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Comparative analysis of circulating dendritic cell subsets in patients with atopic diseases and sarcoidosis

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Abstract

Background: Dendritic cells (DCs) are professional antigen-presenting cells that play a crucial role in the initiation and modulation of immune responses. Human circulating blood DCs are divided into two major subsets: myeloid DCs (mDCs); and plasmacytoid DCs (pDCs). Furthermore, mDCs are subdivided into two subsets: Th1-promoting mDCs (mDC1s); and Th2-promoting mDCs (mDC2s). Although CD1a, CD1c, and CD141 are generally used for classifying mDC subsets, their adequacy as a specific marker remains unclear. We performed this study to compare circulating mDC, pDC, mDC1, and mDC2 subsets between Th1- and Th2-mediated diseases using CD1a and CD141, and to analyze the adequacy of CD1a and CD141 as a marker for mDC1s and mDC2s, respectively.

Methods: Thirty patients with sarcoidosis, 23 patients with atopic diseases, such as atopic bronchial asthma, and 23 healthy subjects as controls were enrolled in this study. Peripheral blood DC subsets were analyzed with flow cytometry according to expressions of CD11c, CD123, CD1a, and CD141. For functional analysis, we measured interleukin (IL) 12p40 levels produced by the sorted mDC subsets.

Results: The sarcoidosis group showed decreased total DC ($P < 0.05$) and mDC counts ($P < 0.05$) compared to controls. The atopy group showed decreased CD1a⁺mDC count ($P < 0.05$), and increased CD1a⁻mDC count ($P < 0.05$) compared to controls. CD141⁺mDC count in the atopy group was higher than controls ($P < 0.05$). Sorted CD1a⁺mDCs produced higher levels of IL-12p40 than CD1a⁻mDCs ($P = 0.025$) and CD141⁺mDCs ($P = 0.018$).

Conclusions: We conclude that decreased count of CD1a⁺mDC and increased count of CD141⁺mDC may reflect the Th2-skewed immunity in atopic diseases. The results of IL-12 levels produced by the sorted mDC subsets suggested the adequacy of CD1a and CD141 as a marker for mDC1 and mDC2, respectively, *in vivo*.

Keywords: Dendritic cells, Peripheral blood, Sarcoidosis, Myeloid DC (mDC), CD1a, CD141

Background

Dendritic cells (DCs) are professional antigen (Ag)-presenting cells (APCs) that originate from bone marrow and play crucial roles in the initiation and modulation of appropriate immune responses by linking innate to adaptive immune response [1,2]. Immature DCs are recruited from the blood circulation to peripheral organs, where they continuously sample the environment for foreign substances. These cells are able to take up and process antigens, and immature DCs develop into

matured DC during this process with the upregulated expressions of major histocompatibility complex (MHC) and costimulatory molecules in inflammatory microenvironments [3]. Subsequently, mature DCs migrate into secondary lymphoid organs and present the processed antigens to naïve T cells for the generation of effector T cells and initiation of adaptive immune responses.

Human blood DCs comprise ~1% of peripheral blood mononuclear cells and have been classically defined as Ag-presenting leukocytes that lack other markers of leukocyte lineages (CD3, 14, 16, 19, 20, and 56) and express high levels of MHC class II (HLA-DR) molecules [4]. CD11c⁺ myeloid DCs (mDCs) and CD123⁺

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plasmacytoid DCs (pDCs) represent the two major DC subsets [5], with each playing distinct and complementary roles in the induction of immune responses. Acting as strong APCs, mDCs are efficient in the uptake, processing, and presentation of foreign antigens. Following Toll-like receptor stimulation, mDCs produce tumor necrosis factor α , and matured mDCs produce proinflammatory cytokines such as interleukin (IL)-12. Conversely, pDCs are less effective in these processes and mainly known for their function in antiviral immunity and rapid production of type I interferon.

DCs are also divided into functional subtypes as a Th1-promoting subtype (DC1s) and a Th2-promoting subtype (DC2s). In the past decade, mDCs and pDCs have been thought to represent DC1s and DC2s, respectively [6,7]. However, recent studies have led to a new theory that both DC1s and DC2s differentiate from mDCs, and in some reports, the terms mDC1 and mDC2 are used to describe the mDC subsets which promote Th1 response and Th2 response, respectively. In fact, mDC1s can be generated by cultivation of monocytes with granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4, whereas mDC2s can be generated by cultivation of monocytes with IL-3 and IL-4 [8,9].

Although distinct markers of DC1 and DC2 have not yet been fully established, CD1c (blood DC antigen (BDCA)-1) and CD141 (BDCA-3) are generally used as markers of mDC1 and mDC2, respectively [10-12]. In a previous study, Th1-inducible DC subtype differentiated from peripheral monocytes by cultivation with GM-CSF expressed higher levels of CD1a than CD1c [9]. Based on these data, we use CD1a as a better marker of mDC1s. In contrast, the adequacy of CD141 as a marker for mDC2 remains controversial [13]. It is known that the expression of CD141 is not specific on mDCs, but also on pDCs [14]. Detailed functional analysis of CD1a⁺mDCs and CD141⁺ mDCs has yet to be fully discussed.

We therefore conducted a study to analyze peripheral blood DC subsets, including mDC1 and mDC2, using CD11c, CD123, CD1a and CD141, in patients with sarcoidosis as a Th1-mediated disease and atopic diseases as Th2-mediated diseases compared to healthy controls. In addition, we measured and compared levels of IL-12p40 produced by sorted mDC subsets to confirm the adequacy of CD1a and CD141 as a specific marker for circulating blood mDC1 and mDC2 subsets, respectively.

Methods

Study subjects

Patients who were referred to our hospital for suspected sarcoidosis or allergic bronchial asthma with or without atopic dermatitis and allergic rhinitis from 2009 to 2011 were enrolled in this study. In all the patients with sarcoidosis, the diagnosis was made from a biopsy obtained

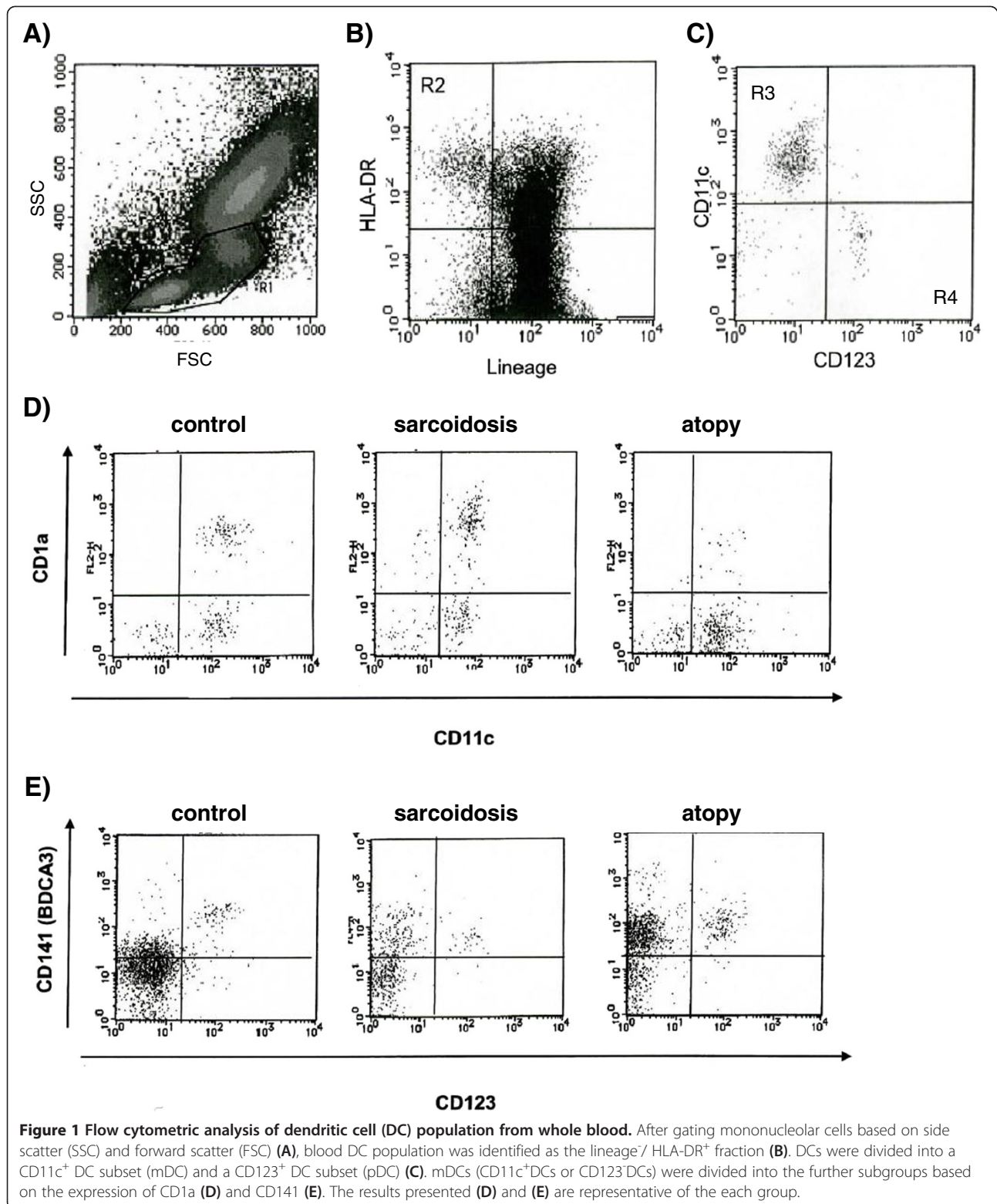
either from lung or lymph nodes and showing non-caseating granulomas, and was made in accordance with Japanese diagnostic criteria [15]. Patients who received any steroid or immunosuppressive treatments were excluded. Clinical stage was classified according to Wurm's radiological staging [16,17]. Patients in the atopy group were defined as having a history of non-treated allergic bronchial asthma and/or atopic dermatitis and/or allergic rhinitis, with positive radioallergosorbent test (RAST; \geq class 2) to house dust mite (HDM). Healthy volunteers with negative RAST to HDM (class 0) were enrolled as the control group. To exclude the influence of smoking on peripheral DC populations, we chose only the subjects who were never-smokers or ex-smokers that had quit smoking at least one year before the enrollment. All the subjects provided informed consent prior to enrollment. The study was approved by the Institutional Ethics Committee and was conducted in accordance with the ethical principles embodied in the Declaration of Helsinki.

Flow cytometric analysis

Twenty milliliters of heparinized blood was obtained for DC isolation. Blood samples were stained with fluorescein isothiocyanate-conjugated monoclonal antibody (mAb) mixture for non-DC lineage leukocytes or lineage-negative (lin⁻) cells with specificity for CD3, CD14, CD16, CD19, CD20, and CD56, peridinin chlorophyll protein-conjugated mAbs against HLA-DR, phycoerythrin-conjugated mAbs against either the surface markers CD123 or CD1a, allophycocyanin (APC)-conjugated CD11c (BD Biosciences, San Jose, CA) or APC-conjugated CD141 (BDCA-3) (Miltenyi Biotec, Bergisch Gladbach, Germany). Following erythrocyte lysis and wash, stained leukocytes were analyzed by flow cytometry (FACS Calibur; BD Biosciences) and acquired data were analyzed using CellQuest Software (BD Biosciences). After gating mononuclear cells based on side scatter and forward scatter (Figure 1A), the blood DC population was identified as the lin⁻/HLA-DR⁺ fraction (Figure 1B). DCs were divided into a CD11c⁺DC subset (mDCs) and a CD123⁺DC subset (pDCs) (Figure 1C). The mDC subset was further subdivided into two subsets based on the expression of CD1a (Figure 1D) and CD141 (Figure 1E). The number of total events was 200,000, and data are expressed as DC counts per 200,000 leukocytes.

IL-12 production by sorted mDC subsets

One hundred milliliters of heparinized peripheral blood was drawn from five healthy volunteers and stained with the panel of mAbs described previously (See 'Flow cytometric analysis'). Peripheral blood DCs are sorted as lin⁻/HLA-DR⁺/CD11c⁺/CD1a⁺, lin⁻/HLA-DR⁺/CD11c⁺/CD1a⁻, lin⁻/HLA-DR⁺/CD123⁻/CD141⁻, and lin⁻/HLA-DR⁺/CD123⁻/CD141⁺ using FACS Aria (BD Bioscience).



The sorted cells were cultured in a 96-well flat-bottom tissue culture plate at 1×10^4 cells/well in medium supplemented with RPMI 1640, 10% fetal bovine serum, penicillin 100 $\mu\text{g}/\text{mL}$, and lipopolysaccharide 100 ng/mL. After cultivation for 48 h, IL-12p40 levels in each supernatant were measured with purchased enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.04 (Graphpad Software, San Diego, CA). Data are summarized as mean with standard deviation (SD) or median with interquartile range (IQR) depending on distribution. Statistical differences in DC counts or ratios between groups were assessed using Kruskal-Wallis test, and subsequent post hoc analyses were performed using Dunn's test. The paired t-test was used to compare mean values between groups. Differences were considered significant for values of $P < 0.05$.

Results

Patient characteristics

Thirty patients with sarcoidosis (13 men, 17 women; mean age, 48.3 ± 15.3 years), 23 patients with atopic diseases (16 men, 7 women; mean age 45.5 ± 14.8 years), and 23 healthy controls (10 men, 13 women; 51.7 ± 14.0 - years) were enrolled in the study (Table 1).

CD11c⁺DC (mDC) and CD123⁺DC (pDC) subsets

All the data for the DC subset are shown in Table 2. In the sarcoidosis group, both total DC [median (IQR): 632.5 (460.0, 941.0)] and mDC [320.0 (226.0, 509.0)]

Table 1 Characteristics of patients and control group

	Sarcoidosis (n = 30)	Atopy (n = 23)	Control (n = 23)
Age: mean \pm SD (range)	48.3 \pm 15.3 (25–72)	45.5 \pm 14.8 (26–70)	51.7 \pm 14.0 (27–67)
Sex (male/female)	13/ 17	16/ 7	10/ 13
stage I	8 (26.7%)	-	-
II	20 (66.7%)		
III	2 (6.7%)		
BA alone	-	7	-
AD alone		2	
AR alone		8	
BA + AD		2	
BA + AR		3	
AD + AR		1	
BA + AD + AR		0	

BA; bronchial asthma, AD; atopic dermatitis, AR; allergic rhinitis.

Table 2 Number of circulating DC and DC subsets in peripheral blood

	Control	Sarcoidosis	Atopy
Total DC	908.0 (661.8, 1097.0)	632.5* (460.0, 941.0)	1100.0 (922.6, 1279.0)
CD11c ⁺ DC (mDC)	481.0 (352.0, 700.0)	320.0* (226.0, 509.0)	457.0 (284.0, 628.3)
CD123 ⁺ DC (pDC)	90.0 (62.8, 143.5)	110.5 (62.5, 168.5)	127.0 (68.0, 268.0)
CD1a ⁺ mDC	351.0 (217.0, 462.0)	238.5 (139.8, 362.8)	215.0* (118.0, 279.0)
CD1a ⁻ mDC	92.5 (53.5, 144.5)	79.0 (44.5, 135.0)	205.0* (93.0, 324.5)
CD141 ⁻ mDC	223.0 (167.0, 355.5)	179.0 (126.0, 310.0)	232.0 (142.0, 299.0)
CD141 ⁺ mDC	90.5 (52.3, 265.3)	116.5 (66.5, 166.3)	261.0* (81.5, 361.3)

Data were expressed as the DC counts per 200,000 leukocytes. Data are presented as median with interquartile range. * $P < 0.05$ vs control.

counts were significantly decreased than controls [total DC: 908.0 (661.8, 1097.0) ($P < 0.05$), mDC: 481.0 (352.0, 700.0) ($P < 0.05$)] (Figure 2). pDC count was not different among the three groups. mDC count was superior to pDC count in all the three groups, and mDC/pDC-ratio did not differ among the groups (data not shown).

CD1a⁺mDC and CD1a⁻mDC subsets

CD1a⁺mDC count was equivalent in the sarcoidosis group: 238.5 (139.8, 362.8) and healthy controls: 351.0 (217.0, 462.0), but was significantly decreased in the atopy group: 215.0 (118.0, 279.0) ($P < 0.05$, Figure 3). CD1a⁻mDC count in the atopy group: 205.0 (93.0, 324.5) was

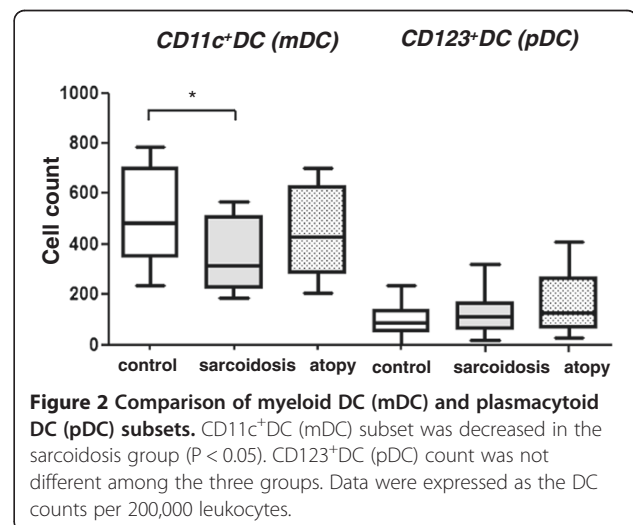
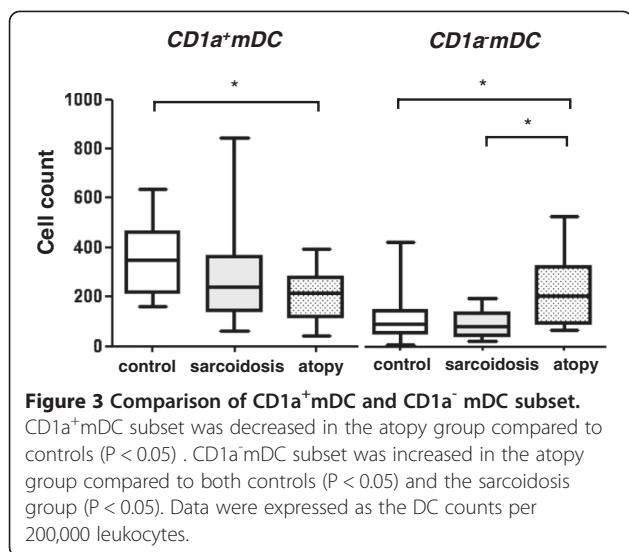


Figure 2 Comparison of myeloid DC (mDC) and plasmacytoid DC (pDC) subsets. CD11c⁺DC (mDC) subset was decreased in the sarcoidosis group ($P < 0.05$). CD123⁺DC (pDC) count was not different among the three groups. Data were expressed as the DC counts per 200,000 leukocytes.



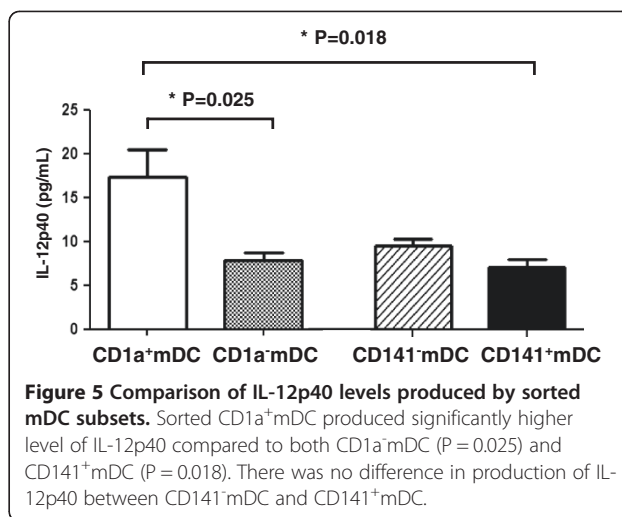
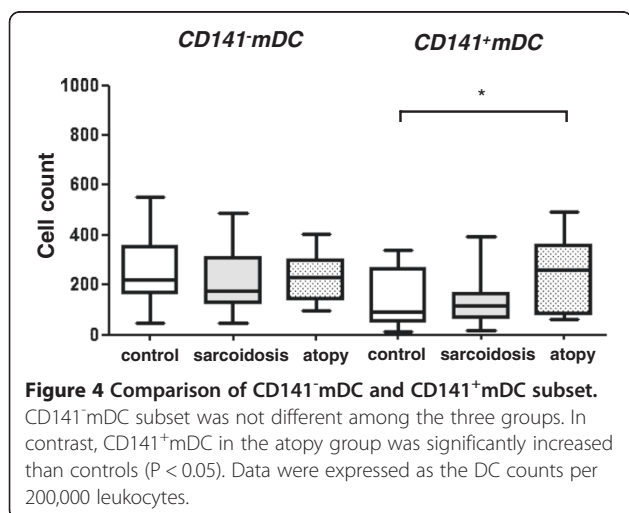
greater than in both controls: 92.5 (53.5, 144.5) ($P < 0.05$) and the sarcoidosis group: 79.0 (44.5, 135.0) ($P < 0.05$).

CD141⁻mDC and CD141⁺mDC subsets

No differences were seen in CD141⁻mDC count among the three groups (Figure 4). In contrast, CD141⁺mDC count was significantly increased in the atopy group compared to controls [atopy: 261.0 (81.5, 361.3), control: 90.5 (52.3, 265.3)] ($P < 0.05$).

IL-12p40 levels produced by sorted mDC subsets

Sorted CD1a⁺mDCs produced significantly higher levels of IL-12p40 (17.3 ± 3.1 pg/mL) when compared with CD1a⁻mDCs (7.8 ± 1.9 pg/mL, $P = 0.025$) and CD141⁺mDCs (6.9 ± 0.8 pg/mL, $P = 0.018$, Figure 5). CD141⁻mDCs showed a trend toward producing higher levels of IL-12p40 (9.3 ± 0.8 pg/mL) when



compared with CD141⁺mDCs (6.9 ± 0.8 pg/mL Figure 5, Table 3), but the difference was not significant ($P = 0.435$).

Discussion

In this study, we have reported differences of peripheral blood DC subsets between sarcoidosis and atopic diseases using four-color flow cytometry, and have analyzed the adequacy of CD1a and CD141 as a marker for mDC1 and mDC2, respectively by the production of IL-12p40. Sarcoidosis patients showed decreased peripheral total and myeloid DC count with similar population of both CD1a⁺ mDC and CD141⁺mDC subsets compared to the control subjects. In contrast, patients with atopic diseases showed lower CD1a⁺mDC count and higher CD141⁺mDC count, which suggests the involvement of CD1a⁻mDCs and CD141⁺mDCs in Th2-polarity in atopic diseases.

In recent years, many studies have examined quantitative alternations of circulating blood DCs in patients with different pathological conditions, for example, Th1-mediated diseases such as viral infections and cancers, or Th2-mediated diseases such as bronchial asthma and atopic dermatitis [18-20]. Most investigations have focused on the proportion of mDC and pDC subsets. After a report describing two further subsets in mDCs, mDC1s as a Th1-promoting mDC subtype and mDC2s as a Th2-promoting mDC subtype [21], an increasing number of studies have been analyzing mDC1/mDC2 subsets using CD1c or CD1a, and CD141 [10,11] has been reported. CD1a and CD1c are the subgroup of CD1, which is structurally and functionally similar to MHC class I and II molecules; however, CD1 presents lipids and glycolipids rather than polypeptides on DCs. CD1 has also evolved a unique path of intracellular trafficking, processing, and loading of lipid antigens. CD141, thrombomodulin, is a glycoprotein on the

Table 3 Comparison of IL-12p40 levels produced by sorted mDC subsets

	CD1a ⁺ mDC	CD1a ⁻ mDC	CD141 ⁻ mDC	CD141 ⁺ mDC
IL-12p40 (pg/mL) (range)	17.3 ± 3.1 (10.1 – 27.2)	7.8 ± 1.9 (5.9 – 10.8)	9.3 ± 0.8 (7.3 – 11.8)	6.9 ± 0.8 (4.0 – 8.5)

Data are shown as mean ± SD.

surface of endothelial cells, and activates protein C as a cofactor of thrombin in the anticoagulant pathway. The immunological function of CD141 on mDCs is unknown. Relatively few studies have analyzed human peripheral blood mDC1 and mDC2 subsets. According to previous studies [10,12] performed *ex vivo*, CD1a⁺mDCs or CD1c⁺mDCs are generally accepted as representing mDC1s and CD141⁺mDCs as representing mDC2s. Hata *et al.* [9] succeeded in making mDC1s and mDC2s from freshly isolated circulating monocytes using either GM-CSF with IL-4 or IL-3 with IL-4, respectively. Furthermore, they confirmed the potential of CD1a as a marker for mDC1s through the differences in expression between mDC1 and mDC2 subsets. Based on these findings and results of previous reports [10-12], we used CD1a and CD141 in this study as a marker of mDC1s and mDC2s, respectively.

Sarcoidosis is a multisystem disorder of unknown etiology characterized by non-caseating granulomas that are composed of epithelioid cells, fibroblasts, and several immune cells such as T cells and histiocytes/macrophages. Lungs, eyes and skin are the most affected organs. In sarcoidosis, lung T cells are shown to spontaneously release high levels of IL-2 and interferon γ [22-24], and studies of T-cell clones from lung parenchyma [25] and of bronchoalveolar lavage fluid (BALF) [26-29] support the opinion of sarcoidosis as a Th1-mediated disease. Similarly, various clinical studies, genetic studies and animal models of allergic diseases support the notion that atopic diseases such as bronchial asthma, atopic dermatitis and allergic rhinitis are Th2-mediated disease.

In the present study, sarcoidosis patients showed decreased total DC and mDC counts compared to healthy controls. Previous studies have identified decreased numbers of total blood DCs and both subsets of CD11c⁺-myeloid DCs and CD11c⁻-lymphoid DCs [30]. Another report showed that sarcoidosis patients tended to show decreased numbers of mDCs [31]. Our result was similar to these previous reports. In sarcoidosis, circulating blood DCs migrate into the affected tissues, contributing to the formation of sarcoid granulomas. This migration and accumulation into local inflammatory tissues may decrease the number of DCs circulating in the blood. Ota *et al.* showed the accumulation of DCs in the lymphocyte layer of sarcoid granulomas [30].

We expected a predominance of CD1a⁺mDC count in peripheral blood in patients with sarcoidosis. Contrary

to our expectation, no such differences in numbers of CD1a⁺mDCs and CD1a⁻mDCs were seen between sarcoidosis and controls. In sarcoidosis, the immunity of circulating blood does not always parallel that of locally affected organs, as seen from granuloma or BALF in lung tissue. For example, the CD4/CD8 ratio in BALF is usually high in sarcoidosis, but is not increased in peripheral blood. Given these findings, immunity of peripheral blood in patients with sarcoidosis is thought not to reflect the Th1/Th2 polarity. There are a few reports demonstrating the expression of CD1a on mDCs in the local inflammatory sites in sarcoidosis. An investigation in the BALF of inflammatory diseases showed an increase of CD1a⁻mDCs in sarcoidosis [32]. Another study of immunohistochemical investigation in muscular sarcoidosis demonstrated that CD1c⁺ mDCs scattered mainly in the lymphocyte layers of granulomas and the endomysium around the granulomas, while CD1c⁺mDCs expressed the mature DC marker CD83, but CD1a positive cells were not found by double immunostaining [33]. Since expression of surface markers on DCs is varied by the existing environment such as in organ tissue or in blood circulation, and also by the involved organs, precise evaluation is not easy. Further investigation is necessary to define the local immunity in sarcoidosis.

In the atopy group, numbers of total DC, mDC, pDC were all equivalent to controls. A previous study reported that total counts of circulating blood DCs were increased in patients with asthma [34]. Some reports have shown that allergen challenge causes a rapid decrease in the circulating mDC count [35] and an accumulation of DCs in airway epithelium [36-38] in patients with allergic asthma, while another report showed a trend toward to decreased number of circulating blood mDCs and a significant increase of pDCs in patients with atopic asthma [18]. The different disease states of the study participants may explain such contradictory results. Contrary to previous reports, which performed allergen challenge in patients with allergic asthma to invoke allergic inflammation, we examined stable atopic patients with no medication in order to exclude any influence of medications. This might have contributed to the unclear differentiation from controls. Actually, Upham *et al.* reported that decreases in circulating mDCs were most marked at 3 and 6 h post-allergen challenge, gradually returning to baseline levels [35].

In the atopy group, CD1a⁺mDC count showed a significant decrease, while CD141⁺mDC count was significantly increased compared to controls. Yerkovich *et al.* reported that after allergen challenge the constitutive expression of CD141 on mDCs was increased in atopic individuals compared to non-atopic subjects [12]. The same group of investigators has also shown that CD141⁺mDCs are associated with Th2 polarizing response, whereas CD141⁻mDCs are associated with a mixed Th1/Th2 response. On the other hands, Jongbloed *et al.* reported that CD141⁺mDCs induce superior Th1 response compared to CD1c⁺DCs [13], and the significance of CD141⁺mDCs is still in dispute. The results of the present study may support the theory that CD1⁺mDCs and CD141⁺mDCs represent mDC1s and mDC2s, respectively. The decreased mDC1s and increased mDC2s may reflect the Th2-skewed immunity in atopic disease.

When discussing the adequacy of CD1a and CD141 as a definite marker for mDC1s and mDC2s, respectively, the ability of sorted CD1a⁺mDCs and CD141⁺mDCs to induce Th1 and Th2, respectively, must be confirmed. However, few reports have examined this area, because these procedures need large amounts of peripheral blood, reducing clinical feasibility. In a previous report, monocyte-derived CD1a⁺mDCs produced high amounts of IL-12, a Th1-inducible cytokine, and induced Th1 when co-cultured with lymphocytes [10]. In our study, sorted CD1a⁺mDCs produced significantly higher levels of IL-12p40 compared to CD1a⁻mDCs. This result suggests that CD1a can offer a marker for human peripheral blood mDC1s. CD141⁺mDCs produced significantly less IL-12p40 compared to CD1a⁺mDCs but no differences in produced IL-12p40 levels were seen between CD141⁺mDCs and CD141⁻mDCs. CD141⁻mDC subset is probably composed of heterogeneous cell populations, and they are not always represent mDC2 subset. So, our results imply the possibility of CD141 as a marker for mDC2, however, further investigation is necessary to clarify the meaning of the expression of CD141 on mDCs as mDC2.

In the present study, we were unable to analyze CD1a/CD141 double positive or double negative mDCs due to technical limitations. There is almost no data about them in human peripheral blood, however, Bratke *et al.* showed that 45% of mDCs were CD1a positive and 78% of mDCs were CD141 positive in the analysis of BALF of never smokers [39]. It may be difficult to completely identify mDC1 and mDC2 only using CD1a and CD141. In future research, we feel it is necessary to find out the number and function of CD1a/CD141 double positive and double negative mDCs in human peripheral blood.

Conclusions

In conclusion, the present study shows, for the first time, human circulating DC subsets including mDC1 and

mDC2 comparing Th1-related to Th2-related disease. These data suggests the involvements of mDC1s and mDC2s in the Th1/Th2-polarity, and the adequacy of CD1a as a marker for mDC1 and the possibility of CD141 as a marker for mDC2.

Abbreviations

DC: Dendritic cell; MHC: Major histocompatibility complex; GM-CSF: Granulocyte macrophage colony-stimulating factor; IL: Interleukin; BDCA: Blood dendritic cell antigen; RAST: Radioallergosorbent test; HDM: House dust mite; HLA: Human leukocyte antigen; APC: Allophycocyanin; SSC: Side scatter; FSC: Forward scatter.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conception and design: YH, YI; acquisition of data: YH, MS, RA, KC; analysis and interpretation of data: YH, MS, YI; drafting of the manuscript: YH, YI; critical revision of manuscript: YI, TF. All authors have read and approved the final manuscript.

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