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ORIGINAL RESEARCH

Exploring Inflammatory Changes in the Peripheral Blood of Type 2 Diabetes Mellitus in China

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Objective: Type 2 diabetes mellitus (T2DM) is a serious, chronic metabolic disease globally and its pathogenesis is not completely understood yet. This study aimed to thoroughly investigate the fluctuation of inflammatory markers in the peripheral blood of patients with T2DM, which are rarely reported.

Methods: Peripheral blood samples from patients with T2DM and healthy individuals, as well as their clinical information, were collected at the Second Affiliated Hospital of Soochow University. Flow cytometry was used to analyse the immune cells and cytokines in the peripheral blood. CCK-8 assay was performed to detect the viability of THP-1 cell after treatment with 5 mM or 50 mM glucose. Flow cytometry, Western Blotting and qPCR were used to analyse the apoptosis of monocytes or THP-1 cells.

Results: The numbers of white blood cells, lymphocytes, and neutrophils substantially increased with elevated IL-6 levels. There was a significant decrease in monocytes due to increased cell apoptosis caused by sustained high glucose stimulation. Hyperglycemia reduced monocyte viability and altered monocyte subgroups by increasing the number of intermediate and non-classical monocytes.

Conclusion: In summary, our work reveals that patients with T2DM do have variations of peripheral inflammation biomarkers, especially monocytes.

Keywords: Type 2 diabetes mellitus, peripheral inflammation, monocytes

Introduction

Diabetes mellitus (DM) is one of the major global diseases and type 2 diabetes mellitus (T2DM) accounts for approximately 95%.[1] T2DM is an organ restricted disease, characterized by a relative β-cell deficit or insulin resistance that lead to inability of pancreatic β-cells to meet the demand for insulin.[2] High glucose is one of its characteristics which has negative impacts on several organs, such as the brain, kidney, and eyes.[3,4] However, to date, there have been no systematic studies on the effects of hyperglycemia on the inflammation in the peripheral blood of T2DM patients.[5]

Inflammation has been a global research topic and plays a pivotal role in T2DM progression, which is often associated with circulating pro-inflammatory cytokines and immune cells,[6,7] but its mechanism still needs to be elucidated. Pro-inflammatory cytokines, like TNF-α, IL-1β and IL-17, are considered the central drivers of the inflammatory cascade, leading to β-cell dysfunction.[8] Immune cells have been shown to be associated with diabetes risk and insulin resistance in some epidemiological studies, which mainly refer to white blood cells, such as circulating Th1 and Th17 lymphocytes, neutrophils, and monocyte-macrophages in the peripheral blood.[9] In addition, previous studies also reported the increase of peripheral dendritic cells in T2DM patients.[7]

Monocytes, together with $CD8^+$ T cells, are the first cells recruited to inflamed pancreatic tissues during the development of T2DM.[10,11] Monocytes are divided into three subgroups and have a relatively unique immunological function. $CD14^+CD16^-$ classical monocytes mainly show anti-inflammatory function. The $CD14^+CD16^+$ intermediate

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and $CD14^{-}CD16^{+}$ non-classical subgroups mainly have pro-inflammatory effects.[12] Studies have shown that monocytes from patients with DM have reduced functions in phagocytosis, chemotaxis, and killing compared to cells from healthy groups.[13,14] However, there have been few reports on the effects of high glucose levels on monocyte classification, cell viability, or survival.

The impact of hyperglycemia on islet tissue and islet β cells is well known, but there is a lack of comprehensive understanding between the inflammation changes in peripheral blood of T2DM patients. In this study, we investigated the inflammatory alterations in the peripheral blood of patients with T2DM and found the variations of peripheral inflammation biomarkers, especially monocytes. Although the number of patients in this study may not be large enough and this study was just conducted in Suzhou area, these results will help to reveal the relationship between hyperglycemia and inflammation in T2DM.

Materials and Methods

Clinical Information of Patients With T2DM

Peripheral blood from sex-matched individuals with T2DM, based on the guidelines of the American Diabetes Association, was collected from the Department of Clinical Laboratory, Second Affiliated Hospital of Soochow University, from January to December 2023. Peripheral blood samples from 515 patients with T2DM and 494 healthy individuals were analyzed. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University (JD-LK-2021–064-01) and was conducted in accordance with the Declaration of Helsinki.

We selected patients with T2DM with exclusion criteria as follows: patients with T1DM, severe hepatopulmonary dysfunction, severe renal insufficiency (glomerular filtration rate (eGFR) < 30 mL/1.73 mm^2), and inflammatory disease; and patients who were unmatched for age and sex.

Flow Cytometry Analysis of Peripheral Immune Cell

Whole peripheral blood (50 μL) from patients with T2DM was stained for neutrophils, lymphocytes, and subsets of monocyte analyzed in flow cytometry. Surface markers included Pe-cy5.5-CD45, APC-CD14, PE-CD16, FITC-CD3, PE-Cy7-CD4, and APC-Cy7-CD8. Each sample was added to 1 mL RBC lysis buffer for 15 min and stained with the mixed markers for another 15 min at room temperature. Then removed the supernatant after centrifugation, and resuspended with 500 μL of Phosphate Buffer Saline (PBS) for analysis. All antibodies used for flow cytometry were purchased from BD Biosciences. The whole peripheral blood from healthy individuals were used as control.

Cytometric Bead Array of Cytokines

Serums from patients with T2DM in pro-coagulant collection tubes were analyzed for cytokines (IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ, and IL-17A) by using a BD™ Cytometric Bead Array kit according to the manufacturer's instruction. The serum from healthy individuals were used as control.

Cell Counting Kit 8 (CCK8) Assay

THP-1 cells were uniformly seeded in a 96-well plate (1×10^4 cells/well). Six wells with THP-1 cells were treated with 50 mM glucose for 24, 48, and 72 h for cell viability using a cell counting kit (CCK)-8 (No. A311-01, Vazyme), according to the manufacturer's protocol. Meanwhile, six wells with THP-1 cells without treated with 50 mM glucose were used as negative controls and six wells with only cell medium were used as blank controls. Optical density at 450 nm was determined using a FLUO Star Omega. Three independent experiments were performed at the same time.

Peripheral Monocyte Separation

Peripheral blood monocytes from patients with T2DM and healthy individuals were separated by using Ficoll-Paque PLUS density gradient medium (Sigma) according to the manufacturer's instructions.

Cell Apoptosis

Monocytes or THP-1 cells (5×10^5 per well) were cultured for 12 h respectively in the presence of 50 mM glucose. The cells were collected and stained with Annexin V and PI (No. A211, Vazyme), according to the manufacturer's instructions. To analyze changes in protein levels, we lysed cultured THP-1 cells for Western blotting. Antibodies were purchased from Cell Signaling Technology. The mRNA levels of apoptosis-associated proteins (cleave-caspase-3, total caspase-3, and Bcl-2) were determined by using qPCR. The forward and reverse primers of Bcl-2, Bax, Bad, Bcl-xl, caspase-3 and GAPDH were synthesized by Tsingke Biotechnology (Table 1). Monocytes or THP-1 cells without treatment of 50 mM glucose were used as control.

Statistical Analysis

GraphPad Prism 8.0 software was used for statistical calculation. The results are expressed as the mean ± SD. Differences were assessed by Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

Clinical Characteristics of Patients With T2DM

The demographic and general characteristics of patients with T2DM are summarized in Table 2. Our data showed that among the 515 enrolled patients, the incidence in males was higher than that of females. Most patients (69.15%) suffered from this disease for more than five years. Half of the patients had high blood pressure or hyperglycemia. In addition, nearly 70% of patients have poor blood sugar control, which can be reflected by the fact that HbA1c is higher than 7%. We then performed a further analysis of the patients' age (Figure 1 and Table 2). We found that the patients with diabetes were younger. Notably, among these 515 patients, the youngest was only 24 years old, and there were five individuals who were younger than 30 years old. Younger onset age may have a substantial association the current dietary habits of human beings.

T2DM Patients Have Variations in Peripheral Immune System

The harm of diabetes does not lie on hyperglycemia itself, but on a series of chronic inflammatory conditions that lead to high morbidity, disability, and mortality. In this study, we found that the total numbers of white blood cells (WBC), lymphocytes (Lym), and neutrophils (Neu) significantly increased, giving rise to inflammation (Figure 2A, left).

Table 1 Primers for qPCR

Gene	Primer	Sequence
Caspase-3	Forward Primer	CTCGGTCTGGTACAGATGTCGATG
	Reverse Primer	GGTTAACCCGGGTAAGAATGTGCA
Bax	Forward Primer	ACCAAGAAGCTGAGCGAGTGT
	Reverse Primer	ACAAACATGGTCACGGTCTGC
Bcl2	Forward Primer	AGATGTCCAGCCAGCTGCAC
	Reverse Primer	TGTTGACTTCACTTGTGGCC
Bcl-xl	Forward Primer	GGAGCTGGTGGTTGACTTTCT
	Reverse Primer	CCGGAAGAGTTCATTCACTAC
Bad	Forward Primer	CAGTGATCTGCTCCACATTC
	Reverse Primer	TCCAGCTAGGATGATAGGAC
Gapdh	Forward Primer	AGGTCGGTGTGAACGGATTTG
	Reverse Primer	TGTAGACCATGTAGTTGAGGTCA

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Table 2 Characteristics of T2DM

Characteristics	Percentage
Sex	
Male	57.15%
Female	42.85%
Age (years)	
0–19	0
20–39	7.98%
40–59	42.02%
60–79	46.64%
80–99	3.36%
Diabetes duration (>5 years)	69.15%
Dyslipidemia	41.61%
Hypertension	54.76%
HbA1c (>7%)	69.13%

Lymphocyte subsets showed no obvious changes (Supplementary Figure 1). Notably, the number and percentage of monocytes (Mon) were considerably significantly reduced (Figure 2A, right). IL-6 markedly increased, but IFN-γ decreased in the peripheral blood of patients with T2DM compared with those in healthy controls. The expression of other cytokines, such as IL-2, IL-4, IL-10, IL-12, IL-17A and TNF-α, was unchanged between patients with T2DM and healthy individuals (Figure 2B and 2C). These variations contribute to the peripheral immune inflammation of T2DM.

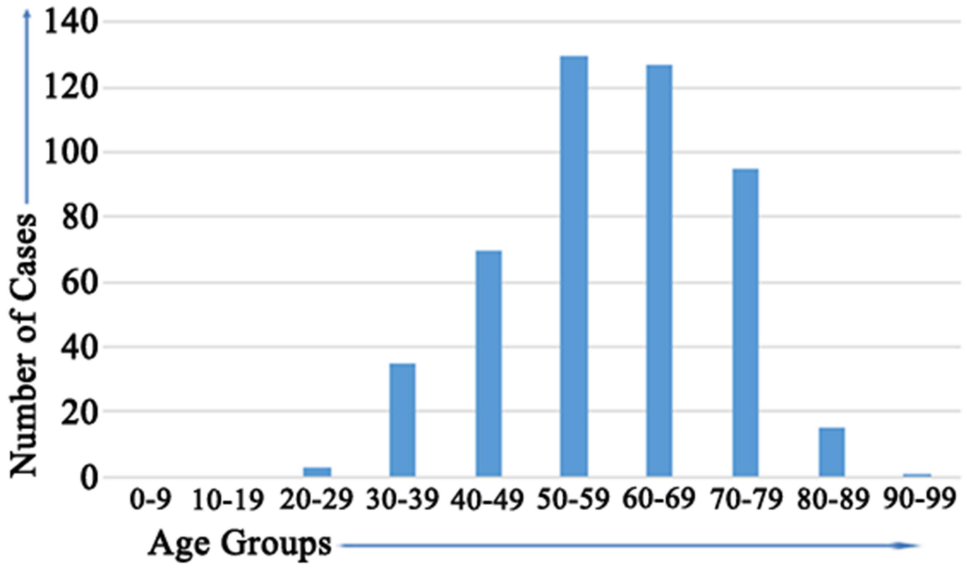

Figure 1 Age distribution of 515 patients with T2DM.

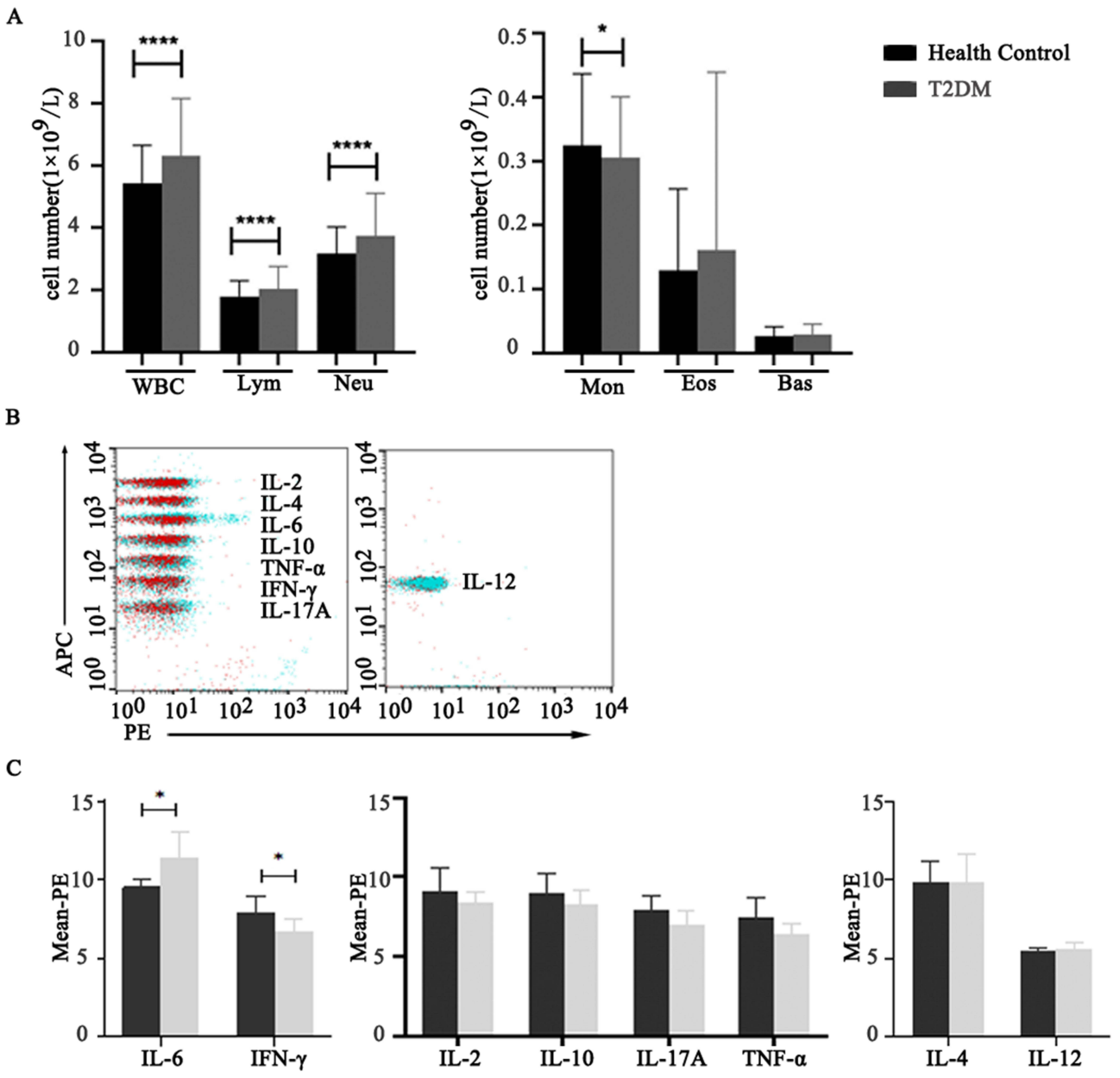

Figure 2 Variations of immune cells and cytokines in peripheral blood from T2DM patients. (**A**) Cell numbers of different peripheral blood subsets between patients with T2DM and health individuals. (**B**) The representative figure from flow cytometric analysis showed the level of IL-2, IL-4, IL-6, IL-10, IL-12, TNF-α, IFN-γ and IL-17A in peripheral blood of patients with T2DM and health individuals. (**C**) The mean of fluorescence intensity of IL-2, IL-4, IL-6, IL-10, IL-12, TNF-α, IFN-γ and IL-17A in peripheral blood of 50 patients with T2DM and 50 health individuals. Bars show the means ± SD. *$P < 0.05$. ****$P < 0.0001$.
Abbreviations: WBC, white blood cells; Lym, lymphocytes; Neu, neutrophils; Mon, monocytes; Eos, eosinophils; Bas, basophils.

Hyperglycemia Affects the Differentiation and Cell Viability of Monocytes

Notably, the number of monocytes in peripheral blood of patients with T2DM decreased. Therefore, we focused on monocytes in future studies. We made distinctions between the following monocyte subgroups: classical monocytes (CM, $CD14^+CD16^-$), intermediate monocytes (IM, $CD14^+CD16^+$), and non-classical monocytes (nCM, $CD14^-CD16^+$). In the peripheral blood of patients with T2DM, the percentage of classical monocytes was decreased, indicating a weakened phagocytic ability. However, IM and nCM levels increased, especially in intermediate monocytes with sharp amplification, which has attracted much attention because of its pro-inflammatory function in the pathophysiology of cardiovascular diseases (Figure 3A). We also found that high concentrations of glucose (50 mM) reduced the viability of monocytic THP-1 cells (Figure 3B).

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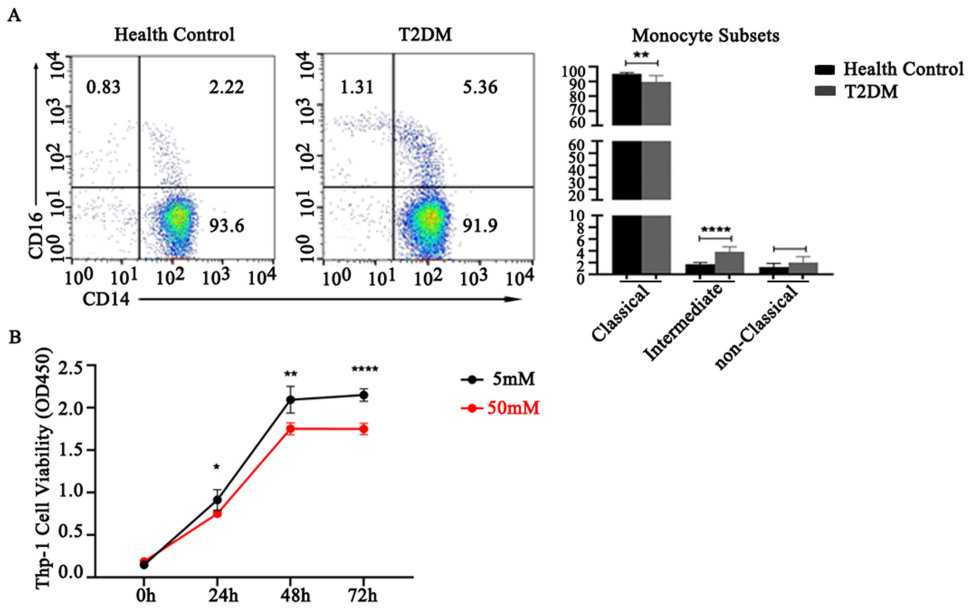

Figure 3 Hyperglycemia affects the differentiation and cell viability of monocytes. (**A**) Percentages of classical, intermediate and non-classical monocytes in peripheral blood of 50 patients with T2DM and 50 health individuals. Bars show the means ± SD. ***P* < 0.01. *****P* < 0.0001. (**B**) THP-1 cell line which stimulated by 5 mM or 50 mM glucose in cell culture medium for different time was used to detect cell viability by CCK-8 staining presented as OD450 values. **P* < 0.05. ***P* < 0.01. *****P* < 0.0001.

Monocyte Cell Apoptosis Augments in T2DM

To assess cell survival, we isolated monocytes from the peripheral blood of healthy controls and patients with T2DM, and then analyzed the combination of annexin V and propidium iodide (PI) after glucose stimulation. For the monocytes from T2DM patients, we found a high percentage of early apoptosis (Annexin-V^+PI^-) with 5 or 50 mM glucose stimulation in culture medium than those from healthy individuals (Figure 4A and 4B). This phenomenon can be explained by the irreversible apoptosis-prone state caused by long-term hyperglycemia. Monocytes from healthy controls showed no palpable changes in cell apoptosis when cultured with 5 or 50 mM glucose for 12 h in vitro, which may be due to the short stimulation time of high sugar. There were no differences in the percentage of Annexin-V^-PI^+ or Annexin-V^+ PI^+ cells among the four groups (Figure 4A and 4B). These monocytes were subjected to Western blotting to detect the levels of apoptosis-related proteins, cleave-caspase-3, total caspase-3, and Bcl-2. Gray analysis showed an increased expression of cleave-caspase-3 and total caspase-3 (Supplementary Figure 2). However, when we cultured the monocyte cell-line, THP-1 for 12 h, we found a significant increase in cell early apoptosis from 4% to 6% (Figure 4C). Meanwhile, mRNA expression of caspase-3 in THP-1 also markedly increased after culturing with 50 mM glucose (Figure 4D). In general, persistent hyperglycemia increased the apoptosis of monocytes.

Discussion

DM is a persistent metabolic disorder that affects millions of people worldwide. T2DM accounts for approximately 95% of diabetes cases, and its pathogenesis may be influenced by complex genetic and environmental factors that are not completely revealed yet.[15,16] At present, it can only be maintained by drugs but still has high morbidity, mortality and complications, which are primarily attributed to sustained hyperglycemia and insulin resistance.[17] As a chronic condition, T2DM tends to increase the risk of several other diseases caused by macrovascular and microvascular damage. Moreover, it also has negative effects on several organs, such as brain, kidney, heart and eyes.[18] T2DM is a serious, chronic metabolic disease, therefore, it is expected to have a higher number of white blood cells, lymphocytes, and neutrophils in the peripheral blood of patients with T2DM. The levels of the pro-inflammatory cytokine IL-6 also significantly increased. However, the number of monocytes decreased and further analysis revealed its impact on monocyte differentiation and cell viability. Hyperglycemia induces monocyte apoptosis by increasing the expression of caspase-3. In summary, patients with T2DM do have variations of inflammatory biomarkers in their peripheral blood.

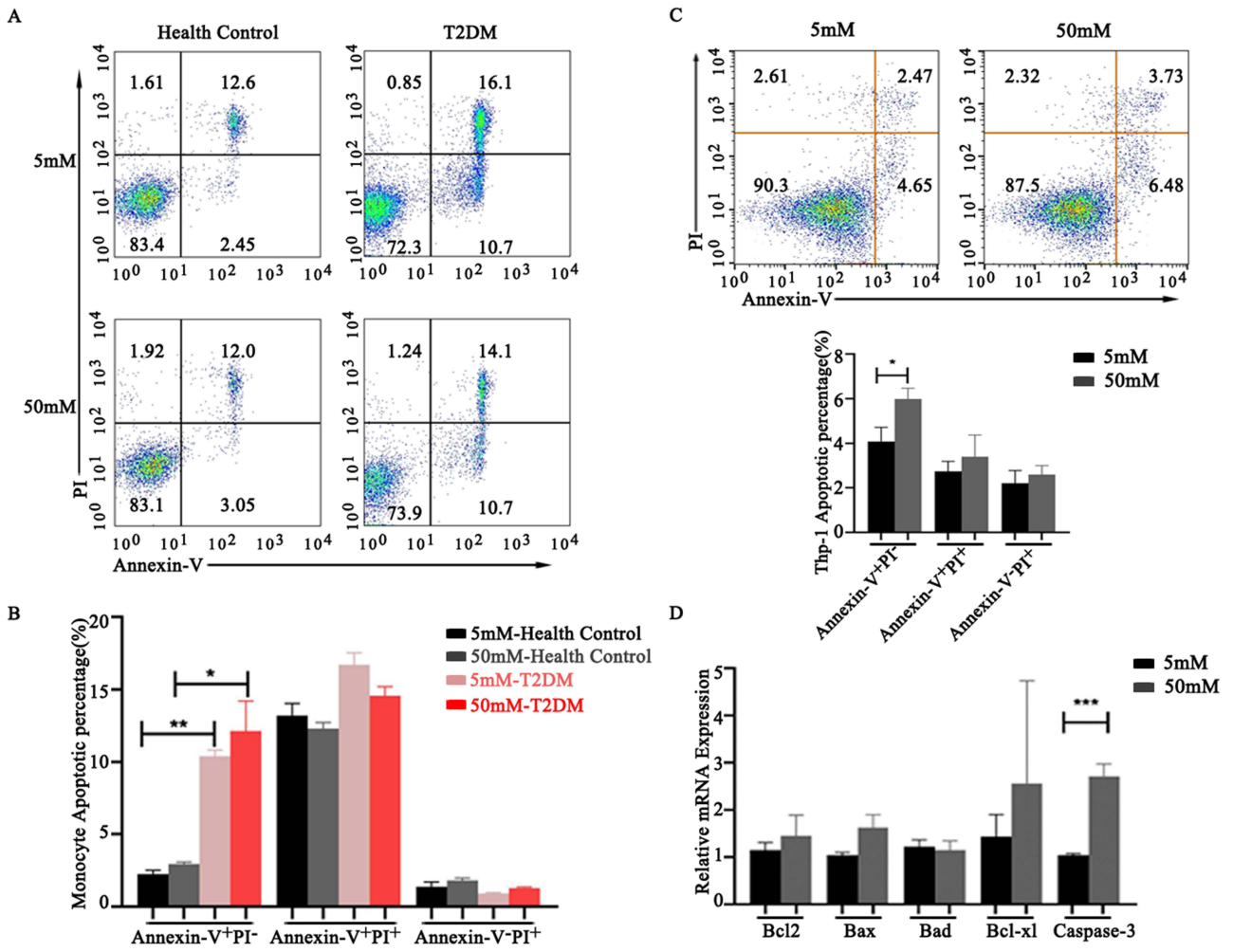

Figure 4 Monocyte cell apoptosis augments in peripheral blood of patients with T2DM. (**A**) Monocytes from T2DM patients' peripheral blood had higher combinations of annexin-v whether there was high concentrations of glucose stimulation or not. (**B**) Histogram analysis showed monocyte apoptotic percentage under different conditions from peripheral blood of 50 patients with T2DM and 50 health individuals. Bars show the means ± SD. *$P < 0.05$. **$P < 0.01$. (**C**) Percentage of THP-1 cell apoptosis with 5 mM or 50 mM glucose stimulation. *$P < 0.05$. (**D**) Real time PCR showed increased expression of caspase-3 in THP-1 cell stimulated with 50 mM glucose. Bars show the means ± SD. ***$P < 0.001$.

Various inflammatory markers are associated with glucose-related disorders.[19] Immune cells in the peripheral blood mainly refer to white blood cells, such as circulating lymphocytes, neutrophils, and monocyte-macrophages.[20] Studies have shown that the risk of diabetes increases with an increased number of white blood cell, which is positively correlated with insulin resistance.[21] Neutrophil dysregulation, characterized by effector dysfunction and inadequate cell death processes that lead to uncontrolled inflammatory responses, can serve as an inflammatory biomarker in patients with diabetes.[22] Other observations also indicate that eosinophils in diabetes, both in the blood circulation and renal tissue, might be associated with the development and severity of diabetic nephropathy.[23] In this study, we found increased leukocytes, lymphocytes, and neutrophils in the peripheral blood of T2DM, but no change in eosinophils or basophils. The increased lymphocytes is closely related to the secretion of cytokines, while the increase of neutrophils may be due to a compensatory increase in dysfunction and inadequate cell death processes. These two cell types account for a large proportion of peripheral blood. Therefore, they are considered the main cells contributing to peripheral immune inflammation.

The peripheral blood is important for the circulation and redistribution of lymphocytes to tissues and organs. Lymphocytes, especially $CD8^+$ T cells, were the first cells found in the inflamed pancreatic tissue during disease development. $CD8^+$ T lymphocytes contribute to persistent low-grade inflammation by secreting pro-inflammatory cytokines and producing cytotoxic molecules, such as granzyme B and perforin.[24] Circulating lymphocytes are increased in patients with T2DM in this

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study, but the percentages of lymphocyte subsets that were mainly divided into $CD4^+$T, $CD8^+$T, NK, and B cells showed no obvious change. Meanwhile, inflammatory responses do not depend on single mediator, but on various markers. Cytokines are one of these markers.[25] We found increased IL-6 levels, which indicates a state of chronic inflammation in the peripheral blood of T2DM patients. In patients with type 1 diabetes mellitus (T1DM), islet autoantigens can stimulate lymphocytes to produce more IL-6, IFN-γ and TNF-α.[26] This was slightly different from what was observed in T2DM.

Monocyte-macrophages were reported as the main effector cells that reduced insulin signaling. Patients with hyperglycemia had more macrophages that infiltrated the adipose tissue and islets, causing inflammation.[27] In this study, we found monocytes were the only cells that were decreased in the peripheral blood of T2DM patients. The reduced number of circulating monocytes in the peripheral blood may have been due to increased tissue settlement. To analyze the reasons for the diminished monocyte counts, experiments demonstrated that hyperglycemia reduced the cell activity and increased the apoptosis of monocytes, which is partially consistent with previous reports that monocytes from patients with T2DM have reduced chemotaxis, phagocytosis, and killing compared to cells from healthy groups. In view of the particularity of this phenotype and the importance of their function, we focused on the analysis of monocyte subsets. $CD14^+CD16^-$ CMs are involved in phagocytosis, pathogen elimination and anti-inflammatory effects. $CD14^+CD16^+$ IMs play an antigen-presenting role that affects $CD4^+$ T cell proliferation and differentiation. $CD14^-CD16^+$ nCM are primarily involved in immune surveillance and tissue repair. The latter two are pro-inflammatory cells.[12] In the present study, the percentage of CM in the peripheral blood from patients with T2DM decreased, while the percentage of IM and nCM increased. However, we did not explore how high glucose affects the differentiation of monocytes. That will be the key points of our future researches.

Conclusion

In this study, we investigated the inflammatory alterations in the peripheral blood of patients with T2DM and found the variations of some peripheral inflammatory cytokines and immune cells, especially monocytes. Although the number of patients in this study may not be large enough and this study was just conducted in Suzhou area, these results will help to reveal the relationship between hyperglycemia and inflammation in T2DM.

Data Sharing Statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Ethics Statement

Informed consent was obtained from all individual participants included in the study. All of the experimental procedures were approved by the ethics committee of the Second Affiliated Hospital of Soochow University (JD-LK-2021-064-01).

Informed Consent

Written informed consent was obtained from all the patients or their guardians.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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