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Type 1 diabetes patients have significantly lower frequency of plasmacytoid dendritic cells in the peripheral blood

Xueqin Chen^{a,b,1}, Levi H. C. Makala^{a,1}, Yulan Jin^a, Diane Hopkins^a, Andy Muir^{a,c}, Nikhil Garge^a, Robert H. Podolsky^{a,d}, and Jin-Xiong She^{a,e,2}

^aCenter for Biotechnology and Genomic Medicine, Medical College of Georgia, 1120 15th Street, CA4124, Augusta, GA, USA

^bDivision of Respiratory Medicine, Wuhan University Renmin Hospital, Wuhan, China

^dDepartment of Medicine, Medical College of Georgia, 1120 15th Street, CA4124, Augusta, GA, USA

^eDepartment of Pathology, Medical College of Georgia, 1120 15th Street, CA4124, Augusta, GA, USA

Abstract

Dendritic cells uniquely orchestrate the delicate balance between T cell immunity and regulation and an imbalance favoring immunogenic rather than tolerogenic DC is believed to contribute to the development of autoimmune diseases such as type 1 diabetes (T1D). In this study, we determined the frequencies of three blood DC subsets (pDC, mDC1 and mDC2) in 72 T1D patients and 75 normal controls using the Miltenyi blood DC enumeration kit. The frequency of blood pDC was found to be negatively correlated with subject age in both normal controls and T1D patients ($p = 0.0007$), while the frequency of mDC1 and mDC2 do not change significantly with subject age. More importantly, the mean frequency of pDC in blood was, after adjusting for age, significantly lower in T1D (mean = 0.127%) than controls (mean = 0.188%) ($p < 6.0 \times 10^{-5}$), whereas no difference was observed for mDC1 and mDC2 between T1D and controls. Furthermore, T1D patients have lower proportion of pDC and higher proportion of mDC1 among the total blood DC population than normal controls. These results indicate that the frequency of blood pDC and the pDC/mDC1 ratio are negatively associated with T1D.

Keywords

Dendritic cells; DC; plasmacytoid DC; myeloid DC; Type 1 diabetes; Autoimmunity

Introduction

The break of tolerance to self antigens in autoimmune diseases including type 1 diabetes (T1D) is largely due to deficient immune regulation [1,2]. Although recent studies have focused on the roles of various types of regulatory T cells (Treg), antigen presenting cells, especially dendritic cells (DC), have long been recognized to play a pivotal role in the pathogenesis/

^cCurrent address: Department of Pediatrics, Emory University, Atlanta, GA.

¹These authors contributed equally to this study.

²Correspondence to: Jin-Xiong She, Ph.D. Center for Biotechnology and Genomic Medicine, Medical College of Georgia, 1120 15th Street, CA4124, Augusta, GA, USA Tel: 706-721-3410 Fax: 706-721-3688 E-mail: jshe@mail.mcg.edu

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protection of autoimmune diseases because they are capable of either priming effector T cells or activating Treg cells depending on the maturation stimuli and/or DC subsets [3-5]. DC comprises a heterogeneous group of cells and the natural DC population includes two distinct subsets, conventional or myeloid DC (mDC) and plasmacytoid DC (pDC). Analysis of DC subsets in human blood has been difficult due to the lack of specific surface antigens. Recently, a number of novel monoclonal antibodies have been developed and used as blood DC markers [6]. These novel antibodies recognize two subsets of myeloid DC. The mDC1 subset is positive for BDCA1 (CD1c) while the mDC2 subset is positive for BDCA3. pDC were originally identified in human [7] and subsequently identified in mice [8,9]. Human blood pDC are specifically recognized as cells positive for BDCA2 and BDCA4.

pDC can produce vast amounts of type I interferons in response to viruses and other stimuli and thus play an important role in antiviral immunity and potentially in autoimmunity [10, 11]. Murine immature pDC, when freshly isolated from mouse secondary lymphoid tissue, are endowed with tolerogenic potential by inducing differentiation of Treg [12]. Immature pDC can also induce allogeneic T cell hyporesponsiveness and prolong heart graft survival when they are derived from bone marrow [13]. Human pDC appear to have an intrinsic capacity to prime naïve T cells to dedifferentiate into regulatory T cells [14].

Although DC are believed to be an important player in T1D pathogenesis, the roles of various DC subsets in T1D has only received some attention in recent years. In the NOD mice, pDC cells are increased after treatment with GCSF, which protects against diabetes [15]. The protective role of pDC has recently been demonstrated in NOD mice [16]. Human studies are more limited due to lack of specific markers and the general difficulties associated with patient-based studies, e.g. high individual variability, ethnic/population differences, variation over time and specimen availability. A early study suggested that T1D patients had higher pDC numbers and secreted more IFN- α than normal controls [17], while a more recent study found no difference in the frequencies of total DC or DC subsets between T1D patients and controls but found reduced IFN- α secretion in T1D patients [18]. These inconsistent results are not surprising as both studies had small sample sizes. In contrast, a recent report using larger sample size suggested that pDC numbers and frequencies were reduced in T1D patients [19]. In order to reconcile these controversial reports, we have undertaken a study on human blood DC subsets in a large cohort of T1D patients using the recently discovered DC surface markers. Our study indicates that the frequency of pDC is significantly reduced in T1D patients while mDC1 and mDC2 subsets are not different between T1D and controls.

Research design and methods

Human subjects

A total of 147 Caucasian subjects were recruited from the Augusta area in Georgia. Among these subjects, 72 were T1D patients and 75 were normal controls (NC). The demographic information for these subjects is presented in Table 1. Diagnosis of T1D was made using the criteria of the American Diabetes Association by physician scientists with extensive experience in type 1 diabetes. All patients used in this study unambiguously have type 1 diabetes as questionable cases were not included in this study. These patients have had diabetes for an average of 5.7 years (range 0 – 35 years). Healthy controls were subjects who had no autoimmune disorders and were negative for the presence of T1D-associated autoantibodies and do not have a family history of T1D. The average age for the control group is 30.6 years. The Medical College of Georgia institutional review board approved the study design and informed consent was obtained from all subjects.

Analysis of human blood DC subsets using DC enumeration kit

Fresh heparinized blood was obtained through venipuncture. Within two hours of blood drawing, an aliquot (300 μ l) of the blood sample was stained with 20 μ l of anti-BDCA cocktail (containing monoclonal antibodies specific for the DC markers BDCA1, BDCA2 and BDCA3 along with CD14 and CD19 antibodies) or 20 μ l of control cocktail containing appropriate isotype controls (Miltenyi Blood DC Enumeration kit — Miltenyi Biotec Inc., Auburn CA, USA). Dead cell detector (10 μ l) was added to both tubes. Following 10 minutes of incubation on ice erythrocytes were lysed. Cells were then washed in PBS containing 1% BSA, 0.1% sodium azide, 2% FCS and 1% human serum. Cells were fixed and analyzed by four-color flow cytometry with the exclusion of B cells, monocytes and dead cells. One million events in a mononuclear gate were collected. With our protocol, the viability of the cells is about 99% and the frequencies of the DC subsets are consistent when the samples are processed within the first few hours of sampling. The relative proportion and the total number of the three DC subsets (mDC1, mDC2 and pDC) were calculated. The total count of each DC subsets could also be calculated for those subjects who had less than one million PBMC in 300 μ l of blood.

Statistical Analysis—Seven continuous variables (% of DC in PBMC, % of pDC in PBMC, % of mDC1 in PBMC, % of mDC2 in PBMC, % of pDC in DC, % of mDC1 in DC and % of mDC2 in DC) were analyzed using analysis of covariance (ANCOVA) with diabetes status as the main factor and age as a covariate. This analysis allows us to control for differences in age between study groups, minimizing the impact of “Age” as a confounding factor because the pDC number has been shown to decrease with age [20]. We tested two different models by assuming similar or different impact of “Age” in the T1D and control subjects. Since the results are very similar from the two models, only results assuming similar impact are presented. Tukey’s honestly significant difference was subsequently used for any analyses that yielded a significant diabetes status effect. For T1D subjects, we also conducted regression analyses, examining the relationship between the continuous variables of interest and duration of T1D. All analyses were carried out using the Proc GLM procedure in SAS.

Results

As shown in Fig. 1, three blood DC subsets (mDC1, mDC2 and pDC) can be clearly identified from whole blood using the Miltenyi Blood DC Enumeration kit. Whole blood samples were analyzed in this study because it requires small blood volume and minimizes sample handling. The frequencies of total blood DC and the three DC subsets in PBMC of normal control and T1D patient groups are shown in Fig. 2. The frequency of total DC, mDC1 or mDC2 did not differ between T1D patients and controls. However, the pDC frequency in PBMC was significantly reduced in T1D patients compared to controls ($p = 0.0018$) (Fig. 2). Because of the age difference between the T1D and control groups, we used ANCOVA to examine the potential impact of various factors such as age and sex on the frequencies of blood DC subsets. Sex of the subjects had no significant impact on the frequencies of total blood DC or DC subsets in PBMC (data not shown). The frequency of mDC1 cells appears to decrease with age (Fig. 3) but this change is not statistically significant ($p = 0.111$). The frequency of mDC2 does not change with subject age ($p = 0.333$) (Table 2 and Fig. 3). In contrast, the frequencies of pDC in PBMC are negatively associated with subject age in both the control and T1D groups ($p = 0.0007$ for the combined dataset). As expected, the frequency of total DC in PBMC also decreases with age and this result can mainly be attributed the pDC subset.

To examine the age impact on the DC phenotypes between T1D and controls, we conducted an ANCOVA analysis with age as the covariate. Table 2 shows the estimated means (least-square [LS]-means) for T1D and controls as well as the p -values. These analyses indicate that the age-adjusted difference between T1D and controls is highly significant for pDC frequency

in PBMC ($p = 6.0 \times 10^{-5}$) and no significant difference was detected for mDC1 or mDC2 after age-adjustment (Table 2 and Fig. 3). The mean pDC frequency (LS-means) is approximately 50% higher in normal controls (0.188%) compared to T1D patients (0.127%) (Table 2).

In addition to the frequencies of the DC subsets in the PBMC, we examined the relative proportion of DC subsets in the total DC population. The pDC proportion (%) in total blood DC decreased with age and the mDC1 proportion increased with age, while the mDC2 proportion did not show a significant change (Table 2 and Fig. 3). In contrast, the age-adjusted mean percentage of pDC in DC and the pDC/mDC1 ratio were lower in T1D patients compared to controls, while the mean percentage of mDC1 was higher in T1D patients than controls (Table 2).

Finally, we examined whether duration of diabetes had any impact on the blood DC phenotypes using two different approaches. First, we conducted regression analyses with T1D subjects to examine the relationship between the frequencies of DC subsets and the duration of diabetes (number of years that a subject has had diabetes). This analysis found no difference between duration of T1D and any of the eight phenotypes examined (data not shown). Second, we used ANCOVA to compare the frequency of DC subsets between controls and new onset T1D patients (defined as diagnosed within one year) or long term diabetic patients (diagnosed for more than one year). None of these analyses yielded a significant difference (data not shown).

Discussion

DC is believed to play a pivotal role in autoimmune diabetes as they are critical initiators and regulators of T cell-mediated immune responses towards immunity to foreign antigens or tolerance/immunity to self antigens. An imbalance favoring immunogenic rather than tolerogenic DC function is believed to contribute to diabetes development in T1D. Most of the evidence supporting a role of DC in T1D comes from the studies of the NOD mouse model. Analyses of human monocytes-derived DC indicated altered frequency, activation or ability to stimulate T cell proliferation in some studies [21-23] but not in other reports [24,25]. Three studies also investigated the endogenous DC phenotype in the peripheral blood of human T1D patients but the results are contradictory. These studies suggested that T1D patients may have higher, unchanged or lower pDC (defined as $\text{Lin}^- \text{CD45}^+ \text{DR}^+ \text{CD123}^+$ cells) in their peripheral blood [17-19]. In our study, we used the Miltenyi Blood DC Enumeration kit, which identifies three DC subsets (pDC, mDC1 and mDC2) using the new BDCA markers. We found that the frequency of pDC in peripheral blood decreases with age in both normal controls, an observation that is consistent with previous reports [20,26], as well as in T1D patients, while no significant correlation with age was observed for mDC1 and mDC2. More importantly, the mean pDC frequency in peripheral blood was significantly lower in T1D patients compared to normal controls after adjusting for age, while no difference was observed for mDC1 and mDC2. As a result of reduced pDC frequency in T1D patients, the frequency for total blood DC and the relative proportion of pDC among total blood DC were both lower in T1D patients than controls, while the relative proportion of mDC1 among total DC was higher in T1D patients. Our study supports the report with larger sample size that T1D patients have lower number and frequency of pDC [19]. A deficiency in pDC appears to be a common characteristic of multiple autoimmune diseases. For example, the number of DC and pDC ($\text{BDCA2}^+ \text{CD123}^+ \text{DR}^+$) was found to be significantly lower in SLE patients compared to controls [27]. The reduced pDC in blood reflects the severity of inflammatory disease in lupus nephritis [28] and the mean pDC count was lower in active than in inactive disease [29].

There is now convincing evidence that the pDC number and frequency are lower in T1D patients than controls. However, this study as well as the previous studies are cross sectional studies and did not examine the natural history of the pDC during the progression to T1D.

Therefore, it is still unclear whether the observed reduction in pDC in the peripheral blood of T1D patients are a consequence of diabetes or the defect may contribute to the development of diabetes. Analyses of pDC in prospective studies will help address this question. This and previous studies in human T1D did not fully address the function of pDC in T1D diabetes. pDC, like mDC, have a functional plasticity in determining the quality of T cell responses, depending on the type of maturation signals [30]. pDC are mostly known to have the ability to produce vast amounts of type I interferons in response to viruses or other stimuli, and thus play an important role in antiviral immunity. It has been recognized that in addition to having an immunogenic function, some pDC subsets may also have a tolerogenic functions [31-34]. The lower pDC number and frequency in diabetic patients suggest that pDC may play an important role in the pathogenesis of T1D. Although functional data is still lacking in humans, studies in the NOD mice indicated that the loss of pDC is associated with acceleration of insulinitis in NOD mice [16]. It has also been shown that increased pDC by GCSF treatment is correlated with reduced diabetes incidence in NOD mice [15]. We believe that the frequency of pDC and the pDC/mDC ratio are critical to the outcome of immune response, even though the absolute pDC numbers may also be important. mDC are potent cells in stimulating T cell proliferation, while pDC may be required for the generation [33,34] and activation [35] of regulatory T cells. Data from our ongoing studies in NOD mice suggest that the proportion of pDC in splenic DC has a major impact on the suppression function of regulatory T cells (data not shown). It will be interesting to determine whether the imbalance of pDC versus mDC in T1D patients observed in our study contributes to the suboptimal suppression function of T cell proliferation in T1D patients [36,37].

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Fig. 1.

Gating strategy used to define pDC, mDC1 and mDC2 for human blood DC. *A:* Peripheral blood mononuclear cells were selected in the R1 gate excluding debris and platelets. *B:* Dendritic cells were selected in the R2 excluding B cells, monocytes and dead cells. *C:* Based on combined R1 and R2 gate, mDC1 and pDC cells were quantified in the R3 and R4 gate respectively. *D.* And mDC2 were quantified in R5.

Fig. 2.

Frequencies of human blood DC subsets in PBMC in T1D and control populations. Data were obtained using the DC enumeration kit. Difference between T1D and controls are tested using Student T test.

Fig. 3. Frequencies of blood DC subsets according to age and diabetes. Slopes were estimated using ANCOVA.

Table 1

Clinical characteristics of blood DC subsets in healthy and diabetic subjects

Variable	AbN	T1D
Total number	75	72
Age (years)*	30.6 ± 9.3	17.9 ± 11.4
Age range	(1 - 65)	(2 - 61)
Male	39	30
Female	36	42
Duration of T1D (years)	N/A	5.7 (0–35)
<30 years of age	42	62
>30 years of age	33	10

Age and duration of disease are presented as means (range).

* The mean age is significantly different between T1D and AbN groups ($p < 0.0001$).

Table 2

Mean percentage of human blood DC subsets in PBMC and relative proportion of DC subsets in T1D versus control subjects.

		LS Means (%)		p value	
NC		T1D	NC-T1D	Age*	
<i>pDC/PBMC</i>	0.188	0.127	6.0×10^{-5}	0.0007	
mDC1/PBMC	0.225	0.214	0.83	0.111	
mDC2/PBMC	0.013	0.013	0.99	0.333	
DC/PBMC	0.426	0.353	0.028	0.006	
<i>pDC/DC</i>	44.3	35.7	0.00018	0.031	
mDC1/DC	52.7	60.7	0.0002	0.067	
mDC2/DC	3.1	3.7	0.098	0.007	
pDC/mDC1	0.69	0.52	0.0006	0.03	

NC = normal controls. LS means are within-group means appropriately adjusted for the other effects in the model (subject age in this case). P values for age are derived from regression analysis for subject age in all subjects (T1D + NC). P-values under "Age" denote p value after ANCOVA.