Diabetic retinopathy (DR) is an important microvascular complication of diabetes mellitus (DM) and a common vision-threatening disease in the working-age population [1]. DR is characterized by retinal microvascular damage leading to vascular leakage and ischemia-induced retinal neovascularization through four classic pathways: the polyol pathway, increased advanced glycation end-product (AGE), protein kinase C (PKC) activation, and the superoxide pathway [2]. Activation of these pathways leads to upregulation of proangiogenic, immunologic, and inflammatory factors such as vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF)-α, Flt-3 ligand (Flt-3L), interleukin (IL)-1β, IL-6, IL-8, sIL-2Rα, transforming growth factor (TGF)-α, tumor necrosis factor (TNF)-α, TNF-β, and VEGF were measured with Luminex multiplex bead immunoassay. The levels of these cytokines were investigated according to the DR stage.

**Results:** The plasma level of ten cytokines—MCP-1, IL-6, IL-7, IL-9, IL-13, IL-15, IL-17, sCD40L, sIL-2Rα, and TNF-β—increased significantly in the diabetic group compared to the controls. The Flt-3L, IL-1Ra, IL-3, IL-5, and IL-12 (p40) levels were lower in the diabetic group than in the control group. The TNF-α plasma level was significantly elevated in patients with proliferative diabetic retinopathy (PDR) compared with the levels in patients with non-proliferative diabetic retinopathy (NPDR) and patients with no apparent diabetic retinopathy (NDR).

**Conclusions:** TNF-α might be involved in the progression of DR, especially in the pathogenesis of PDR. TNF-α is a potential cytokine for the prognosis of DR and might act as a therapeutic target in DR.

Inflammation is the potential component of DR investigated in the present study. Current discussions regarding inflammation include molecular changes and mechanisms. Many inflammatory cytokines have been found to be upregulated in the serum, vitreous humor, aqueous humor, and tear samples of patients with DR [6-10]. Studies aimed at understanding the mechanisms of inflammation have done by investigating cell signaling, animal models, and gene knockout. Results of these studies support the hypothesis that DR is a low-grade subclinical inflammatory disease.

Many cytokines play roles in DR and have multiple interactions that impact the pathogenesis of the disease. Therefore, studying only one cytokine is not enough. Multiplex bead array assay (MBAA) is a new technology that effectively allows multiple cytokines to be assayed from a single sample. Measurements using MBAA show excellent correlation with enzyme-linked immunosorbent assay (ELISA) data and reduce time, cost, sample volume, and sampling error [11]. This development is important, since other studies that have measured cytokines in the vitreous humor, aqueous humor, tears, and especially plasma of patients with DR have shown...
conflicting results [12]. To assess the roles of plasma cytokines in DR, we used MBAA to investigate the plasma levels of 39 cytokines in diabetic patients with DR compared with normal controls.

METHODS

This study adhered to the ARVO statement on human subjects and was performed in compliance with the tenets of the Declaration of Helsinki and approved by the internal Ethics Committees of the First Affiliated Hospital of Nanjing Medical University. Written informed consent was obtained from every individual involved in this study.

Study population: This study recruited 59 patients with type 2 diabetes and 19 non-diabetic controls from the first affiliated hospital of Nanjing Medical University. The male to female ratio and mean±SD age in diabetic subjects was 23/36 and 61.8±5.3 years, and in the control group was 12/17 and 62.9±5.0 years. Dilated ophthalmic eye examinations, color fundus photography, and fluorescein fundus angiography were conducted on all subjects. According to the Diabetic Retinopathy Disease Severity Scale [13], the 59 diabetic patients were classified into three groups: 19 with no apparent retinopathy (NDR), 19 with non-proliferative diabetic retinopathy (NPDR), and 21 with proliferative diabetic retinopathy (PDR). The male to female ratio and mean±SD age was 9/10 and 64.1±4.0 in NDR, 6/13 and 60.6±5.7 in NPDR, and 8/13 and 60.8±5.5 in PDR.

At the time of recruitment, all the diabetic and control subjects were in well monitor and control of their state of health. Detailed demographic information was collected from every subject, such as family history, duration, current medication, height, weight, body mass index (BMI = weight/height²), blood pressure, and blood and urine samples. Fasting serum glucose, glycosylated hemoglobin (HbA₁c), serum lipid, and serum creatinine levels were also recorded. Subjects with other systemic diseases apart from diabetes or with other ocular conditions, such as glaucoma, infection, uveitis, and previous ocular surgery history, were excluded.

Plasma collection: Blood samples (5 ml) were obtained from each subject by cubital venipuncture using anticoagulant tubes. After centrifugation at 1500 ×g for 15 min, the plasma samples were aliquoted and stored at −80 °C until further analysis.

Cytokine analysis: The plasma samples were analyzed using the MILLIPLEX Human Cytokine/Chemokine panel (catalog number MPXHCYTO60KPMX39 (premixed); Millipore Corporation, Billerica, MA). The kit can detect the following 39 cytokines simultaneously in a single sample: EGF, eotaxin, FGF-2, Flt-3L, fractalkine, G-CSF, GM-CSF, GRO, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1Ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-1β, sCD40L, sIL-2Ra, TGF-α, TNF-α, TNF-β, and VEGF.

The samples were brought to room temperature and added in duplicate to 96-well filter-bottom plates. Antibody-coated capture beads were added to the wells and incubated on a plate shaker at 4 °C overnight. After a washing step, the beads were further incubated with biotin-labeled secondary antibody for 1 h and then incubated with streptavidin–phycocyanin for 30 min. Then 100 μl of sheath fluid were added to each well, and the samples were analyzed using Luminex laser-based fluorescent analytical test instrumentation (Luminex 200, Austin, TX). Cytokine concentrations were determined from standard curves prepared on each plate and expressed as picogram per milliliter (pg/ml).

Statistical analysis: The data were analyzed using the Statistical Package for Social Sciences (IBM SPSS Statistics 19.0, New York, NY). Differences between samples from two groups, such as diabetics and controls, were analyzed using the two-tailed Student t test or the two-tailed Mann–Whitney test, depending on normality assumptions and the homogeneity of variances. One-way ANOVA and the Kruskal–Wallis H test were used to evaluate differences among multiple groups, such as different degrees of DR severity. Spearman’s rank order correlation was used to determine correlations between and to assess the association of clinical characteristics with cytokine levels. Univariate and multivariate logistic regression analysis was used to assess the risk factors for DR and PDR, which was estimated with odds ratios (ORs) with 95% confidence intervals (CIs). For all tests, p values less than 0.05 were considered significantly different.

RESULTS

Patients’ clinical characteristics: A total of 78 subjects with diabetes (cases, n = 59) and without diabetes (controls, n = 19) were included in the analysis. Clinical characteristics of the enrolled patients are shown in Table 1. The mean ± standard deviation (SD) age was 62.9±5.0 years in the controls, 64.1±4.0 years in the NDR group, 60.6±5.7 years in the NPDR group, and 60.8±5.5 years in the PDR group, with no significant difference among the groups (p = 0.093). The male to female ratio was 12:7 in the controls, 9:10 in the NDR group, 6:13 in the NPDR group, and 8:13 in the PDR group, with no significant difference (p = 0.232). The BMI was 21.9±1.6 in the controls, 23.0±2.5 in the NDR group, 23.2±2.8 in the NPDR group, and 24.0±2.4 in the PDR group, with no significant difference (p = 0.058). In the diabetic
group, the mean±SD duration was 9.0±6.5 years in the NDR group, 11.3±6.5 years in the NPDR group, and 12.3±6.5 years in the PDR group (p = 0.266). The mean±SD HbA1c level was 7.0±1.6% in the NDR group, 7.5±1.6% in the NPDR group, and 7.8±2.0% in the PDR group (p = 0.434; Table 1).

**Expression of cytokines in plasma:** The plasma levels of ten cytokines—MCP-1 (p = 0.020), IL-6 (p = 0.000), IL-7 (p = 0.000), IL-9 (p = 0.002), IL-13 (p = 0.000), IL-15 (p = 0.000), IL-17 (p = 0.004), sCD40L (p = 0.007), sIL-2Rα (p = 0.049), and TNF-β (p = 0.000)—were significantly higher in the diabetic group compared to the control group. Five factors—Flt-3L (p = 0.001), IL-1Ra (p = 0.000), IL-3 (p = 0.000), IL-5 (p = 0.000), and IL-12 (p40); (p = 0.000)—had lower levels in the diabetic group compared to the controls. When we subdivided the diabetic group into NDR, NPDR, and PDR groups, 13 of the 15 cytokines—all except MCP-1 (p = 0.061) and sIL-2Rα (p = 0.123)—were significantly higher in the diabetic group compared to the control group. Five factors—Flt-3L (p = 0.001), IL-1Ra (p = 0.000), IL-3 (p = 0.000), IL-5 (p = 0.000), and IL-12 (p40); (p = 0.000)—had lower levels in the diabetic group compared to the controls.

When we subdivided the diabetic group into NDR, NPDR, and PDR groups, 13 of the 15 cytokines—all except MCP-1 (p = 0.061) and sIL-2Rα (p = 0.123)—differed significantly among the control, NDR, NPDR, and PDR groups (p<0.05). TNF-α was also significantly different among the four groups (p = 0.005; Appendix 1). When multiple comparisons were examined, the TNF-α level was significantly higher in the diabetic group compared to the NDR group (p = 0.009 and 0.001, respectively). There were no significant differences between any other two groups (PDR and control, p = 0.192; NDR and control, p = 0.055; NPDR and control, p = 0.245; NPDR and NDR, p = 0.401). The mean±SD TNF-α levels were 9.5±2.0 pg/ml in the control group, 7.1±1.8 pg/ml in the NDR group, 8.1±4.2 pg/ml in the NPDR group, and 11.1±4.5 pg/ml in the PDR group (Figure 1). There were no significant differences in the other 13 cytokines among the diabetic groups (NDR, NPDR, and PDR).

**Logistic regression analysis:** We subdivided the diabetic group into two groups: NDR (n = 19) and DR (NPDR and PDR groups, n = 40). A logistic regression analysis was used to assess the risk factors of DR, which included age, duration, HbA1c, and all the cytokines examined. A univariate analysis was used to screen out age, IL-2, IL-3, TNF-α, and TNF-β as related factors to DR; the ORs were 0.865 (p = 0.023), 0.451 (p = 0.038), 3.250 (p = 0.041), 1.301 (p = 0.039), and 0.078 (p = 0.016), respectively. A multivariate logistic forward regression analysis then excluded other factors except age (OR = 0.800, p = 0.023; Table 2). To evaluate the risk factors of PDR, we subdivided the diabetic group into two groups: no PDR (NDR and NPDR, n = 38) and PDR (n = 21). A univariate analysis was used to screen TNF-α, and IL-17 as related factors to PDR; the ORs were 1.31 (p = 0.013) and 0.45 (p = 0.038), respectively. A multivariate logistic forward regression analysis then excluded other factors except TNF-α (OR = 1.28, p = 0.025; Table 3).

**Correlation analysis:** The correlation between TNF-α and other cytokines was analyzed in the diabetic group and in the PDR group. TNF-α levels of the diabetic group correlated significantly with three cytokines: GRO (r = 0.419, p = 0.001), MIP-1β (r = 0.383, p = 0.004), and IL-8 (r = 0.377, p = 0.005; Figure 2). Furthermore in the PDR group, TNF-α levels correlated significantly only with GRO (r = 0.572, p = 0.008; Figure 3).

**DISCUSSION**

In our study, the plasma levels of ten cytokines—MCP-1, IL-6, IL-7, IL-9, IL-13, IL-15, IL-17, sCD40L, sIL-2Rα, and TNF-β—were higher in the diabetic group than in the control group (n = 59) than in the control group (n = 19), while five cytokines—Flt-3L, IL-1Ra, IL-3, IL-5, and IL-12 (p40)—had lower levels in the diabetic group compared to the controls. Although 13 of the 15 cytokines were significantly different among the three groups, there was no significant difference in these cytokines between any two diabetic groups (NDR, NPDR, and PDR) according to the multiple comparisons performed. When the diabetic groups 

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**Table 1. Clinical characteristic of the enrolled patients, shown in different groups.**

| Characteristics       | Control | NDR    | NPDR   | PDR    | P value |
|-----------------------|---------|--------|--------|--------|---------|
| Gender (male/female)  | 12/7    | 9/10   | 6/13   | 8/13   | 0.232   |
| Age (years)           | 62.4±5.0| 64.1±4.0| 60.6±5.7| 60.8±5.5| 0.093   |
| BMI (kg/m²)           | 21.9±1.6| 23.0±2.5| 23.2±2.8| 24.0±2.4| 0.058   |
| Duration (years)      | N.A.    | 9.0±6.5| 11.3±6.5| 12.3±6.5| 0.266   |
| HbA1c (%)             | N.A.    | 7.0±1.6| 7.5±1.6| 7.8±2.0| 0.434   |

N.A.=Not available. The mean ± standard deviation (SD), age, male to female ratio, and BMI in the control, NDR, NPDR, and PDR groups were not significantly different among the groups (p=0.093, 0.232, and 0.058, respectively). In the diabetic group, the mean±SD duration and HbA1c level were also not significantly different among the NDR, NPDR, and PDR groups (p=0.266 and 0.434, respectively).
Figure 1. Bar graph of tumor necrosis factor-α plasma levels in the control, no apparent diabetic retinopathy, non-proliferative diabetic retinopathy, and proliferative diabetic retinopathy groups. The tumor necrosis factor (TNF)-α level was significantly higher in the proliferative diabetic retinopathy (PDR) group compared with the non-proliferative diabetic retinopathy (NPDR; p=0.009,*) and no apparent diabetic retinopathy (NDR; p=0.001,**) groups.

| Variate | Univariate logistic regression analysis | Multivariate logistic forward regression analysis |
|---------|---------------------------------------|-----------------------------------------------|
|         | OR  | 95%CI  | P          | Variate in the equation | OR  | 95%CI  | P          |
| Age     | 0.87 | 0.76–0.98 | 0.023* | Age          | 0.80 | 0.66–0.97 | 0.023* |
| IL-2    | 0.45 | 0.21–0.96 | 0.038* | IL-2         | 0.59 | 0.22–1.54 | 0.281  |
| IL-3    | 3.25 | 1.05–10.06 | 0.041* | IL-3         | 2.01 | 0.39–10.50 | 0.406  |
| TNF-α   | 1.30 | 1.01–1.67 | 0.039* | TNF-α        | 1.40 | 0.93–2.10 | 0.106  |
| TNF-β   | 0.08 | 0.01–0.62 | 0.016* | TNF-β        | 0.09 | 0.01–1.30 | 0.077  |

OR=odds ratios, CI=confidence intervals. *P value was significant (p<0.05) We subdivided the diabetic group into two groups: NDR (n=19) and DR (n=40). Univariate analysis screened age, IL-2, IL-3, TNF-α, and TNF-β as related factors to DR; OR values were 0.87 (p=0.023), 0.45 (p=0.038), 3.25 (p=0.041), 1.30 (p=0.039), and 0.08 (p=0.016), respectively. After that, multivariate logistic forward regression analysis excluded other factors except age (OR=0.80, p=0.023).

| Variate | Univariate logistic regression analysis | Multivariate logistic forward regression analysis |
|---------|---------------------------------------|-----------------------------------------------|
|         | OR  | 95%CI  | P          | Variate in the equation | OR  | 95%CI  | P          |
| TNF-α   | 1.31 | 1.06–1.61 | 0.013* | TNF-α        | 1.28 | 1.03–1.58 | 0.025* |
| IL-17   | 0.45 | 0.21–0.96 | 0.038* | IL-17        | 0.55 | 0.25–1.25 | 0.256  |

OR=odds ratios, CI=confidence intervals. *P value was significant (p<0.05) We subdivided the diabetic group into two groups: no PDR (n=38, NDR and NPDR) and PDR (n=20). Univariate analysis screened TNF-α and IL-17 as related factors to PDR; OR values were 1.31 (p=0.013) and 0.45 (p=0.038), respectively. After that, multivariate logistic forward regression analysis excluded other factors except TNF-α (OR=1.28, p=0.025).
Figure 2. Scatterplot of concentration of tumor necrosis factor-α and the three related cytokines (interleukin-8, macrophage inflammatory protein-1β, and growth-related oncogene) in the diabetic group. The vertical axis is on a logarithmic scale. The best fit line is created from logarithmic regression.

Figure 3. Scatterplot of tumor necrosis factor-α and growth-related oncogene levels in the proliferative diabetic retinopathy group. The best fit line represents linear regression.
were subdivided into NDR (n = 19) and DR (n = 40), there were no significant differences in any of the 15 cytokines between the groups. Thus, although these 15 cytokines play roles in DM, they do not contribute to DR.

TNF-α is the only cytokine assessed in this study that showed a relationship with DR. TNF-α was significantly higher in the PDR group compared with the NPDR and NDR groups. In addition, this was the first time the difference in TNF-α levels in PDR plasma was significant using the MBAA method. Lange [14] detected 42 cytokines (MBAA) in the plasma of PDR and control groups; the concentration of TNF-α was 4.94 (1.6–9.14) pg/ml in the PDR group and 6.45 (1.6–11.56) pg/ml in the controls, with no significant difference. Chen [15] (MBAA) also reported that TNF-α plasma levels were not significantly different between patients with DM and controls or within the different DR stages. However, studies support the results of the current study. Koleva-Georgieva [16] (using ELISA) reported that patients with PDR had significantly higher plasma TNF-α values compared with patients with NDR. Evidence from other studies also supports our results. Kuo [17] reported that the sTNF-R1 and sTNF-R2 levels were highly correlated with the DR severity in patients with DM. Ben-Mahmud et al. [18] found that plasma or serum from diabetic patients with retinopathy increases the TNF-α-associated activity of cultured human myelocytic cells. In Paine et al.’s gene polymorphism analyses [19], the genetic variant TNF-α-238A was a potent risk factor for pathogenesis of PDR. Huang et al. [20], using the TNF-α knockout strain of mice, demonstrated that TNF-α has no effect on early breakdown of the blood–retinal barrier (BRB) in DR, but that it is essential for progressive BRB breakdown. All of these studies support the conclusion that TNF-α influences DR, especially in the progressive stages (PDR).

In this study, the average concentration of TNF-α in the control group was also at a relatively high level, higher than that in the NDR and NPDR groups, although without significant differences. We think that one reason for the variability of TNF-α expression in the control group is the sampling error, and another might be the effect of other undetected complications in the TNF-α level. The 19 non-diabetic controls were carefully screened to exclude any other systemic or eye disease; however, the screening did not completely eliminate the effect of some unknown status on the plasma level of TNF-α, since features of inflammation are displayed years before the onset of clinical manifestation. Enlarged sample size may reduce the impact of these potential elements and help us to get more credible results.

Many studies have investigated the mechanisms of action of TNF-α. TNF-α is the prototypical member of a family of cytokines that also include Fas ligand (FasL), CD40 ligand (CD40L), and TNF-related apoptosis-inducing ligand (TRAIL). It can induce apoptosis, differentiation, cell activation, and inflammation [21]. Aveleira [22] demonstrated that TNF-α increased the permeability of bovine retinal endothelial cells, decreased the protein and mRNA content of the tight junction proteins ZO-1 and claudin-5, and altered the cellular localization of these tight junction proteins. Preventing nuclear factor-kappa B (NF-κB) activation reduced TNF-α-stimulated permeability. Finally, inhibiting PKC reduced NF-κB activation and completely prevented alterations in the tight junction complex and cell permeability induced by TNF-α in cell culture and rat retinas. Joussen [21] found that the TNF-α inhibitor etanercept suppressed caspase activation, retinal cell injury, and apoptosis in short-term diabetic rats. Pericyte and endothelial cell loss was also reduced in long-term hypergalactosemic mice.

Univariate logistic regression analysis screened out age, TNF-β, and IL-2 as protective factors, while IL-3 and TNF-α were risk factors for DR. However, multivariate logistic forward regression analysis excluded the other factors, with the exception of age (OR = 0.800, p = 0.023), which is a protective factor in DR. The younger the patients with DM are, the more easily they may suffer DR. A clinic-based cross-sectional study in Singapore [23] also reported that younger age was a risk factor for vision-threatening retinopathy (multivariate OR = 0.97, p = 0.000). When considering the risk factors of PDR, a multivariate logistic forward regression analysis showed TNF-α (OR = 1.28, p = 0.025) as a risk factor in PDR. This result also supports our conclusion that TNF-α has a role in PDR. Because the pathogenesis of DR is complicated, a vast network of multiple factors affects DR [24]. Larger sample sizes would help better assess whether the cytokines studied are credible risk factors.

We are not aware of a previously reported association of TNF-α with GRO in the plasma of diabetes. GRO is a chemokine belonging to the CXC family, which recruits neutrophils and basophils and involves in the action of inflammation and angiogenesis [25]. Sajadi [26] found an elevated GRO level in the plasma of diabetic patients compared to the controls, and Lange [14] reported an increased expression of GRO in the vitreous of patients with PDR. From these reports and the result of our study, we assume that GRO may also be an influence factor in DR, especially in PDR, because it correlated with TNF-α, which was found to have a role in PDR, although the GRO itself in our study was not with significant difference among groups. There are evidence that
the PKC and NF-κB pathways may be the common method for mediating the secretion or the mRNA expression of GRO and TNF-α [22,27]. A study in human vascular endothelial cells (ECs) revealed how TNF-α and GRO correlated; the study found that TNF-α stimulates GRO release from human ECs through c-Jun NH2-terminal kinase (JNK)-mediated GRO mRNA expression and p38 mitogen-activated protein kinase (MAPK)- and phosphatidylinositol 3-kinase (PI3K)-mediated GRO secretory processes [28]. Further investigation is needed to determine their relationship and to seek out the mechanisms in DR models.

VEGF has been extensively studied in the pathogenesis of DR. The concentration of VEGF in aqueous and vitreous humor in DR has been reported to be elevated in most studies, while there has been no consensus on the plasma level of VEGF [12]. Our results showed no significant differences among groups. In recent years, intravitreal injections of different types of anti-VEGF have been used in PDR and diabetic macular edema (DME). However, the effect of this treatment is not perfect, and response to the anti-VEGF treatments has been variable and transient. The regression rate of new vessels following anti-VEGF treatment has been shown to range from 62% to 87.5%; the average time before retinal neovascularization recurs has ranged from 2 weeks to 3 months [29,30]. This clinical observation indicates that many other factors in addition to VEGF might have roles in this process. Sfikakis et al. [31] conducted a double-blind, placebo-controlled, crossover, 32-week study in which patients with DME received the TNF-α inhibitor infliximab intravenously. The excess visual acuity in the infliximab-treated eyes was greater than that in the placebo-treated eyes, and the infliximab treatment was well tolerated. However, Pulido [32] and Wu [33] treated DME intravitreally with two TNF-α inhibitors, adalimumab and infliximab, and neither treatment appeared to benefit the affected eyes. TNF-α inhibitors might be useful therapeutic agents in treating DME in the future, either alone or in combination with the currently used anti-VEGF drugs. More well-designed trials are needed.

In conclusion, the increased plasma level of TNF-α in PDR indicates that TNF-α is involved in the pathogenesis of PDR. It is a potential cytokine as a therapeutic target of DR. Larger samples are needed to determine more related factors and their correlations with each other.

**APPENDIX 1. PLASMA CONCENTRATION OF 39 CYTOKINES OF EACH CLASSIFICATION.**

To access the data, click or select the words “Appendix 1.” *p value was significant(p<0.05). aThe data of the concentrations of the 20 cytokines was catagorical variable, the mean rank was used to describe the data. P1. P values between control and DM. P2. P values among control, NDR, NPDR and PDR. †Significantly increased in DM group compared to the control. ‡Significantly decreased in DM group compared to the control.

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