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Imbalance of the two main circulating dendritic cell subsets in patients with myasthenia gravis

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ABSTRACT

Although it is well documented that circulating dendritic cells (DCs) have specialized features during many kinds of physiological and pathological conditions, there are few reports about the features of DCs in the peripheral blood of myasthenia gravis (MG) patients. We investigated the quantitative and component features of DCs and their implications in MG. Peripheral blood samples from different kinds of MG patients were collected and their clinical characteristics were recorded. Using flow cytometry, we distinguished circulating DC subsets [plasmacytoid DCs (pDCs) and myeloid DCs (mDCs)] and enumerated their densities in peripheral blood. Absolute numbers of circulating pDCs were significantly decreased in naïve MG patients compared with healthy controls, resulting in a markedly lower ratio of the pDC to mDC percentage in total circulating DCs (pDCs/mDCs), suggesting an imbalance in the proportions of the two main circulating DC subsets. The clinical status of MG patients was improved after drug treatment, together with increased pDCs/mDCs. In a longitudinal follow-up, we observed that circulating mDCs were significantly reduced after 1 month of therapy with a corticosteroid and immunosuppressant, resulting in recovery of pDCs/mDCs. Although the exact meaning of the proportion change in circulating DC subsets is unknown, pDCs/mDCs might reflect the balance between the autoimmune response and immune tolerance of a patient. Moreover, changes in pDCs/mDCs during treatment might be a promising marker to predict the efficacy of a specific drug used for MG patients.

1. Introduction

Dendritic cells (DCs), which link innate and adaptive immune responses, are rare in human peripheral blood [1,2]. Circulating DCs play a critical role in shaping anti-tumor and anti-infection responses by continually replenishing the pool of tissue-residing DCs [1,2]. Usually, circulating DCs are divided into two major subsets, myeloid (m)DCs and plasmacytoid (p)DCs [3]. mDCs can be further divided into CD1c + mDCs and CD141 + mDCs, according to their surface markers.

It is well documented that circulating DC numbers may be altered during the course of acute and chronic infections [4–8], autoimmunity [9,10], tumors [11], and transplantation [12,13]. In addition, circulating DC numbers decrease throughout life, which might contribute to the decline in immune functions observed in the aged population [14].

Myasthenia gravis (MG) is a typical autoimmune disease with self-reactive antibodies targeting acetylcholine receptor (AChR), muscle-specific kinase or some other components in the neuromuscular junction [15]. MG patients usually have muscle fatigue, and symptom fluctuation is a typical feature of this disease [16]. Very few studies have focused on the features of peripheral blood DCs in MG patients. In one study, the investigators examined peripheral blood DC subtypes in general MG patients with thymoma [17]. They found that the number of circulating DCs and their subsets in generalized MG patients with

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thymoma were significantly lower than those in controls before or after treatment. In another study that focused on the hyperplasic thymus of MG patients, Weiss et al. also found a significantly decreased number of mDCs in the peripheral blood of MG patients [18]. However, the detailed features of circulating DCs in non-thymoma MG patients are unclear. It is unknown whether such features of circulating DCs change at various stages or clinical situations of MG, or whether they can be used as indicators to predict the outcomes of pharmaceutical treatments.

In the current study, cross-sectional and longitudinal assessments were performed for 112 MG patients, including patients with different clinical statuses or in different disease courses, and the results were compared with 39 healthy controls. Of interest was enumeration of circulating DCs, the ratio of different DC subset percentages in total circulating DCs, and their changes during the course of pharmaceutical treatments. The features of circulating DCs were also used for various analyses to identify factors suitable for clinical implementation to predict the clinical outcome of a specific drug.

2. Patients and methods

2.1. Subjects

MG patients who visited the first affiliated hospital of Sun Yat-sen University were enrolled (total number = 112). Information on the different MG patient groups is provided in Table 1. Peripheral blood samples were collected from outpatients or patients during hospitalization. For all enrolled MG patients, antibodies against AChR were assessed by an enzyme-linked immunosorbent assay kit (RSR Limited, Cardiff, UK). An antibody titer higher than 0.45 nmol/L was considered to be positive. In addition, anti-MuSK antibodies were measured in serum samples that were negative for anti-AChR antibodies. The demographic information, Myasthenia Gravis Foundation of America (MGFA) subtype, and anti-AChR antibody titer were recorded. Some enrolled MG patients were assessed for their quantitative myasthenia gravis (QMG) score.

Naïve patients were defined as MG patients that did not receive steroid or immunosuppressant treatments or those without steroid or immunosuppressant treatments for at least 3 months but no limitation on anticholinesterase inhibitor use. Completely stable remission (CSR) patients were defined according to the post-intervention status-MGFA standard [19]. Before a regular drug treatment, all CSR patients had been thymectomized. Infection was confirmed by infection symptoms, a raised white blood cell number or procalcitonin, or radiological indications. Usually, the infected MG patient presented with severe myasthenic symptoms and had a higher QMG score than during their previous follow-up. Most of these patients required hospitalization to treat the infection and myasthenic symptoms.

Peripheral blood samples of healthy controls were blood donations collected from healthy individuals (n = 39, age range: 18–55 years).

Table 1
Characteristics of MG patients with different clinical statuses.

Group	n	Female (%)	Age (y)	Anti-AChR antibody + (%)	Figures
Naïve MG patients	42	18(42.86)	37 (5–77)	39(92.86)[a]	1, 2, 4
Thymecotomized	14				
Non-thymecotomized	28				
Naïve MG patients (matched)[b]	27	13(48.15)	34 (14–55)	25(92.59)	2
Naïve MG patients (count)[b]	19	9(47.37)	31 (17–54)	17(89.47)	3
CSR patients	13	4(30.77)	35(23–57)	8(61.54)	1, 2, 3
Infected patients[c]	6	4(66.67)	35(8–70)	5(83.33)	4
Pred	28	17(60.71)	38(4–68)	20(71.43)	4
Pred + LEF	18	13(72.22)	37 (18–63)	14(77.78)	4
Pred + LEF (followed)	5	3(60)	32(16–61)	4(80)	5

[a] Only one naïve MG patient was positive for anti-MuSK antibodies (1 in 3, 33.33%).

[b] MG patients in this group were also included in the "naïve MG patient" group. LEF, leflunomide.

[c] Five patients in this group had pneumonia and one had an upper respiratory infection.

2.2. Processing of blood and thymus samples

All blood and thymus samples were collected according to Institutional Review Board guidelines of the first affiliated hospital of Sun Yat-sen University.

Red blood cells were directly lysed in the samples. The remaining white blood cells were washed twice and stained with monoclonal antibodies (mAbs).

Normal human thymuses were obtained from donors who underwent corrective cardiac surgery. Thymuses from MG patients were obtained during therapeutic thymectomy. At the first affiliated hospital of Sun Yat-sen University, all MG patients had stable or subtle symptoms (QMG score <8 or without bulbar and respiratory symptoms) before the thymectomy procedure. Therefore, most MG patients had underwent treatments including IVIg, corticosteroids, or immunosuppressants. Only thymus tissue reported as thymic hyperplasia by pathological observation was analyzed. Tissue sections of normal thymus were prepared from 14 to 28-year-old patients (average: 21.14 years, n = 6). The tissue sections of hyperplastic thymus were prepared from 16 to 24-year-old MG patients (average: 21.22 years, n = 8). Representative tissue blocks of each sample were embedded in paraffin.

2.3. Flow cytometry antibodies

FITC-, PE-, Percp/Cy5.5-, APC-, PE/Cy7-, or AF700-conjugated anti-lineage cocktails (CD3, CD14, CD19, CD20, and CD56), CD123, CD11c, CD141, CD1c, CD16, and HLA-DR mAbs against human antigens were purchased from Biolegend (San Diego, CA, USA).

2.4. Flow cytometry

After red blood cell lysis, blood samples were incubated with mAb cocktails for 30 min on ice, washed three times in phosphate buffered saline with 0.5% bovine serum albumin, and then analyzed on a CytoFlex S (Beckman Coulter, Brea, CA, USA). All collected data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

To calculate the exact density of DCs in peripheral blood, we used a fixed blood volume, usually 200 µl, to perform red blood cell lysis. Before the flow cytometric analysis, stained cells were resuspended in 200 µl FACS buffer. The CytoFlex S flow cytometer can draw a fixed volume from different samples. By taking advantage of this feature, the absolute number of circulating DCs in a known volume can be determined, and the density of circulating DCs can be calculated.

2.5. Immunohistochemistry

Sections (4 µm thick) of paraffin-embedded thymus fragments were stained. The sections were deparaffinized by a typical procedure. Then, the sections were incubated in 3% H_2O_2 for 15 min at room temperature, followed by blocking buffer to block non-specific antigen binding.

For immunohistochemical staining, the sections were incubated with an antibody against human CD123 (Gene Company, Shanghai, China) for 90 min at room temperature. After the first antibody labeling, the tissue was incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated polyclonal secondary antibodies (Gene Company) for 30 min at room temperature. The staining was developed using 3,3′-diaminobenzidine (Gene Company). Finally, the sections

were counterstained in Mayer's hematoxylin (BASO, Zhuhai, China), rinsed in water, and mounted in Arabian gum (BASO).

Calculation of the pDC density in the thymus was based on the following method. Unlike the usual method (five samples zones were randomly chosen to represent the whole tissue section), we imaged an entire typical thymus tissue section. Because CD123+ pDCs were clearly seen as brown dots, it was easy to count pDCs in the whole tissue. Using Nikon NIS Elements software, the total number of pDCs in the tissue and the tissue area were calculated. The pDC density in the thymus was the quotient of the number of pDCs and the area of the tissue.

2.6. Statistics

Results are shown as means ± standard error of the mean unless stated otherwise. In bar graphs, results are expressed as means of different values from different samples, and error bars represent the standard deviation. Significance was evaluated using the two-tailed unpaired *t*-test, one-way ANOVA for unpaired data, or the two-tailed paired t-test for paired data.

3. Results

3.1. Percentages of circulating DC subsets in MG patients are different from those in healthy controls and CSR patients

After red blood cell lysis and mAb staining, white blood cells were analyzed by flow cytometry. Peripheral blood mononuclear cells (PBMCs; including lymphocytes and monocytes) were gated for further analysis, although DCs were mainly present in the lymphocyte position. DCs were present in the lineage-HLA-DR+ cell position. CD16+ cells among lineage-HLA-DR+ cells were non-classical monocytes. Therefore, we excluded them (Fig. 1A). Lineage-HLA-DR + CD16- cells were divided into four groups by CD1c and CD141 (Fig. 1A, B). CD1c + CD141+ cells were called CD1c + mDCs, and CD1c-CD141+ cells were pDCs (also CD123+, see Supplementary Fig. 1). CD141+ + cells were CD141+ mDCs, which were a minimal subset of circulating DCs.

In the peripheral blood of naïve MG patients, three DC subset percentages (pDCs, CD1c + mDCs, and CD141+ mDCs) were 32.64% ± 1.81, 62.18% ± 1.82, and 5.17% ± 0.53 of total circulating DCs, respectively. These percentages of circulating DC subsets were different from those in HCs (40.45% ± 1.82, 54.45% ± 1.89, and 5.10% ± 0.39) and CSR patients (42.53% ± 3.92, 51.47% ± 3.91, and 6.00% ± 0.62) (Fig. 1C). Precisely, the percentage of pDCs in naïve MG patients was lower than those in healthy controls and CSR patients, but the peripheral blood of naïve MG patients contained a higher percentage of CD1c + mDCs (Fig. 1C).

3.2. The ratio of circulating pDC to circulating mDC percentages (pDCs/mDCs) is much lower in naïve MG patients, and, pDCs/mDCs is even lower in naïve MG patients after age matching

In this study, to describe the constitution of circulating DC subsets simply, CD1c + mDCs (Lineage-HLA-DR + CD16-CD1c + CD141+ cells) and CD141+ mDCs (Lineage-HLA-DR + CD16-CD141+ + cells) were combined, and showed as mDCs. In addition, we used pDCs/mDCs to represent the DC subset composition balance. pDCs/mDCs was the ratio calculated from the percentage of pDCs divided by the percentage of mDCs in total circulating DCs. MG patients had lower pDCs/mDCs (0.54 ± 0.04) compared with pDCs/mDCs in healthy controls (0.74 ± 0.06) (Fig. 2A). pDCs/mDCs recovered in CSR MG patients to 0.9 ± 0.15 (Fig. 2A), which was consistent with the changes in the percentages of DC subsets (Fig. 1C).

Considering the influence of age on pDCs/mDCs (Supplementary Fig. 2D), we adjusted the analyses of naïve MG patients depending on their age. The range of healthy individuals was 18–55 years of age. However, enrolled naïve MG patients had a wider range of 5–77 years of age. To match the age of healthy individuals, we screened out MG patients whose ages were below 14 years or above 55 years. After the moderation, the gap between pDCs/mDCs of healthy individuals and MG patients became even wider. pDCs/mDCs in naïve MG patients changed to 0.45 ± 0.03 (Fig. 2B).

3.3. Fewer pDCs exist in the peripheral blood of MG patients

The imbalance between circulating DC subsets in naïve MG patients reflected by pDCs/mDCs led us to count circulating DCs to interpret the reason. We determined whether reduced pDCs or increased mDCs played a more critical role in the decrease of pDCs/mDCs in naïve MG patients. There was no difference in the circulating mDC density among healthy individuals (9355 ± 719 per ml blood), naïve MG patients (9251 ± 650 per ml blood), and CSR MG patients (8916 ± 1374 per ml blood) (Fig. 3A). However, the density of circulating pDCs in naïve MG patient (4635 ± 432 per ml blood) was obviously decreased compared with healthy individuals (6009 ± 449 per ml blood) and CSR MG patients (6710 ± 756 per ml blood) (Fig. 3B).

Nearly 55% of MG patients with antibodies against AChR had thymus hyperplasia [18]. In our previous and other studies, more mDCs were found in the hyperplastic thymus of MG patients [20,21]. Based on this observation, Nagane et al. believed that such mDC enrichment in the thymus was derived from peripheral blood, which exacerbated hyperplasia in the MG patient thymus [21]. Therefore, it is reasonable to speculate that there might also be aggressive pDC enrichment in the hyperplastic thymus because of their migration from peripheral blood to the thymus. We calculated the densities of pDCs in normal and hyperplastic thymuses. To our surprise, we did not find enrichment of pDCs in hyperplastic thymus tissue from MG patients (Fig. 3B, C). There was no difference in the density of pDCs between normal (163 ± 28.23 per mm^2) and hyperplastic (146.8 ± 22.52 per mm^2) thymuses.

The typical structure of thymus hyperplasia, the germinal center, was easily found in the MG patient thymus (Fig. 3C). In the eight hyperplastic thymus samples, germinal centers were found in five. In total, 15 germinal centers were found in all eight tissue sections.

3.4. MG patients with different clinical situations have various pDCs/mDCs, and higher pDCs/mDCs generally reflect a better outcome

By calculating the density of pDCs in the thymus of MG patients, our observations indicated that there were less circulating pDCs in naïve MG patients. It was difficult to determine why there were less pDCs in the peripheral blood of MG patients. From a different viewpoint, we considered that pDCs/mDCs might be different in MG patients with a different clinical situation or at a different stage of the disease, because it appeared in the CSR MG patients mentioned above. The typical symptom of MG is fluctuating weakness of muscles. Most MG patients experienced exacerbation and remission of myasthenia during the disease course. MG patients usually experience exacerbation upon infection, and remission in patients occurs through therapy by medicine or other treatments such as intravenous immunoglobulin therapy and plasma exchange. Therefore, we collected blood samples while MG patients were experiencing infection or during drug treatments. Then, pDCs/mDCs of these MG patients were compared with those of naïve MG patients.

MG patients with infection-induced exacerbation had lower average pDCs/mDCs (0.42 ± 0.06) but not statistically different (Fig. 4A). However, patients under drug treatment (prednisone, 0.65 ± 0.06; prednisone + leflunomide, 0.75 ± 0.08) had significantly higher pDCs/mDCs than naïve MG patients (Fig. 4A). It was interesting that patients treated with steroids and immunosuppressants had the highest average pDCs/mDCs.

In further analysis, MG patients undergoing prednisone treatment were divided into three groups depending on their QMG score changes

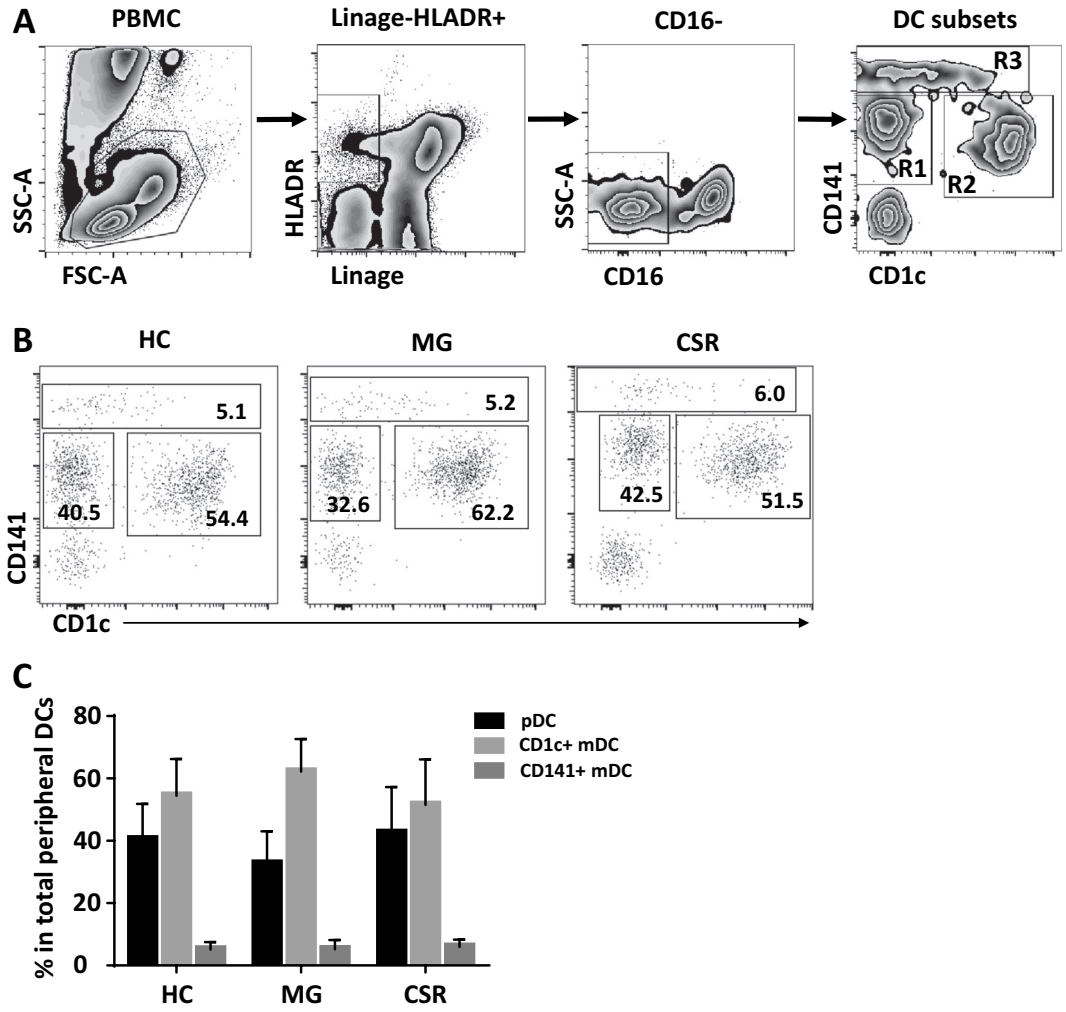

Fig. 1. Proportions of peripheral blood DC subsets in healthy controls (HC), naïve MG patients (MG), and completely stable remission MG patients (CSR).
A) After red blood lysis, whole white blood cells were analyzed by flow cytometry. The gating strategy is shown. The density plot clearly showed three subsets of peripheral DCs (Lin-HLA-DR + CD16-): R1, pDCs (CD141 + CD1c-); R2, CD1c + mDCs (CD1c+); R3, CD141+ mDCs (CD141++). B) Typical composition of the three subsets in total peripheral DCs shown by flow cytometry in representative healthy donors (n = 39), naïve MG patients (n = 42), and completely stable remission MG patients (n = 13). Numbers in gates represent the percentage of each subset in total peripheral DCs. C) The grouped graph shows the percentage of the three different subsets in total peripheral DCs (sum of the total number of pDCs, CD1c + mDCs, and CD141+ mDCs) in health controls, naïve MG patients, and CSR MG patients.

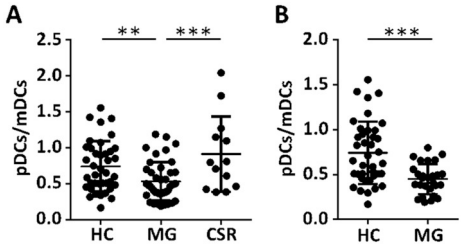

Fig. 2. Comparison of pDCs/mDCs between naïve MG patients and healthy individuals before and after age matching.
A) The percentage of mDCs was calculated as the percentage of CD141+ mDCs plus the percentage of CD1c + mDCs. pDCs/mDCs were calculated in healthy controls (HC), naïve MG patients (MG), and CSR MG patients (CSR). B) Age-matched naïve MG patients (n = 27) had much lower pDCs/mDCs. **p < 0.01; ***p < 0.001.

during the treatment: patients with a QMG score decrease (delta QMG is "+"), patients with a QMG score increase (delta QMG is "−"), and patients with no change in QMG score (delta QMG is "0"). After the steroid treatment, only patients with decreased QMG scores improved their pDCs/mDCs (Fig. 4B).

3.5. A decline of circulating mDCs in MG patients under treatment is responsible for the obvious increase of pDCs/mDCs

Naïve MG patients and drug-treated MG patients had different pDCs/mDCs. After steroid or immunosuppressant treatments, the pDCs/mDCs ratio was increased significantly, indicating that the steroid or immunosuppressant treatments had an effect on the DC density in peripheral blood. Next, we investigated the exact changes in circulating DC subsets. We followed five MG patients during their treatment for 1 month. All five patients were treated with prednisone and an immunosuppressant (leflunomide, LEF) [22]. Blood samples were collected before and after the 1 month of therapy. The quantities of circulating pDCs and mDCs were evaluated by flow cytometry.

All five patients exhibited significant pDCs/mDCs increases (mean, 0.36 to 0.88) after 1 month of treatment (Fig. 5A) and their mDC density decreased obviously (mean, 11,079 to 3336 per ml blood) (Fig. 5C). However, the density of pDCs did not correspondingly increase. Instead, in four patients, there was a slight decrease (mean, 3711 to 2580 per ml blood) (Fig. 5B).

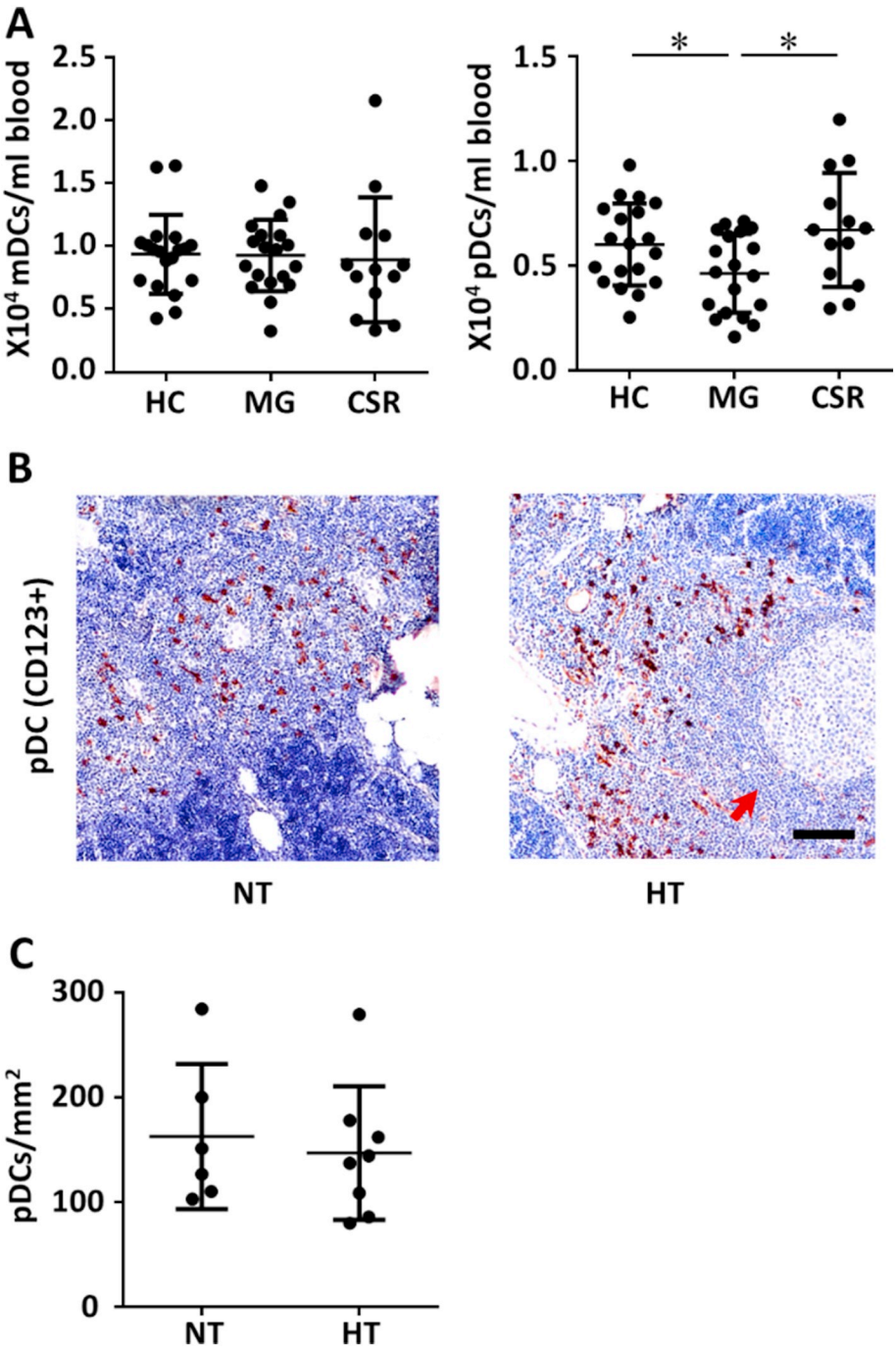

Fig. 3. pDC densities in the peripheral blood and hyperplastic thymus of MG patients.
A) Absolute numbers of mDCs (left) and pDCs (right) in peripheral blood were calculated in healthy controls (HC) (n = 19), MG patients (MG) (n = 19), and CSR MG patients (CSR) (n = 12). B) CD123 was used to mark pDCs in tissue sections of the thymus by immunohistochemistry. The density of brown dots in the thymus (not including atrophic areas such as fat tissue) represents pDCs. Left, representative section from healthy donors. Right, representative section from MG patients. The germinal center (right, arrow), a typical structure in the hyperplastic thymus, was found in the thymus of MG patients. Bar, 100 μm. C) pDC densities were calculated in tissues from eight MG patients with a hyperplastic thymus (HT) (average age = 21.22 years) or normal thymus (NT) from six patients with cardiac disease (average age = 21.14 years). *$p < 0.05$.

4. Discussion

Three types of DCs (pDCs and two types of mDCs) exist in human blood [3]. Lineage-HLA-DR + CD16+ cells were identified as a specialized subgroup of monocytes called non-classical monocytes [23]. Using cell surface markers CD141 and CD1c, we distinguished lineage-HLA-DR + CD16- cells (most were circulating DCs) into four populations. Three populations clearly reflected circulating DC subsets: CD303+ pDCs (we used CD123), CD1c + mDCs, and CD141+ mDCs [24]. CD123 is a more popular cell surface marker to label pDCs in flow cytometry, immunohistochemistry, and immunofluorescence [25].

Because of the small number of DCs in circulating peripheral blood, it is difficult to obtain enough DCs to study their functions. Only few reports have described limited functional features of circulating DCs [26–28]. Most studies of circulating DCs have focused on enumeration of DC subsets or the constitution of DC subsets. In these studies, investigators have correlated the features of these circulating DCs to different disease stages or performed longitudinal studies during a long disease course including drug or surgical treatments [11,29,30].

It is relatively difficult to perform flow counting methods. In contrast, the ratio of pDC to mDC percentages in total circulating DCs can be calculated providing that the blood DC subsets can be distinguished properly. Several studies have reported increases in the ratio of the pDC to mDC percentage in peripheral blood from pregnant women [31] and patients with asthma [32]. It is well documented that pDCs have the ability to induce regulatory T cells (Tregs) [33,34]. Non-activated pDCs might cause immune tolerance and/or immunosuppression. Therefore, it is reasonable that pregnant women have higher pDCs/mDCs [31].

In line with these observations indicating a tolerogenic role of pDCs, an increase in the pDC number is often associated with a poor outcome

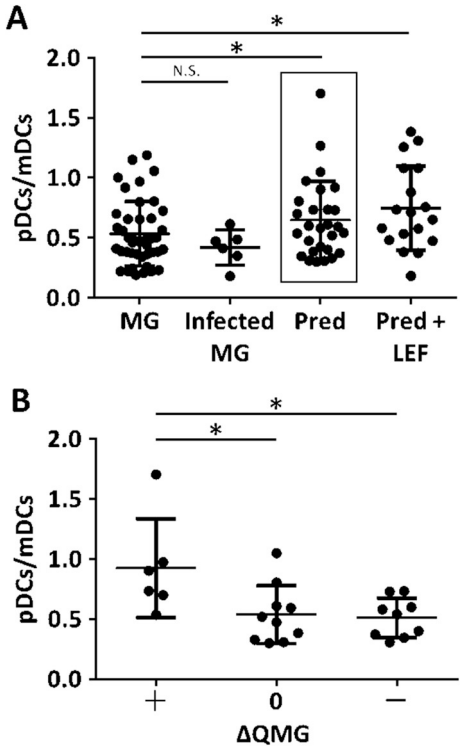

Fig. 4. MG patients with different clinical situations have various pDCs/mDCs. Peripheral blood samples were collected from MG patients with different clinical situations, such as patients with infections (n = 6) and patients under corticosteroid (prednisone, Pred) (n = 28) treatments or corticosteroid and immunosuppressant (leflunomide, LEF) treatments (n = 18).
A) MG patients in these groups had different pDCs/mDCs. Infection exacerbated myasthenia in MG patients and reduced pDCs/mDCs. In contrast, Pred or Pred and immunosuppressant treatments alleviated the clinical symptoms and raised pDCs/mDCs. B) pDCs/mDCs of MG patients under therapy with steroids (black box in A), but with a different response, were analyzed further (n = 25, three patients were excluded because of incomplete data). Patients with a positive response had higher pDCs/mDCs than those without a response or a negative response. +, QMG score decreased after treatment. 0, QMG score did not change after treatment. −, QMG score increased after treatment. *$p < 0.05$; N.S., not significant.

of solid tumor patients [35]. Conversely, mDCs that encounter pathogen-associated molecular patterns or damage-associated molecular patterns mature and trigger an adaptive immune response [2] and occasionally an autoimmune response [36]. In terms of Tregs [37], the balance between pDCs and mDCs probably represents an equilibrium of the immune response and tolerance.

After preliminary analysis, we found that naïve MG patients had a reduction of pDCs/mDCs in peripheral blood, indicating significant alterations in the constitution of circulating DC subsets. Age-matched MG patients had even lower pDCs/mDCs. Although Zhang et al. found no significant differences in the ratio of mDC to pDC percentages between generalized MG patients with thymoma and controls before and after the operation [17], our results showed a decrease of pDCs/mDCs in naïve MG patients. We believe that this decrease may because of the severe autoimmune response in MG patients. It is worth mention that we used healthy adults as control, different from the control used in Zhang's research. In which, patients with thymoma but without MG were used as control. Various kinds of controls are chosen for different purposes. Here we used healthy adults as a standard reference for the density and composition of peripheral DCs.

In this study, we used a specialized flow cytometer to count circulating DCs and observed a decrease in the density of pDCs in the peripheral blood of MG patients. Moreover, the density of mDCs was similar between MG patients and healthy controls. These findings indicated that the reduction of pDCs/mDCs was the outcome of the low density of circulating pDCs in MG patients. In lupus and psoriasis patients, the number of pDCs decreases peripherally, together with an increase in skin lesions [38,39]. This observation suggests migration of pDCs to other sites, possibly the thymus, which is considered to be the major site for autoimmune antibody production in MG patients. However, we did not find that pDCs gathered in the hyperplastic thymuses of MG patients. As reported in patients with early stage breast cancer [11], apoptosis or a generated decrease might be the cause of the decline in circulating pDCs of MG patients.

We observed that, in general, MG patients with a better clinical status (both single and combined treatments) had significantly higher pDCs/mDCs, but MG patients with infections had a lower pDCs/mDCs, although not significant. Studies focusing on the circulating DC quantity found the same trend in patients with inflammatory bowel disease or human immunodeficiency virus infection [40,41]. Unlike the anti-AChR autoantibody titer [42], the pDCs/mDCs reflected the clinical severity of naïve MG patients using the QMG score (Supplementary Fig. 2F). Although this result was not consistent with the comparative analysis of pDCs/mDCs and MGFA subtypes (Supplementary Fig. 2B), we speculated that the different statistical pattern should take charge of the different statistical result (one-way ANOVA analysis for MGFA subtypes, and correlation analysis for QMG scores).

We found that drug treatment increased pDCs/mDCs. We further evaluated the difference in pDCs/mDCs of the corticosteroid treatment group. As shown in tuberculosis patients [8], we found that successful steroid therapy significantly increased pDCs/mDCs. However, in patients without a response to steroid treatment, no significant longitudinal variations in pDCs/mDCs were observed. In a longitudinal observation of five MG patients, who received 1 month of steroid and leflunomide treatments [22], we observed that the treatments changed pDCs/mDCs by decreasing the density of mDCs but not by increasing the density of pDCs. This result indicates that the steroid and leflunomide treatments were responsible for the decline in circulating mDCs but had a limited influence on the density of pDCs. Previous studies have reported that steroids and immunosuppressants block the

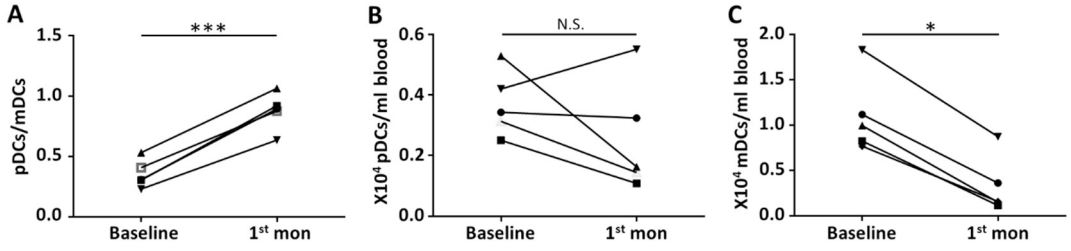

Fig. 5. Change in the density of mDCs in peripheral blood of MG patients treated with corticosteroids and immunosuppressants.
A) After a 1-month treatment course, pDCs/mDCs in different patients were increased significantly and consistently.B) In the same course, the density of pDCs did not significantly change, although a decrease in the mean was observed. C) There was a dramatic decrease in the density of mDCs after the 1-month treatment course. *$p < 0.05$; ***$p < 0.001$; N.S., not significant.

differentiation of human CD34+ progenitors [43] and induce circulating DC apoptosis *in vitro* [44,45].

Our longitudinal observation indirectly suggests that a low number of circulating pDCs is not the main reason for MG. Similarly, the steroid or immunosuppressant treatments did not ameliorate myasthenia by removing circulating mDCs in MG patients. However, this finding suggests that such treatments, especially an effective treatment, will improve the clinical status of MG patients by correcting the immune equilibrium. In a number of clinical transplant settings, an increase in the pDC/circulating DC ratio correlates with improved graft survival [46,47]. The effects of systemic chemotherapy of breast cancer on circulating DCs suggests a specific restraint on key elements of the immune response, rather than the generalized suppression of hematopoiesis [11]. Our results also suggest that the steroid and immunosuppressant used for the MG patients played a role in moderating the immune system. Therefore, correcting the immune status of MG patients should be the main goal of corticosteroid and immunosuppressant therapies. To monitor the immune balance, pDCs/mDCs appear to be a promising marker.

The reduction of pDCs in naïve MG patients and the reduction of mDCs in MG patients under drug treatment indicate that circulating DCs might not be directly involved in the pathogenesis of MG but could be used as a sensitive marker to reflect the autoimmune response intensity. Furthermore, pDCs/mDCs, representing the constitution of circulating DC subsets, can be used as a sensitive marker to predict the outcome of a drug treatment. Our results uncovered more features of circulating DCs in autoimmune diseases, especially MG. In addition, the longitudinal study showed that pDCs/mDCs were increased by effective treatment undertaken by MG patients. We consider that these features of circulating DCs can be used as a biomarker to predict the outcomes of specific drug therapies. However, there are still many features and roles of DCs, which are unclear in the pathogenesis of MG. Future experiments or observations will be required to resolve these issues.

Author Contributions

P.C., Y.L., H.R., and W.L. contributed to the study concept, design, and writing of the manuscript. P.C. and Y.L. performed acquisition of data, statistical analysis, and drafted the manuscript. P.C., Y.L., X.L., H.H and X.H. performed blood sample collection, processing and analysis by flow cytometry. H.H., Y.L., and Z.C. collected and processed the thymus samples. L.Q., C.O., Z.H., and Z.L. contributed to the collection of clinical information of patients with myasthenia gravis. All authors approved the final version of this manuscript.

Potential conflicts of interest

None.

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Appendix A. Supplementary data

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