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Expansion of Myeloid-Derived Suppressor Cells Correlates with Renal Progression in Type 2 Diabetic Nephropathy

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ABSTRACT

Type 2 diabetic nephropathy (T2DN) progresses with an increasingly inflammatory milieu, wherein various immune cells are relevant. Herein, we investigated the levels of myeloid-derived suppressor cells (MDSCs) and their clinical implication in patients with T2DN. A total of 91 subjects (T2DN, n=80; healthy, n=11) were recruited and their PBMCs were used for flow cytometric analysis of polymorphonuclear (PMN-) and monocytic (M-) MDSCs, in addition to other immune cell subsets. The risk of renal progression was evaluated according to the quartiles of MDSC levels using the Cox model. The proportion of MDSCs in T2DN patients was higher than in healthy individuals (median, 6.7% vs. 2.5%). PMN-MDSCs accounted for 96% of MDSCs, and 78% of PMN-MDSCs expressed Lox-1. The expansion of PMN-MDSCs was not related to the stage of T2DN or other kidney disease parameters such as glomerular filtration rate and proteinuria. The production of ROS in PMN-MDSCs of patients was higher than in neutrophils of patients or in immune cells of healthy individuals, and this production was augmented under hyperglycemic conditions. The 4th quartile group of PMN-MDSCs had a higher risk of renal progression than the 1st quartile group, irrespective of adjusting for multiple clinical and laboratory variables. In conclusion, PMN-MDSCs are expanded in patients with T2DN, and may represent as an immunological biomarker of renal progression.

Keywords: Diabetic nephropathy; End-stage renal disease; Immunology; Monocyte; Myeloid-derived suppressor cell

INTRODUCTION

Diabetic nephropathy (DN) is an important health condition because it is the leading cause of end-stage renal disease worldwide (1). The proportion of patients with DN has

been increasing and reached up to 50% of patients with end-stage renal disease (2), with frequently encountering risks of higher morbidity and mortality (3,4). When these burdens in addition to socioeconomic illness are considered (5), development of an appropriate therapeutic approach for DN is a critical issue, yet there are no reversing or targeting agents except for supportive care such as glycemic control and anti-hypertensive agents.

The disease progression of DN can be classified by decreased kidney function (graded 1–5) and proteinuria (graded A1–3) (6). These progressions are clinically heterogeneous and may have different courses depending on patient status (7). This feature may be because the mechanism of DN is heterogeneous and not determined, and includes metabolic, hemodynamic, and oxidative stress processes, which interact with one another (8). The inflammatory milieu is related to the progression of DN, and several immune cell subsets play roles in DN, contributing to dysregulated metabolism, cell stress, and chronic inflammation (9). Tracking immune cell subsets may be helpful because specific subsets may underlie the progression of DN and thus represent potential novel targets to abrogate the disease.

Myeloid-derived suppressor cells (MDSCs) are a regulatory immune cell subset with the ability to suppress other immune cells including T, B, and NK cells (10). MDSCs are expanded in the tumor environment and can promote the severity and metastasis of tumors (11). There are 2 representative subtypes of MDSCs: polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSC (M-MDSCs). Intriguingly, their detection is not limited to patients with tumors and they are frequently observed in several pathologic conditions such as autoimmune diseases (12,13). Patients with type 1 diabetes mellitus display an increased number of MDSCs in peripheral blood (14), but this cell type has not been comprehensively investigated in type 2 DN (T2DN). Herein, we investigated the expansion of the MDSC subset and its relationship with progression in a cohort of patients with T2DN.

MATERIALS AND METHODS

Study subjects

The study protocol complied with the ethical principles of the Declaration of Helsinki and received full approval from the Institutional Review Boards of Seoul National University Hospital (H-1702-049-831). A total of 80 patients who had been clinically diagnosed with T2DN were recruited from July 2017 to February 2018. To compare the proportion of immune subsets, 11 healthy individuals without evidence of kidney disease, hypertension, or diabetes mellitus were recruited during the same period. All study subjects provided written informed consent for the donation and use of their specimens in the present study.

Study variables and outcome

Clinical data on age, sex, and medications such as metformin, sulfonylurea, meglitinide, thiazolidinedione, dipeptidyl peptidase-4 inhibitor, glucagon-like peptide-1 receptor agonist, and insulin were collected. Laboratory findings, including serum creatinine, glucose, hemoglobin A1c, and random urine protein-to-creatinine ratio (uPCR) were obtained. The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation (15). Renal progression as a primary outcome was defined when a doubling of serum creatinine, ≥50% decrease of eGFR, or end-stage renal disease (i.e., dialysis or kidney transplantation) occurred during the follow-up period.

Analysis of immune cells in peripheral blood samples

PBMCs were immediately isolated from heparinized whole blood by density gradient centrifugation with Ficoll-Paque (GE Healthcare, Chicago, IL, USA). Cells were washed, resuspended in staining buffer consisting of 2% horse serum and 0.05% sodium azide, blocked with anti-human Fc receptor binding inhibitor (eBioscience, San Diego, CA, USA), and then incubated with primary Abs. Samples were processed using a BD Fortessa™ machine (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo software (FlowJo, Ashland, OR, USA). Singlet cells were selected based on the scatter profiles, and dead cells were stained with DAPI (Molecular Probes, Eugene, OR, USA) and excluded from the analysis. The Abs used for flow cytometry are listed in **Supplementary Table 1**.

ROS production

Production of ROS in PBMCs and neutrophils from T2DN patients (n=18) and healthy individuals (n=8) were measured using the oxidation-sensitive dye, DCFDA (Invitrogen, Carlsbad, CA, USA). In brief, cells were labeled for surface markers, washed, and incubated at 37°C in serum-free RPMI media with 3 μM of DCFDA for 30 min. ROS production was measured by flow cytometry and presented as mean fluorescence intensity. In another experiment, PMN-MDSCs from patients with T2DN were cultured with indicated glucose concentrations for 12 h and their ROS production was measured as stated above.

Statistical analysis

All analyses and calculations were performed using SPSS (version 23.0; IBM Corp., Armonk, NY, USA) and GraphPad Prism (version 7.0; GraphPad Software, Inc., La Jolla, CA, USA). Categorical and continuous variables are expressed as proportions and the means±SD for normally distributed variables and as the median with interquartile range for non-normally distributed variables. The normality of distribution was analyzed via the Kolmogorov-Smirnov test. The χ^2 test was used to compare categorical variables (Fisher's exact test, if not applicable). The Student's t-test or the Mann-Whitney U test was used to compare continuous variables with or without normal distributions, respectively. The correlation coefficient between continuous variables was measured using Pearson's correlation test. Kaplan-Meier survival curves were constructed and compared using the log-rank test. A Cox proportional hazards regression model was applied to calculate hazard ratios of renal progression. All p-values were 2-sided, and values <0.05 were considered significant.

RESULTS

Baseline characteristics

Table 1 shows baseline characteristics of patients with T2DN. The mean age was 69±9 years, and 63.7% were male. The median value of eGFR was 36.9 ml/min/1.73 m^2 (17.1–54.2 ml/min/1.73 m^2). When peripheral blood immune subsets were analyzed (**Table 1** and **Supplementary Table 2**), the main subsets were CD3$^+$ T cells, NK cells, and monocytes. Most MDSCs belonged to the PMN subset, whereas the proportion of M-MDSC was less than 1% of immune cells. The 78% of PMN-MDSC additionally expressed Lox-1 (i.e., Lox-1$^+$ PMN-MDSCs).

Comparison of immune cell subsets between patients and healthy individuals

Gating strategies for immune cell subsets are shown in **Fig. 1A**. While the proportion of M-MDSCs did not differ between T2DN patients and healthy individuals except for those

Table 1. Baseline characteristics and immune profiling according to the progression of diabetic nephropathy

Variables	Total (n=80)	Non-progression (n=60)	Progression (n=20)	p
Age (years)	69.0±8.8	69.8±8.5	66.5±9.1	0.159
Male (%)	63.7	63.3	65.0	0.893
Weight (kg)	68.1±11.7	69.9±11.8	62.6±9.7	0.002
Comorbidities (%)				
History of ischemic heart disease	23.8	21.7	30.0	0.448
History of stroke	8.8	8.3	10.0	0.819
Diabetic retinopathy	32.5	31.7	35.0	0.783
Laboratory findings				
Creatinine (mg/dl)	1.7 (1.2–2.9)	1.5 (1.1–2.2)	3.6 (2.7–5.4)	<0.001
eGFR (ml/min/1.73 m²)	36.9 (17.1–54.2)	43.3 (29.8–61.4)	13.7 (9.8–21.8)	<0.001
Random uPCR (g/g)	1.0 (0.2–4.1)	0.5 (0.1–1.8)	5.7 (2.1–9.0)	<0.001
Fasting glucose (mg/dL)	136.3±41.1	141.6±43.4	120.2±28.4	0.015
Hemoglobin A1c (%)	7.0±1.2	7.2±1.2	6.5±1.1	0.033
Medications (%)				
Metformin	50.0	55.0	35.0	0.121
Sulfonylurea	36.3	33.3	45.0	0.347
Meglitinide	2.5	3.3	0	0.408
Thiazolidinedione	2.5	3.3	0	0.408
DPP4 inhibitor	56.3	53.3	65.0	0.362
GLP1 receptor agonist	0	0	0	NA
Insulin	31.3	31.7	30.0	0.889
Immune cell subset (%)				
CD3$^+$ T cells	40.5±10.7	40.5±10.7	40.7±11.0	0.943
CD3$^+$ CD4$^+$ T cells	25.1±8.4	25.4±8.3	24.5±8.7	0.679
CD3$^+$ CD8$^+$ T cells	11.1±5.4	10.9±5.2	11.7±5.9	0.603
CD3$^+$ CD4$^+$ CD25$^+$ CD127$^{low/-}$ Treg cells	0.8±0.5	0.8±0.5	0.7±0.5	0.691
CD3$^+$ CD56$^+$ NKT cells	3.4 (1.8–5.1)	3.4 (1.8–5.3)	3.2 (1.4–4.4)	0.420
CD19$^+$ B cells	6.3±3.6	6.8±3.9	4.7±1.7	0.022
CD56$^+$ NK cells	16.7±9.2	17.7±9.6	13.8±7.5	0.068
CD14$^+$ monocytes	15.0±6.6	14.7±6.8	15.9±6.0	0.463
M-MDSCs	0.2 (0.1–0.3)	0.2 (0.2–0.3)	0.2 (0.1–0.3)	0.356
PMN-MDSCs	6.5 (3.7–11.9)	5.6 (3.5–10.5)	9.7 (4.4–22.5)	0.016
Lox-1$^+$ PMN-MDSCs	5.1 (3.0–9.6)	4.5 (2.7–8.5)	8.8 (3.9–16.7)	0.004

DPP4, dipeptidyl peptidase-4; GLP1, glucagon-like peptide-1.

with stage 4 T2DN, the proportion of PMN-MDSC was significantly higher in patients with T2DN than in healthy individuals (**Fig. 1B**). Lox-1, which has been known to distinguish the population of human PMN-MDSCs from neutrophils in cancer patients, was further analyzed (16,17). The proportion of Lox-1$^+$ PMN-MDSCs was elevated in patients with T2DN compared with healthy individuals, except for stage 3a. The overall trend in the absolute cell number results was similar to that in the proportion results, which is shown in **Supplementary Fig. 1**.

When other immune cell subsets were evaluated, the proportions of total, CD4$^+$, and CD8$^+$ T cells decreased in T2DN patients compared with healthy individuals (**Fig. 1C**). Other T cell subsets such as Treg and NKT cells did not differ between patients and healthy individuals (**Fig. 1D**). The proportion of B cells decreased in patients with advanced stages of T2DN, whereas that of NK cells increased in patients with early stage T2DN compared with healthy individuals (**Fig. 1E**). The calculated proportions of immune cell subsets are shown in **Supplementary Table 2**. When the relationships between MDSC and the parameters of kidney dysfunctions were explored, none of the MDSC subtypes had a linear relationship with the levels of eGFR (**Fig. 2A-C**) and uPCR (**Fig. 2D-F**). Collectively, PMN-MDSCs, but not M-MDSCs, were expanded in patients with T2DN, although stage of chronic kidney disease and levels of eGFR and uPCR did not correlate with this expansion.
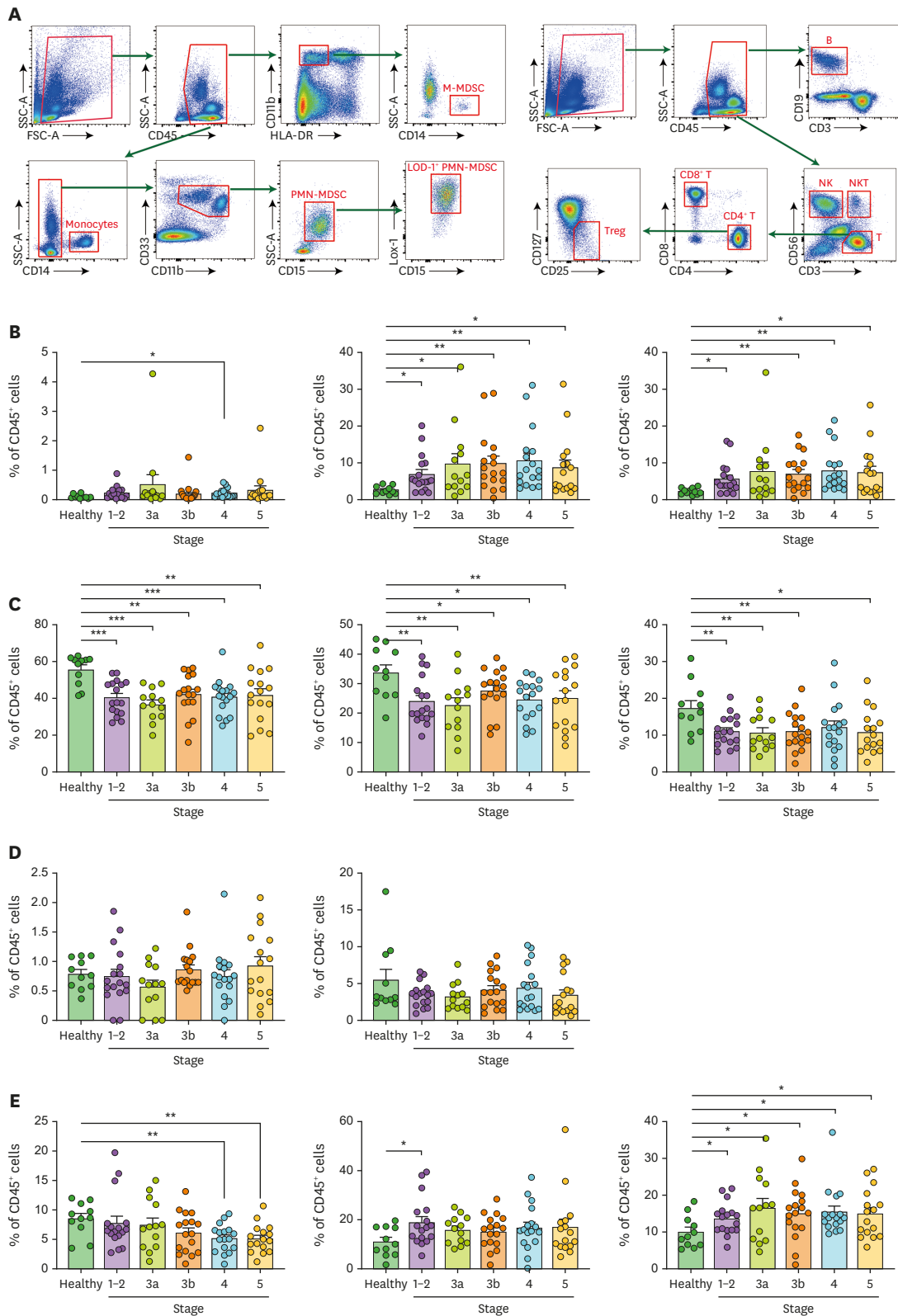
Figure 1. Profiling of immune cell subsets of patients with T2DN. (A) Gating strategies for peripheral blood immune cells. Proportions of (B) M-, PMN-, and Lox-1+ PMN-MDSCs, (C) total, CD4+, and CD8+ T cells, (D) regulatory and NK T cells, (E) B and NK cells, and monocytes in patients and healthy individuals. *p<0.05; **p<0.01; ***p<0.001.
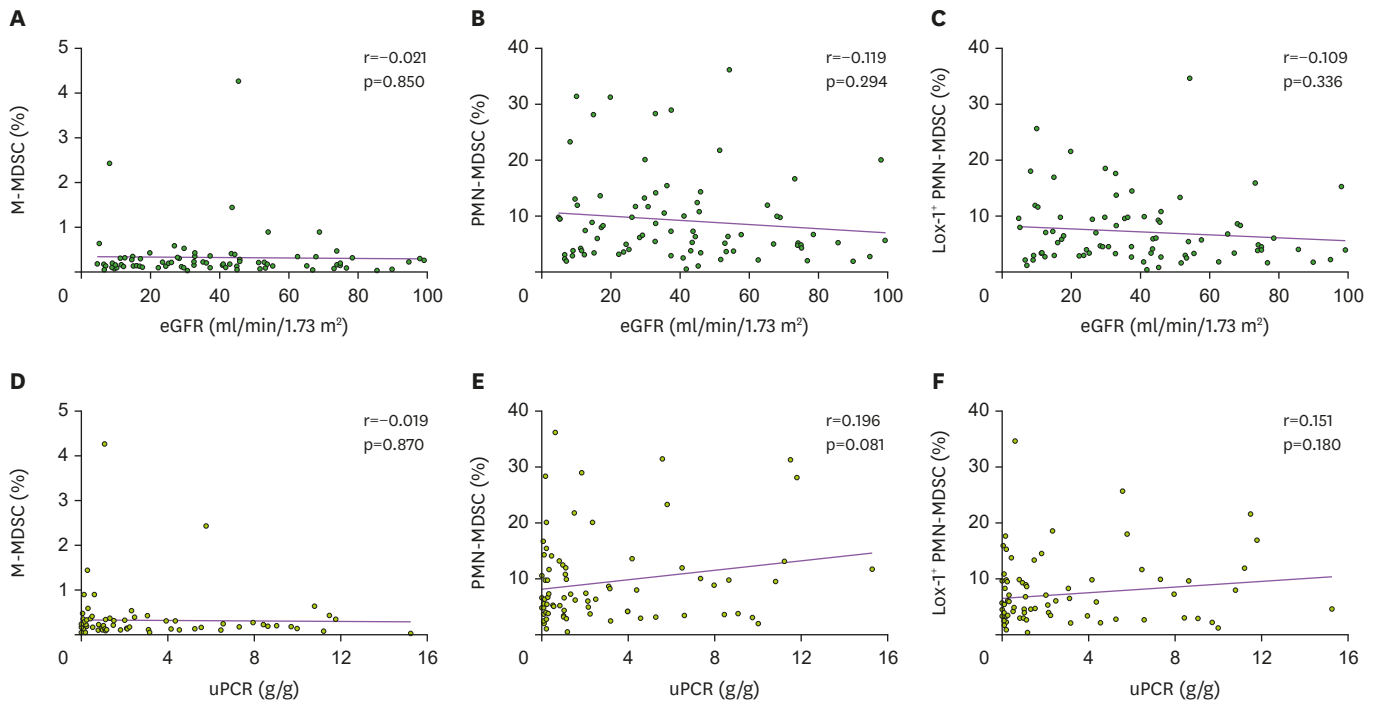
Figure 2. Correlation between MDSCs and kidney parameters: eGFR (A-C) and random uPCR (D-F). Correlation of eGFR with (A) M-MDSCs, (B) PMN-MDSCs, and (C) Lox-1^{+} PMN-MDSCs. Correlation of uPCR with (D) M-MDSCs, (E) PMN-MDSCs, and (F) Lox-1^{+} PMN-MDSCs.

ROS production in PMN-MDSC

As a representative function of PMN-MDSC, we evaluated the production of ROS (18). ROS production was higher in PMN-MDSCs from T2DN patients than in neutrophils from both patients and healthy individuals (**Fig. 3A**). When PMN-MDSCs from patients with T2DN were cultured with high concentrations of glucose, ROS production was further augmented depending on the glucose concentrations (**Fig. 3B and C**). Collectively, ROS production in PMN-MDSCs of T2DN patients was elevated compared with neutrophils, and this might be enhanced by the diabetic condition.
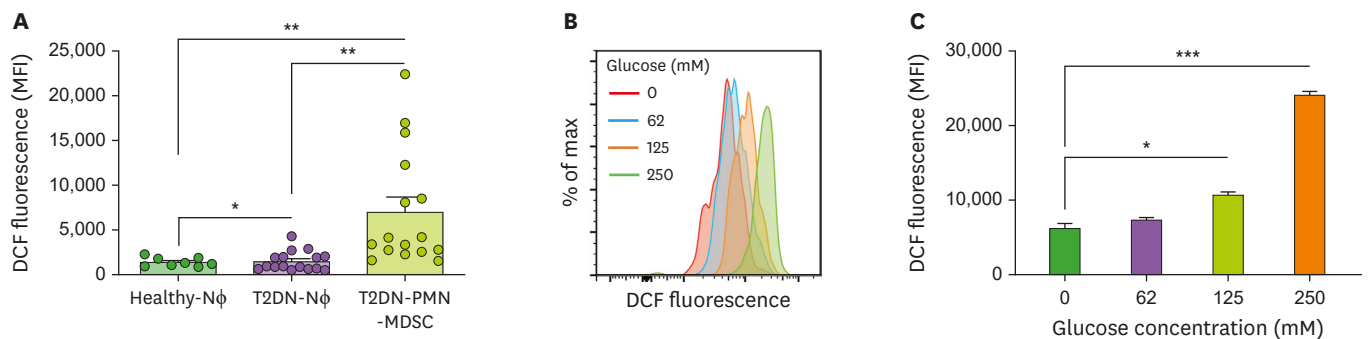

Figure 3. Production of ROS. (A) Comparison of ROS production between neutrophils and PMN-MDSC from patients with T2DN and neutrophils from healthy individuals. (B) Representative histogram and (C) bar graph for DCF fluorescence in cultured PMN-MDSCs with indicated glucose concentrations.
Nϕ, neutrophil; MFI, mean fluorescence intensity; DCF, dichlorofluorescein.
*p<0.05; **p<0.01; ***p<0.001.

Table 2. Renal progression-predicting model with MDSCs

Variables	Quartiles	Model 1		Model 2		Model 3		Model 4	
		HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p
M-MDSCs	1st Q	1 (Reference)		1 (Reference)		1 (Reference)		1 (Reference)	
	2nd Q	1.05 (0.26–4.20)	0.949	0.83 (0.20–3.54)	0.804	1.01 (0.16–6.21)	0.994	0.73 (0.128–4.22)	0.729
	3rd Q	1.64 (0.46–5.82)	0.446	1.30 (0.36–4.79)	0.689	1.22 (0.28–5.43)	0.793	1.95 (0.35–10.99)	0.451
	4th Q	1.59 (0.45–5.64)	0.477	1.90 (0.51–7.13)	0.340	1.63 (0.35–7.63)	0.534	1.41 (0.26–7.52)	0.690
PMN-MDSCs	1st Q	1 (Reference)		1 (Reference)		1 (Reference)		1 (Reference)	
	2nd Q	0.96 (0.19–4.79)	0.963	0.74 (0.14–3.96)	0.726	0.83 (0.13–5.21)	0.840	0.83 (0.12–6.04)	0.857
	3rd Q	1.69 (0.40–7.12)	0.472	1.02 (0.23–4.44)	0.983	0.82 (0.14–4.95)	0.827	1.08 (0.20–6.00)	0.927
	4th Q	3.07 (0.83–11.37)	0.093	4.40 (1.07–18.06)	0.040	4.02 (0.84–19.29)	0.082	5.95 (0.97–36.61)	0.054
Lox-1+ PMN-MDSCs	1st Q	1 (Reference)		1 (Reference)		1 (Reference)		1 (Reference)	
	2nd Q	0.71 (0.12–4.29)	0.708	0.55 (0.09–3.39)	0.521	0.44 (0.07–2.86)	0.391	0.51 (0.05–5.24)	0.567
	3rd Q	1.49 (0.35–6.28)	0.587	1.28 (0.28–5.92)	0.749	3.71 (0.48–28.66)	0.209	3.11 (0.43–22.27)	0.259
	4th Q	4.03 (1.09–14.93)	0.037	6.85 (1.54–30.38)	0.011	12.32 (1.78–85.24)	0.011	37.69 (2.45–579.44)	0.009

Model 1: Unadjusted. Model 2: Adjusted for age, sex, and eGFR. Model 3: Adjusted for other clinical and laboratory variables plus Model 2. Model 4: Adjusted for other immune cell subset plus Model 2.
HR, hazard ratio; CI, confidence interval; Q, quartile.

Effect of PMN-MDSCs on renal progression

Renal progression occurred in 20 patients during the median period of 17 months (7–20 months; maximum 2 years). The progression group had higher levels of serum creatinine and uPCR, but lower levels of eGFR, glucose, and hemoglobin A1c than the non-progression group (**Table 1**). The medications used did not differ between the 2 groups. In terms of immune cell subsets, the proportion of total or Lox-1+ PMN-MDSCs was higher in the progression group than in the non-progression group. The proportion of B cells was also different between the 2 groups. To calculate hazard ratios for renal progression, we divided patients into quartiles of each MDSC subset. **Fig. 4** shows Kaplan-Meier curves of renal progression according to the quartiles of MDSCs. The 4 quartile groups of M-MDSC had a similar rate of renal progression (**Fig. 4A**), but the 4th quartile group of PMN-MDSC had a higher rate of renal progression than the lower 3 quartile groups (**Fig. 4B**). This trend was similar to the results for the quartile groups of Lox-1+ PMN-MDSC (**Fig. 4C**). To identify independent relationships, stepwise multivariate Cox models were applied (**Table 2**). High levels of PMN-MDSCs seemed to correlate with the risk of renal progression independently of other variables, although the significances were marginal. Lox-1+ PMN-MDSCs had a more independent relationship with renal progression than PMN-MDSCs. Levels of M-MDSC were not associated with renal progression.
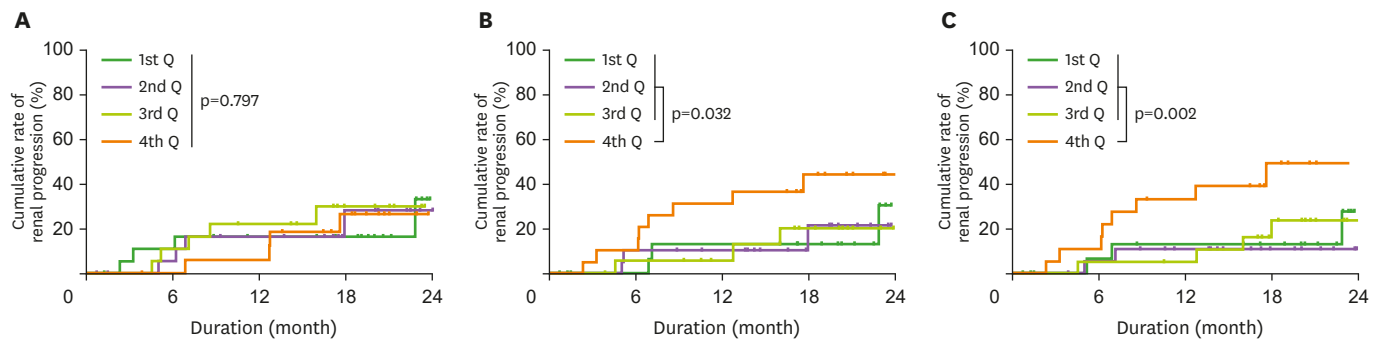

Figure 4. Cumulative rate of renal progression according to the quartiles of (A) monocytic myeloid-derived suppressor cells, (B) polymorphonuclear myeloid-derived suppressor cells, (C) Lox-1+ polymorphonuclear myeloid-derived suppressor cells.
Q, quartile.

DISCUSSION

T2DN is regarded as an inflammatory disease, and renal function is aggravated by inflammatory insults. However, this inflammatory milieu generated by several immune cell subsets in T2DN has not been fully explored. The present study focused on MDSCs and identified that PMN-MDSCs were primarily expanded in patients with T2DN and their ROS production as a functional capacity was potentially maintained and augmented by the diabetic condition. Higher expansions of total and Lox-1+ PMN-MDSCs were associated with worse renal outcome than less expanded counterparts, which suggests that PMN-MDSCs could be an immune biomarker or a therapeutic target in T2DN.

MDSCs are one type of immunosuppressive cell (19). These cells are frequently expanded in tumor conditions and their unique functions, such as production of ROS, arginase, nitric oxide, and anti-inflammatory cytokines, and the expression of inhibitory immune checkpoint molecules promote tumor progression (11). Interestingly, the presence of MDSCs has been documented in several non-tumorous conditions such as autoimmune or chronic inflammatory diseases (12,13). Inflamed tissues may attract the migration of MDSCs although the linking molecules have not been comprehensively identified (20). In accordance with these observations, a preclinical model under diabetic condition displays an enriched MDSC subset (14), and thus, suggests MDSCs could be a therapeutic tool (21). Diabetic patients have shown an expansion of MDSC (14), but this issue has been documented primarily in type 1 diabetes. One study focused on 24 patients with type 2 diabetes, but these patients did not receive oral anti-diabetic drugs (i.e., taking insulin alone) and did not have T2DN, and thus, may not be representative of patients with treated type 2 diabetes or T2DN (22). Furthermore, that study did not differentiate target cells into M-MDSCs and PMN-MDSCs in the analyses. The indicated proportion of MDSCs seemed to be approximately 6%–7% (the study did not report the exact proportion), and this was similar to the findings in the present study (median, 6.7% [3.9%–12.3%]). The present study further identified that 96% of MDSCs were the PMN-subset; and 78% of PMN-MDSCs expressed Lox-1. Their functional capacity, illustrated using ROS production, was augmented by hyperglycemic conditions. These results have clinical implications because therapeutic targeting can be further focused on PMN-MDSCs in T2DN.

Preclinical models have found that MDSCs regulate the severity of kidney injury. In acute renal inflammatory models such as ischemia-reperfusion injury (23) and doxorubicin-induced glomerulonephritis (24), MDSCs were expanded in both blood and injured kidneys and their adoptive transfer reduced renal damage. The expansion of MDSCs was also identified in a chronic fibrosis model using adenine (25), and renal fibrosis was reduced by the infusion of MDSCs in a streptozotocin-induced DN model (26). One human study of 49 patients with end-stage renal disease found that PMN-MDSCs were primarily elevated in these patients and the M-MDSC subset was intriguingly elevated after a session of hemodialysis alone, although the underlying mechanism was not provided (27). The present study increases the knowledge of MDSCs in kidney diseases, specifically in T2DN, which is the most common cause of end-stage renal disease (1).

The presence or expansion of MDSCs has frequently been associated with poorer outcomes of patients with cancer (28). In the present study, high expansion of PMN-MDSCs correlated with subsequent renal progression, although the increase in MDSCs was not dependent on the stage of T2DN or other parameters of kidney dysfunction such as eGFR and uPCR. When their anti-inflammatory functions are considered, this relationship seems to be

counterintuitive. Some hypotheses may be suggested regarding this reversed relationship. The anti-inflammatory capacity of PMN-MDSCs in T2DN might be insufficient to maintain kidney function, because several other inflammatory cells, cytokines, adipokines, and chemokines would participate in and aggravate the renal inflammatory milieu (9,29). Additionally, the capacity of PMN-MDSCs might be altered in T2DN compared with tumorous conditions. Particularly, uremic conditions are known to blunt the anti-inflammatory function of immune cells (30). This potential alteration of functions has been much explored in the Treg subset. The number of Tregs is infrequently elevated in the diabetic state (31,32), and their functions are reduced in chronic kidney disease irrespective of stage (33,34). To improve the anti-inflammatory function of PMN-MDSCs and make them a potential therapeutic target in T2DN, other solutions may be needed such as expansion of *ex vivo* or *in vivo* cytokine-augmented MDSCs (14,26).

Understanding of the inflammatory milieu in T2DN is essential to develop immune cell-targeting therapy for prevention of renal damage. The present study identifies the expansion of PMN-MDSCs in T2DN, and their high expansion is related to renal outcome. These results will form the basis of future studies to understand the pathophysiology of human T2DN and to develop immune cell-targeting therapy.

ACKNOWLEDGEMENTS

SUPPLEMENTARY MATERIALS

Supplementary Table 1
Abs used in this study

Click here to view

Supplementary Table 2
Mean proportion of immune cell subset among CD45$^+$ cells

Click here to view

Supplementary Figure 1
Cell number of MDSCs in patients with T2DN. (A) M-MDSC. (B) PMN-MDSC. (C) Lox-1$^+$ PMN-MDSC.

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