Increased Toll-like Receptor-2 Expression on Nonclassic CD16+ Monocytes from Patients with Inflammatory Stage of Eales' Disease

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PURPOSE. To identify the distribution, differential Toll-like receptor (TLR) expression, and functional contribution of monocyte subpopulations in the inflammatory stage of Eales' disease (ED).

METHODS. Peripheral blood mononuclear cells were isolated from nine patients during the inflammatory stage of ED and nine age- and sex-matched healthy controls. The expression of CD14, CD16, TLR-2, and TLR-4 on monocytes was measured by flow cytometry. The CD14+, CD16−, and CD16+ monocyte populations were sorted on the basis of magnetic-activated cell-sorting methodology, and levels of cytokines were measured by ELISA.

RESULTS. In ED patients, the number of circulating monocytes was significantly expanded compared with that in controls ($P = 0.01$), with a marked increase in the nonclassic CD16+ subset, which showed an activated phenotype in patients that correlated with levels of serum proinflammatory cytokines and clinical progression. A higher expression of cell surface TLR-2 ($P = 0.02$), but not TLR-4, was found in monocytes of patients with ED. Furthermore, TLR-2 was expressed at higher levels on CD16+ monocytes than on CD16− monocytes in patients, whereas no significant variation was found in TLR-4 expression on different monocyte subsets. Peptidoglycan-induced TNF-α expression correlated with TLR-2 expression in monocytes isolated from controls ($r = 0.85$, $P = 0.0061$), but not in monocytes isolated from ED patients ($r = 0.553$, $P = 0.1328$).

CONCLUSIONS. These results indicate that in the pathogenesis of ED, TLR activation and increased numbers of nonclassic CD16+ monocytes are crucial regulators, along with the secretion of proinflammatory cytokines that perpetuate the inflammatory process in the retina. (*Invest Ophthalmol Vis Sci.* 2011; 52:6940–6948) DOI:10.1167/iovs.11-7834

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Eales' disease (ED) is a prototype inflammatory disorder in which immunogenic inflammation of the peripheral retina leads to nonperfusion and neovascularization. At the proliferative or advanced stage of the disease, newly formed retinal vessels are prone to develop vitreous hemorrhages resulting in profound visual loss.[1,2] Although the clinical characteristics of the disease are well recognized, its etiopathogenesis is so far not well defined. However, several lines of experimental evidence link ED with Gram-positive bacteria, *Mycobacterium tuberculosis*, and other nontuberculosis mycobacteria.[3–6] Two studies have demonstrated a statistically significant higher phenotype frequency of human leukocyte antigen (HLA) in ED patients than in healthy controls[7] and have identified the presence of inflammatory cells in the epiretinal membrane of patients with ED (Badrinath SS, et al. *IOVS* 1990;31:ARVO Abstract 33). Thus, it can be hypothesized that individuals with a predisposition to HLA, predominantly HLAB5 (B51), DR1, and DR4, may develop retinal vasculitis as a consequence of cell-mediated immunologic tissue damage triggered by a sequestered mycobacterial antigen in an inactive form that clinically presents as ED.[8]

Even though most patients with ED lack clinical evidence of infection, laboratory and clinical findings suggest the presence of systemic inflammation in the acute phase of this disease.[9,10] Therefore, it is probable that both innate and adaptive immune systems are involved in the development of ocular inflammation. The ability of the innate immune system to recognize microbial component–associated molecular patterns have highlighted the central role played by one group of pattern recognitions receptors (PRRs), the Toll-like receptors (TLRs), in microbial recognition and host defense.[11] Members of the TLR family are expressed in a variety of cells of the innate immune system[12] and orchestrate the intracellular inflammatory signaling pathways after recognition of pathogen-associated molecular patterns (PAMPs), such as bacterial cell wall components, dsRNA, and bacterial DNA bacterial flagellin.[13] Although TLR expression is primarily associated with monocytes/macrophages and dendritic cells, other cells of the adaptive immune system and nonimmune cells have also been shown to express TLRs.[14] In fact, TLRs are capable of forming an important bridge between the innate and adaptive immune response by regulating expression of costimulatory molecules on antigen-presenting cells, to drive T-cell activation, and by creating a cytokine milieu, to conduct differentiation of T-cells into the desired subset.[15]

Irrespective of any past effort to identify TLR involvement in pathogenesis of ED, mycobacterial involvement in the pathogenesis of the same is well studied. TLR-2 is a highly relevant TLR in the *Mycobacterium*-associated pathogenic outcome and responds to various lipoproteins and cell wall compo-

nents, such as peptidoglycan (PGN),[16-18] whereas an undefined heat-labile cell–associated mycobacterial factor has been found to be the ligand for TLR-4—hence, the relevance of studying TLR-2 and-4 in this respect.[16,19]

Monocytes, too, are crucial for the immune and inflammatory response, and by using the PRRs, they produce a large number of functionally distinct host cytokines.[20] Research interest in monocyte heterogeneity has gained strong thrust in the last decade past decade, as a subset-specific contribution of monocytes to immune stimuli has been postulated.[21,22] Presently, three human monocyte subsets are defined, by virtue of differential surface expression of LPS receptor CD14 and FcγIII receptor CD16—namely, CD14^{++}CD16^{−} (monocytes expressing CD14 but not CD16), CD14^{++}CD16^{+} (monocytes expressing CD14 and CD16), and CD14^{+}CD16^{+} (monocytes expressing CD16 and low CD14) cells, of which the latter two subsets are summarized as CD16^{+} monocytes and have been found to be a minor monocyte population in healthy individuals.[23]

Although, TLR expression is known to differ among classic and minor monocyte populations in peripheral blood,[24] no study to date has documented any alteration of the monocyte subpopulations and the mechanisms underpinning changes in blood monocyte TLR expression in the pathogenesis of ED. Therefore, the present study was conducted to identify the distribution, differential TLR expression, and functional contribution of monocyte subpopulations, as well as the cytokine production pattern of isolated monocytes after interaction of a clinically relevant bacterial antigen with TLR in the inflammatory stage of ED.

METHODS

Study Subjects

Nine (eight men, one woman) patients, who received a diagnosis of ED were recruited from the retina research clinic at the Regional Institute of Ophthalmology, Kolkata, India, from 2007 through 2010. All nine patients were in the active stage of ED, as characterized by acute inflammation of veins in the periphery of the retina. Among them, two patients (one man, one woman) had inflammation of the small-caliber vessels, with superficial retinal hemorrhage; two patients (both men) had large-caliber vessel inflammation, with superficial retinal hemorrhage; and the remaining five male patients had large-caliber vessel inflammation with macular edema. The patients received no medication before sample collection.

Diagnosis was made for all patients by dilated fundus examination with direct and indirect ophthalmoscopy, slit lamp biomicroscopy with +90 D and three-mirror lens, stereoscopic color fundus photography, and fluorescein angiography. Other detailed ophthalmic examinations included visual acuity determination by ETDRS chart and anterior segment evaluation by slit lamp examination. The location and extent of retinal involvement by vasculitis was documented in all patients by digital color fundus photography and fluorescein angiography.

Nine (seven men, two women) age- and sex-matched healthy adults, without any history suggestive of ED, attending the outpatient department of the same institute for treatment of visual difficulty due to refractive errors without any other systemic inflammatory or ocular disease provided blood samples and constituted our healthy control group.

Clinical history was obtained from all participants for the presence of any associated systemic diseases and use of anti-inflammatory medication. Participants were eligible for this study if they had no smoking and/or alcohol addiction, had not received any immunosuppressive therapy, and did not have any coexisting chronic inflammatory disease or any other recent infection 6 months before collection of the samples. Subjects with a history of systemic disorders such as, diabetes

mellitus, hypertension, collagen vascular disease, HIV, symptomatic arthritis, symptomatic malignancy, sarcoidosis, Behçet's disease, systemic lupus erythematosus, Coats' disease, or syphilis were also excluded from the study. The study protocol complied with the Declaration of Helsinki and was approved by the Institution's Ethics Committee. Informed consent was obtained from each subject.

Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood was collected in an EDTA-coated vial on the day of diagnosis of ED and from healthy control subjects. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation (Ficoll; GE Healthcare, Piscataway, NJ, with Histopaque-1077; Sigma-Aldrich, St. Louis, MO). Isolated PBMCs were washed with RPMI1640 medium (Invitrogen-Gibco, Grand Island, NY). This cell fraction contained monocytes and lymphocytes and was adjusted to 1×10^6 monocytes/mL, viability of the cells confirmed by trypan blue exclusion assay. Expression of CD14, CD16, TLR-2, and TLR-4 was determined, and the remaining cell suspension was used for isolation and culture for CD14^{+} monocytes and different monocyte subpopulations, as required.

Flow Cytometry

Four-color flow cytometry was performed on freshly isolated PBMCs, to investigate the cell surface expression of CD14, CD16, TLR-2, and TLR-4, on monocytes of patients with ED and healthy controls. Briefly, PBMCs were washed twice with phosphate-buffered saline (PBS; 0.02 M, pH 7.2) and resuspended in PBS containing 2% heat-inactivated fetal bovine serum (FBS). Fifty microliters of PBMC suspension (1×10^6 cells/mL) was incubated with primary antibodies for 30 minutes at room temperature: fluorescein isothiocyanate (FITC)– conjugated anti-CD14 (clone 61D3, eBioscience, San Diego, CA), phycoerythrin cyanine 5 (PECy5)– conjugated anti-CD16 (clone 3G8, BioLegend, San Diego, CA), allophycocyanin (APC)-conjugated anti-TLR-2 (clone TL2.1; eBioscience), and phycoerythrin (PE)-conjugated anti-TLR-4 (clone HTA125, eBioscience), along with isotype-matched antibody controls. After they were washed with PBS, the cells were resuspended in 400 μL PBS containing 2% FBS, and counts were acquired on a four-parameter flow cytometer (FACSCalibur; BD Biosciences, San Diego, CA). The monocytes were gated on the basis of parameters of forward and side light scatter, and acquisition was performed on 5000 gated events. Data analysis was performed with system-associated software (CellQuest Pro; BD Biosciences). The absolute count of monocytes was obtained with a hemocytometer counting chamber, and the monocyte subpopulation counts were calculated with the percentage distribution obtained from flow cytometry. To measure TLR fluorescence in monocytes, the cells were plotted on the basis of their characteristic linear forward and side scatter and further gated with CD14 and/or CD16 positivity, and then TLR fluorescence was measured on a logarithmic scale in the FL2 channel (TLR-4) and FL4 channel (TLR-2). The mean channel fluorescence intensity (MFI) derived from the fluorescence histogram was used to determine the extent of cell surface TLR expression and expressed as the MFI of specific subtracted from the MFI of respective isotype control (i.e., MFI-specific staining minus MFI isotype).

Monocyte Separation and Culture

After isolation of PBMCs from healthy controls and ED patients by density gradient, the monocytes were isolated from the PBMCs by incubation with magnetic-activated cell sorting (MACS) beads coated with a monoclonal antibody against human CD14 (Miltenyi Biotec, Bergisch Gladbach, Germany). Immunomagnetic separation was performed according to the manufacturer's instructions. Purity >90% was confirmed by flow cytometry. The cells were adjusted to 1×10^6 monocytes/mL and cultured in RPMI supplemented with 100 U/mL penicillin/100 μg /mL streptomycin and 10% FBS for 18 hours at 37°C in 5% CO_2, with or without stimulation by 5 μg/mL PGN of *Staphylococcus aureus* (InvivoGen, San Diego, CA), and the cell supernatants

were collected and stored at −20°C until used for detection of cytokines.

Separation and Culture of Monocyte Subpopulation

CD14$^+$CD16$^-$ and CD14$^+$CD16$^+$ monocytes were selectively isolated from purified CD14$^+$ monocytes (isolated by CD14 microbead and MACS methodology) of three ED patients by using a CD16$^+$ monocyte isolation kit (Miltenyi Biotec) and MACS methodology, according to the manufacturer's instructions. Purity >90% was confirmed by flow cytometry analysis. CD14$^+$CD16$^-$ and CD14$^+$CD16$^+$ monocytes were separately cultured in RPMI supplemented with 100 U/mL penicillin/ 100 μg/mL streptomycin and 10% FBS for 48 hours at 37°C in 5% CO$_2$ without any stimulation, and the cell supernatants were collected and stored at −20°C until used for cytokine detection.

Measurement of Cytokines

The presence of cytokines in the culture supernatants and serum was measured by enzyme-linked immunosorbent assay (ELISAs for TNF-α, IL-6, IFN-γ, and IL-10; ImmunoTools, Friesoythe, Germany; for IL-1β and TGF-β; R&D System, Inc., Minneapolis, MN). Measurements were performed in duplicate with 100-μL sample volume, according to the manufacturer's instruction.

Determination of Erythrocyte Sedimentation Rate and High-Sensitivity C-reactive Protein

The erythrocyte sedimentation rate (ESR) was determined by using the method of Westergren within 2 hours of obtaining the blood. Briefly, 2 mL of EDTA anticoagulant blood was added to 0.5 mL of 0.85% of sodium chloride, and the contents of the test tube were mixed gently for 2 minutes. A standard 200-mm, Westergren tube was filled to the 0 mark, set in a vertical position, and left for 1 hour. The level to which the red cell column fell at the end of 1 hour was noted, and the result expressed as millimeters in the first hour.

Serum high-sensitivity C-reactive protein (hsCRP) was then measured (Magiwel Enzyme Immunoassay; United Biotech, Inc., Mountain View, CA), per the manufacturer's instructions.

Inhibition of TLR-2 Expression by siRNA

Human TLR-2-specific or nonspecific control siRNA was transfected into purified CD14$^+$ monocytes isolated from four healthy controls with a lipophilic transfection reagent (Lipofectamine 2000; Invitrogen), according to the protocol of the manufacturer. Briefly, the purified monocytes (0.5×10^6 cells/mL) were plated in a 24-well plate and incubated at 37°C, 5% CO$_2$ for 24 hours. Before the transfection, cultures were washed, and medium was replaced by 400 μL/well of serum-free RPMI1640 medium (without antibiotics). Serial dilutions of siRNA were transfected at 10 to 80 nM, with the transfection reagent (1 μL/mL). The transfection reactions were supplemented with 100 μL of FBS after 8 hours and changed to fresh culture medium containing 10% FBS in the next morning. After 48 hours of transfection, cells from

some wells were harvested and examined for surface TLR-2 expression by flow cytometry. The remaining cells were stimulated with PGN (5 μg/mL) for 18 hours, and cell culture supernatants were collected to measure TNF-α by ELISA.

Statistical Analysis

Age and sex differences between the patients and control subjects were investigated by Student's t-test and χ^2 test, respectively. The significance of differences of measured parameters between corresponding groups of observations was evaluated by the Mann-Whitney U test, and all values are expressed as the mean ± SD. To examine correlations, we used Spearman's rank correlation coefficient on the logged data (Graph-Pad, San Jose, CA). Acceptable significance was recorded when values reached $P < 0.05$. To determine the minimum number of patients, yet appropriate sample sizes for our experiments, we undertook power analysis calculations (PS Power and Sample Size Calculation software version 3.0.34; http://biostat.mc.vanderbilt.edu/ wiki/Main/PowerSampleSize/ open source software developed by William DuPont and Walton Plummer, Jr., Department of Biostatistics, Vanderbilt University, Nashville, TN). First, based on our previous studies, we estimated the effect size among different experimental units. Thereafter, the sample size was set to nine per group to reject the null hypothesis at a 5% significance level, ensuring at least 80% power for each experiment.

RESULTS

Nine patients with the inflammatory stage of ED satisfying the inclusion and exclusion criteria were studied and compared with nine healthy age- and sex-matched controls. Demographic and clinical characteristics indicative of disease progression are summarized in Table 1.

Increase in Total Circulating Monocytes in Patients with the Inflammatory Stage of ED, along with a Shift toward the Nonclassic CD16$^+$ Monocyte Subset

Monocytes were analyzed after exclusion of other peripheral mononuclear cells shortly after the isolation by side scatter and forward scatter (Fig. 1A); a higher number of circulating blood monocytes were found in patients with the inflammatory stage of ED than were found in healthy controls (Figs. 1B, 1C).

CD14^{++}CD16$^-$ (R1), CD14^{++}CD16$^+$ (R2), and CD14$^+$CD16$^+$ (R3) monocytes were distinguished by the surface expression pattern of the LPS receptor CD14 and the FcγIII receptor CD16 (Fig. 1D). In healthy controls, CD14^{++}CD16$^-$ monocytes (R1) constituted the major population (69.96% in the representative subject shown), whereas CD14^{++}CD16$^+$ (R2) and CD14$^+$CD16$^+$ (R3) were the minor populations (Fig. 1D). However, in a

TABLE 1. Demographic and Clinical Characteristics of the Study Participants

| Characteristics	Patients with ED in Inflammatory Stage	Healthy Controls	*P*
Ratio of men to women	8:1	7:2	NS
Age, y	30.2 ± 3.8	30.4 ± 8.6	NS
IL-6, pg/mL	36.15 ± 18.46	6.28 ± 1.71	0.0004
TNF-α, pg/mL	21.06 ± 6.72	9.09 ± 1.01	<0.0001
hsCRP, mg/L	2.89 ± 0.63	0.89 ± 0.19	<0.0001
Total leukocytes, cells/μL	8679 ± 2405	6772 ± 1130	0.03
Lymphocyte count, cells/μL	1843 ± 395	2150 ± 538	NS
ESR, mm/h	38 ± 12	21 ± 14	0.013

Data are expressed as the mean ± SD.
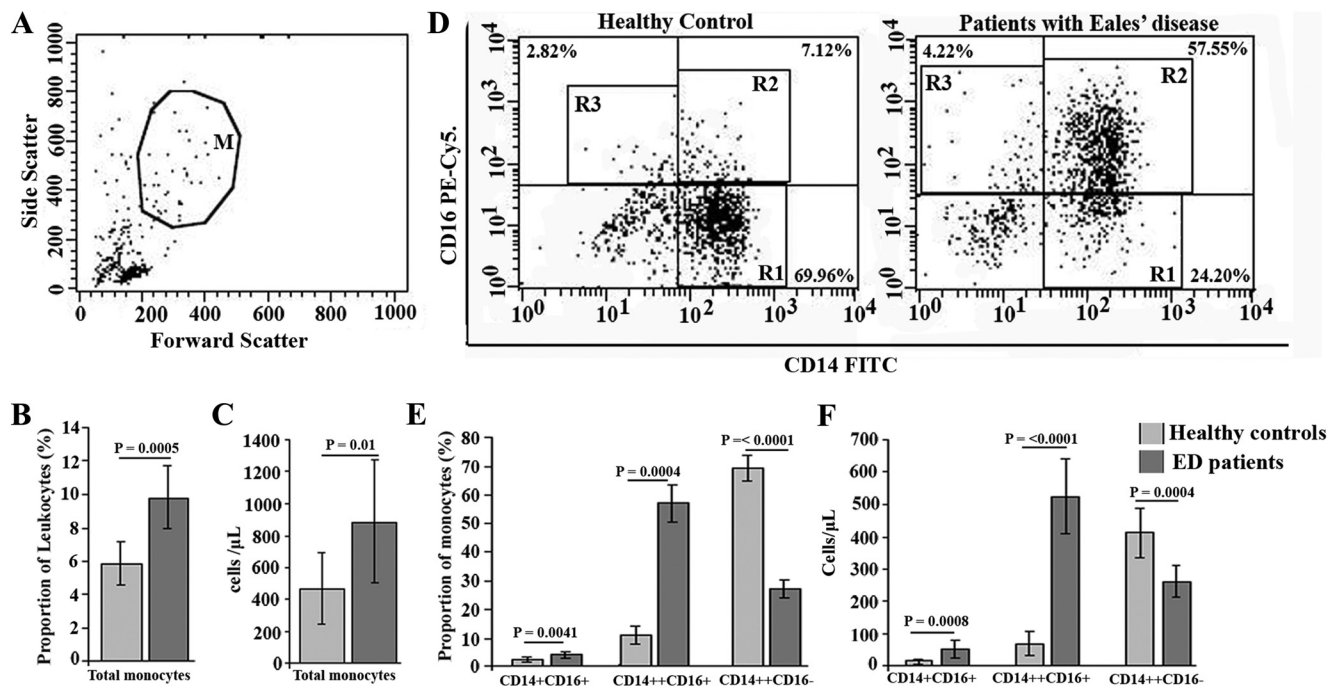
FIGURE 1. Flow cytometric determination of monocyte cell subsets among PBMCs in study participants. (**A**) Representative dot plot depicting placement of the primary gate around the monocyte population based on forward and side scatter. (**B**) Proportional and (**C**) absolute monocyte counts were obtained with a hemocytometer counting chamber. (**D**) Representative flow cytometry plots displaying an increase in $CD14^{++}CD16^{+}$ (R2) and $CD14^{+}CD16^{+}$ (R3) cells among the PBMCs of patients with ED, compared to those of healthy controls. Cumulative data for (**E**) the proportions and (**F**) absolute counts of $CD14^{+}CD16^{+}$, $CD14^{++}CD16^{+}$, and $CD14^{+}CD16^{-}$ monocyte subsets from healthy controls ($n = 9$) and patients ($n = 9$). $P < 0.05$ indicates significance.

patient with ED, $CD14^{++}CD16^{+}$ (R2) was predominant, being 57.55% versus 7.12% in the control (Fig. 1D).

On average, the percentage of $CD14^{+}CD16^{+}$ and $CD14^{++}CD16^{+}$ monocytes was increased in patients compared with healthy controls ($3.99\% \pm 0.84\%$ vs. $2.38\% \pm 0.83\%$; $P = 0.0041$ and $56.83\% \pm 6.7\%$ vs. $10.92\% \pm 2.98\%$; $P = 0.0004$, respectively). In contrast, the proportion of $CD14^{++}CD16^{-}$ monocytes significantly decreased in ED patients compared with controls ($27.09\% \pm 3.26\%$ vs. $69.06\% \pm 4.47\%$; $P \leq 0.0001$; Fig. 1E). Significant increases in the $CD14^{++}CD16^{+}$ ($P \leq 0.0001$) and $CD14^{+}CD16^{+}$ ($P = 0.0008$) monocyte subsets were also observed for absolute cell counts, along with a decrease in $CD14^{++}CD16^{-}$ monocytes ($P = 0.0004$) when compared with the count in the healthy subjects (Fig. 1F).

When the absolute number of total monocytes and different subsets were related to demographic data, serum inflammatory cytokine levels, serum hsCRP level, total leukocyte count,

lymphocyte count, and ESR, the $CD14^{++}CD16^{+}$ monocyte subset showed a significant correlation with serum CRP ($r = 0.95$; $P = 0.0004$) and ESR ($r = 0.52$; $P \leq 0.001$), which are accepted clinical indicators of the disease. The $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{+}$ monocyte subsets also correlated with serum TNF-α ($r = 0.784$; $P = 0.003$ and $r = 0.4692$; $P = 0.009$, respectively), but not with age, sex, and other studied parameters, whereas the $CD14^{++}CD16^{-}$ monocytes showed an inverse correlation with these markers (Table 2).

Increased Expression of TLR-2 on Isolated Total Monocytes and CD16⁺ Monocyte Subsets in Patients with ED

The intensity of TLR-2 and -4 expression in total and different subsets of the monocyte population was measured shortly after isolation. Figure 2A shows a representative histogram profile of

TABLE 2. Correlation Analyses between the Absolute Number of Total Monocytes or the Subpopulation of Monocytes and Demographic Data, Inflammatory Cytokine Levels, and Other Blood Cells

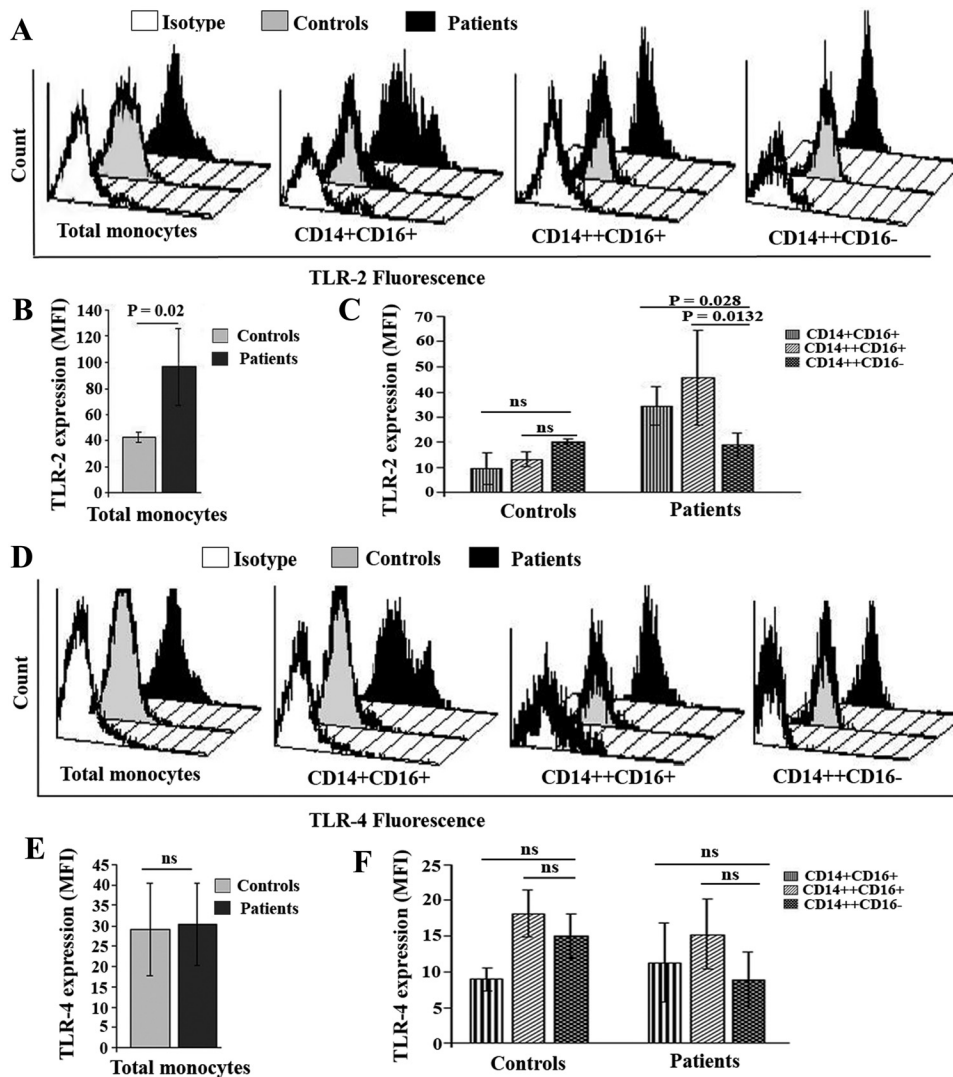|	Total Monocytes		$CD14^{++}CD16^{+}$ Monocytes		$CD14^{+}CD16^{+}$ Monocytes		$CD14^{++}CD16^{-}$	
	r	P	r	P	r	P	r	P
Age, y	–	NS	–	NS	–	NS	–	NS
Sex	–	NS	–	NS	–	NS	–	NS
IL-6, pg/mL	0.961	<0.001	–	NS	–	NS	–	NS
TNF-α, pg/mL	0.894	<0.001	0.784	0.003	0.4692	0.009	−0.618	0.001
hsCRP, mg/L	0.85	0.0061	0.95	0.0004	0.374	NS	−0.82	0.0051
Total leukocytes, cells/μL	0.9667	0.0002	–	NS	–	NS	–	NS
Lymphocyte count, cells/μL	−0.8167	0.0108	–	NS	–	NS	–	NS
ESR, mm/h	0.462	0.006	0.52	<0.001	–	NS	–	NS

FIGURE 2. Flow cytometric analysis of TLR-2 and -4 expression on monocytes isolated from patients with ED and healthy controls. (**A**) Representative histograms showing TLR-2 expression in total and different subsets of monocyte population. (**B**) TLR-2 expression in total monocytes was higher in patients with ED than in healthy controls. (**C**) TLR-2 fluorescence intensity on CD16$^+$ monocytes was significantly higher than that of CD16$^-$ monocytes in patients with ED, but no significant subset-specific differences in TLR-2 expression were found in healthy controls. (**D**) Representative histograms show TLR-4 expression in total and different subsets of the monocyte population. (**E**) No significant differences were found between patients and health controls in TLR-4 expression on total monocytes. (**F**) There were no differences in TLR-4 fluorescence intensity on different monocyte subsets in both groups. Fluorescence intensity of cell surface TLR was expressed as the difference between the mean fluorescence intensity (MFI) of staining with anti-TLR-2 or anti-TLR-4 antibody and isotype-matched control antibody (MFI$_{specific staining}$ − MFI$_{isotype}$). $P < 0.05$ indicates significance. ns, nonsignificant.

TLR-2 expression on gated monocytes and the different cell subsets from healthy controls and patients with ED. A significantly higher level of TLR-2 expression, as measured by MFI, was observed on the total monocytes of patients in the active stage of ED, compared with age- and sex-matched healthy controls ($P = 0.02$; Fig. 2B). When comparison was made between different monocyte subsets in patients, the average MFI of TLR-2 on CD14$^+$CD16$^+$ and CD14^{++}CD16$^+$ monocytes were significantly higher than that of CD14^{++}CD16$^-$ monocytes in patients with ED ($P = 0.028$ and $P = 0.0132$, respectively; Fig. 2C). Conversely, in healthy controls, a higher TLR-2 expression was found in CD14^{++}CD16$^-$ monocytes, but the difference in TLR-2 intensity in the different subsets was statistically nonsignificant (Fig. 2C).

In contrast to TLR-2, staining for TLR-4 revealed low fluorescence intensity in both study groups, and no statistically significant intergroup differences and intragroup monocyte-subset–specific differences were found (Figs. 2D–F).

In addition, in patients with ED, the intensity of TLR-2 expression in total monocytes showed a positive correlation with serum hsCRP level ($r = 0.843$; $P = 0.007$), whereas no such correlation was found in expression of TLR-4 on total monocytes and serum hsCRP ($r = 0.414$; $P = 0.21$; data not shown).

Increased TLR-2 Induced Cytokine Production by Monocytes Isolated from ED and Differential Cytokine Secretion in the Monocyte Subsets

The increased baseline TLR-2 expression on monocytes from patients with ED may be due to a previous contact with endotoxin. Hence, we investigated whether TLR-2 engagement results in increased cytokine production. We isolated circulating monocytes by human CD14-coated microbeads via MACS methodology in cells from patients and healthy controls, and then pro- and anti-inflammatory cytokine production was determined after cells were cultured with or without PGN.

In the absence of stimulation, monocytes from ED patients had increased mean levels of proinflammatory (TNF-α, IL-6, IFN-γ, and IL-1β) and anti-inflammatory (IL-10 and TGF-β) cytokines compared with monocytes isolated from healthy controls (Fig. 3A). After activation with PGN, mean levels of all cytokines increased in both groups compared with unstimulated cells, within each group, but no statistically significant differences were observed after PGN induction, when the two groups were compared (Fig. 3B).

Because of the marked accumulation of CD16$^+$ monocytes in inflammatory phases of ED, we further sought to define the likely function of this subset in the pathogenesis of this disease.
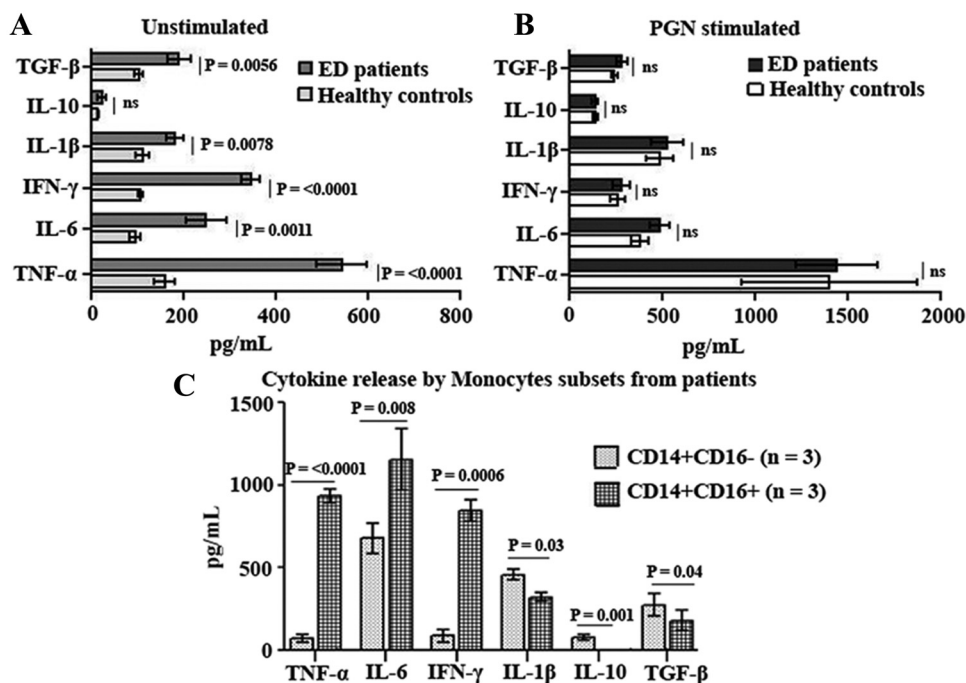
FIGURE 3. Monocytes were functionally active in patients with a differential release of distinct cytokines by monocyte subpopulation. (**A**) Cytokine release of isolated monocytes from healthy controls and patients with ED (18-hour culture) without stimulation and (**B**) after stimulation with 5 μg/mL PGN for 18 hours. (**C**) Cytokine release by purified monocyte subsets after 48-hour culture without stimulation. $P < 0.05$ indicates significance. ns, nonsignificant.

CD16$^-$ and CD16$^+$ monocytes were isolated from three patients by MACS methodology, and baseline cytokine secretion was measured after 48 hours of culture without any stimulation. The CD16$^+$ monocytes were the major producers of TNF-α, IL-6, and IFN-γ, compared with the CD16$^-$ subgroup ($P \leq 0.0001$, $P = 0.008$, and $P = 0.0006$, respectively). CD16$^-$ monocytes, on the other hand, were the main producers of IL-1β ($P = 0.03$) and TGF-β ($P = 0.04$). Moreover CD16$^-$, but not CD16$^+$, monocytes were the only producers of the anti-inflammatory cytokine IL-10 (Fig. 3C).

Relationship between TLR-2 Expression and Ligand-Induced Cytokine Production

Spearman's rank correlation analysis was performed to determine the relationship between surface TLR-2 expression on total isolated circulating monocytes and response to the TLR-2 ligand. A significant positive correlation was noted between cell surface TLR-2 expression and PGN-induced TNF-α production ($r = 0.85$; $P = 0.0061$; Fig. 4A) by monocytes isolated from healthy controls. However, no significant correlation was found between TLR-2 expression and PGN-induced TNF-α production ($r = 0.553$; $P = 0.1328$; Fig. 4B) by total monocytes isolated from ED patients.

Diminished Activation by PGN Resulting from Forced Reduction of TLR-2 Expression

Since the relationship of TLR-2 expression on monocytes and activation by PGN was not consistent between healthy controls and ED patients, experiments were performed to directly determine the effects of forced reduction of TLR-2 expression on PGN-induced activation. Forty-eight hours after introducing siRNA into monocytes isolated from four different healthy controls, cell surface TLR-2 expression was significantly reduced by TLR-2-specific siRNA ($P = 0.003$ at concentration of siRNA 20 nM, $P = 0.002$ at concentration of siRNA 40 nM, and $P \leq 0.001$ at concentration of siRNA $\geq$60 nM, when compared with nontransfected controls), but not by control siRNA (Fig. 4C). PGN induced TNF-α production demonstrated a significant dose-dependent reduction ($P = 0.0016$ at concentration of siRNA 20 nM and $P \leq 0.001$ at a concentration of siRNA $\geq$

40 nM when compared with nontransfected controls) after the introduction of TLR-2-specific siRNA into monocytes isolated from healthy controls, but no reduction was detected in the nonspecific control siRNA (Fig. 4D). These results demonstrate that the forced reduction of TLR-2 expression in monocytes resulted in diminished PGN mediated secretion of TNF-α.

DISCUSSION

The notion that inflammatory mechanisms play an important role in the progression of the pathologic angiogenesis, including retinal neovascularization, has emerged as a major paradigm shift in our understanding of disease pathogenesis. In an effort to eliminate invading pathogens, inflammation is usually the final common pathway used, and both innate and adaptive immune responses are known to trigger inflammation. Considering the significant impact of ED, including visual disability and blindness, investigation of the basic mechanisms underlying the ocular inflammation linked with this disease is warranted. Association of systemic inflammation with the inflammatory stage of ED has been established by the presence of elevated levels of C-reactive protein[9] or circulating IL-6 as a marker.[25] However, the findings of association between systemic and local inflammation in ED is limited. Only one study has described the association between increased levels of TNF-α with severity of retinal periphlebitis in patients with ED.[26] In concurrence, we also noted that high levels of systemic inflammatory markers correlated with an increased severity of retinal inflammation in patients with ED (data not shown). However, inflammation is not only a phenomenon of plasma acute-phase proteins but also of alteration in circulating pools of blood immune cells, including monocytes.

In this study, we demonstrated that number of monocytes are increased in circulation during the active stage of ED; strikingly, we observed a strong shift toward the CD16$^+$ monocytes, particularly the CD14^{++}CD16$^+$ monocyte subset. These CD16$^+$ cells have a distinct phenotype and are efficient producers of proinflammatory cytokines on TLR engagement,[27] whereas they weakly secrete the anti-inflammatory cytokine IL-10.[28] They also have high endothelial affinity conferred by
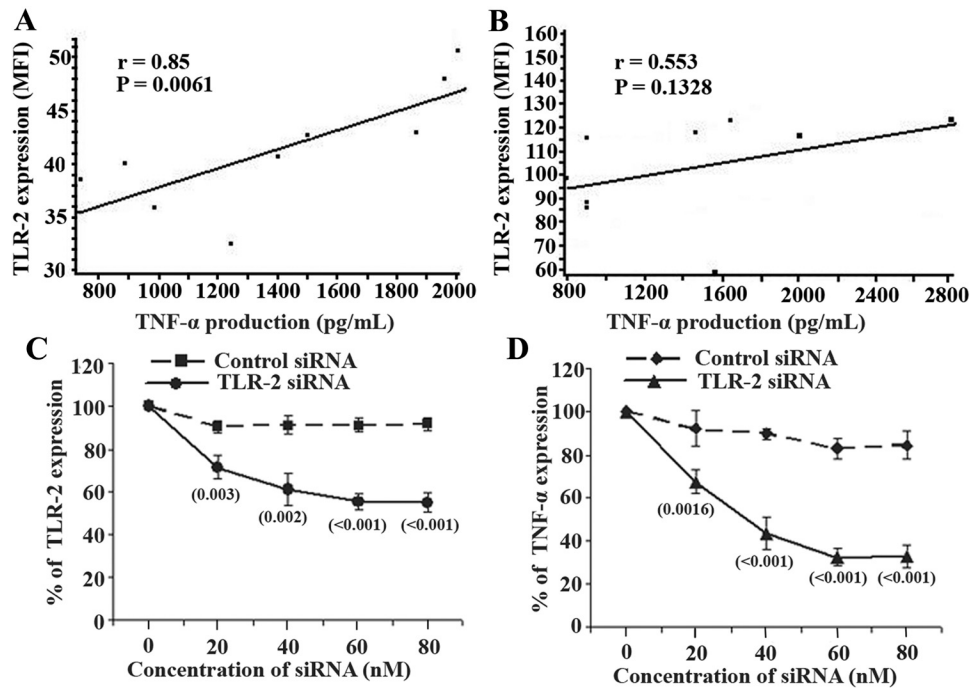
FIGURE 4. Relation between TLR-2 expression and PGN-induced TNF-α production in monocytes. Correlation of the cell surface expression of TLR-2 with production of TNF-α after PGN stimulation in monocytes isolated from (**A**) healthy controls (*n* = 9) and from (**B**) patients with ED (*n* = 9). The *solid line* represents the linear regression curve of best fit. (**C**) Monocytes isolated from healthy controls (*n* = 4) were transfected with different concentrations of control siRNA or TLR-2-specific siRNA and examined for cell surface expression of TLR-2. (**D**) TNF-α production by different concentrations of control or TLR-2-specific siRNA–transfected monocytes, isolated from healthy controls, after 5 μg/mL PGN stimulation. The cell surface TLR-2 expression was determined by flow cytometry analysis, and TNF-α concentration was determined by ELISA. Results are presented as the percentage of TLR-2 expression and the percentage of TNF-α production normalized to the nontransfected control (100%). The *P* values in parentheses indicate a significant difference (*P* <0.05) between the transfected and nontransfected controls.

their surface expression of chemokine receptors and adhesion molecules such as CX3CR1, CCR5, VLA-4, and ICAM-1.[29–31] Furthermore, the "patrolling" behavior of the mouse counterpart of CD16^{+} monocytes has been described by investigators, who found that these monocytes crawl along the endothelium and, on inflammatory stimulation, rapidly gain access to the endothelium and home to sites of endothelial activation in a CX3CR1-dependent manner. There, they secrete MMP-9, CCL-2, and IL-6 with the ability to propagate further vascular damage through the recruitment of T-lymphocytes and additional monocytes.[32,33]

Endothelial dysfunction, a concomitant effect of endothelial cell activation, is the ultimate fate of many vasoproliferative retinal disorders, including ED.[34] The evidence makes a good case for a probable role of CD16^{+} monocytes in this disease. Although, the involvement of MMP-9[35] and inflammatory cytokines[36] has been established in the pathogenesis of ED, no previous reports have confirmed the involvement of cell adhesion molecules. Keeping in mind their important role in inflammatory ocular neovascularization events,[37] participation of these adhesion molecules with immunopathogenesis of ED cannot be ruled out and is a subject of ongoing research for better understanding of the pathologic course of these diseases. The associated data from the present study (i.e., relative abundance of CD16^{+} monocytes) correlated with circulating proinflammatory cytokines and parameters indicative of disease progression, whereas CD14^{++}CD16^{−} monocytes showed an inverse correlation with these markers, and in view of the endothelial activation capability of these minor subsets, sup-

port the relevance of CD16^{+} monocytes to the inflammatory stage and may be suggested to be stimulators of endothelial damage in the advanced stage of ED.

In addition, elevated levels of cell surface TLR-2 expression on systemic circulating monocytes, but not TLR-4, was found in the inflammatory stage of ED. Interestingly, expression of TLR-2 on various subsets of monocytes from ED patients was different from that of healthy controls. TLR2 expression was significantly higher in CD16^{+} compared with CD16^{−} monocytes in ED patients. In contrast, the intensity of TLR-4 was similar in both monocyte subsets from ED patients and healthy controls. In addition, the intensity of the TLR-2, but not the intensity of the TLR-4, expressed on total monocytes was found to correlate with serum CRP level in ED patients. Taken together, the data suggest that TLR-2 expression may be enhanced during CD16^{+} monocyte maturation in vivo in association with disease activity, but TLR-4 expression remained unchanged. These observations further support the proposed concept of *M. tuberculosis* association and suggest involvement of TLR-2 activation by respective microbial products or their PAMPs in the pathogenesis of ED.

Because we assumed that the increased TLR-2 expression on monocytes isolated from patients was a result of previous contact, we proceeded to determine whether TLR-2 engagement results in increased cytokine expression by monocytes. We also wanted to determine the functional contribution of different monocyte subsets in the pathogenesis of ocular inflammation in ED. Accordingly, cytokine production was mea-

sured in total monocytes, isolated from patients and matched controls, with and without PGN stimulation, and in monocyte subsets in ED patients without PGN stimulation.

At baseline, total monocytes from ED patients secreted significantly higher amounts of inflammatory mediators than healthy subjects. These findings are in good agreement with the hypothesis that increased productions of inflammatory cytokines are a crucial mechanism in development of periretinal neovascularization and worsen outcome of ED.[34] Further dissecting the diverse functional capacities of both monocyte subsets in vitro confirmed that the CD16^{+} monocyte subset is the main source of proinflammatory mediators, thereby perpetuating the chronic inflammatory response in the retina. This conclusion is corroborated by correlating between circulating CD16^{+} monocyte counts and levels of serum TNF-α in ED patients.

After PGN stimulation, cytokine production by monocytes increased in all study groups, and no significant intragroup differences were found. It is thought that monocytes isolated from patients are inherently more responsive than those obtained from healthy controls, due to the possibility that the cell surface expression of TLR controls the ligand-induced downstream response. Forced diminution of TLR-2 expression on monocytes in our study resulted in a decreased response to PGN, also supporting the hypothesis that increased expression of TLR-2 results in an enhanced response to TLR-2 ligand. Conversely, when we examined the relationship of surface expression of TLR-2 on monocytes with the levels of cytokines induced by stimulation with TLR-2 ligand, a significant positive association was found in monocytes isolated from healthy controls but not in monocytes from patients. The data revealed that the PGN-induced responses of monocytes isolated from patients, although increased compared with those of unstimulated monocytes in vitro, were less than would have been expected of monocytes isolated from healthy controls at comparable levels of cell surface TLR-2.

These findings are of relevance, given the further evidence supporting the role of a microbial trigger and, in particular, Gram-positive bacteria in the development of ED. As all study patients had active inflammation at the time of sampling, it is feasible that prior in vivo exposure to potential endogenous TLR-2 ligands induced tolerance to repeat stimulation,[38,39] possibly accounting for the lack of association between TLR-2 expression and response to TLR-2 ligand in ED patients. The existence of TLR-2 ligand in ocular tissue of ED patients has not yet been established. Analysis of vitreous collected from ED patients at the time of vitrectomy by molecular biology techniques has detected the presence of *Mycobacterium* DNA suggesting the existence of *Mycobacterium*-derived ligands.[5,6] However, reduced response to PGN is a transient state, and the present study did not demonstrate a dynamic change in TLR function in ED. It is conceivable that the kinetics of TLR-2 expression, along with cytokine production, is different at later stages of progression of ED and should be investigated in the future.

In summary, the findings of the present study indicate that nonclassic CD16^{+} monocytes are crucial regulators in the pathogenesis of ED and act by secreting an abundance of cytokines perpetuating the retinal inflammatory process. Furthermore, this study also implies a role for TLR-2 activation in the pathogenic outcome of ED. Demonstration of increased TLR-2 expression on systemic immune cells in ED together with a functional state of reduced TLR-2 ligand response provides further evidence of the role of *Mycobacterium* in the development of Eales' disease.

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