Programmed death 1 (PD-1) is involved in the development of proliferative diabetic retinopathy by mediating activation-induced apoptosis

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Purpose: Recent studies revealed that immunological mechanisms play a prominent role in the pathogenesis of proliferative diabetic retinopathy (PDR). Given the importance of the immune response in PDR and the significance of the programmed death 1 (PD-1) pathway as an immune regulatory pathway, the aim of this study is to determine the expression and functional characteristics of the PD-1 pathway in peripheral blood lymphocytes from patients with PDR.
Methods: Peripheral blood lymphocytes were obtained from patients with PDR, age-matched patients with diabetes mellitus and no diabetic retinopathy (DM-NDR), and controls. The mRNA expression of PD-1 and its ligands were determined using real-time PCR. The frequencies of PD-1 and its ligands, activation-induced apoptosis, IFN-γ, and IL-4 were determined by flow cytometry.
Results: The PD-1 mRNA expression markedly decreased, while the frequency of PD-1$^+$ cells increased in the PDR group compared with the DM-NDR and control groups. The expression of PD-ligand 1 (PD-L1) mRNA and PD-L1$^+$ cells in the PDR group was lower than that in the other two groups. In the PDR group, the frequency of Annexin V$^+$PI$^-$ and Annexin V$^+$PI$^-$PD-1$^+$ cells increased, while the frequency of Annexin V$^+$PI$^-$PD-L1$^+$ cells decreased. Although their expression was upregulated, the ratio of PD-1$^+$ IFN-γ$^+$ to PD-1$^+$IL-4$^+$ cells in the PDR group was not significantly different to that in the DM-NDR and control groups.
Conclusions: These results suggest that PD-1 is involved in the development of PDR by mediating activation-induced apoptosis.

Diabetic retinopathy (DR) is a major cause of blindness worldwide. It has been reported that the occurrence and progression of DR may be related to hyperglycemia, oxidative stress, the accumulation of polyols and advanced glycation end products, apoptosis, and cytokines [1]. Recently, there is an accumulating body of evidence to suggest that immunological mechanisms play a role in the pathogenesis of DR, particularly in proliferative diabetic retinopathy (PDR). The involvement of the immune response in DR is supported by the presence of antipericyte and antiendothelial cell autoantibodies in the circulation [2] and the abnormal expression of T cells in the vitreous and epiretinal membranes of patients with PDR [3,4]. An imbalance of activated immune cells in

patients with DR is implicated to impair the immune privilege of the retina [3,5,6]. The upregulation of inflammatory cells and proinflammatory factors, leading to persistent low-grade inflammation and an influx of leukocytes, contributes to DR-associated damage [7]. The clinical application of nonsteroidal anti-inflammatory drugs and corticosteroids in the treatment of DR also indicates that an immune inflammatory response exists in DR [8-10].

As a member of the CD28/B7 family and co-inhibitory molecules, programmed death 1 (PD-1) and its ligands (PD-Ls), PD-L1 and PD-L2, have been demonstrated to play an important role in autoimmune disease, organ transplant rejection, microorganism infection, and tumor immune escape [11,12]. PD-1 is not only a major regulator of apoptosis, which can impact antiviral T cells in chronic infections [13], but it also has significant effects on cytokine production, such as IFN-γ, TNF-α, and IL-2 [14]. Our previous study demonstrated that CD4$^+$PD-1$^+$ T cells acted as regulatory

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cells during the induction of an anterior chamber-associated immune deviation [15]. PD-1/PD-L interactions exert a vital and diverse range of immunoregulatory roles in T cell activation, cytokine production, apoptosis induction, and immune-mediated tissue damage [11,16,17]. PD-1/PD-L1 interactions play a substantial role in regulating autoreactive T cells to maintain immune privilege in the eye and to protect the eye from activated T cells [15,18,19]. However, PD-1/PD-L2 is critical in regulating immune responses to environmental antigens [20].

Given the importance of the immune response in PDR and the significance of PD-1/PD-Ls as an immune regulatory pathway, we hypothesized that the PD-1 pathway may be involved in the development of PDR. The purpose of this study was to investigate the expression and functional characteristics of the PD-1 pathway in peripheral blood lymphocytes obtained from patients with PDR.

METHODS

This study was approved by the Research Ethics Committee of Guangdong General Hospital and conducted in accordance with the Declaration of Helsinki. Written informed consent was received from all subjects.

Subjects: Two hundred and thirty-two subjects were recruited from Guangdong General Hospital (Guangzhou, China), Henan Eye Hospital (Zhengzhou, China), and the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China). The subjects were divided into three groups: PDR group (79 patients), diabetes mellitus and no diabetic retinopathy (DM-NDR) group (60 patients), and control group (93 cases). Type 2 diabetes patients with DR absence were enrolled in the DM-NDR group. Patients in the PDR group included those with type 2 diabetes and high-risk or advanced PDR, as follows: 1) the presence of neovascularization at the disc (NVD) at more than a quarter of the disc area, 2) the presence of NVD associated with vitreous or preretinal hemorrhage, 3) neovascularization elsewhere (NVE) with more than a half-disk area associated with vitreous or preretinal hemorrhage, or 4) PDR associated with severe vitreous hemorrhage or tractional retinal detachment. Controls consisted of age-related healthy volunteers. The diagnosis of type 2 diabetes was performed according to the criteria recommended by American Diabetes Association criteria (2010) [21]. DR was diagnosed according to the International Clinical Diabetic Retinopathy Severity Scale [22]. Exclusion criteria included a history of infection or chronic disease or a family history of immune-mediated disease, which could possibly influence the immune response (e.g., uveitis, chronic hepatitis, HIV infection, coronary heart disease, tumors).

Routine examinations consisted of a general medical history, laboratory examinations (including urinalysis, routine blood analysis, and biochemical examination), and an ophthalmic examination (including best-corrected visual acuity, intraocular pressure measured by a non-contact tonometer, anterior and posterior segment examinations at the slit-lamp using a 90-Diopter lens). Fundus color photography, fundus fluorescein angiography, and B-scan ultrasonography were performed when necessary.

Analysis of PD-1, PD-L1, and PD-L2 mRNA by fluorescent quantitative real-time PCR: In total, 2 ml of fresh peripheral whole blood was placed into tubes containing EDTA, and lymphocytes were collected using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Total RNA was extracted from the lymphocytes using TRIzol (Molecular Research Center, Cincinnati, OH) and was subsequently quantified by absorbance at 260 nm. Then, cDNA was synthesized with the PrimeScript RT regent Kit (TaKaRa, Dalian, China). Fluorescent quantitative real-time PCR was performed using SYBR Premix Ex Taq (TaKaRa) on the MJ Research Opticon 2 real-time PCR unit (MJ Research, Quebec, Canada) following the manufacturer's instructions. The following primer pairs were used (Invitrogen, Shanghai, China): Human-rat-β-actin: 5′-GCC AAC ACA GTG CTG TCT G-3′, 5′-TAC TCC TGC TTG CTG ATC CA-3′; Human-PD-1: 5′-CCC TGG TGG TTG GTG TCG T-3′, 5′-GCC TGG CTC CTA TTG TCC CTC-3; Human-PD-L1: 5′-TGG TGT AGC ACT GAC ATT CA-3, 5′-TCC AAT GCT GGA TTA CGT CT-3′; and Human-PD-L2: 5′-TTC ATA GCC ACA GTG ATA GC-3, 5′-GGT TCA GAT AGC ACT GTT CA-3′.

The protocol included an initial denaturation step at 95 °C for 120 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. Data analysis was based on the $2^{-\Box Ct}$ method with normalization of the raw data to β-actin housekeeping genes. All PCR assays were performed in duplicate.

Analysis of PD-1-, PD-L1-, and PD-L2-positive cells and activation-induced apoptosis by flow cytometry: Lymphocytes were collected from 4 ml of peripheral whole blood containing heparin by Ficoll-Paque PLUS and resuspended in RPMI 1640 plus 10% fetal calf serum (Gibco, Carlsbad, CA). The cells were then stimulated with phytohemagglutinin (PHA, Sigma, St. Louis, MO), a mitogenic stimulant of lymphocytes and potent apoptogen [23], at the concentration of 40 mg/l for 48 h at 37 °C in an atmosphere containing 5% CO_2. After washing in PBS, 100 μl cells (1×10^6 cells/ml) were incubated with 10 μl mouse anti-human PE-conjugated anti-PD-1 monoclonal antibody (mAb) or anti-PD-L1 mAb or anti-PD-L2 mAb (eBioscience, San Diego, CA) for 15 min

in the dark at room temperature, respectively. PE-conjugated mouse lgG1k (eBioscience) served as an isotype control.

Apoptosis was determined using the Annexin V-FITC Apoptosis Detection Kit (Bender Medsystems, San Diego, CA) according to the manufacturer's instructions. Cells in 150 µl were incubated with 5 µl Annexin V-FITC for 10 min at room temperature and then resuspended in 150 µl binding buffer (1×). The cells were added to 5 µl propidium iodide (PI) and analyzed by direct triple immunofluorescence with a FACS Calibur flow cytometer (BD Bioscience, San Jose, CA). Data management was performed with CELL Quest™ software (BD Bioscience).

Intracellular cytokine analysis in lymphocytes by flow cytometry: Lymphocytes were collected from 4 ml peripheral whole blood using Ficoll-Paque PLUS. As phorbol-12-my-ristate-13-acetate (PMA)-Ionomycin is a potent stimulator of IFN-γ and IL-4 in lymphocytes and monensin as a secretion inhibitor can retain cytokines intracellularly after fixation and permeabilization [24], cells were stimulated with 50 µg/l PMA (ENZO, Farmingdale, NY), l mg/l Ionomycin (ENZO), and 1.7 mg/l monensin (ENZO) for 4 h at 37 °C in a 5% CO_2 humidified atmosphere. Cells cultured with 1.7 mg/l monensin alone were used as an unstimulated control group. After washing twice with PBS, 100 µl cells (1×10^6 cells/ml) were stained with 10 µl mouse anti-human PE-conjugated anti-PD-1 mAb (BD Bioscience) for 15 min in the dark at room temperature. Subsequently, the cells were treated with the Fix & Perm Reagent (eBioscience) and then incubated with mouse anti-human FITC-conjugated anti-IFN-γ mAb or anti-IL-4 mAb (eBioscience) for 30 min in the dark at room temperature, respectively. PE-conjugated mouse IgG1k (eBio-science) and FITC-conjugated mouse IgG1k (eBioscience) served as isotype controls. Cells were washed twice with PBS and flow cytometric measurement was performed within 4 h.

Statistical analysis: All values are present as mean ± standard deviation (SD) or median and interquartile range (IQR). A Pearson chi-square test was used to compare the proportion of males and females. The Kruskal–Wallis test was used to compare mRNA expressions, as the data was not normally distributed (Kolmogorov–Smirnov test). An independent-samples *t* test was used to assess the cytokine expression under stimulation and no stimulation. A one-way ANOVA was used to assess the significance of differences between groups. Data were considered significant at $p < 0.05$.

RESULTS

The baseline characteristics of enrolled subjects: The number, sex, and age data of subjects in each group are summarized in Table 1. There were no significant differences in the mean age and sex ratio among the PDR, DM-NDR, and control groups.

Expressions of PD-1, PD-L1, and PD-L2 mRNA in lymphocytes: PD-1, PD-L1, and PD-L2 mRNA was detected in all groups using real-time PCR. The relative expression of PD-1

TABLE 1. BASELINE CHARACTERISTICS OF THE ENROLLED SUBJECTS.				
Experiment	**Group**	**Number**	**Sex (male/female)**	**Age (mean±SD)**
mRNA analysis	PDR	41	16/25	55.41±12.39
	DM-NDR	27	10/17	58.59±13.34
	Controls	59	30/29	53.51±10.68
	P value		0.356	0.184
Apoptosis analysis	PDR	18	6/12	49.13±11.15
	DM-NDR	15	7/8	51.07±9.36
	Controls	15	9/6	49.13±11.15
	P value		0.309	0.398
	Cytokine analysis			
Stimulated	PDR	15	8/7	52.47±6.37
	DM-NDR	13	5/8	56.92±7.31
	Controls	14	7/7	54.50±10.62
	P value		0.717	0.374
Unstimulated	PDR	5	2/3	50.20±4.32
	DM-NDR	5	4/1	55.80±9.26
	Controls	5	1/4	57.20±14.64
	P value		0.153	0.541

TABLE 2. THE RELATIVE EXPRESSION OF PD-1, PD-L1, AND PD-L2 MRNA IN LYMPHOCYTES.			
Group	PD-1 (×10E-5)	PD-L1 (×10E-5)	PD-L2 (×10E-5)
PDR	1.7 (4.7–0.9)	5.5 (8.7–3.3)	4.7 (9.0–2.3)
DM-NDR	2.9 (6.7–2.5)	8.6 (13.2–5.1)	7.4 (10.8–4.0)
Control	4.4 (7.0–2.3)	9.7 (16.1–6.8)	5.2 (10.0–3.1)
P value	0.002	0.001	0.212

Data were shown as median (Q3-Q1) of relative expression of mRNA and analyzed by Kruskal–Wallis test.

and PD-L1 mRNA in patients with PDR was significantly lower than that in the DM-NDR and control groups (PD-1: $p = 0.002$; PD-L1: $p = 0.001$). The PD-L2 mRNA expression was also low in the PDR group, but it was not significantly different to that in the other two groups ($p = 0.212$; Table 2).

Frequencies of PD-1-, PD-L1-, and PD-L2-positive cells on lymphocytes: The frequencies of PD-1-, PD-L1-, and PD-L2-positive cells on lymphocytes were subsequently observed. As shown in Figure 1, a statistical analysis showed a significantly higher frequency of PD-1$^+$ cells in the PDR group than in the DM-NDR and control groups ($F = 10.227$; $p = 0.035$ versus DM-NDR; $p = 0.000$ versus control). In contrast, the PDR group had a lower frequency of PD-L1$^+$ cells compared with the other two groups ($F = 10.688$; $p = 0.000$ versus DM-NDR; $p = 0.018$ versus control). However, PD-L2$^+$ cells on lymphocytes were not detected in any of the three groups.

Expressions of PD-1 and PD-L1 on activation-induced apoptotic lymphocytes: We further evaluated the expressions of PD-1 and PD-L1 on activation-induced apoptotic lymphocytes, as PD-1/PD-L has been demonstrated to induce apoptosis in lymphocytes. As shown in Figure 2, a higher expression of Annexin V$^+$PI$^-$ cells was observed in the PDR

group than in the DM-NDR and control groups ($F = 11.323$; $p = 0.012$ versus DM-NDR; $p = 0.000$ versus control). The frequency of Annexin V$^+$PI$^-$PD-1$^+$ cells increased, while Annexin V$^+$PI$^-$PD-L1$^+$ cells decreased in the PDR group, which was significantly different to that in the DM-NDR and control groups (Annexin V$^+$PI$^-$PD-1$^+$ cells: $F = 15.236$; $p = 0.003$ versus DM-NDR; $p = 0.000$ versus control. Annexin V$^+$PI$^-$PD-L1$^+$ cells: $F = 19.605$; $p = 0.000$ versus DM-NDR; $p = 0.040$ versus control).

Expressions of IFN-γ and IL-4 on PD-1-positive cells: In view of the upregulation of PD-1-positive cells and Annexin V$^+$PI$^-$PD-1$^+$ cells, we further examined the expression of IFN-γ and IL-4 on PD-1-positive cells with or without PMA-Ionomycin stimulation using flow cytometry. The results showed that compared with unstimulated lymphocytes, the expressions of IFN-γ and IL-4 were significantly increased on stimulated lymphocytes in each group (IFN-γ: $p = 0.000$, 0.000, 0.000; IL-4: $p = 0.001$, 0.047, 0.001, respectively), which suggested that stimulation with PMA and Ionomycin was effective (Table 3). As shown in Figure 3, under stimulation, the frequencies of IFN-γ$^+$, IL-4$^+$, PD-1$^+$ IFN-γ$^+$, and PD-1$^+$IL-4$^+$ cells in the PDR group were the highest, respectively. An analysis of the ratio of IFN-γ$^+$ cells to IL-4$^+$ cells revealed the ratio was markedly higher in the PDR group than in the
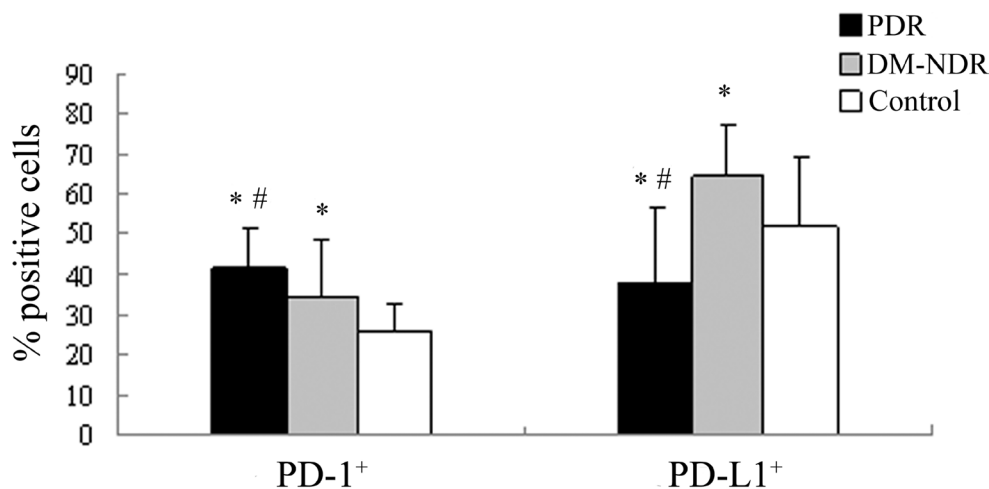

Figure 1. The frequencies of PD-1- and PD-L1-positive cells on lymphocytes were evaluated by flow cytometry. All data shown represent the mean ± SD of at least three independent experiments. *p<0.05 compared with the control group; #p<0.05 compared with the DM-NDR group.
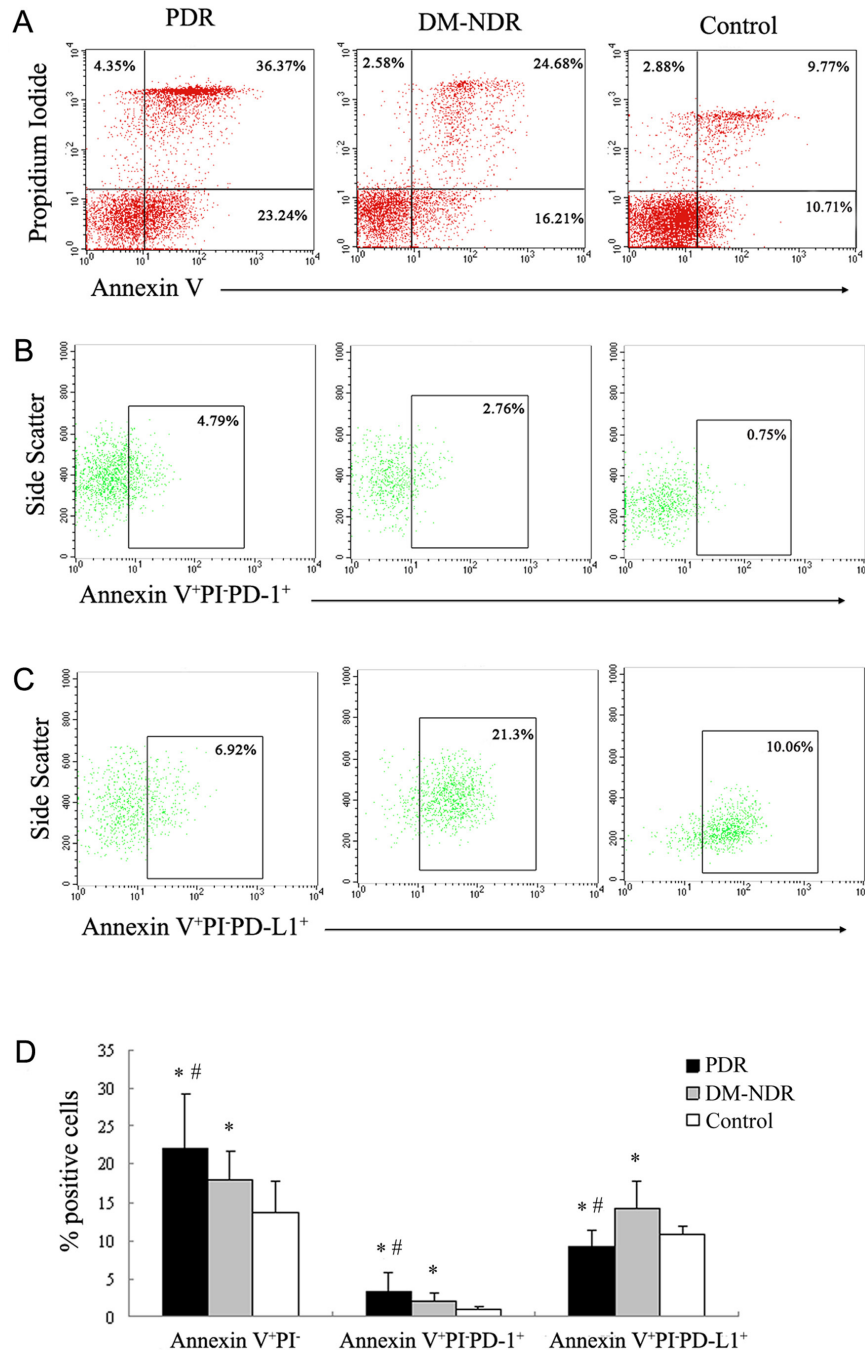
Figure 2. Apoptosis and the expressions of PD-1 and PD-L1 on activation-induced apoptotic cells were evaluated by flow cytometry. **A**: Cells considered to be undergoing apoptosis were positive for Annexin V, but negative for PI (the lower right part). **B**: The gated cells represent the expression of Annexin V⁺PI⁻PD-1⁺ cells. **C**: The gated cells represent the expression of Annexin V⁺PI⁻PD-L1⁺ cells. Results of a representative experiment are shown. **D**: Apoptosis and the expressions of PD-1 and PD-L1 on activation-induced apoptotic lymphocytes are shown by a histogram. All data shown represent the mean ± SD of at least three independent experiments. *p<0.05 compared with the control group. #p<0.05 compared with the DM-NDR group.

control group (F = 2.296, p = 0.046). However, the ratio of PD-1$^+$IFN-γ$^+$ cells to PD-1$^+$IL-4$^+$ cells was not significantly different among the groups (F = 0.346, p = 0.328).

DISCUSSION

In this study, we analyzed the expression and functional characteristics of the PD-1 pathway in patients with PDR. Our study revealed the mRNA expression of PD-1 significantly decreased. In the PDR group, a higher expression of PD-1$^+$, Annexin V$^+$PI$^-$, and Annexin V$^+$PI$^-$PD-1$^+$ cells was observed, but the frequency of PD-L1$^+$ and Annexin V$^+$PI$^-$PD-L1$^+$ cells was lower. The ratio of PD-1$^+$IFN-γ$^+$ to PD-1$^+$IL-4$^+$ cells was not significantly different between the groups, although their expressions were upregulated in the PDR group. These results suggest PD-1 can mediate activation-induced apoptosis in the development of PDR.

Diabetes mellitus is a complex metabolic disease that can have devastating effects on multiple organs in the body, of which PDR is a highly serious microvascular complication. In the present study, peripheral whole blood was chosen as the research tissue because abnormalities in various biochemical, hemorheological, and immunological elements in systematic diseases can be examined using it. The procedure of GE Ficoll-Paque PLUS is a generally acceptable method for the preparation of viable lymphocytes in high yield, so it was used to isolate lymphocytes from peripheral whole blood in this study. Moreover, the enrolled subjects in each group were age-matched to reduce the impact of age on the results. We first determined the expression of PD-1 and its ligands. The significant changes in both the mRNA expressions of PD-1 and PD-L1 and the frequencies of PD-1- and PD-L1-positive cells were observed in the PDR group, which suggests the PD-1/PD-L1 pathway may be involved in the pathological process of PDR.

Apoptosis of lymphocytes is an important homeostatic mechanism in the immune system. Cellular apoptosis induced by hyperglycemia is crucial for the initiation of diabetic pathologies [25]. The frequency of early apoptosis in retinal microvascular cells may predict the development of histologic lesions of retinopathy in diabetes [26,27]. Activation-induced apoptosis, one pathway of apoptosis, is induced by antigen stimulation under particular conditions [28]. It is a major mechanism for generating T-cell tolerance [29] and has been found to be involved in chronic hepatitis, systemic lupus erythematosus (SLE), and Parkinson's disease [30-32]. PD-1 was originally identified as a molecule linked to the in vitro induction of apoptotic cell death in murine lymphoid cell lines and its stimulation can initiate the apoptotic cascade [33]. The PD-1/PD-L pathway has been demonstrated to influence T cell activation by causing activation-induced cell death [34]. Therefore, we evaluated the expressions of PD-1 and PD-L1 on activation-induced apoptotic lymphocytes. Our results demonstrated a higher expression of activation-induced apoptotic lymphocytes in patients with PDR, which seems to be an indication of enhanced susceptibility to PDR. However, the frequency of PD-1$^+$ and AnnexinV$^+$PI$^-$PD-1$^+$ cells increased, while the frequency of Annexin V$^+$PI$^-$PD-L1$^+$ cells decreased. These results suggest that PD-1, but not PD-L1, is involved in the regulation of lymphocyte activation and the development of PDR by mediating activation-induced apoptosis.

The analysis of cytokine production levels is recognized as an important tool for describing the course of an immune response and for discovering pathophysiological mechanisms in immune diseases [35]. The higher frequencies of PD-1$^+$ cells and Annexin V$^+$PI$^-$PD-1$^+$ cells in the PDR group observed in this study indicate PD-1 may be a significant molecule in PDR. Consequently, we examined the expression of IFN-γ and IL-4, respectively, on PD-1$^+$ cells. Higher frequencies of IFN-γ$^+$, IL-4$^+$, PD-1$^+$ IFN-γ$^+$, and PD-1$^+$IL-4$^+$ cells in the PDR group were found in this study. IFN-γ is primarily thought to be a pro-inflammatory cytokine released by activated T cells and it was found to be increased in the retina of diabetic rats [36]. IL-4 is critical for the upregulation of costimulatory molecules, which in turn allow the activation and expansion of alloreactive CD4 T cells [37]. Therefore, our

Positive cells	Group	Stimulated	Unstimulated	t	p value
IFN-γ$^+$ cells	PDR	20.26±5.26	1.30±0.66	−13.638	0.000
	DM-NDR	14.91±6.86	1.25±0.43	−7.144	0.000
	Control	10.59±4.87	1.17±0.98	−6.850	0.000
IL-4$^+$ cells	PDR	4.77±1.46	2.01±1.06	−3.882	0.001
	DM-NDR	3.63±1.13	2.34±1.14	−2.157	0.047
	Control	3.67±1.24	1.37±0.55	−3.966	0.001

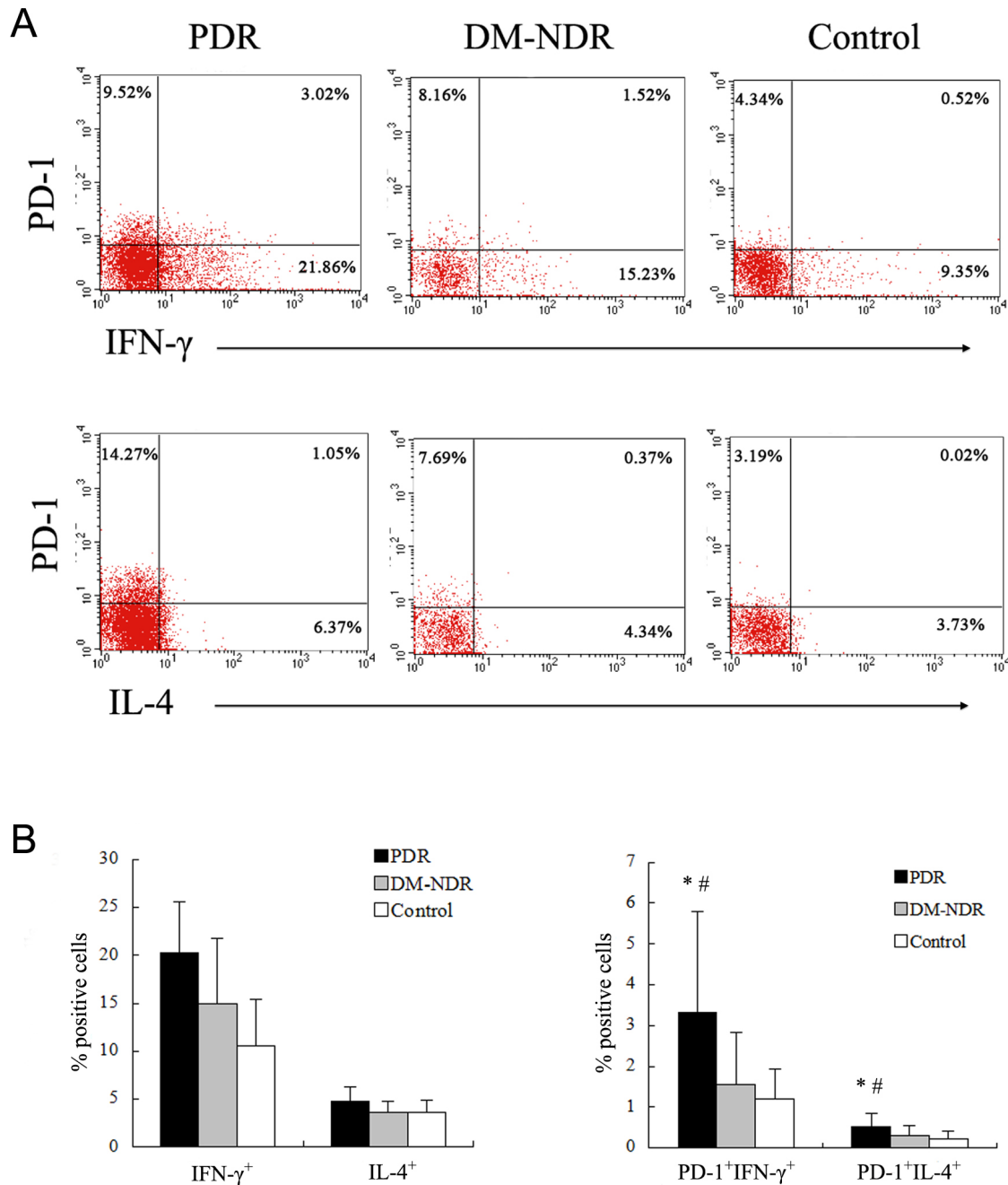TABLE 3. THE FREQUENCY OF IFN-ɣ+ AND IL-4+ CELLS ON STIMULATED AND UNSTIMULATED LYMPHOCYTES (%, MEAN±SD)

Figure 3. The expressions of IFN-γ and IL-4 on stimulated lymphocytes were evaluated by flow cytometry. **A**: The frequencies of IFN-γ and IL-4 were detected by flow cytometry. Results of a representative experiment are shown. **B**: The frequencies of IFN-γ and IL-4 are shown by a histogram. All data shown represent the mean ± SD of at least three independent experiments. *p<0.05 compared with the control group; #p<0.05 compared with the DM-NDR group.

results support the concept of an immune-mediated inflammatory response in PDR.

It is commonly believed the Th1/Th2 balance plays a crucial role in the maintenance of immune homeostasis. Previous studies found a high Th1/Th2 ratio in patients with Graves' ophthalmopathy, multiple myeloma, and SLE with nephritis [38-40], which indicated that a Th1/Th2 imbalance might be involved in these immune diseases. Th1 cells produce mainly IFN-γ, whereas Th2 cells mainly produce IL-4. We assessed IFN-γ and IL-4 production at the single

cell level by flow cytometry to determine the clinical significance of the Th1/Th2 ratio analysis. In this study, we found that the ratio of IFN-γ+ to IL-4+ cells in the PDR group was increased, implying a Th1/Th2 imbalance and Th1-dependent immune response might play an important role in the pathogenesis of PDR. However, the ratio of PD-1+ IFN-γ+ to PD-1+IL-4+ cells in our study was not significantly different between the groups. We hypothesize that PD-1 may be involved in the development of PDR without affecting the balance between Th1 and Th2. Because there is a significant limitation—that is, lymphocyte subsets such as CD4+ T cells, which are more sensitive to Th1/Th2 balance in some immune responses [32], were not determined—this speculation needs to be confirmed by further studies.

In conclusion, the present study investigated the expression and functional characteristics of the PD-1 pathway in peripheral blood lymphocytes from patients with PDR. PD-1+ cells and activation-induced apoptosis triggered by PD-1 were upregulated in PDR patients. However, the ratio of PD-1+IFN-γ+ to PD-1+IL-4+ cells was not significantly different between the groups, although their frequencies were increased in PDR patients. These results suggest that PD-1 is involved in the development of PDR by mediating activation-induced apoptosis. Considering the main pathological lesions of DR in the retina, studies on ocular tissue, such as vitreous humor and epiretinal membranes, are going to be performed.

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