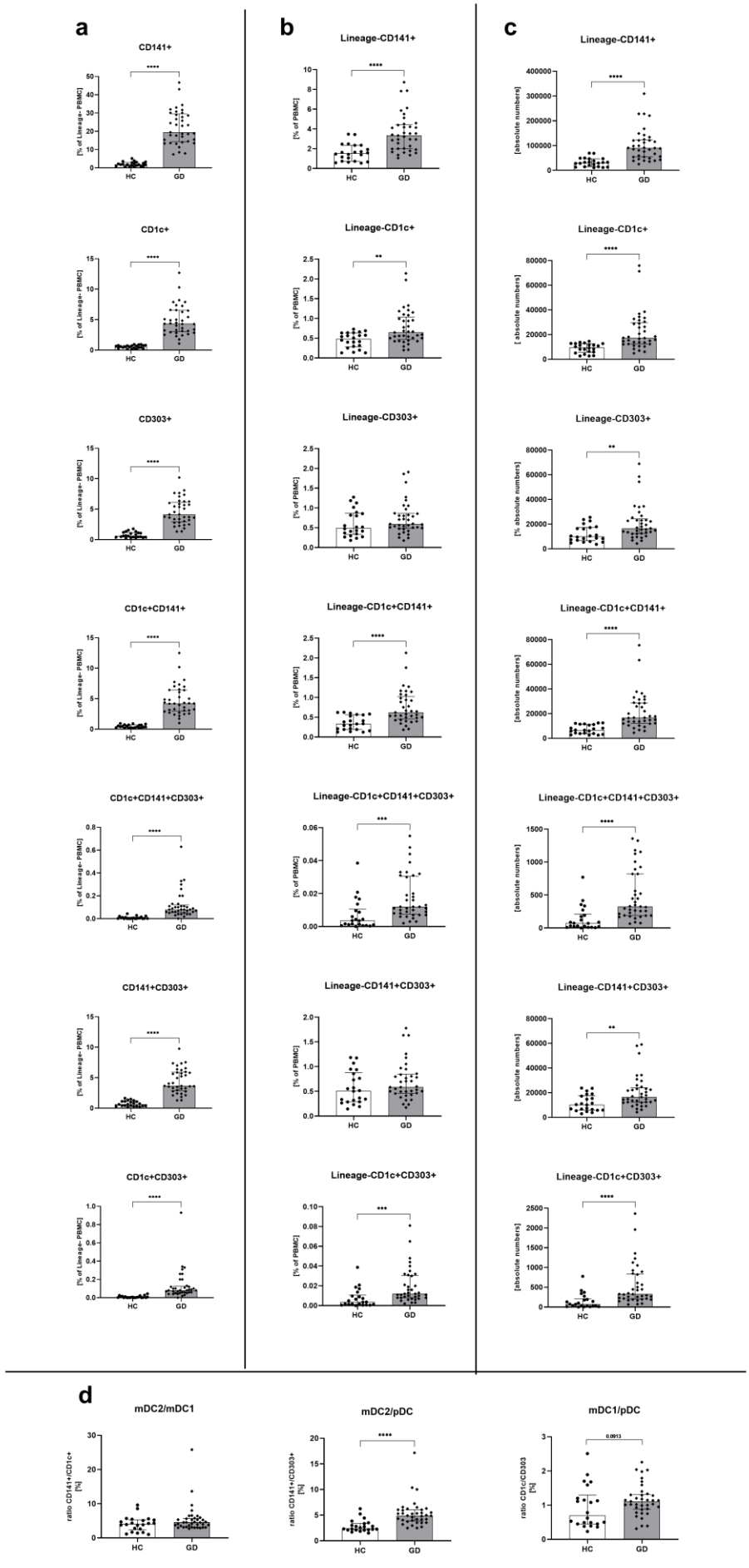

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Fig. 1 Dendritic cell-related differences among Graves' disease patients and healthy control group. Acquired data were analyzed in the context of DC subsets frequency within Lineage-negative PBMC (a), the total pool of PBMC (b), as an absolute number of cells (c), and the ratio between studied DCs (d). Data presented on each graph as median with interquartile range.

Treatment with methimazole influences changes in dendritic cells distribution in Graves pediatric patients.

We found that methimazole implementation in Graves' disease patients did not lead to general significant changes in all DC subsets within the first 3 months of the therapy. An exception were subtypes of DC with surface expression of CD303 marker characteristic for plasmacytoid DCs, even in combination with surface expression of CD1c and/or CD141 typical for myeloid DCs. Reduction of these populations in the course of treatment was reported both at frequency and absolute number levels. In addition, such direction of changes was maintained until further time points were reported, especially in reference to CD1c+CD303+ and CD1c+CD141+CD303+ dendritic cells. Similarly, a slight change was also observed in the CD303+ pDCs analyzed as the frequency within Lineage- PBMC. Noteworthy, considering mDC-related markers, CD1c+ mDC1s seemed to respond to the methimazole regimen with a decline in frequency and number of these cells after 1 year of therapy significantly. No differences were found in dendritic cells expressing CD141 marker (Fig. 2, a-c).

Furthermore, ratio analysis indicated that changes in myeloid mDC2 and plasmacytoid pDC are more profound in the course of treatment with methimazole applied. Moreover, a higher decline in pDCs than mDC2s might be responsible for the increased ratio of mDC2/pDC after 3 months of therapy. In accordance, less significantly increased mDC1/pDC ratio at 3rd month of methimazole application suggest a higher role of changes in CD141+ mDC2 and CD303+ pDC in the course of Graves' disease management (Fig. 2d).

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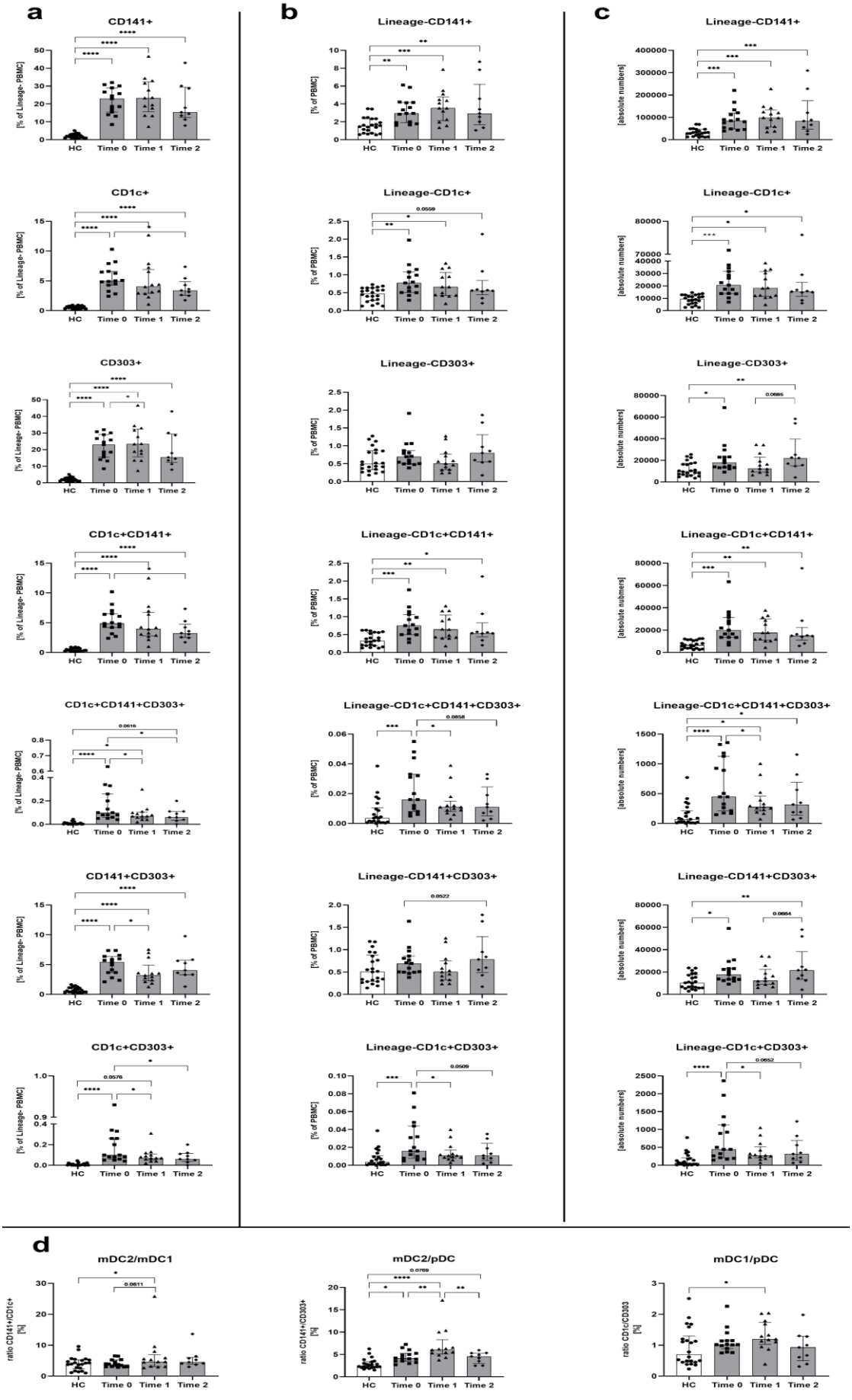

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Fig. 2 Changes in dendritic cell-related parameters in the course of Graves' disease patients treatment. Acquired data were analyzed in the context of DC subsets frequency within Lineage-negative PBMC (a), the total pool of PBMC (b), as an absolute number of cells (c), and ratio between studied DCs (d). Data presented on each graph as median with interquartile range.

Dendritic cells demonstrate to correlate with disease-related clinical parameters in a treatment-dependent manner.

Prior application of methimazole, analyzed subtypes of dendritic cells were found to correlate with TSH levels negatively, and on the contrary, a positive correlation was found in reference to fT4, fT3, and TRAb. It is worth noting that none of the population exhibited dominance at that time point in their association with clinical parameters. Despite the relatively sustainable phenomenon observed before treatment, 3 months of methimazole seemed to cause critical changes within dendritic cell populations. CD303+ pDC and CD141+ mDC2 subtype only correlated with TSH, and importantly, unlike at time 0, here positive link was found. In addition, a subtype of CD1c+ mDC1 was demonstrated to change its role in reference to fT3 levels with a shift from positive to negative correlation at 3rd month of therapy. Following 1 year of treatment, correlations between DC-related parameters and clinical data were comparable to those observed before therapy implementation. However, contrary to the positive correlation of DCs with TRAb at time 0, here changes within dendritic cells seemed to be associated with the completely different response of TSH-R antibodies. Similarly, the correlation of CD303+ pDC subtype with clinical data was affected in the course of treatment as that population was the only among others demonstrating negative correlation with fT4 and fT3 levels (Fig. 3; Supp. Tab. 2).

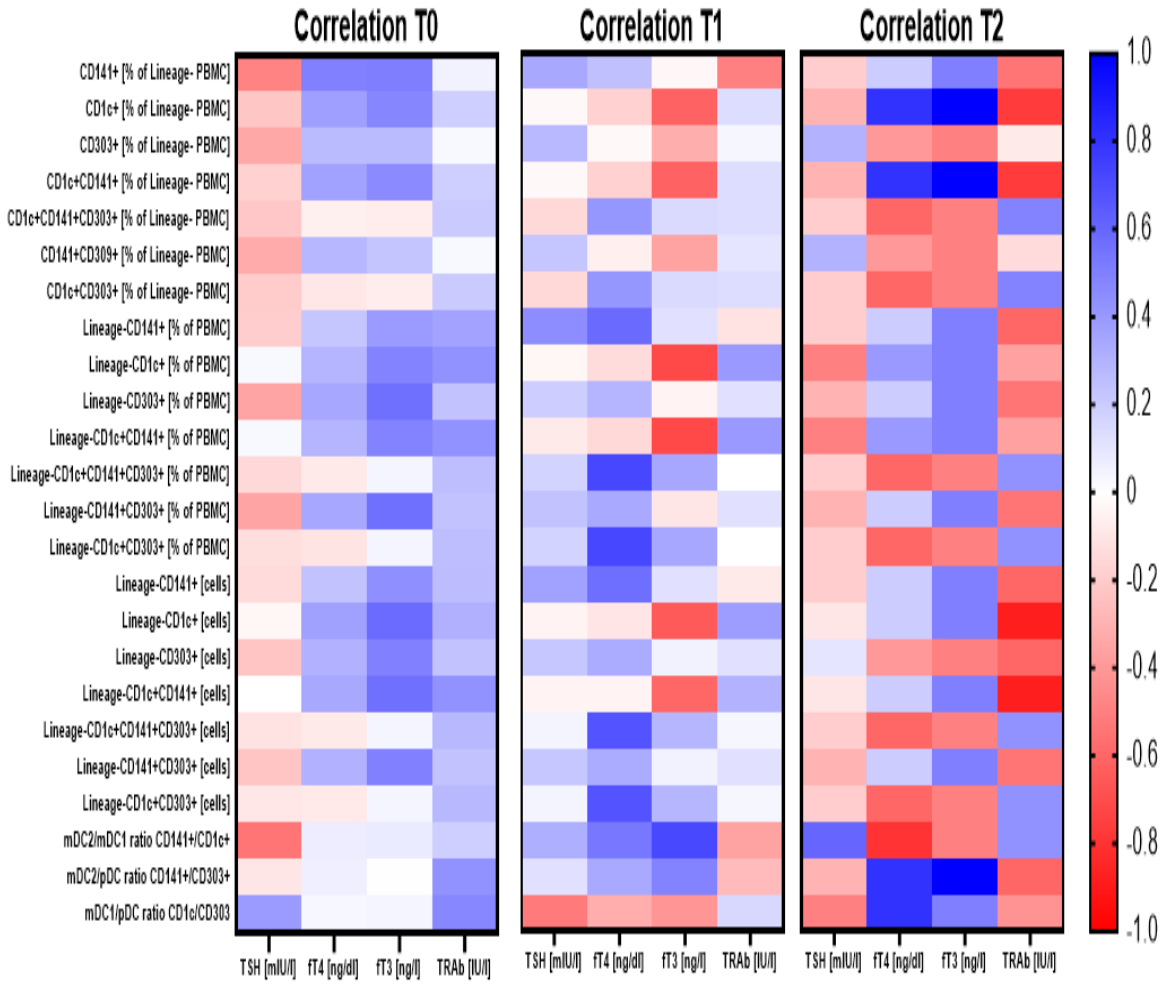

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Fig. 3 Demonstration of correlations between dendritic cell-related parameters and critical clinical analysis results, prior to and in the treatment course. Correlation analysis demonstrated for three time-points for Graves' disease pediatric patients: before treatment (T0), after 3 months (T1) and 1 year (T2) of the treatment. Strength of correlations demonstrated through color gradient – blue for positive correlations and red for negative correlations.

4. Discussion

Graves' disease is characterized by disturbances in immune tolerance. Reactive autoantibodies, especially TSHR antibodies (TRAb), influence the course and the clinical manifestation of the disease [17]. Dendritic cells can create tolerance mechanisms in various ways, while lack of self-tolerance is one of the causes of autoimmune processes. Accordingly, an increasing number of dendritic cells reports suggested potential involvement in autoimmune diseases by modifying tolerance enrolling lymphocytes [18]. The mechanisms of dendritic cells participation in autoimmune processes does not seem to be associated only with changes in distribution of circulating and tissue-resident dendritic cells. Notably, impairment of DCs function may result in the break-down of self-tolerance, leading to autoimmune disorders [8]. Dendritic cells contribution to autoimmunity processes occurs through two main cellular mechanisms. On one hand, DCs are responsible for maintaining immune tolerance (immunosuppression effect) via induction of regulatory T cells phenotype through differentiation from CD4+ naïve T lymphocytes, associated inter alia with higher production of TGF-β. On the other hand, dendritic cells can promote adaptive self-reactive responses and thus cause loss of tolerance. Lack of response to the antigen also affects the inhibition of T cells activity, CD8+ cytotoxic phenotypes especially [19]. Rönnblom et al. proved that pDCs may induce Th1-related reactions, produce type I INF, and thereby, lead to the autoimmune phenomenon in the pathogenesis of systemic lupus erythematosus [20]. Moreover, dendritic cells ability to present the antigen (APCs), here in context of autoimmune disorders - autoantigens, might be a feature responsible for effector cells induction that consequently acquire autoreactive status. Noteworthy, despite numerous partial information on DC in autoimmunity, there are only a few studies aimed directly at the DCs role in GD. To date, our study is the first research that focuses on pediatric population of Graves' disease in context of dendritic cells and their relation to the treatment effects.

In our research, we found a significant increase in the number of dendritic cells in GD patients compared to the healthy control group (Fig. 1). Demonstrated and increased percentage of circulating DCs might be directly related to the allegedly increased proportion of DCs involved in antigen, and thus induction of lymphocytes producing anti-TSH-R autoantibodies. Dominant subpopulation of DCs presenting antigens to lymphocytes are plasmacytoid DCs, characterized by the presence of the CD303 molecule on their surface [21]. In our GD patients we found significantly higher levels of CD303-positive DCs cells at time of admission when compared to control group. Furthermore, slight decrease in the frequency of CD303+ DCs in the course of treatment suggest that methimazole can effectively reduce the percentage of pDCs population. Presumably such decline in pDC might be associated with better outcome of the treatment, what can be supported by the fact that negative correlation was found between the number of CD303-positive DCs and the TSH level (thyrotropic hormone). Noteworthy, in accordance with link between pDCs and TSH levels, tendency for positive correlation was found between that population and fT3 and fT4 levels. Therefore, methimazole-induced reduction of pDCs might also be associated with lower levels of fT3 and fT4. In addition, mechanism of thyroid function restoration did not seem to be associated directly with tolerance induction as we did not find any correlation between CD303-positive dendritic cells and the level of anti-TSH-R autoantibodies (TRAb).

As indicated above, the data on DCs role in Graves are scarce, however, increased numbers of pDCs were reported at the onset of other autoimmune diseases, including type 1 diabetes mellitus [18]. In non-obese diabetic mice model, presence of special DC phenotype - CD103+, was associated with occurrence of pancreatic islets-reactive CD4+ T cells initiating autoimmune cascade. Interestingly, ablation of these CD103+ DC gene lead in consequence to proper immune tolerance and lack diabetes-related autoimmune features [22]. Similarly to diabetes, dermal autoimmune

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disorder - psoriasis, an increase of DCs was detected in the epidermis and dermis of affected patients [23].

It is worth noting that plasmacytoid dendritic cells (pDC) by secretion of interferons, type I interferons predominantly, can induce maturation of myeloid DC into fully active mDCs. Such mechanism could explain autoimmune inflammation in systemic lupus erythematosus where pDC-related production of type 1 interferons caused mDC maturation, and consequently their increased interaction with the lymphocytes [24]. Mature mDCs can interact with CD4+ lymphocytes triggering an inflammatory activity of Th1 and Th17 subtypes, and enhancing activity of cytotoxic CD8+ lymphocytes. Additionally, some data suggest that mutual interactions between mDC and B lymphocytes might be responsible for induction of autoantibodies secretion [25-26].

Myeloid/classical dendritic cells, comprised of mDC1 (CD1c+) and mDC2 (CD141+) subsets, are predominantly responsible for the presenting antigens to T cells [21]. Here, we observed statistically significant differences in mDCs in patients with GD compared to controls. Furthermore, methimazole treatment led to slight decrease in mDC1 and mDC2 subsets. Interestingly, methimazole allowed to stabilize the ratio of, both mDC1/pDC and mDC2/pDC, causing their gradual decrease through 3 months up to a year after therapy initiation. This value shows a downward trend to reach the level observed in the control group (HC). Noteworthy, we did not observe such type of changes in context of mDC2/mDC1 ratio during whole period of treatment. Only slight tendency for an increase was demonstrated at 3rd month of methimazole application. In general, reported differences are very interesting in context of significant phenotype shifts between dendritic cells subsets, which in fact, contribute differently to the phenomenons occuring in the Graves' progression. In addition to mDC-related events, strong positive correlation of CD141+CD1c+ DC population frequency with fT3 and fT4 levels observed after one year of treatment might indicate their potential use as markers of favorable control of the disease with methimazole treatment.

Treatment with methimazole (MMI) was found to demonstrate unambiguous effects on studied DCs. Implementation within first 3 months of therapy did not lead to crucial changes within all dendritic cell subsets. In contrary's subsets demonstrating CD303 expression (pDCs) showed a decrease in their number. Treatment extended over 3 months contributed to significant decline in mDC2 and pDCs number, with higher decrease observed in pDCs than mDC2 which probably led to the increased ratio of mDC2/pDC. Less pronounced increase in mDC1/pDC ratio at 3rd month of methimazole application suggests more essential role of shifts between CD141+ mDC2 and CD303+ pDC than CD1c+ mDC1 in the course of Graves' disease management. Effects of prolonged treatment with anti-thyroid drugs and changes in levels of DCs might indicate immunomodulatory role of methimazole. MMI is often postulated as a therapeutic with direct immunosuppressive facilities according to the number and functions of various types of immunocompetent cells in peripheral blood [27]. It is proved that methimazole reduces serum levels of cytokines and may influence a shift from Th1 towards Th2 responses in GD [28-29]. Crescioli et al., for the first time evidenced that MMI decreases CXCL10 protein in thyrocytes , which is involved in Th1 immune responses [30]. Bossowski et al. took upon the topic of effector T lymphocytes, Th17 predominantly, in autoimmune thyroid diseases. They demonstrated a significantly lower frequency of cells with CD4+IL17+ phenotype in newly diagnosed GD, and its normalization after 2–3 months of methimazole therapy [31]. Here, we demonstrated for the first time that methimazole properties affects significantly also phenotype of dendritic cells and shifts between studied subsets. The mechanism leading to the observed effects requires further investigation, however, considering influence of Graves' therapy on adhesion molecules one of the possible explanations arises. Adhesion molecules like VCAM-1 and ICAM-1 are markedly elevated in sera of patients with GD, and importantly, decline after therapy application [32-33]. In accordance, decreased number of DCs occurring in response to GD therapy might be associated with changes in these molecules and consequently affected migration.

Regarding the relationship between DCs and thyroid hormone levels before and after MMI treatment, we found a positive correlation between all analyzed subtypes of DCs versus fT4, fT3 and TRAb levels, with concomitantly negative correlation of CD303+ pDCs with TSH concentration. As commonly observed elevated levels of fT4, fT3, and TRAb and low concentration of TSH were detected in studied group of pediatric GD at the admission. In context of high levels of DCs subsets

before treatment, that phenomenon could be partially explained with fT3 concentrations. Levels of fT3 were found to affect maturation of peripheral blood monocytes into functional DCs., and further their activation and changes in expression of MHC II and costimulatory molecules [34-37]. Noteworthy, we demonstrated link between, inter alia, fT3 and DCs, thus reduction of these cells in the course of methimazole must at least to a certain degree be associated with that hormone.

Cumulatively, pediatric patients with GD may demonstrate various deficiencies in their immunological system. Circulating antibodies and infiltration of autoreactive lymphocytes initiate the cascade of autoimmunization. DCs classified as immunogenic or tolerogenic exhibit dual nature activating either autoreactive effector or regulatory T cells [38-40]. Here, we demonstrated that Graves' disease in children is associated with increased frequencies of circulating dendritic cells. Interestingly, apart from previously reported effects, we found that also in context of DCs methimazole can influence changes in these cells. Moreover, not only declines in DCs levels were reported but also shifts between myeloid or plasmacytoid DCs phenotypes in response to the therapy. However, it is worth noting that direct role of dendritic cells in etiology of AITD remains uncertain, thus further investigations are required for better determination of mechanism behind changes in DCs in the course of Graves' disease.

5. Conclusions

Disturbances in immune tolerance are one of the main causes leading to the development of autoimmune diseases. Considering partial information indicating potential involvement of dendritic cells in autoimmune diseases, studies on patients with Graves' disease are of great importance and might contribute significantly to scientific knowledge in that field. In our study for the first time we found that pediatric GD is associated with significantly higher levels of three main subsets of DCs. Moreover, we showed crucial correlations of these cells with thyroid function-related parameters, and in addition, favorable effects of methimazole on the observed phenomenon. We believe that further comprehensive of analyzes within individual subpopulations of dendritic cells might allow to establishing direct role of DCs in disease progression and response to treatment, and thus establish novel potential biomarkers or targets for the immunotherapeutic development.

Supplementary Materials:

Author Contributions: BA, GK and MM have made substantial contribution to the project concept. GK and SA have designed the work and supervised project implementation. GK, AS and SK have performed the experiments, acquired and analyzed data. BA, GK, SA and SK summarized the data and prepared the manuscript. All authors read and approved the final manuscript.

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