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The Association of Monocyte Subtypes Frequency and Serum TNF-α and TGF-β Levels with Diabetic Wound Grade

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ABSTRACT

The exact mechanisms underlying impaired wound healing in diabetes are not fully understood. In this study, we aimed to investigate the effect of classical and non-classical monocyte ratios along with TNF-α and TGF-β plasma levels on diabetic wound healing.

Twenty-four patients with confirmed type 2 diabetes and twenty healthy controls were enrolled in this study. The peripheral blood mononuclear cells (PBMC) isolation was performed by Ficoll-Paque density gradient centrifugation method. The frequency of different subsets of monocytes was characterized in diabetic patients and healthy controls using flow cytometry. TNF-α and TGF-β plasma levels were measured by the enzyme-linked immunosorbent assay (ELISA) method.

We found a significant difference in the frequency of classical and non-classical monocytes in healthy controls and diabetic patients. The plasma level of TNF-α was higher in diabetic patients than in healthy controls, and its level was associated with wound grade. Moreover, the plasma level of TGF-β was lower in diabetic patients rather than healthy controls. Also, our data showed a higher percentage of non-classical monocytes as wound grade increased.

In conclusion, the wound healing process is affected by diabetes via changes in non-classical and classical monocyte percentages, which may be the result of TNF-α increase and TGF-β levels decreasing in diabetic patients' plasma.

Keywords: Diabetes mellitus; Inflammation; Monocytes; Type 2; Wound healing

INTRODUCTION

Diabetes is known as the most common metabolic disease all over the world.[1] Diabetes mellitus (Type 2 diabetes) is one of the most challenging metabolic disorders in which an increase in blood glucose leads to disorders in carbohydrates, lipids, and protein metabolism with improper insulin production.[2] Systemic inflammation as an important complication in diabetic individuals was reported previously in white non-smokers.[3] There have been numerous studies on the relationship between type 2 diabetes and inflammation, and it has been observed that inflammatory biomarkers

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include C-reactive protein (CRP), interleukin-6, and TNF-α increase in diabetes.[4] Another important complication of type 2 diabetes is diabetes-related foot issues and delayed healing of diabetes-related foot ulcers (DRFUs) which contribute to significant disability in paitents with diabetes.[5] Around the world, more than one-third of diabetes patients will encounter diabetic foot ulcers (DFUs), which can be complicated and may require new strategies for high-quality treatment.[6] Various types of cells in the skin help to heal wounds. These include mast cells, neutrophils, lymphocytes, monocytes, macrophages, keratinocytes, fibroblasts, and endothelial cells.[7] Monocyte/Macrophage function is important in host defense, tissue debridement, and cell regulatory functions, and investigations have reported the role of these cells in normal wound healing, collagen deposition, angiogenesis, and wound closure.[8,9] It should be noted that the monocyte phenotype is important in the pathogenesis of infectious diseases and inflammatory conditions, and studies have shown that monocyte anti-inflammatory phenotype $CD16^+$ and $CD163^+$ expression is altered in diabetic retinopathy.[10] Human monocytes are classified into three main populations: classical $CD14^{++}CD16^-$ monocytes, intermediate $CD14^{++}CD16^+$ monocytes, and non-classical $CD14^+CD16^+$ monocytes.[11] Intermediate monocytes through the secretion of pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin (IL)-1β, have been confirmed to promote atherosclerosis. However, it has been reported that the non-classical monocytes are the most pro-inflammatory cells in response to Toll-like receptors (TLRs) stimulation in vitro.[12] Sepsis is a life-threatening syndrome mostly caused by bacterial infection, while Systemic Lupus Erythematous (SLE) is a chronic autoimmune disease of unclear etiology. In a study, a significant increase in both 'intermediate' and 'non-classical' subsets was detected in sepsis patients, whereas only 'non-classical' monocytes were increased in SLE patients compared with normal controls. They also reported that, following treatment of sepsis patients, 'non-classical' inflammatory monocytes returned to normal levels.[13,14] Classical monocytes can undergo apoptosis or differentiate into non-classical monocytes after a period of blood circulation. The polarization status of circulating monocytes in type 2 diabetes has not been fully determined and further investigations have been done on the expression of related genes.[15]

Another factor in wound healing in type 2 diabetes is growth factors, such as TGF-β, which are important for all phases of wound healing through the regulatory effects on cell proliferation, differentiation, extracellular matrix production, and modulation of the immune response.[16] Moreover, along with TGF-β, the role of TNF-α in insulin resistance and inflammatory reactions in type 2 diabetes has also been reported. [17] Impaired wound healing is a major complication of diabetes and is the most common cause of lower limb amputation. Wound healing requires the coordination of complex biological and molecular events and the two-way interaction between the nervous system and the immune system, which play an important role in wound healing through the secretion of neuropeptides and cytokines from various cells, including immune cells and keratinocytes[18,19]. In this study, we aimed to determine the effects of circulating non-classical and classical monocyte ratios along with TNF-α and TGF-β plasma levels on diabetic wound grade.

MATERIALS AND METHODS

Patient and Study Design

In this cross-sectional study, twenty-four patients with confirmed type 2 diabetes and suffering from foot ulcers who referred to Imam Khomeini Hospital, Ardabil, Iran, were enrolled from 2020 to 2021. The previous study results were used to determine the study's sample size. [20] The patients who met the following criteria were included in the study; subjects with diabetes and a foot ulcer diagnosed based on clinical guidelines, subjects with a diabetic foot infection (DFI), and individuals aged 18 years or older. Patients with other inflammatory diseases, acute illnesses, chronic inflammatory diseases, a history of cancer, hepatic, renal or autoimmune illnesses, type 1 diabetes and type 2 diabetes patients with morbid obesity were excluded (supplementary Figure 1). The inclusion criteria for the control group were based on fasting blood sugar (FBS) test results , with individuals having FBS level below 99 mg/dL, as defined by the World Health Organization criteria, were eligible for enrollment [21]. Individuals with type 1 diabetes and type 2 diabetes and other inflammatory disorders, who had a history of surgery or severe trauma and pregnant women were not included in the study as a control group.

Diabetic foot infection was evaluated in the following grades: Grade 0: No purulent discharge, erythema, induration, sensitivity to touch, or pain, and local warmth is the same compared to the lateral part

of the leg. Grade 1: Mild, non-purulent discharge (pink in color and with noticeable hardness), erythema, induration, tenderness, and mild pain, and the local warmth is slightly increased compared to the lateral part of the leg. Grade 2: Moderate non-purulent discharge (pale red color with distinct edges and with noticeable hardness), erythema, induration, tenderness, and moderate pain. Local warmth is moderately increased compared to the lateral part of the foot. Grade 3: Purulent discharge, severe erythema (red to dark red color), induration, throbbing, and severe pain. Local warmth is greatly increased compared to the lateral part of the leg.

Sample Collection

After 12-hrs of fasting, baseline venous blood samples were collected in heparinized (for PBMC and flow cytometry), ethylenediaminetetraacetic acid (EDTA)-treated (to measure glycated hemoglobin and the full blood count) and untreated tubes (to assess routine biochemical parameters, TNF-α and TGF-β) from both patients and the control group.

PBMC Isolation and Flow Cytometer

Flow cytometry immunophenotyping to classify and characterize monocyte subpopulations was accomplished less than 4 h after blood sampling. Fresh blood samples were collected from patients and healthy controls. PBMCs were isolated using the Ficoll-Paque (Histopaque Germany) density gradient centrifugation method. Following isolation, Trypan blue staining was used for viability calculation. Afterward, flow cytometry was used for phenotypes determination of isolated monocytes. For each experiment 1,000,000 cells were used that were first rinsed with phosphate-buffered saline (PBS), and then were stained with CD16-Fluorescein-5-isothiocyanate (FITC), CD14-Allophycocyanin (APC), HLA-DR- PE-Cyanine7 monoclonal antibodies (BioLegend, USA) for 30 min at 4°C and darkness. Also, an isotype control was used for each antibody to decrease nonspecific binding. When incubation time was completed, 1-2 ml FACS buffer was used for washing (5 minutes at 4°C at 300 g). Finally, the cells were assessed using the MACSQuant 10 Analyzer (Miltenyi Biotec, Germany), and the data were analyzed using FlowJo software 7.6.

Enzyme-Linked Immunosorbent Assay (ELISA)

Commercial TGF-β and TNF-α Enzyme Linked Immunosorbent Assay (ELISA) kits were used to measure TGF-β and TNF-α serum levels according to company protocol (Gmbh Ulm, Germany, Cat No). Briefly, in a 96-well ELISA plate, 50µl of standard and 50 µL of Streptavidin-Horse Radish peroxidase (HRP) were added as standard solution wells and 40µl sample plus 10 µL of anti- TGF-β and TNF-α Biotin-labeled antibody with 50 µL of Streptavidin-HRP were added as sample wells. The plate was gently shaken, washing solution was added and then incubated for 1 hour at 37°C. In the next step, 100 µL of chromogen solution was added and the plate incubated for 10 minutes at 37°C. Finally, 50µl of stop solution was added to stop the reaction and each well absorbance was measured with plate reader at 450 nm. The experiment was performed in duplicate.

Compliance with Ethical Standards

The protocol of the study was approved by the ethical committee of Ardabil University of Medical Sciences (IR.ARUMS.REC.1399.154). Informed consent was obtained from participants. The procedure of the study was explained to all enrolled cases, and if anyone had questions, they were kindly answered.

Statistical Analysis

SPSS 16.0 software was used for data analysis. The results were shown as the mean±standard deviation (SD). Parametric analysis of normally distributed variables was performed via the One-way ANOVA and Student's t-test. Moreover, the Mann–Whitney –test was used for not normally distributed data. The Kolmogorov-Smirnov test was used (along with a degree of freedom parameter) to test for normality The correlation between variables assessed using the Spearman correlation test. $p<0.05$ was statistically considered to be a significant value.

RESULTS

Demographic Data of Study Population

In our study, 41.7% and 45% of case and control subjects were female. The mean age was 63.79±2.1 years in the patient and 65.58±12.34 years in the control groups. Moreover, FBS level was 287.33±27.47 mg/dL in the patient group. The demographic characteristics of study population, including age, gender, FBS, family history, wound grade, wound location, underlying diseases,and HbA1C level are shown in more detail in Table 1.

Classical, Non-classical and Intermediate Monocytes Percentages in Diabetic Patients and Healthy Controls

Monocytes were gated based on HLA-DR expression and plotted in a CD14 versus CD16 graph to categorize monocyte subpopulations as follows: classical monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{++}CD16^{+}$), and non-classical monocytes ($CD14^{-}CD16^{++}$) (Supplementary Figure 2). As shown in Figure 1A, there is a significant difference in classical monocyte levels in healthy control (80.89%) and diabetic patients (58.73%). Control subjects showed a higher level of classical monocytes in comparison with diabetic patients ($p\leq0.001$). Also, diabetic patients showed higher levels of non-classical monocytes (26.6%) in comparison with control subjects (1.55%) ($p\leq0.001$). However, there was no significant difference between the two study groups (17.5% in healthy control vs. 15.23% in diabetic patients) in intermediate monocyte level ($p\geq0.05$).

TNFα and TGFβ Plasma Levels in Diabetic Patients and Healthy Controls

The plasma level of TNF-α was higher in diabetic patients (13.2±6.4pg/mL) in comparison with healthy control (6.98±2.2 pg/mL) which was statistically significant ($p\leq0.001$). Moreover, the plasma level of TGF-β was lower in diabetic patients (68.28±39.62 pg/mL) compared to healthy controls (132.6±103.8 pg/mL) which was statistically significant ($p\leq0.05$) (Figure 1B).

Statistical analysis showed a significant decrease in the percentage of classical monocytes in grade 3 and 4 wound groups compared to the control group ($p\leq0.01$). However, there was no significant difference between the wound grade groups ($p\geq0.05$). In the case of non-classical monocytes, a significant increase was observed in wound grade groups compared to the control group ($p\leq0.001$). Moreover, the difference between grade 2 and grades 3 and 4 was significant ($p\leq0.001$ and $p=0.028$, respectively). There was no significant difference between grades 3 and 4 ($p=0.71$) (Figure 2). Regarding the non-classical to classical monocyte ratio, the comparison between the control group with grade 2 ($p=0.004$) as well as grade 4 ($p\leq0.001$) shows a significant difference.

Table 1. Demographic, laboratory and clinical Characteristics of study individuals

	Category	Patients N (%) / Mean±SD	Healthy N (%) / Mean±SD
Gender	Female	10 (41.7%)	9 (45%)
	Male	14 (58.3%)	11 (55%)
Age		63.79±2.1	65.58±12.34
FBS		287.33±27.47	89.76±13.83
HbA1C	Rang:7-14	11.5±1.6	-
Family history	Yes	7 (29.2%)	8 (40%)
	No	17 (70.8%)	12 (60%)
Underlying disease	Yes	13 (54.2%)	-
	No	11 (45.8%)	-
Wound degree	1	0(0%)	-
	2	13 (54.2%)	-
	3	6 (25%)	-
	4	5 (20.8%)	-
Wound location	Left leg	9 (37.5%)	-
	Right leg	12(50%)	-
	Both leg	3(12.5%)	-

FBS: Fast blood sugar; HbA1C: hemoglobin A1c

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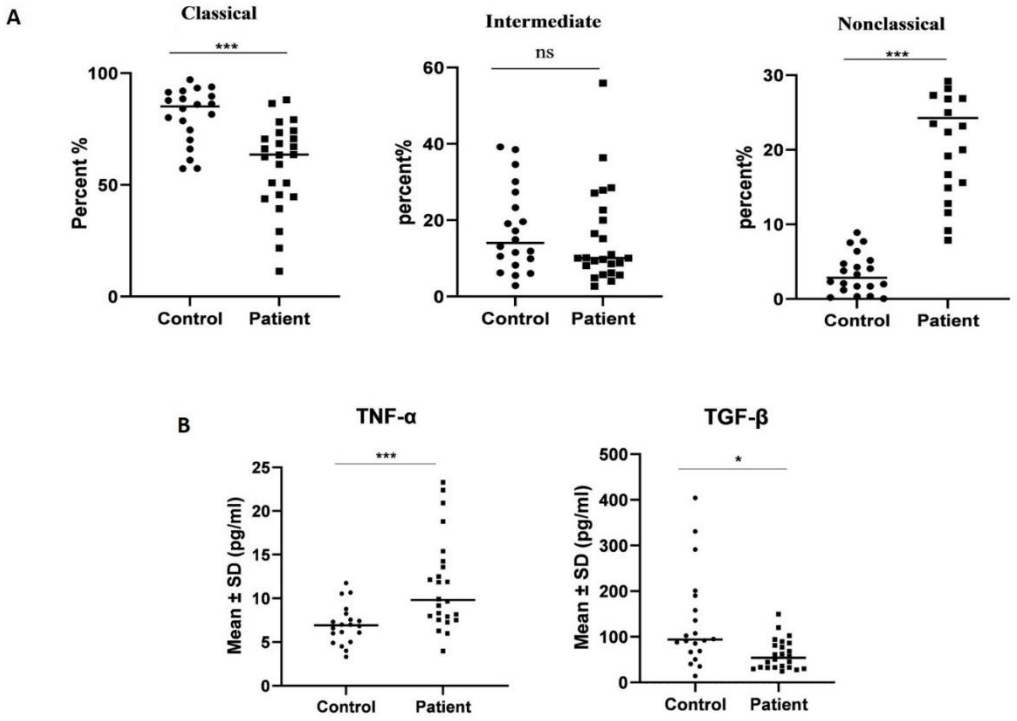

Figure 1. A: Statistical analysis for flow-cytometry assay of isolated cell subsets between groups. Percentage of classical, non-classical and intermediate monocytes in diabetic patients and healthy controls were provided. B: TNFα and TGFβ Plasma levels in study individuals. (*= $p \leq 0.05$,= $p \leq 0.01$, ***= $p \leq 0.001$).**

Association of Monocyte Subtypes, TNFα and TGFβ Plasma Levels with Diabetic Wound Grade

The percentage of classical, intermediate, non-classical monocytes, and TNF-α and TGF-β plasma levels, were analyzed according to diabetic wound grade and are shown in Figure 2. According to our results, the average percentage of classical monocyte subtype in the control group was 80.89±12.97%, and in wound grade 2 it was 65. 1±18.8%, wound grade 3 was 54.6±14.5% and wound grade 4 was 52.9±15.6%. The average of intermediate subtype in the control group was 17.50±11.23%, and in wound grade 2 it was 15.9±15.3%, in wound grade 3 was 11.5±8.5%, and in wound grade 4 was 17.6±8.5%. Moreover, the average of non-classical subtype in the control group was 1.55±1.37%, and in wound grade 2 it was 18.8±6.5%, wound grade 3 was 33.8±11.3%, and wound grade 4 was 29.4±13.1%.

The percentage of intermediate monocytes did not show a significant difference across any of the groups ($p \geq 0.05$). The comparison of TGF-β levels between the control group and grades 2 and 3 ($p=0.70$) showed a decrease, but it was not statistically significant, except for grade 4 ($p \leq 0.05$). Moreover, the difference between grade 2 and grades 3 and 4 was not significant ($p=0.99$ and $p=0.96$, respectively). Regarding the plasma level of TNF-α, it was significantly increased in patients compared to the control group. The comparison between the control group with grade 2 ($p=0.02$) and 4 ($p \leq 0.001$) showed a significant difference; however, grade 3 and the control group did not show a significant difference ($p=0.14$). Moreover, the difference between grade 2 and 3 was not significant ($p=0.99$); but, there was a significant difference between grade 2 and 4 as well as between grade 3 and 4 ($p \leq 0.001$). Additional details are shown in Figure 2.

The Correlation of TNFα and TGFβ Plasma Levels with Monocyte Subgroups

Correlation analysis showed that in the patient group, there was a significant negative correlation between the plasma level of TNF-α with the percentage of classical monocytes ($p=0.028$, r=-0.448). Additionally, there was a significant positive correlation with the percentage of non-classical monocytes ($p=0.013$, r=0.500) as well as the ratio of non-classical to classic monocytes ($p=0.005$,

r=0.558). Furthermore, there was a positive correlation between the plasma level of TGF-β and the percentage of classical monocytes (p=0.004, r=0.561), and a negative correlation with the percentage of intermediate monocytes (p=0.012, r=-0.503). Further details are provided in Table 2.

Table 2. Correlation of TNFα and TGFβ plasma levels with monocytes subtypes percent

		Classic		Non-classic		Intermediate		Non classic to Classic	
		Pearson Correlation	*p*	Pearson Correlation	*p*	Pearson Correlation	*p*	Pearson Correlation	*p*
TNF-α	Healthy	0.252	0.283	- 0.101	0.673	- 0.268	0.253	- 0.157	0.508
	Patient	- 0.448	**0.028**	0.500	**0.013**	0.178	0.406	0.558	**0.005**
TGF-β	Healthy	0.324	0.164	0.269	0.251	0.099	0.676	0.236	0.27
	Patient	0.561	**0.004**	- 0.313	0.136	- 0.503	**0.012**	- 0.198	0.199

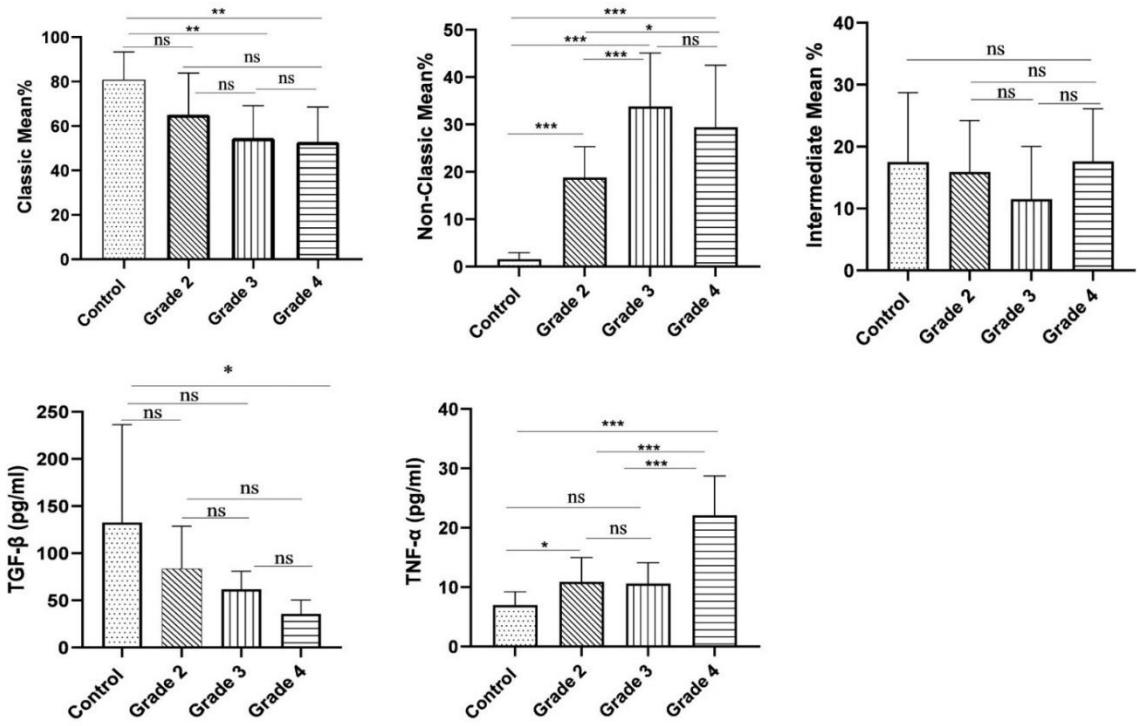

Figure 2. Statistical analysis of classical, intermediate, non-classical monocyte percentage and plasma level of TNFα and TGFβ in control groups and different wound grades (ns: non-significant, *= p≤0.05,= p≤0.01, ***= p≤0.001).**

DISCUSSION

Monocytes and lymphocytes, particularly monocytes, might play an important role in the impaired wound healing mechanisms observed in pathological conditions, including diabetes. In this regard, we investigated the percentage of classical, intermediate, and non-classical monocytes in diabetic patients and healthy controls. Moreover, we evaluated the monocyte percentages according to wound grade in diabetic patients. Our data revealed a significant difference between healthy controls and diabetic patients. Healthy

controls showed higher levels of classical monocytes than diabetic patients, while diabetic patients showed higher levels of non-classical monocytes compared to healthy controls. However, there was no significant difference between the two groups in intermediate monocyte levels. Moreover, the percentage of classical monocytes was inversely associated with wound grade. Also, our data showed an increase in the percentage of non-classical monocytes as wound grade increased. In the case of the non-classical to classical monocyte ratio, the comparison between the control group and grade 2 as well as grade 4 showed a significant difference. However, the difference between grade 2 and grades 3 and 4 was not significant. There was no significant difference between grades 3 and 4. Though, Wildgruber et al reported a reduction in the percentage of non-classical monocytes in advanced stages of peripheral artery occlusive disease patients. This suggests that the percentage of non-classical monocytes may vary depending on the disease, and different findings may be attributed to the specific disease type.[22]

There was a negative correlation between the plasma level of TNF-α and the percentage of classical monocytes, as well as a positive correlation with the percentage of non-classical monocytes and the ratio of non-classical to classical monocytes. Additionally, there was a positive correlation between the plasma level of TGF-β and the percentage of classical monocytes, and there was a negative correlation with the percentage of intermediate monocytes. These findings suggest that TNF-α and classical monocytes may act in opposing direction in the context of diabetic wound healing. The non-classical population has been characterized as being involved in complement and Fc gamma-mediated phagocytosis and adhesion, which may play an important role in vascular diseases. A higher percentage of the non-classical subset has also been reported previously in all sepsis patients with diabetes compared to non-diabetic patients, obese patients, and with age-related diseases, which aligns with our results.[23-25] It appears that patients with a higher grade of diabetic wounds have a lower proportion of classical monocytes and a higher proportion of non-classical monocytes. These findings suggest that diabetes may influence the selection of non-classical monocytes, indicating that under diabetic conditions, the monocyte differentiation trend shifts toward non-classical monocytes, potentially contributing to a more complicated wound healing process. However, the effect of diabetes on the phenotype of monocytes remains controversial. In this regard, Fadini et al reported no significant differences in classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical ($CD14^{+}CD16^{+}$)monocytes in type 2 diabetes patients with the control group.[10] Conversely, in the study by Alvarado et al the percentage of classical monocytes ($CD14^{++}CD16^{-}$) was reported to be lower in type 2 diabetes patients, which correlated with a poor prognosis.[26].

Other cells of the immune system have also been investigated in previous studies. It has been reported that the percentages of two macrophage subtypes, M1 and M2 have distinct effects on wound aggravation and the healing process.[27] Under normal conditions, macrophages convert from M1 to M2 type; however, in diabetic wounds, a deficiency in this transitionis observed, leading to a reduced collagen deposition, impaired angiogenesis, and, consequently, delayed wound healing.[28]

Cytokines and growth factors are critical mediators in the wound healing process, particularly during the inflammatory phase. However, the presence of diabetes can significantly disrupt this delicate balance, adversely affecting wound repair. In diabetic conditions, excessive production of TNF-α, a key pro-inflammatory cytokine, has been observed in non-healing ulcers. This overproduction has been linked to impaired wound healing, as demonstrated in type 2 diabetes mouse models. These findings highlight the detrimental impact of chronic inflammation and dysregulated cytokine activity on the wound healing process in individuals with diabetes, potentially contributing to the persistence of chronic wounds.[29,30] Our findings revealed a statistically significant increase in plasma TNF-α levels in diabetic patients compared to healthy controls. This aligns with evidence that elevated glucose levels in diabetes can stimulate the production of pro-inflammatory cytokines. These cytokines play a crucial role in promoting systemic inflammation, which is a hallmark of diabetes and contributes to its associated complications. The observed correlation between hyperglycemia and heightened cytokine activity underscores the complex interplay between metabolic dysregulation and immune system activation in diabetic patients.[31,32]

There is a significant relationship between TNF-α and diabetic wounds. The elevation in TNF-α plays a critical role in hindering cutaneous wound healing and reducing collagen production, which can further complicate wound recovery in diabetes.[33,34] Moreover,

increased levels of TNF-α led to impaired fibroblast proliferation and increased apoptosis of fibroblasts, keratinocytes, and endothelial cells in vitro, which led to a deficiency in diabetic wound healing.[35] Also, TNF-α is involved in other diabetic complications such as diabetic retinopathy and nephropathy.[36,37] In accordance with our results, Siqueira et al reported increased TNF-α in diabetic wounds in an animal model.[35] Conversely, decreased TGF-β1 production has been associated with impaired wound healing in diabetic rats and foot ulcers in diabetic patients. TGF-β is known to stimulate the expression of PDGF (platelet-derived growth factor), as demonstrated in previous studies.[37,38] This relationship suggests a potential association between the observed reduction in PDGF levels and diminished TGF-β activity in individuals with diabetes. Such a connection could have implications for understanding the underlying molecular mechanisms in diabetic conditions, particularly in the context of impaired growth factor signaling. Consistent with our results, previous studies have also demonstrated a reduction in TGF-β levels in diabetic patients and wound ulcers.

Considering the role of other immune cells, including neutrophils, in the production of inflammatory and anti-inflammatory cytokines, it is suggested that future studies evaluate not only monocytes but also variations in other inflammatory cells. Additionally, the association between these cells and related cytokines with the clinicopathological characteristics of diabetic patients should be investigated.

Type 2 diabetes has a profound impact on the human immune system, particularly in altering the subsets of monocytes, which are key players in immune response and tissue repair. Studies have shown that diabetes induces changes in the distribution of classical and non-classical monocytes, two major subsets with distinct roles in inflammation and wound healing. In diabetic individuals, the percentage of non-classical monocytes is often increased, while classical monocytes may be reduced. This shift in monocyte subsets could be linked to an imbalance in cytokine levels, specifically the elevation of TNF-α and the decrease in TGF-β levels in the plasma of diabetic patients. These cytokine changes can influence monocyte differentiation, activation, and migration, which in turn affects wound healing and the resolution of inflammation. Given the pivotal role of monocytes in tissue repair and immune regulation, it can be concluded that the heterogeneity of human monocytes is an important factor in the pathogenesis of complications associated with type 2 diabetes, including impaired wound healing, chronic inflammation, and increased susceptibility to infections.

STATEMENT OF ETHICS

This study was approved by the Ethics Committee of Ardabil University of Medical Sciences, Ardabil, Iran (IR.ARUMS.REC.1399.154).

FUNDING

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CONFLICT OF INTEREST

The authors declare they have no financial/non-financial competing interests or other interests that might be perceived to influence the interpretation of the article.

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DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

AI ASSISTANCE DISCLOSURE

Not applicable.

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