

# CD33<sup>+</sup> HLA-DR<sup>-</sup> Myeloid-Derived Suppressor Cells Are Increased in Frequency in the Peripheral Blood of Type1 Diabetes Patients with Predominance of CD14<sup>+</sup> Subset

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## Abstract

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**Keywords:** Myeloid-derived suppressor cells; Type 1 Diabetes; Diabetic nephropathy; CD 14<sup>+</sup>; MDSCs subsets

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**INTRODUCTION:** Type 1 Diabetes Mellitus (T1D) is an autoimmune disease that results from the destruction of insulin-producing beta cells of the pancreas by autoreactive T cells. Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells that can potentially suppress T cell responses.

**AIM:** To detect the presence of MDSCs in T1D and compare their percentage in T1D versus healthy individuals.

**METHOD:** Thirty T1D patients were included in the study. Diabetic patients with nephropathy (n = 18) and diabetic patients without nephropathy (n = 12). A control group of healthy individuals (n = 30) were also included. CD33<sup>+</sup> and HLA-DR<sup>-</sup> markers were used to identify MDSCs by flow cytometry. CD14 positive and negative MDSCs subsets were also identified.

**RESULTS:** MDSCs was significantly increased in T1D than the control group and diabetic patient with nephropathy compared to diabetic patients without nephropathy. M-MDSCs (CD14<sup>+</sup> CD33<sup>+</sup> HLA-DR<sup>-</sup>) were the most abundant MDSCs subpopulation in all groups, however their percentage decrease in T1D than the control group.

**CONCLUSION:** MDSCs are increased in the peripheral blood of T1D with a predominance of the CD14<sup>+</sup> MDSCs subset. Future studies are needed to test the immune suppression function of MDSCs in T1D.

## Introduction

Autoimmune Diabetes (AID) also known as type 1 diabetes (T1D) usually develops as a result of the triad of environmental factors, genes and immune system interaction [1]. Several genes like cytotoxic T-lymphocyte antigen 4 (CTLA4), major histocompatibility complex class II (MHC II), and others drive an immune response that controls the T1D outcome. The over-reactive immune system also occurs in AID patients [2]. Subsequently, loss of insulin-producing pancreatic  $\beta$  – cells, caused by infiltrating immune cells, results in hypoinsulinemia, hyperglycemia and fatal complications. To date, no means for preventing or curing AID exists [3]. Numerous strategies have been developed, trying to restore physiological insulin production in diabetic patients [4]. Despite some progress, restoring self -

tolerance or specifically correcting autoimmunity remains a crucial step toward reversing AID. In this respect, research and clinical interest in regulatory Treg and MDSC have increasingly grown [5][6][7].

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells that are defined by their myeloid origin, immature state and ability to suppress T cell responses potently. Under normal conditions, they differentiate into dendritic cells, macrophages and granulocytes. However, in pathological states such as infection, inflammation or cancer, there is an arrest of differentiation of this population of cells resulting in their accumulation [8][9][10]. The composition and percentage of MDSCs were found to vary according to disease nature. However, MDSC of different origins has potent suppressive activities [11][12].

Human MDSCs were initially defined as HLA-DR<sup>+</sup>CD33<sup>+</sup> or CD14<sup>+</sup>CD11b<sup>+</sup> cells, with both phenotypes identifying cell populations with T cell suppressive activity [13][14]. Recently, other markers have defined MDSC subsets, including CD14-positive monocytic MDSC (Mo-MDSC) and CD15-positive granulocytic MDSC (G-MDSC). The expression of CD14 on MDSCs as a Mo-MDSC marker was initially controversial but has now become accepted. The CD14 expression is difficult to be used as a lineage marker as low levels of this marker are expressed on polymorphonuclear leukocytes (PMNs). Therefore, differentiation between CD14<sup>bright</sup> versus CD14<sup>low</sup> in the assessment of MDSCs is crucial [15].

Accumulative evidence shows that T- cells play a pivotal role in AID pathogenesis through the secretion of inflammatory cytokines and destruction of  $\beta$ -cells [16]. Accordingly, the importance of studying MDSCs emerges since several therapeutic strategies targeting MDSCs could inhibit immune responses in the setting of autoimmune disease [17].

This study aimed to determine the frequency of MDSCs and study MDSCs subsets in type1 autoimmune diabetes and to evaluate the effect of diabetic nephropathy on the frequency of MDSCs for later studying of their suppressor activities on T cells and their potential therapeutic use in AID.

## Material and Methods

### Subjects

This study enrolled thirty type 1 diabetic patients from the diabetes outpatient clinic of National Research Center (NRC) and thirty age - and gender-matched healthy controls. In particular, inclusion criteria for patients were age range 20 - 60 years. The first group of patients included 12 T1D without diabetic nephropathy. Diagnosis of diabetes was based on criteria of the American Diabetes Association [18]

The second group of patients included 18 T1D with diabetic nephropathy. Diagnosis of diabetic nephropathy is based on detection of abnormal levels of urinary albumin in diabetic patients combined with exclusion of other causes of albuminuria. Albumin measurements are defined as follows [19]:

1. Normal albuminuria: urinary albumin excretion < 30 mg/24h;
2. Microalbuminuria: urinary albumin excretion in the range of 30 - 299 mg/24h;
3. Macroalbuminuria: urinary albumin excretion  $\geq$  300 mg/24h.

Exclusion criteria included Type 2 diabetes, any febrile illness during the last three months, chronic inflammatory/rheumatic disease or other chronic kidney disease.

Clinical data were collected from patients' records. Informed consent was obtained from each subject. Approval for the study was obtained from the Ethics Committee of National Research Center, Cairo, Egypt (Number of the approval: 15160).

### Samples

From each subject, 3 ml venous blood was taken and divided into three sterile tubes. Two tubes were sent to the Clinical Chemistry laboratory in NRC for the determination of fasting blood sugar level; two hours postprandial blood sugar test, glycated haemoglobin level. The third tube was sent to the flow cytometry unit in Kasr El Aini hospital for immunofluorescent staining for the determination of myeloid-derived suppressor cells.

Samples were kept at room temperature and were not shaken. They were analysed within 24 hours of venipuncture.

Microalbuminuria was defined by urinary albumin excretion of at least 30 mg in a 24 - hour period

### Flowcytometry

First, isolation of human PBMCs was performed using a Ficoll - Hypaque density gradient before antibody staining. Second, 100  $\mu$ l of test sample was incubated with 20  $\mu$ l of specific monoclonal antibodies: CD33 PE (Beckman Coulter, USA), CD14 - FITC (Beckman Coulter, USA) and HLA - DR PC5 (Beckman Coulter, USA) at room temperature in the dark, same species isotypes served as a negative control.

After 20 min of incubation, 2 ml of Phosphate Buffer Saline were added to each tube of monoclonal treated cells. The mixtures were then centrifuged for 5 min at 150 xg at room temperature followed by discarding the supernatant and resuspending the pellet in 3 ml PBS. Cell analysis was performed using CYTOMICS FC 500 Flow Cytometer (Beckman Coulter, FL, USA) and analysed using CXP Software version 2.2.

### Statistical analysis

The data were analysed using Microsoft Excel 2010 and statistical package for social science (SPSS version 24.0) for Windows (SPSSIBM, Chicago, IL). Results were expressed as mean  $\pm$  SD with 95% confidence interval using the range for quantitative variables, and using the frequencies and

percentage for qualitative ones; a p-value < 0.05 was considered statistically significant. Spearman's rank correlation coefficient (r) was done to show the correlation between different parameters in this study. Diagnostic parameters of subjects were compared using the non-parametric Wilcoxon – Mann - Whitney U - test, whereas the parametric parameters were compared using the Paired samples (t) test. Also, Chi-square ( $\chi^2$ ) test was used for comparison of categorical data. Whenever the expected values in one or more of the cells in a 2 x 2 tables were less than 5, Fisher exact test was used instead and using linear by linear association in larger than 2 x 2 cross - tables.

## Results

### Characteristics and clinical data

A total number of 30 T1D patients were included in the study. Diabetic patients with nephropathy represented 60% (n = 18) and diabetic patients without nephropathy represented 40% (n = 12). A control group of healthy individuals (n = 30) were also included. Demographic and laboratory data of Patients and controls are shown in Table 1. Human MDSCs are immature myeloid cells that express the cell surface antigen CD33 and weakly express HLA-DR being a mature myeloid cell marker [20]. Accordingly, we used the surface markers CD33<sup>+</sup> within HLA-DR<sup>+</sup> neg cells to identify MDSCs and compared the frequency of MDSCs in the peripheral blood of the two groups of patients - diabetes with nephropathy (Figure 1a) and diabetes without nephropathy (Figure 1b) - and of the control group as well (Figure 1c).

To determine the percentage of MDSCs in patients, acquired cells were first gated based on the expression of HLA DR (PC5). The PC5 subset was comprised of HLA DR<sup>+</sup> cells. Within this population, the fraction of cells expressing CD33 (PE) and CD14 (FITC) was determined.

**Table 1: Demographic and laboratory data of the two groups of patients and control**

Descriptive parameters		Control N=30	Diabetic patients Without nephropathy N=12	Diabetic patients With nephropathy N=18
Age	Range	28 - 55	35 - 55	28 - 56
	Mean ± SD	38.3 ± 5.3	49.1 ± 5.3	40.0 ± 8.7
Sex	Female	21 (70.0%)	9 (75.0%)	15 (83.3%)
	Male	9 (30.0%)	3 (25.0%)	3 (16.7%)
FBS mg/dl	Range	80-120	86-243	98-272
	Mean ± SD	95.6 ± 13.8	(125.4 ± 47.7)	(123.3 ± 40.3)
PP.BS mg/dl	Range	90-150	110-242	120-250
	Mean ± SD	119.6 ± 20.6	(152.9 ± 32.3)	(155.1 ± 27.9)
Hba1C %	Range	3-5.5	4.5-7.0	5.1-7.2
	Mean ± SD	4.22 ± 0.79	(5.7 ± 0.7)	(5.8 ± 0.6)
Microalbumin mg/24h	Range	-----	16.5-29.0	185-310
	Mean ± SD	-----	(23.97 ± 2.9)	(253.8 ± 33.8)

FBS: Fasting blood sugar, PP: BS = postprandial blood sugar, SD = standard deviation.

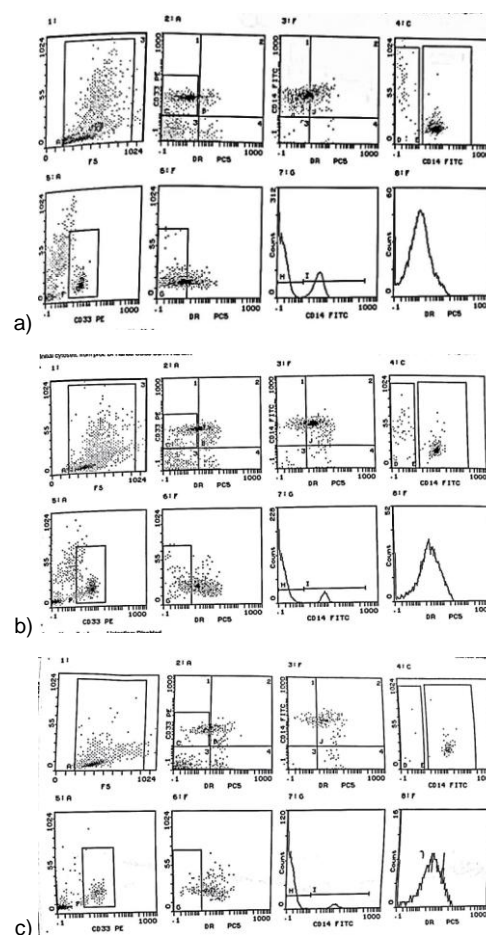


Figure 1: a) Flowcytometry analysis of the percentage of CD33<sup>+</sup> HLA-DR<sup>+</sup> MDSCs in T1D patients with diabetic nephropathy; b) Flowcytometry analysis of the percentage of CD33<sup>+</sup> HLA-DR<sup>+</sup> MDSCs in T1D patients without diabetic nephropathy; c) Flowcytometry analysis of the percentage of CD33<sup>+</sup> HLA-DR<sup>+</sup> MDSCs in healthy control group

### Increased frequency of CD33<sup>+</sup> HLA - DR<sup>+</sup> MDSCs in the peripheral blood of T1D patients

The frequency of total MDSCs was increased in the peripheral blood of type 1 diabetes patients compared to the control group with a highly significant difference (Figure 2), (Table 2).

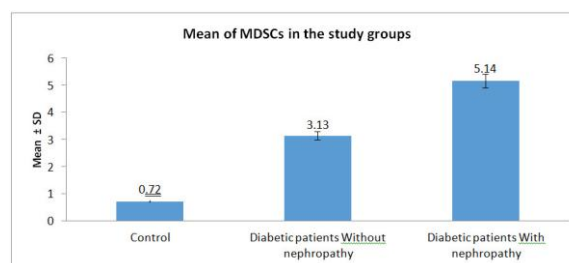


Figure 2: Mean of MDSCs in the study groups

Then, we subdivided the group of diabetic patients according to nephropathy into two groups: Diabetic patients without nephropathy (n = 12), and Diabetic patients with nephropathy (n = 18), and we compared the frequency of total MDSCs among

the two groups and the control group. We found that the frequency of total MDSCs in a diabetic patient with nephropathy was increased than diabetic patients without nephropathy with a highly significant difference ( $P$  - value < 0.001) (Figure 2), (Table 2).

**Table 2: The frequency of MDSCs in both groups of T1D and the control group. Percentage of the two subpopulations of MDSCs CD14<sup>+</sup> and CD14<sup>-</sup> in both patients group compared to the control group**

Descriptive parameters		Control	Diabetic patients		Total
		N = 30	Without nephropathy N = 12	With nephropathy N = 18	Diabetic Patients
MDSCs	Range	0.3-1.01	2.4-3.8	3.0-10.3	2.4-10.3
	Mean ± SD	0.72 ± 0.24	(3.13 ± 0.5)	(5.14 ± 2.3)	(4.4 ± 2.07)
CD14	Range	98.9-99.7	96.0-97.5	87.5-99.5	87.5-99.5
	Mean ± SD	99.3 ± 0.3	(96.9 ± 0.6)	(95.4 ± 3.8)	(96.5 ± 3.02)
CD14 <sup>+</sup>	Range	0.1-1.1	2.5-4.0	0.52-12.5	0.52-12.5
	Mean ± SD	0.62 ± 0.33	(3.09 ± 0.56)	(4.6 ± 3.8)	(3.98 ± 3.0)

The total population of CD33<sup>+</sup> HLA-DR<sup>-</sup> MDSCs obtained from peripheral blood of T1D patients were further divided into two subpopulations of cells expressing CD14<sup>+</sup> and CD14<sup>-</sup>. We compared the two subsets in the two groups of diabetic patients in relation to the control group. Then, we compared the percentage of these 2 MDSCs subsets in the two groups of diabetic patients (Table 2).

Analysis of the previous results of MDSC subsets (CD14<sup>+</sup> and CD14<sup>-</sup>) demonstrated that all healthy individuals in the control group, diabetic patients with nephropathy and diabetic patients without nephropathy had higher numbers of CD14<sup>+</sup> MDSC and lower number of CD14<sup>-</sup> MDSCs.

The percentage of CD14<sup>+</sup> subset tended to decrease in the T1D patients compared to the control group even though their absolute number increased due to the increase of total MDSCs. On the contrary, Percentage of CD14<sup>-</sup> subset has increased in T1D patients than the control group.

Among the diabetes patients, the percentage of CD14<sup>+</sup> subset was less in diabetic patients with nephropathy than diabetic without nephropathy. On the contrary, the percentage of CD14<sup>-</sup> was increased in diabetic patients with nephropathy than diabetic without nephropathy (Figure 3), (Table 2).

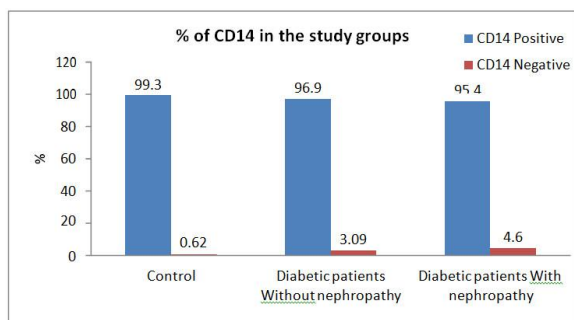


Figure 3: Comparison of CD14<sup>+</sup> and CD14<sup>-</sup> subsets among the three tested groups

In Brief, comparing diabetic patients with and without nephropathy to the control group demonstrated a highly significant difference in the frequency of total MDSCs and a significant difference in the proportion of CD14<sup>+</sup> and CD14<sup>-</sup> subset of MDSCs among the three groups.

### Correlation between CD33<sup>+</sup> HLA - DR<sup>-</sup> MDSCs and HbA1C

We examined the possible correlations between CD33<sup>+</sup> HLA - DR<sup>-</sup> MDSCs and HbA1C in T1D patients. As shown in Figure 4, CD33<sup>+</sup> HLA - DR<sup>-</sup> MDSCs were positively correlated with levels of HbA1C ( $r = 0.702$ ,  $P < 0.001$ ) (Figure 4).

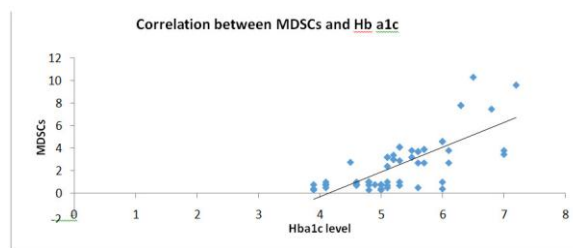


Figure 4: Positive correlation (direct proportion) between MDSCs and HbA1c ( $r = 0.702$ ;  $P < 0.001$ )

### Correlation of CD33<sup>+</sup> HLA - DR<sup>-</sup> MDSCs, microalbumin

We examined the possible correlations of CD33<sup>+</sup> HLA - DR<sup>-</sup> MDSCs, levels of microalbumin in T1D patients. As shown in Figure 5, CD33<sup>+</sup> HLA-DR<sup>-</sup> MDSCs were positively correlated with levels of microalbumin ( $r = 0.814$ ,  $P < 0.001$ ) (Figure 5).

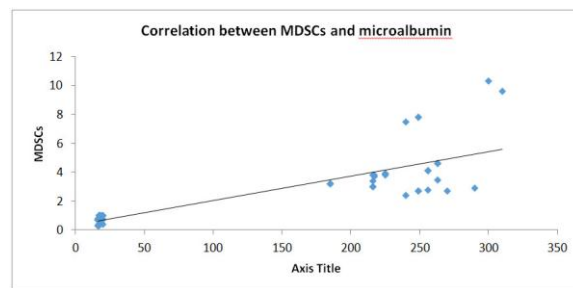


Figure 5: Positive correlation (direct proportion) between MDSCs and microalbumin ( $r = 0.814$ ;  $P < 0.001$ )

## Discussion

MDSCs are a heterogeneous population of cells that may inhibit the immune response, which makes them important goals for the treatment of autoimmune diseases [21]. Some studies focused on the importance of MDSCs, and they suggested the possibility of their use in the prevention and

treatment of T1D due to their potential suppressor function [22]. On the contrary, MDSCs was found at different stages of differentiation, accumulating in various pathological conditions like inflammation and autoimmune diseases [21]. These findings appear to be ambiguous. In our work, we studied the frequency of MDSCs in T1D patients.

According to *Kracht et al.* destruction of Beta-islets of the pancreas and development of autoimmune T1D and the antitumour immunity may share some common pathways and may differ in others [23]. Environmental factors have an important role in the aetiology of T1D as well as the antitumor immune response. The subsequent inflammation induces massive destruction of the target cells. Both beta cells and tumour cells respond to inflammatory signals by releasing proinflammatory cytokines such as (IL-1b), chemokines (CCL2, CXCL10) [24][25], and by producing neoantigens that are recognised by a tumour - specific or autoreactive T cells [26][27]. Overexpression of human leukocyte antigen (HLA) is an early indicator of islet distress, seemingly before insulinitis in the case of T1D [28]. While tumours can evade immune detection by downregulation of HLA class I or direct inhibition of lymphocytes [29]. The hyperexpression of HLA class I by beta - cells together with the production of chemo-attractants, results in amplification of the immune response [30]. This, in conjunction with signals produced by infiltrating immune cells (CD8 and CD4 T cells), that lead to a microenvironment comparable to antitumor response. The microenvironment of progressive tumours contains numerous cells that promote tumour growth. Among these cells, we can find MDSCs that prevent cytotoxic T cell activity [31]. In case of T1D, Whitfield - Larry *et al.*, reported an increased frequency of MDSCs in T1D patients in contrast to the islet of diabetic Non- Obese Diabetic (NOD) mice that showed fewer MDSCs suggesting an underlying defect in immune suppression [22]. Even though studies of MDSCs in autoimmunity in mice are frequent, only a few data about MDSCs in human autoimmune diseases are available.

Our work reveals a highly significant increase in the frequency of CD33<sup>+</sup> HLA-DR<sup>-</sup> MDSCs in the peripheral blood of T1D patients. The underlying mechanism of this increase, however, is not well known. In a previous study, *Haile et al.* had described an increased frequency of peripheral MDSCs in Crohn's disease and ulcerative colitis [32]. A strong association reported in their study was that most of their patients followed an immunosuppression regimen. In our study, none of the T1D patients was on immunosuppressive treatment, yet our results similarly show an increased frequency of peripheral MDSCs. Therefore, we suggest that the high frequency of peripheral MDSCs might be a general finding in autoimmune diseases and not a result of the use of immunosuppressive drugs.

A possible explanation of MDSCs increase in T1D raised when a previous cancer study suggested that some factors produced by cancer cells and inflammatory cells may have a role in the increased frequency of MDSCs [33][34]. These factors included three cytokines known to be important in the pathogenesis of T1D which are IFN - gamma, IL-1beta, and IL-6 [35][36]. So, increased frequency of peripheral MDSCs in T1D patients is likely a result of the elevation of these cytokines.

Some recent studies reported a relation between end-stage renal disease (ESRD) and expansion of MDSCs [37], so we investigated the effect of renal complication of T1D known as diabetic nephropathy on the frequency of MDSCs and whether MDSCs would significantly differ in T1D without nephropathy than in T1D with diabetic nephropathy. We found a highly significant increase of MDSCs in diabetic patients with nephropathy than in those without nephropathy.

Human MDSCs can be identified as CD33<sup>+</sup> HLA-DR<sup>-</sup>, and further divided into 2 subsets: granulocytic CD14<sup>-</sup> and monocytic CD14<sup>+</sup> MDSCs [38][39]. In our study, we analysed the peripheral blood of T1D patients with and without diabetic nephropathy for the presence of 2 recently described MDSCs subpopulations (G-MDSCs and M-MDSCs). Our data demonstrate that MDSCs show highly significant increase in the peripheral blood of patients with T1D compared to healthy controls. M-MDSC phenotype (CD14<sup>+</sup> CD33<sup>+</sup> HLA-DR<sup>-</sup>) were the most abundant MDSCs subpopulation in the blood of healthy control group, T1D with and without nephropathy. To our knowledge, this is the first study that compares the difference in the percentage of MDSCs subsets (CD14<sup>+</sup> and CD14<sup>-</sup>) in T1D and stating the decrease in the percentage of CD14<sup>+</sup> and the increase in CD14<sup>-</sup> in T1D compared to the control group.

In conclusion, this study reports an increase in MDSCs in the peripheral blood of T1D patients versus healthy control with a predominance of the CD14<sup>+</sup> MDSCs subset. Considering the immunosuppressive effect of MDSCs, we realise that continuous monitoring of the composition of these cells in T1D patients may be highly rewarding. Further future studies are needed to test the immune suppression function of MDSCs in T1D and their potential use as targets to inhibit the immune response in autoimmune disease.

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