Identification of intraocular inflammatory mediators in patients with endophthalmitis

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Purpose: Endophthalmitis is mediated by inflammatory cytokines. We employed a quantitative antibody array, which profiles protein expression and function in a high-throughput manner, to identify inflammatory mediators in the infectious aqueous and vitreous humor from patients with endophthalmitis.
Methods: In this prospective study, aqueous humor (AH) and vitreous humor (VH) samples were obtained from 30 patients with endophthalmitis and were collected during anterior chamber paracentesis and vitrectomy. Control samples were obtained from 32 healthy donors. We examined the expression of 20 inflammatory mediators in AH and VH using a quantitative antibody protein array. Hierarchical cluster analysis based on the expression of the quantified cytokines was applied to identify the specificity of endophthalmitis disease. Validation analysis using enzyme-linked immunosorbent assay (ELISA) was performed to confirm the expression of the cytokines identified in the AH and VH samples.
Results: Our results demonstrated elevated expression of interleukin (IL)-1β, IL-6, and macrophage inflammatory protein (MIP)-3α in AH or VH from patients with endophthalmitis. The concentration of IL-17 was upregulated in AH from the patients. The expression of IL-2, IL-5, IL-21, and transforming growth factor (TGF)-β1 was downregulated in AH from the patients. The cluster analysis demonstrated that the cytokine profile expression in AH or VH significantly differed between the patients with endophthalmitis and the healthy controls. Confirmation with ELISA validated the increase in IL-1β, IL-6, and MIP-3α in the AH and VH samples from the patients with endophthalmitis.
Conclusions: Increased expression of IL-1β, IL-6, IL-17, and MIP-3α and decreased expression of IL-2, IL-5, IL-21, and TGF-β in the AH and VH suggests an abnormal cytokine profile in patients with endophthalmitis. Knowledge of this will aid in the diagnosis of infectious endophthalmitis.

Endophthalmitis often occurs after the introduction of an infectious agent into the interior of the eye and results in acute inflammation. Infectious agents generally gain access to affected eyes via the following routes: a consequence of intraocular surgery (post-operative), access through a penetrating injury of the globe (post-traumatic), or from hematogenous spread of microbes into the eye from a distant anatomic site (endogenous) [1,2]. Infectious endophthalmitis is a particularly devastating complication of penetrating ocular trauma. It has been reported that endophthalmitis occurs in approximately 3% to 17% of open globe injuries [3-5]. Several studies have shown that the specific effects of microbial toxins on tissues and cells and the presence of certain organisms in immunologically privileged areas stimulate intraocular inflammation [6-8]. Pathogen-associated molecule pattern (PAMP) molecules, as well as the growing

organisms themselves, come in contact with and collectively stimulate resident immune cells to produce proinflammatory cytokines or other inflammatory mediators. Induction of these cytokines further initiates a cascade of inflammatory events, including increased permeability of the blood–ocular fluid barrier, which results in an influx of additional soluble mediators and the recruitment of inflammatory cells to the site of infection. Inflammatory cells produce additional inflammatory cytokines that mediate ocular inflammatory immune responses [2, 9,10]. The essential roles of inflammatory mediators in the development of endophthalmitis have been determined in animal models. However, the role of proinflammatory cytokines in human infectious endophthalmitis is not fully understood.

Recent studies have focused on the levels of various inflammatory mediators in intraocular (aqueous and vitreous) humor as markers for the activity and severity of ocular inflammation [11-17]. Post-traumatic intraocular infection severely influences the intraocular humor, which is the one of most vital targets of intraocular infection. However, only limited information is available about the profile

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of proinflammatory mediators present in the intraocular humor from patients with endophthalmitis. In this study, we employed a high-throughput quantitative antibody protein array that allowed us to obtain cytokine expression levels from small-volume aqueous humor (AH) and vitreous humor (VH) samples in a highly sensitive manner. This allowed the comparison between patients with endophthalmitis and healthy controls. Additionally, we compared the inflammatory cytokines between AH and VH to identify their respective specific markers. These results were validated using traditional enzyme-linked immunosorbent assay (ELISA) techniques.

Our data provide insight into the identification of inflammatory mediators in the anterior chamber and vitreous cavity in patients with endophthalmitis and healthy controls. Significantly increased expression of interleukin (IL)-1β, IL-6, and macrophage inflammatory protein (MIP)-3α was observed in the AH and VH from patients with endophthalmitis. However, increased IL-17 and decreased transforming growth factor (TGF)-β1, IL-2, IL-5, and IL-21 were observed only in AH from patients with endophthalmitis. Cluster analysis of these cytokines in the aqueous or vitreous humor distinguished the patients with endophthalmitis from the control group. The ELISA further demonstrated that the production of IL-1β, IL-6, and MIP-3α was elevated in aqueous humor and vitreous humor from patients with endophthalmitis. All these results indicated that the specific expression of these cytokines in infectious intraocular humor is associated with the development of endophthalmitis and identified the specific markers responsible for the infectious intraocular inflammation and potential therapeutic targets.

METHODS

Patients: This study was a prospective study. Thirty patients with endophthalmitis (30 eyes) following open globe injuries were examined: 20 men and 10 women with an average age of 37.4 years. Thirty-two healthy individuals, 18 men and 14 women aged 36.5 years on average, were included in this study. All study subjects were recruited from Zhongshan Ophthalmic Center, Sun Yat-sen University (Guangzhou, P.R. China) from May 2010 to June 2013. The diagnosis of endophthalmitis following open globe injuries (including ocular penetrating injury and intraocular foreign body introduction) was based on the positive outcomes of vitreous or aqueous humor bacteria culture and direct smear staining. All patients with endophthalmitis had bacterial infections (Figure 1A) and underwent complete ophthalmological examinations, including visual acuity, slit-lamp biomicroscopy, direct ophthalmoscopy, ultrasonography, orbital X-ray

film, or orbital computed tomography. Thirty patients with endophthalmitis showed the following clinical ocular manifestations: corneal edema (100%), anterior chamber inflammation or hypopyon (100%), vitritis or vitreous opacification (100%), or retinitis or retinal periphlebitis (57%; Figure 1B, Table 1). Patients who had positive bacterial pathogenic tests combined with open globe injuries and typically clinical manifestations of endophthalmitis were included in this study. Suspicious patients who had negative bacterial pathogenic outcomes, patients with endophthalmitis without a history of open globe injury, and patients with fungal endophthalmitis were excluded from this study. Patients with endophthalmitis were examined and diagnosed by the same experienced ophthalmologist at Zhongshan Ophthalmic Center (Liwen He). All patients received pars plana vitrectomy and anterior chamber paracentesis within 4 to 10 days after trauma when the characteristic clinical symptoms and signs of endophthalmitis were observed. The AH samples from patients with endophthalmitis were collected before vitrectomy. All aqueous samples (50–100 μl) were collected aseptically in a syringe with a 30 G needle and were then transferred into a presterilized Eppendorf tube for further tests. Then, patients underwent anterior chamber irrigation. We then collected the VH samples from the vitreous and performed vitrectomy and intra-vitreous antibiotic injection. Vitreous samples from patients with endophthalmitis were obtained during vitrectomy using 23-gauge needles attached to syringes from the pars plana incision before injection of balanced salt solution. Humor samples were deposited in an Eppendorf tube for subsequent procedures. Cadaveric AH and VH were obtained from 32 donors without systemic inflammatory or ocular diseases and served as the control group. All control samples from cadaveric eyes were collected within 8 h (4–10 h) after death. The supernatants of AH and VH were collected by centrifugation at 300 ×g for 10 min and 5000 ×g for 30 min, respectively, at 4 °C and stored at −80 °C until tested. All AH and VH samples were divided into two groups: One group (15 patients and 16 normal controls) was for quantitative cytokine antibody array assay, and the other (15 patients and 16 normal controls) was for ELISA.

Quantitative cytokine antibody array: The production of 20 cytokines or chemokines in the supernatants of vitreous and aqueous was quantitatively detected using Quantitative Cytokine Quantibody Human Array (RayBiotech, Norcross, GA). These molecules included granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-28, IFN-γ, MIP-3α, TGF-β1, tumor necrosis factor (TNF)-α and TNF-β. Multiplex antibodies against different cytokines were spotted onto the high-throughput cytokine
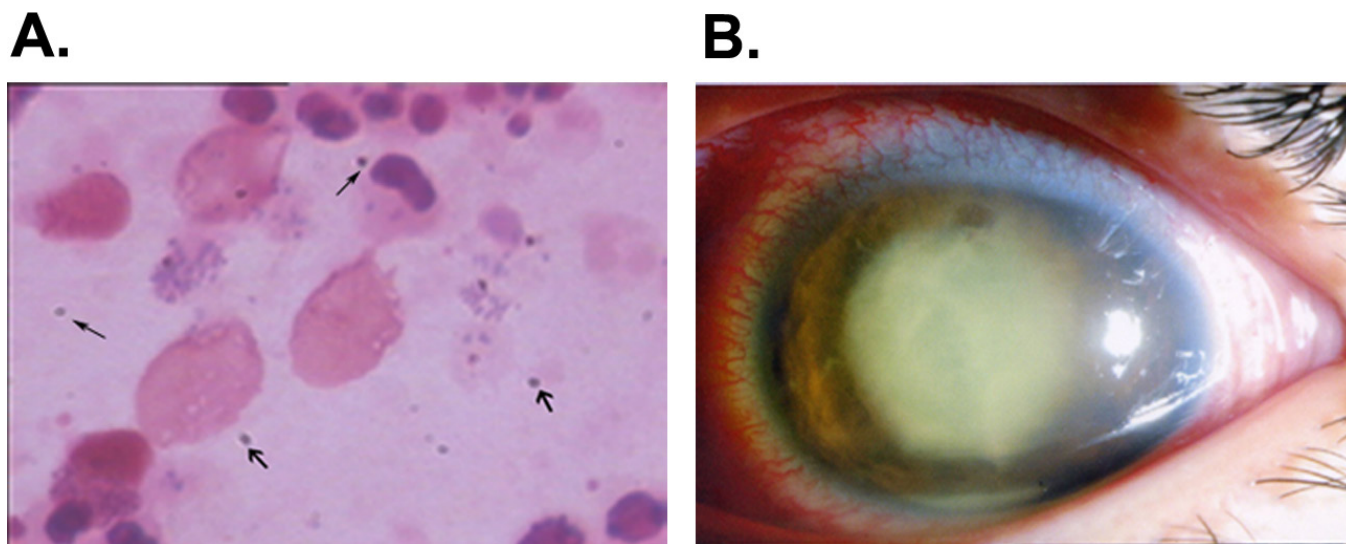
A.

B.

Figure 1. Anterior segment ocular manifestations and Gram staining of patients with endophthalmitis. **A**: Identification of bacteria strain by Gram staining. Gram-positive coccus indicated by arrow. Magnification, ×100. **B**: Anterior chamber inflammation and hypopyon in patients with infectious endophthalmitis following post-traumatic globe injury.

array according to the manufacturer's protocol. Fluorescence intensities (green fluorescence, Cy3 channel, 532-nm excitation) were captured using an Axon GenePix 4000B laser scanner (Molecular Devices, Sunnyvale, CA). Images were analyzed with the Quantitative Cytokine Antibody Array software program (RayBiotech), and the cytokine concentrations were quantified according to the standard curve calibrated from the same array.

Enzyme-linked immunosorbent assay: The concentrations of IL-1β, IL-2, IL-5, IL-6, IL-17A, IL-21, MIP-3α, and TGF-β1 in AH and VH were validated using human ELISA kits according to the manufacturer's instructions (RayBiotech). The detection limits of the IL-1β, IL-2, IL-5, IL-6, IL-17A, and MIP-3α ELISA kits were 15 pg/ml and that of the IL-21 kit was 30 pg/ml.

Cluster analysis: The hierarchical cluster method (R statistical language package stats) was used for clustering and visualization. Cytokine values were log-transformed and subjected to hierarchical correlation clustering using Ward's method, which minimizes within-cluster variance. The patients and the cytokines were clustered to obtain a heatmap. Cluster analysis was performed with R 2.15 (R Development Core Team [R Foundation for Statistical Computing], 2012). R scripts were used to construct trees and heatmaps and are available upon request.

Ethical statement: This study adhered to the ARVO statement on human subjects and was approved by the Ethics Committee of Sun Yat-sen University (2011KYNL013). All

of the procedures were performed according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all patients.

Statistical analysis: Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA and the Wilcoxon rank-sum test. The data were analyzed using SPSS13.0 for Windows XP (SPSS Science, Chicago, IL). A p value of less than 0.05 was considered statistically significant.

RESULTS

Amelioration of intraocular inflammation and vision improvement after anterior chamber irrigation, vitrectomy, and intravitreous antibiotic injection treatment: Prior to vitrectomy, 30 patients with endophthalmitis had anterior chamber inflammation, hypopyon, vitritis or vitreous opacity, and low visual acuity (range from 5.00 to 2.00; mean: 3.29±1.08, in terms of LogMAR). The post-surgery vision of patients with endophthalmitis improved markedly (range from 5.00 to 1.40; mean: 2.48±1.12, in terms of LogMAR, p=0.008; Table 1).

Identification of inflammatory cytokines in AH of patients with endophthalmitis: To perform a more comprehensive study, we investigated the expression level of a wider range of cytokines in the aqueous humor from patients in a highly sensitive manner. Our data demonstrated that the levels of IL-1β, IL-6, IL-17A, and MIP-3α were significantly upregulated in the aqueous humor from the patients with

TABLE 1. OCULAR MANIFESTATION OF PATIENTS WITH ENDOPHTHALMITIS.

Patient number	Corneal edema	KPs	Aqueous cell	Aqueous flare	hypoyon	Vitreous cell	Vitreous haze	retinitis	Retinal periphlebitis	Visual acuity (in terms of LogMAR)	
										Before vitrectomy	After vitrectomy
1	1+	-	1+	1+	3mm	1+	4+	-	-	4	2
2	3+	2+	2+	2+	1mm	1+	2+	-	-	3	3
3	2+	2+	3+	2+	1mm	2+	2+	+	+	2	1.4
4	1+	1+	1+	1+	2.5mm	1+	2+	+	-	4	3
5	3+	-	1+	1+	0.5mm	1+	3+	-	-	3	1.7
6	1+	-	1+	1+	1mm	2+	3+	-	+	3	2
7	1+	1+	1+	1+	4mm	1+	4+	+	-	5	5
8	2+	3+	3+	1+	3mm	1+	4+	+	+	2	1.7
9	2+	2+	3+	2+	1.5mm	1+	2+	-	-	4	3
10	1+	3+	3+	1+	3mm	1+	3+	-	-	2	1.8
11	1+	1+	2+	2+	3mm	2+	2+	-	-	4	3
12	1+	-	1+	1+	0.4mm	1+	3+	+	-	3	1.4
13	1+	-	2+	2+	0.5mm	1+	2+	-	+	2	1.4
14	1+	-	1+	2+	1mm	1+	2+	+	-	4	2
15	3+	2+	1+	2+	2mm	1+	3+	+	+	3	3
16	1+	3+	3+	2+	3mm	1+	3+	-	+	2	1.7
17	1+	1+	1+	1+	2.5mm	1+	4+	+	-	4	3
18	2+	1+	1+	1+	4mm	1+	4+	-	+	5	5
19	2+	-	1+	2+	0.4mm	1+	3+	+	+	3	2
20	2+	1+	1+	1+	1mm	2+	3+	-	+	4	2
21	3+	2+	3+	2+	0.5mm	1+	2+	-	-	2	1.4
22	3+	3+	3+	1+	2mm	1+	4+	-	-	2	1.7
23	2+	1+	1+	1+	4mm	1+	4+	+	-	4	4
24	2+	1+	1+	1+	2.5mm	1+	2+	-	+	5	3
25	2+	-	1+	1+	1mm	1+1	3+	-	-	4	2
26	3+	2+	2+	3+	0.5mm	+1	2+	-	-	2	1.5
27	2+	1+	2+	3+	4mm	1+	4+	-	-	5	5
28	2+	1+	3+	2+	0.5mm	1+	2+	+	+	4	3
29	1+	1+	1+	1+	0.5mm	3+	3+	-	-	3	2
30	1+	2+	3+	2+	2mm	1+	3+	-	+	2	1.7

KPs means the count of fresh keratic precipitates throughout the corneal. Fresh KPs less than 10 is generally considered as +, 11–30 as 2+, 31–50 as 3+, and 31–50 as 3+, and

more than 50 as 4+. Cells in field of aqueous less than 1 is generally considered as 0, 1–5 as 0.5+, 6–15 as 1+, 16–25 as 2+, 26–50 as 3+, and more than 50 as 4+. No flare in anterior chamber is generally considered as 0, faint as 1+, iris and lens details clear as 2+, iris and lens details hazy as 3+, fibrinous exudate as 4+. clear is generally considered as 0 in grades of vitreous haze, trace as 0.5+, few opacities, mild blurring of optic nerve and retinal vessels as 1+, significant blurring of optic nerve and retinal vessels, but still visible as 2+, optic nerve visible, borders blurred, no retinal vessels seen as 3+, dense opacity obscuring optic disc head as 4+. No Corneal edema as-; limitations of a corneal haze like edema, corneal endothelium smooth, iris texture is still as 1+; light gray corneal edema, corneal endothelial roughness, iris blur as 2+; white diffuse corneal edema, corneal endothelium appear before the crack-like, depending on iris texture is unclear as 3+, milky corneal edema, eye structure is unclear as 4+. No Cells in field of vitreous cells as -, occasionally see cells as +, 1~9 as ++, 1O~30 as +++, 31 to 100 as ++++, numerous cells as +++++. logMAR=logarithm of minimal angle of resolution.
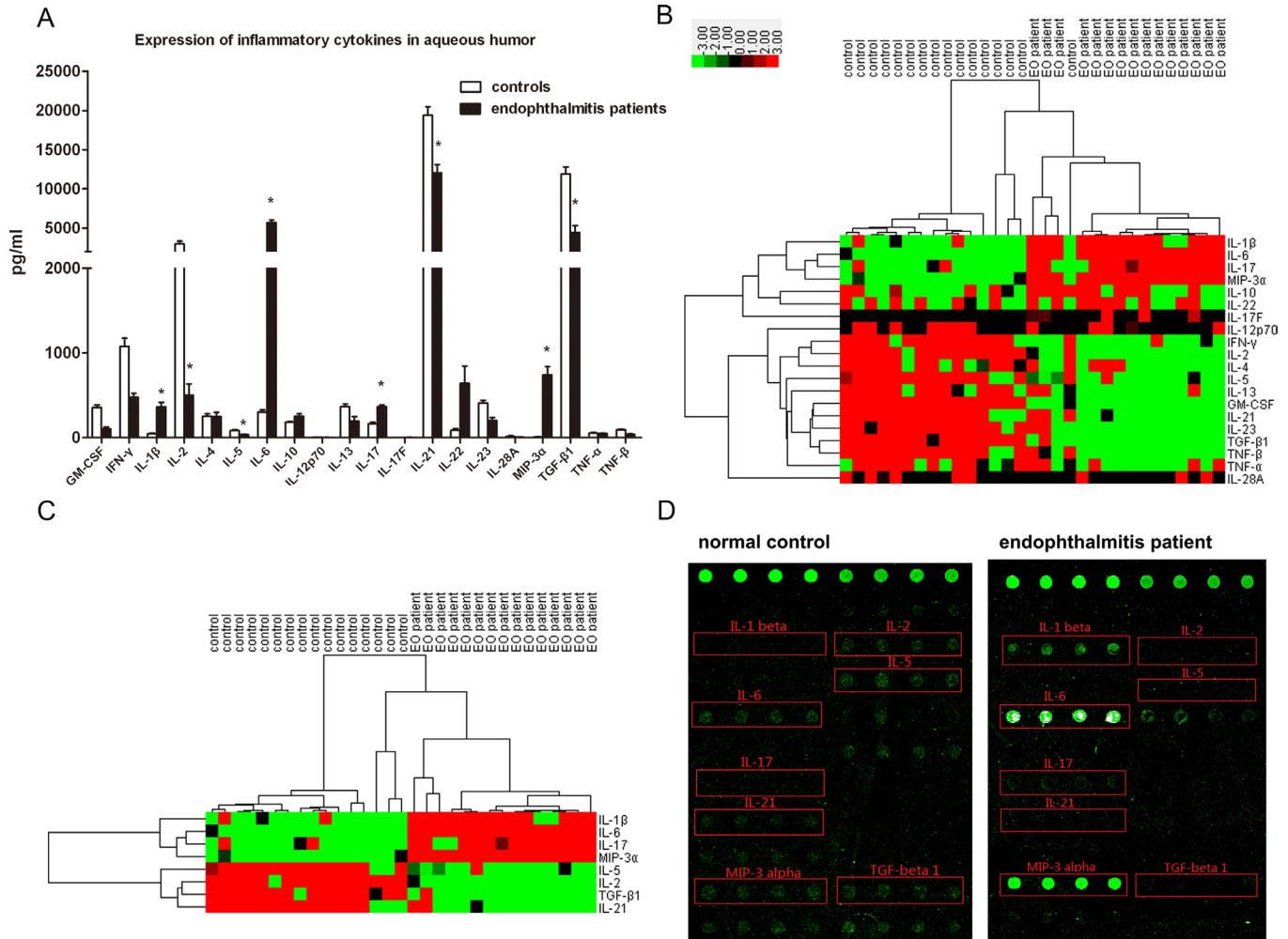
Figure 2. The cytokine profile in the aqueous humor from patients with endophthalmitis (n=15) and controls (n=16). Abbreviations: IFN-γ represents interferon gammar; IL represents interleukin; MIP represents Macrophage inflammatory protein; GM-CSF represents granulocyte-macrophage colony-stimulating factor; TGF represents transforming growth factor; TNF represents tumor necrosis factor. **A**: The levels of GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-28, IFN-γ, MIP-3α, TNF-α, TGF-β1, and TNF-β were detected using an antibody cytokine array. Data are represented as mean±SD *$p<0.05$. **B**: Heatmap of cluster analysis. Cluster analysis was performed with data from 20 cytokines and represented in a dendrogram. The horizontal bar on the left of the heatmap indicates cytokine expression. Higher expression is displayed in red, and lower expression is displayed in blue. Upregulation or downregulation of cytokines is indicated on a scale from red to green, respectively. The vertical lines indicate patients and controls. **C**: A heatmap of the cluster analysis was performed with eight of the chosen 20 cytokines. The differentially expressed cytokines were clearly associated with endophthalmitis with bacterial infection. **D**: Representative fluorescent mean intensities images for antibody array of AH. Additional cytokines in patients with endophthalmitis is marked in red.

endophthalmitis (n=15) compared to that of the healthy controls (n=16). In contrast, the expression of IL-2, IL-5, IL-21, and TGF-β was significantly decreased in the aqueous humor from the patients compared with that of the healthy controls (Figure 2A and Table 2).

The cluster analysis grouped the samples or cytokines based on cytokine levels (non-supervised analysis). The sample and cytokine cluster analyses were combined to visualize them as a heatmap (Figure 2B,C). Figure 2B shows

a cluster heatmap of the pan 20 cytokines. Expression of eight of the 20 cytokines differed significantly between the patients with endophthalmitis and the healthy controls. Figure 2C shows that the sample dendrogram can be divided into two principle clusters that largely segregate into healthy controls and patients with endophthalmitis. The horizontal subgroup was divided into elevated levels of the inflammatory cytokines IL-1β, IL-6, IL-17A, and MIP-3α and decreased expression of IL-2, IL-5, IL-21, and TGF-β. Figure 2D illustrates

TABLE 2. CYTOKINE CONCENTRATIONS (PG/ML) IN AQUEOUS HUMOR FROM ENDO-
PHTHALMITIS PATIENTS AS COMPARED WITH NORMAL CONTROLS.

Name of target	Endophthalmitis (pg/ml)	Control (pg/ml)	Fold change	P value
GM-CSF	106.48	355.57	0.30	<0.001
IFNg	478.65	1078.8	0.44	<0.001
IL-1b	363.27	47.63	7.63	<0.001
IL-2	501.38	2972.3	0.17	<0.001
IL-4	249	251.79	0.99	0.962
IL-5	36.59	83.34	0.44	0.001
IL-6	5701.8	302.43	18.85	<0.001
IL-10	253.51	184.47	1.37	0.033
IL-12p70	8.5	9.97	0.85	0.751
IL-13	198.18	367.51	0.54	0.011
IL-17	365.15	166.68	2.19	<0.001
IL-17F	0	0	0.00	0
IL-21	12,061	19,406	0.62	<0.001
IL-22		94.67	0.00	0.024
IL-23	220.75	409.83	0.54	<0.001
IL-28A	0	29.25	0.00	0.893
MIP-3a	741.45	9.23	80.33	<0.001
TGF-b1	4436.5	11,880	0.37	<0.001
TNFa	48.85	54.79	0.89	0.62
TNFb	37.96	94.38	0.40	<0.001

IFNγ=interferon gammar; IL=interleukin; MIP=Macrophage inflammatory protein; GM-CSF=granulocyte-macrophage colony-stimulating factor; TGF=transforming growth factor; TNF=tumor necrosis factor.

images captured of the antibody array that compare the patients with endophthalmitis and the healthy controls.

Patients with endophthalmitis have an inflammatory cytokine profile in the vitreous humor: The cytokine profile of the vitreous humor in the patients with endophthalmitis is still unclear. To determine the cytokine profile in the VH of patients with endophthalmitis, we employed the antibody cytokine array. These data indicated that the production of IL-1β, IL-6, and MIP-3α was significantly upregulated in the vitreous humor from patients with endophthalmitis (n=15) compared with that of the healthy controls (n=16; Figure 3A and Table 3).

Cluster analysis of the antibody array results in VH showed a significant difference in the expression of three of the 20 inflammatory mediators between patients with endophthalmitis and healthy controls. These results were combined and visualized as a heatmap (Figure 3B,C). Figure 3B shows the cluster heatmap of the pan 20 cytokines. Figure 2C shows that three (IL-1β, IL-6, and MIP-3α) of the 20 inflammatory mediators were identified to have specific expression only

in patients with endophthalmitis. The sample dendrogram was found to be markedly divided into two principle clusters that clearly separate in healthy controls and patients with endophthalmitis. Figure 3D illustrates antibody arrays and the images captured of the vitreous samples from patients with endophthalmitis and healthy controls.

Comparison of inflammatory cytokine profiles between AH and VH from patients with endophthalmitis: The production of IL-1β, IL-6, and MIP-3α was elevated in AH and VH from patients with endophthalmitis. In the AH, the concentration of IL-17A was significantly increased; however, the expression levels of IL-2, IL-5, IL-21, and TGF-β were decreased. We did not observe this profile in the VH.

Validation of cytokine profiles in patients with endophthalmitis using ELISA: To confirm the results of multiplex detection of the quantitative antibody array, we performed single-target ELISAs to validate the expression of the cytokines. Similar to the antibody array, elevated levels of IL-1β, IL-6, and MIP-3α in AH (Figure 4A) and VH (Figure 4B) from patients with endophthalmitis were confirmed with ELISA.

The levels of IL-2, IL-5, IL-17A, IL-21, and TGF-β in the patients with endophthalmitis and the healthy controls were below the limit detectable with ELISA.

DISCUSSION

In this study, we identified the inflammatory mediators in the AH and VH from patients with endophthalmitis after post-traumatic open globe injury. Quantitative cytokine antibody array analysis demonstrated that compared to healthy controls, patients with endophthalmitis presented with significantly upregulated expression of IL-1β, IL-6, IL-17, and MIP-3α but significantly lower levels of TGF-β, IL-2, IL-21, and IL-5 in the AH. In addition, patients with endophthalmitis had significantly higher concentrations of IL-1β, IL-6, and MIP-3α in the VH. Cluster analysis verified that the expression of IL-1β, IL-6, IL-17, MIP-3α, TGF-β, IL-2, IL-21, and IL-5 in AH and IL-1β, IL-6, and MIP-3α in VH was strongly associated with endophthalmitis. The expression of this cytokine profile was validated with ELISA and demonstrated an increase in IL-1β, IL-6, and MIP-3α in AH and VH from patients with endophthalmitis.

Post-traumatic infectious endophthalmitis is the most challenging and devastating consequence of open globe injury and often leads to rapid loss of vision and blindness when early intervention and appropriate therapy are not received [9,18-20]. Prompt diagnostic and therapeutic
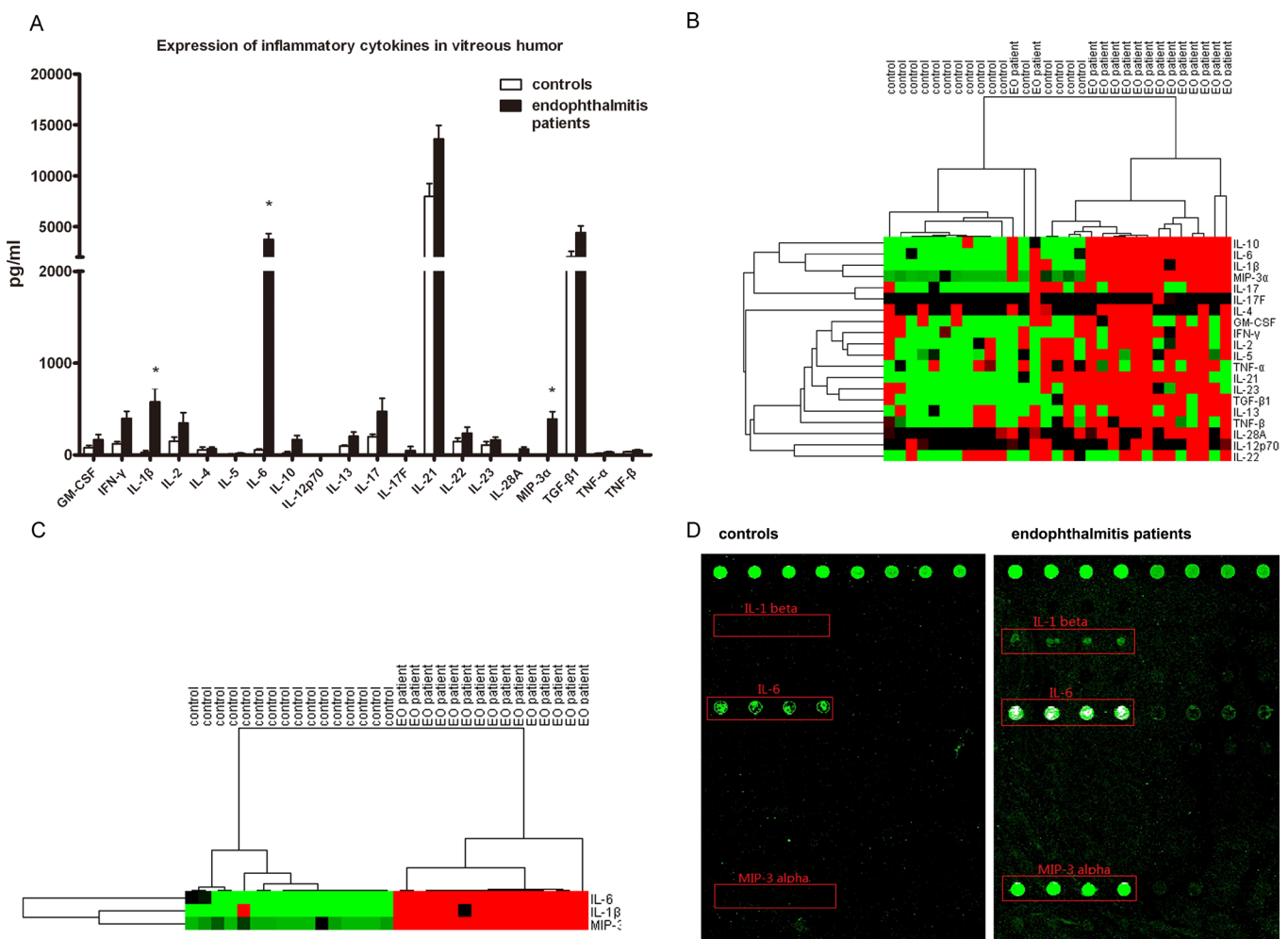

Figure 3. The cytokine profile in the VH from patients with endophthalmitis (n=15) and controls (n=16). **A**: The expression of 20 cytokines was detected using antibody cytokine array. The interleukin (IL)-1β, IL-6, and macrophage inflammatory protein (MIP)-3α levels were significantly upregulated in the patients with endophthalmitis compared to the healthy control samples. Data are represented as mean±SD *p<0.05. **B**: Heatmap of cluster analysis. Cluster analysis was performed with the 20 chosen cytokines. The horizontal bar on the left of the heatmap indicates the cytokine expression. **C**: Heatmap of cluster analysis of three of the 20 cytokines. The endophthalmitis group and the control group completely segregated into two principle clusters. **D**: Representative fluorescent signal images for the antibody array in the vitreous humor (VH). The additional cytokines are marked in red.

TABLE 3. CYTOKINE CONCENTRATIONS (PG/ML) IN VITREOUS HUMOR FROM ENDOPHTHALMITIS PATIENTS.

Name of target	Endophthalmitis (pg/ml)	Control (pg/ml)	Fold change	P value
GM-CSF	162.55	79.28	2.05	0.808
IFNγ	397.79	119.33	3.33	0.003
IL-1β	577.17	28.07	20.56	0.005
IL-2	346.16	145.76	2.37	0.125
IL-4	67.42	53.83	1.25	0.682
IL-5	18.48	10.15	1.82	0.461
IL-6	3740.67	53.97	69.31	<0.001
IL-10	164.85	0		0.008
IL-12p70	11.23	11.8	0.95	0.941
IL-13	201.85	94.78	2.13	0.048
IL-17	472.25	197.83	2.39	0.157
IL-17F	0	0	0.00	0
IL-21	13,614.87	7965.29	1.71	0.004
IL-22	233.61	151.26	1.54	0.32
IL-23	161.38	107.24	1.50	0.367
IL-28A	64.3	0		0.016
MIP-3α	392.94	0.75	523.92	<0.001
TGF-b1	4413.77	2026.59	2.18	0.011
TNFα	32.16	16.22	1.98	0.113
TNFβ	52.3	34.69	1.51	0.021

IFNγ=interferon gammar; IL=interleukin; MIP=Macrophage inflammatory protein; GM-CSF=granulocyte-macrophage colony-stimulating factor; TGF=transforming growth factor; TNF=tumor necrosis factor.

management is vitally important to prevent the occurrence of infectious endophthalmitis. Diagnostic bacterial culture of ocular samples, including AH, VH, and conjunctival tissue, is the best approach for diagnosis. In the literature, the positive culture rates vary from 24% to 95% [21]. The smear positivity of endophthalmitis is 52.5–76.6% [22,23]. Patients whose results culture and smear test results were positive were included in the present study to avoid potential bias. We combined the positive culture or smear test and open globe history and clinical manifestations to avoid false-positive results of pathogenic tests. In view of the low culture and smear positivity, there is a great need for a faster, more reliable diagnostic tool. Previously, only one report investigated the expression of cytokines in patients with endophthalmitis, focused only on IL-6, IFN-γ, TNF-α, and GM-CSF in the AH and indicated only the upregulation of IL-6 [24]. In this study, we proposed that determination of the inflammatory mediator profile in AH and VH from patients with endophthalmitis could potentially be that tool to meet this diagnostic need. We observed a consistent significant difference in the expression of several cytokines (significantly upregulated IL-1β, IL-6, IL-17, and MIP-3α and downregulated TGF-β,

IL-2, IL-21, and IL-5) in AH from the patients with endophthalmitis compared to the healthy controls. Hierarchical cluster analysis demonstrated the expression of an additional eight cytokines in AH that completely segregated between the patients with endophthalmitis and healthy control groups. This suggests a correlation between the specific expression of these cytokines and endophthalmitis. The elevated level of IL-6 in the AH from patients with endophthalmitis in this study was consistent with the results in the report by Feys et al. [24]. It has been reported that bacterial infection induces the release of proinflammatory cytokines (e.g., TNF-α and IL-1β), and these cytokines induce other proinflammatory cytokines (e.g., IL-6) [25]. This robust immune response induces a disturbance in the balance between pro- and anti-inflammatory cytokines. Accumulated evidence supports the idea that IL-6 exerts a dual role in type 1/type 2 helper (Th1/Th2) cell differentiation [26,27] and could be induced by infection and trauma leading to inflammation [28,29]. IL-6 could induce the production of IL-17A and IL-1β. In contrast, IL-6 suppresses TGF-β-induced Treg differentiation [30]. Several reports have indicated that the cytokines in endophthalmitis are secreted by macrophages, lymphocytes,

natural killers, endothelial cells, and other immune cells [10,31]. Toll-like receptor (TLR) signaling activation initiates host immune defense mechanisms against invasive microbes in humans as well as in nonhuman species. Singh et al. and Kumar et al. have demonstrated that TLR selective activation induces IL-1β and IL-6 upregulation in in vitro and in vivo animal model experiments [32,33]. This response is similar to the results in the AH and VH samples from patients with endophthalmitis identified in the current study, implying that TLR activation may contribute to increases in IL-1β and IL-6 expression in endophthalmitis. The production of IL-17A may further promote sustained production of inflammatory cytokines, such as IL-1β, TNF-α, IL-6, and the chemokine MIP-3α (CCL20), which strongly induces the migration of Th17 cells [34]. It has been demonstrated that MIP-3α secretion is dependent on IL-6 activation, which is dependent on the activation of nuclear factor-κB (NF-κB) and the signal transducer and activator of transcription 3 (STAT3) pathway [35]. Moreover, our results confirmed that the expression of TGF-β, IL-2, IL-21, and IL-5 was decreased in endophthalmitis AH. The

determination of the role of these cytokines requires further investigation in animal models of endophthalmitis.

Our vitreous data from the antibody array and cluster analysis demonstrated that the expression of IL-1β, IL-6, and MIP-3α was significantly elevated in patients with endophthalmitis and are consistent with the results of the AH data. Although absent in the reports of clinical trials, Petropoulos and Giese et al. also confirmed the upregulated expression of IL-1β and IL-6 in an endophthalmitis animal model [9,10]. To verify the accuracy of the quantitative cytokine antibody array, we then tested the cytokine concentrations using ELISA. The ELISA results further indicated that these three cytokines were specifically upregulated in AH and VH from the patients with endophthalmitis compared to the healthy controls. The additional cytokines identified by the antibody array were below detectable levels. However, the concentrations of certain cytokines, such as IL-21, were high using the quantitative antibody array assay, which was not consistent with the ELISA results, implying that the exact results require more than one method for verification. Moreover, MIP-3α was elevated in the AH and VH from patients with
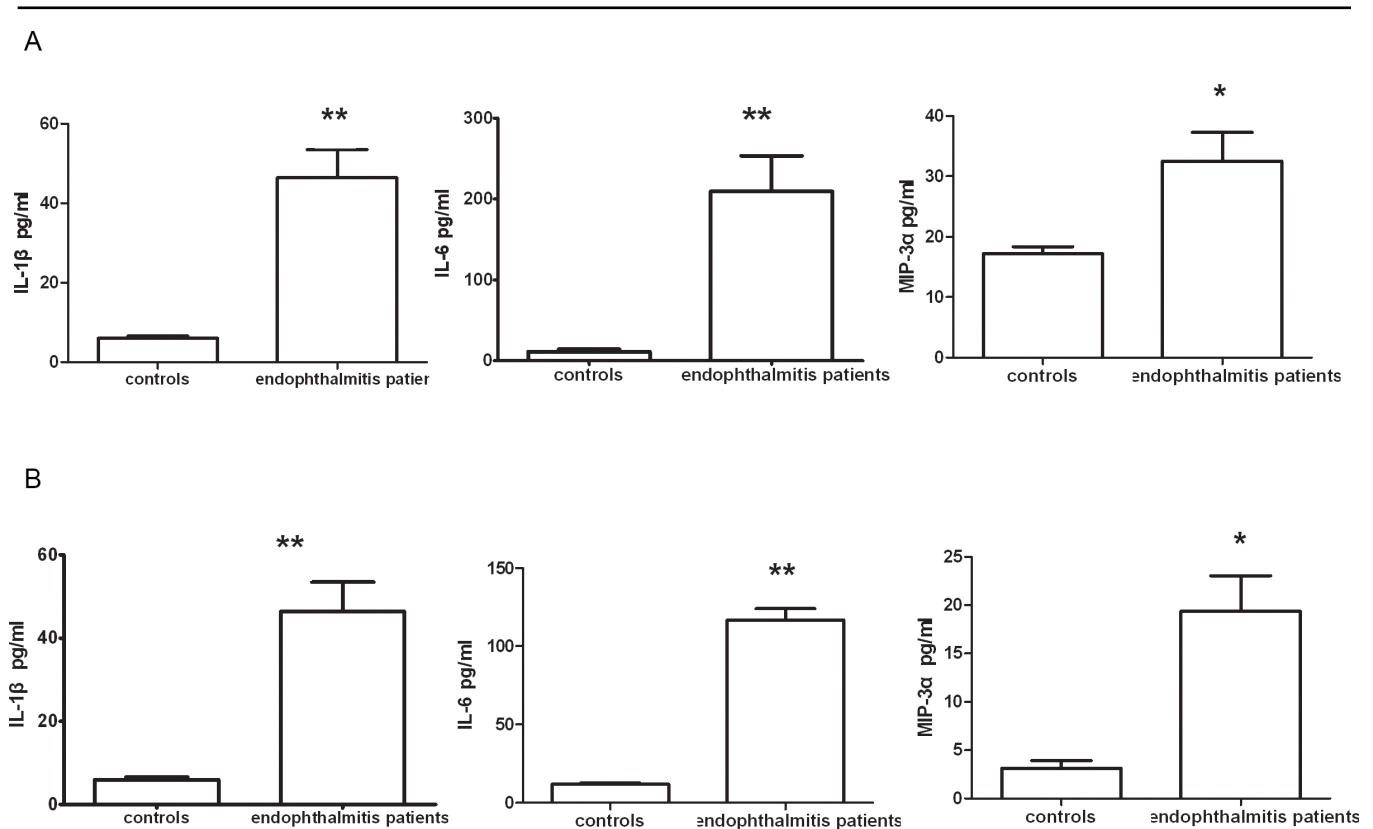

Figure 4. Validation analysis with ELISA of the AH and VH from patients with endophthalmitis (n=15) and healthy controls (n=16). **A**: The expression of interleukin (IL)-1β, IL-6, and macrophage inflammatory protein (MIP)-3α was significantly upregulated in the aqueous humor (AH) from patients with endophthalmitis. **B**: The production of IL-1β, IL-6, and MIP-3α was also elevated in vitreous humor (VH) from the patients compared to the health control samples. Data are represented as mean±SD *p<0.05, **p<0.001.

endophthalmitis and is a chemoattractant that induces IL-17 production [36,37]; IL-17 was significantly elevated only in AH. The levels of IL-17 in the VH from the patients with endophthalmitis (472.25±143.10) were also higher than those in from the healthy controls (197.83±25.69; the fold change of patients versus controls was 2.39), although there was no significant difference between the two groups. These results indicate that the elevation of IL-17 in the AH and VH from patients with endophthalmitis was in accordance with the upregulation of MIP-3α. These results suggest that IL-1β, IL-6, and MIP-3α may be potential biomarkers for endophthalmitis diagnosis.

In summary, all results from the antibody array and ELISA suggest that IL-1β, IL-6, and MIP-3α are potential biomarkers useful for endophthalmitis diagnosis. However, the roles and the associated mechanisms of the cytokine network involved in endophthalmitis require further investigation in animal models. Our study provides a new means for improving the diagnosis yield of endophthalmitis as well as other ocular inflammatory or infectious diseases and determining certain biomarkers for diagnosis and potential therapeutic strategies for the treatment of endophthalmitis.

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