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Toll-like receptors in acute post-cataract surgery endophthalmitis

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Abstract

Objective To evaluate the expression of toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4) on CD14 + cells in vitreous and blood of post-cataract surgery acute endophthalmitis.

Design This prospective case–control pilot study enrolled 16 patients of post-cataract surgery endophthalmitis. All the cases were subjected to 23 G pars plana vitrectomy (PPV). Ten patients undergoing 23 G PPV for non-infectious conditions were taken as controls.

Methods 23 G PPV was performed, and three undiluted vitreous samples were collected in heparinized syringes from the cases and the controls. Simultaneous venous blood sample was taken, and flow cytometry was performed to detect the expression of TLR2 and TLR4 in vitreous and blood samples. The vitreous and blood samples were incubated with fluorescein isothicyanate (FITC) conjugated anti-TLR2 monoclonal antibody Alexafluor (AX) 647 and anti-TLR4 monoclonal antibody phycoerythrin. Data acquisition was done on a pre-calibrated flow cytometer. TLR analysis of the acquired flow cytometry data was then performed. Mean channel fluorescence intensity (MFI) derived from fluorescence histogram was used to study the level of cell surface TLR expression. MFI was calculated as a ratio and recorded as the MFI of the TLR2 or -4 antibody divided by the MFI of the isotype-matched negative control antibody. Core vitrectomy was done as per the comfort of the surgeon, and intravitreal antibiotics vancomycin (1 mg/0.1 ml) and ceftazidime (2.25 mg/0.1 ml) were injected. The cytological examination was done on vitreous and blood sample.

Statistical analysis The median TLR 2 and TLR4 values between cases and controls were compared by Mann–Whitney U test. Spearman's rank correlation test was used to assess the correlation between TLR expression and disease activity.

Results Vitreous cytology evaluation showed the presence of neutrophils (81.25%, $n = 13$), monocytes (68.75%, $n = 11$) and lymphocytes (62.50%, $n = 10$). The level of expression of TLR2 in vitreous showed a statistically significant correlation with an increase in the time interval of cataract surgery and intervention for endophthalmitis ($p < 0.05$), but the same was not observed for TLR4. A drift toward higher level of expression of TLR2 and TLR4 in vitreous was observed in patients with poor outcome.

Conclusion TLR2 levels increase with the delay in presentation; thus, TLR2 ligands in vitreous could serve as a good target for the treatment of endophthalmitis.

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Keywords Endophthalmitis · Toll-like receptors · Innate immunity · Vitreous biopsy · Post-cataract surgery endophthalmitis · TLR-4 and TLR-2 · Vitreous cytology in endophthalmitis

Introduction

Postoperative endophthalmitis is one of the most dreaded complications after cataract surgery. In view of the poor prognosis of these cases [1], there has been an increasing interest to understand the basis of pathogenesis of the disease to improve the outcome by targeting the critical areas. It is well understood that in endophthalmitis it is not only the infection but the associated inflammation that leads to visual loss due to structural damage in the eye due to inflammation. Toll-like receptors (TLRs) play a pivotal role in the innate immunity. TLR has been implicated in the recognition of the microbial agent by pathogen-associated molecular patterns (PAMP) which further signals for the host defense responses against the pathogen. This involves the cascade of events involving inflammatory cytokines. The role of toll-like receptors in endophthalmitis is well defined in the experimental mice models, and it has been proven from the biopsy samples of the retinal tissues [2]. In real-life scenario, in endophthalmitis eyes, fragile retina makes the retinal biopsy technically challenging and impractical. The present study was planned to determine the in vivo level of expression of TLR 2 and 4 in the cells present in vitreous and the serum of the post-cataract surgery endophthalmitis. The hypothesis tested in the present study was that if TLR2 and 4 can be assessed from the vitreous samples of endophthalmitis patients, then accordingly these can be targeted in the treatment of endophthalmitis.

Material and methods

Study design

This was a single-center prospective pilot case–control study and was approved by the institutional research board and ethical committee. The informed consent was taken in all cases as per the principles of International Declaration of Helsinki. The study enrolled 16 eyes of 16 patients of endophthalmitis, occurring within six weeks after surgery for senile cataract, presenting to the Retina Clinic of a tertiary eye care centre. For the purpose of this study, post-cataract surgery endophthalmitis was defined as inflammation of posterior segment as evidenced by slit-lamp biomicroscopy, ophthalmoscopy and ultrasound, with or without hypopyon. All cases presented after 24 h of surgery and had localized corneal edema, unlike the one seen in TASS. All had vision $\leq$ logMAR 2.0 and were subjected to 23 G pars plana vitrectomy (PPV) with broad-spectrum intravitreal antibiotics as initial treatment. Ten patients undergoing 23 G PPV for non-infectious conditions like retinal detachment and macular hole, during the same duration, were taken as controls.

We excluded the eyes with baseline vision of no perception of light, complicated cataract, corneal oedema or keratitis precluding vitreous surgery, patients who underwent any additional surgery in the same eye during follow-up period, patients with retinal detachment or choroidal detachment and patients with immunocompromised diseases like diabetes mellitus, a history of cancer, prolonged use of steroids or other immunosuppressive drugs.

A detailed history was taken in all cases. The history was obtained regarding the time gap between the cataract surgery and the symptoms of endophthalmitis, and also the time gap between the surgery and the withdrawal of vitreous sample. The symptoms of endophthalmitis included pain, redness and decrease in vision. History of any systemic disease like diabetes mellitus, cancer, tuberculosis and use of corticosteroids was recorded. The detailed ophthalmological examination included thorough slit lamp biomicroscopy and indirect ophthalmoscopy with + 20 D lens. Visual Acuity assessment was done on the Snellen visual acuity chart, and the observed values were converted to logMAR scale for statistical analysis. If the participant could not identify the largest letter on the chart at 6 m (m), the distance was reduced to 1 m. If he/she could not still recognize the largest optotype, Visual acuity (VA) was classified using the semi-quantitative ordinal scale of counting fingers (CF), hand motions (HM), and perception of light (PL). HM and PL were noted at a distance of 30 cm. The vision of hand motions was done moving the hands in a vertical direction and horizontal direction. Similar reply 8 out of 10 times was taken as reliable.

LogMAR 2 to 2.6 was assigned to vision counting fingers at various distances of 1 to 6 m and log MAR 2.7 for vision HM and logMAR 2.8 for vision PL [3].

The anterior chamber reaction and flare evaluated according to Standard Uveitis Nomenclature (SUN) classification. The media clarity was graded from I–V using an indirect ophthalmoscope (IDO) as in EVS study [4].

Ultrasound *B* scan was done to confirm the vitreous involvement in all cases. All the eyes underwent 23 G PPV within 24 h of presentation to our hospital. At the start of the PPV, three undiluted vitreous samples of 0.5 ml each were collected under guarded aspiration in 1 cc heparinized syringes on the aspiration tubing of the cutter with air infusion in the posterior segment. The samples were taken to the microbiology, cytology and haematology laboratories immediately in syringe plugged with sterile rubber cork so as to ensure no contamination of the samples. A venous blood sample was also taken from the arm in sterile anticoagulant ethylene diamine tetra acetic acid (EDTA)-coated tubes and sent to the haematology laboratory for evaluation of TLR2 and TLR4 [5].

Core vitrectomy was done as per the comfort of the surgeon, and intravitreal antibiotics vancomycin (1 mg/0.1 ml) and ceftazidime (2.25 mg/0.1 ml) were injected. Postoperatively, all patients received oral tablet levofloxacin 500 mg for 5 days, topical eye drops vancomycin 5%, topical ceftazidime 5% and cycloplegics. Any other intraoperative and postoperative complications, if noted, were managed according to the standard protocol.

The cytological examination was done on vitreous and blood sample. Two smears were prepared from the vitreous sample. One smear was fixed in absolute alcohol and was subjected to Hematoxylin and Eosin staining. The other smear was air-dried and subjected to May Grunwald Giemsa staining. Smears were evaluated for the presence of neutrophils, monocytes and lymphocytes. A similar procedure was done for the blood sample.

Flow cytometry was performed on a freshly isolated blood sample and vitreous sample to investigate for TLR2 and TLR4 expression on Cluster of Differentiation positive (CD14 +) monocytes. 100 µl of the blood sample was incubated with anti-TLR2 monoclonal antibody conjugated with fluorescein isothicyanate(FITC) fluorescent dye Alexafluor (AX) 647 and anti-TLR4 monoclonal antibody conjugated with FITC dye PhycoErythrin (PE) for 30 min in dark. (Fig. 1) Further, 2 ml of flow cytometry lysing solution (FACS; BD Biosciences) was added and incubated for 10–12 min. The sample was spun at 1200 to 1300 rotations per minute for 3 to 5 min. The supernatant was discarded. The test tube with the remaining pellet was used for flow cytometry. Data acquisition was done on a pre-calibrated flow cytometer. A similar procedure was done for the vitreous sample also. Isotype-matched antibody controls were used to detect nonspecific staining (Fig. 2).

TLR analysis of the acquired flow cytometry data was then performed. The cytometry results were obtained in the form of fluorescence histogram. Monocytes were identified with the help of their characteristic forward and side-scatter flow cytometry profiles and CD14 (a monocyte marker). The histogram showed two peaks, blue colored which is the level of expression of TLR2 and TLR4 in vitreous/ blood depending upon the sample being analyzed (M1) and green colored which is isotype-matched antibody controls to detect nonspecific staining (M2). Mean channel fluorescence intensity (MFI) is calculated by the ratio of these two peaks [5]. MFI indirectly equals the level of receptor density on the cell surface. MFI of 1 represents no significant expression and MFI of more than 1 indicated higher expression of receptors.

The vitreous sample sent for microbiological evaluation, included smear examination with potassium hydroxide for fungus, gram staining for bacteria and culture for both bacteria and fungus. The smear

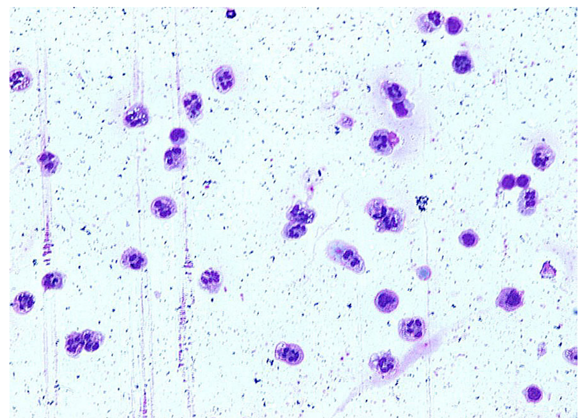

Fig. 1 Smear prepared from vitreous fluid showing neutrophils (red arrow head) and monocytes (green arrow head)Hematoxylin & Eosin, 400X maginification

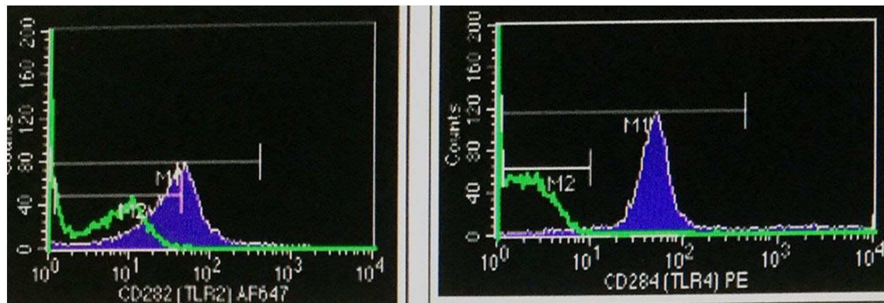

Fig. 2 Fluorescence histogram showing TLR2 (left) and TLR 4(right) expression in vitreous M1 represents level of expression in vitreous(blue peak) and M2 represents level of expression in isotype-matched antibody control (green peak). M1/M2 = MFI

and culture sensitivity reports were used to further guide the treatment. Laboratory confirmed growth was defined as at least semi-confluent growth on a solid medium, any growth on two or more media, or growth on one medium supported by positive gram stain [6].

Patients were followed-up as per the institute's protocol. At each visit to the hospital, the patient was subjected to a detailed anterior and posterior segment examination including visual acuity assessment. The eyes were examined for the visual outcome at three months, and the expression of TLR was correlated with the final outcome. A successful functional outcome was taken as pre-study defined criteria of best-corrected visual acuity (BCVA) $\geq$ 20/400 (logMAR value $\leq$ 1.3) [7], and a successful anatomical outcome was taken as preserved anatomy of the globe and no sign of active inflammation at follow-up.

Statistical analysis

The presence or absence of endophthalmitis was considered as the primary explanatory variable. TLR2 and TLR 4 status in vitreous and blood was considered as primary and secondary outcome variables, respectively. Age, gender, hypopyon, visual acuity at 3-month follow-up period were considered as other explanatory variables. Descriptive analysis of all the parameters was done using a median and interquartile range for quantitative variables, frequency and percentage for categorical variables. As the data obtained was skewed, the medians and distributions of MFI value of TLR 2 and TLR4 values between cases and controls were compared by Mann–Whitney U test and independent-sample median test. A similar comparison was done in TLR 2 and TLR 4 values between both genders and between people with good and poor visual outcome. The association between quantitative explanatory variables like visual acuity, hypopyon and TLR values was done by Spearman's rank correlation coefficient (r-value). An r value of $r < 0.31$ was taken as modest correlation, 0.32 to 0.55 was taken as moderate correlation, and > 0.55 was interpreted as strong correlation. P-value < 0.05 was considered statistically significant. IBM SPSS version 21 was used for statistical analysis (Fig. 3).

Results

The mean age of cases was 55.50 years (range 12.75–66.50 years) and in controls, it was 54.50 years (range 35.50–60.00 years). Both groups were age and sex-matched. The time interval between cataract surgery and vitreous sample withdrawal for endophthalmitis varied between 2.00 days to 2.00 months. (Mean value = 8.94 days). All patients had poor visual acuity and the details of the initial presentation are as in Tables1 and 2.

Vitreous cytology evaluation showed the presence of neutrophils (81.25%, n = 13), monocytes (68.75%, n = 11) and lymphocytes (62.50%, n = 10). The details of the MFI of TLR2 and TLR4 in vitreous and blood of cases and controls are shown in Table 3.

In vitreous, MFI of TLR2 in CD14 + cells was > 1 in 93.75% cases (n = 15) and < 1 in 6.66% (n = 1) cases (range −0.88 to 5.04, median value −1.22). The MFI of TLR4 in CD14 + cells in vitreous was > 1 in all 100% (n = 16) patients (range −1.01 to 9.74, median value −1.85).

In blood, MFI of TLR2 in CD 14 + cells was more than 1 in 100% (n = 16) cases (range-1.02 to 6.15, median value −1.26). MFI of TLR4 in CD 14 + cells

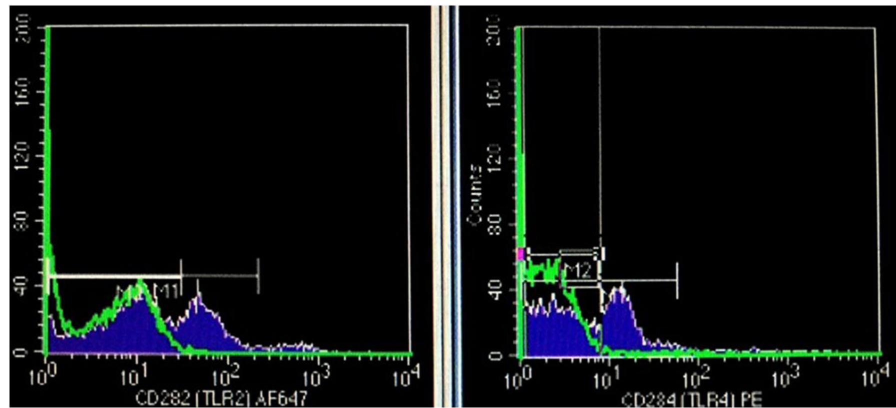

Fig. 3 Fluorescence histogram showing TLR2 (left) and TLR 4(right) expression in blood. M1 represents level of expression in blood (blue peak) and M2 represents level of expression in isotype matched antibody control (green peak). M1/M2 = MFI

Table 1 Baseline variables in study and control group

	Study group (%)	Control group
Visual acuity		
Light perception LP HMCF	2(12.5%)	0
CFCF	6(37.5%)	0
CFto20/200	8(50%)	0
> 20/200	0	1(10%)
	0	9(90%)
Anterior segment inflammation		
Cells + 4	16(100%)	0
Hypopyon	16(100%)	0
Media clarity		
≥ GradeII	0	10(100%)
GradeIII	3(18.8%)	0
GradeIV	13(81.2%)	0
Vitritis	16(100%)	0
Any systemic ailment (DM, cancer, uveitis)	0	0

in blood was > 1 in 87.5% cases ($n = 14$) and < 1 in 12.5% cases ($n = 2$) (0.65 to 17.14, median value-1.58) (Table 2). The median value of MFI of TLR2 and TLR 4 in CD 14 + cells of blood in cases and controls was comparable (Table 3, $p > 0.05$).

The level of expression of both TLR2 and TLR4 was not statistically significantly associated with the demographic profile of the patient. A weak correlation was present between the vitreous TLR2 and TLR4 with visual acuity at presentation ($r = 0.190$ and 0.498, respectively) which was not statistically significant (p-value = 0.498 and 0.804, respectively). The correlation between MFI of vitreous TLR2 and TLR 4 to other baseline variables was not statistically significant (0.456). There was a significant correlation between the time interval from cataract surgery to intervention for endophthalmitis with the level of expression of vitreous TLR2.($r = 0.577$) (p-value = 0.024). The correlation between MFI of vitreous TLR4 with the time interval from cataract surgery to intervention for endophthalmitis was weak ($r = 0.086$) which was not statistically significant (p-value = 0.761). (Table 4).

The microbiological evaluation of vitreous showed growth of organisms in 3 (18.75%) endophthalmitis patients. The organisms were methicillin-sensitive *Staphylococcus aureus* ($n = 1$) and *Staphylococcus epidermidis* ($n = 2$). Successful final functional outcome was seen in 50% ($n = 8$) of patients. The median value of MFI TLR2 and TLR4 in vitreous of endophthalmitis patients did not correlate with the visual outcome.

Table 2 TLR 2 and TLR 4 Mean fluorescence index in vitreous study group

	Age	Sex	Time interval between surgery and PPV	Visual acuity at presentation logMAR value	Microbial culture	N	M	L	MFI TLR2	MFI TLR4	Successful visual outcome VA $\geq$ 20/200	Successful anatomical outcome
1	52	F	7 days	2.7	No growth	+	+	+	1.11	2.16	no	yes
2	62	F	15 days	2.7	No growth	+	+	–	2.83	1.14	no	yes
3	21	M	2 days	2.7	No growth	+	+	+	1.28	2.14	yes	yes
4	55	F	4 days	2	No growth	+	+	+	1.18	2.61	yes	yes
5	60	F	6 days	2.3	*Staphylococcus aureus*	+	+	–	2.32	5.57	yes	yes
6	70	M	60 days	2.7	*Staphylococcus epidermidis*	+	+	+	4.41	4.17	no	yes
7	55	M	7 days	2	*Staphylococcus epidermidis*	+	+	+	1.27	1.39	yes	yes
8	48	F	7 days	2	No growth	+		+	1.25	4.7	no	yes
9	45	M	7 days	2.7	No growth		+		1.03	3.65	no	yes
10	68	M	5 days	2.8	No growth	+		+	1.06	1.47	yes	yes
11	56	F	4 Days	2.8	No growth	+	+	+	2.4	1.12	no	yes
12	50	F	4 days	2.7	No growth	+	+		1.6	5.04	no	yes
13	70	M	4 days	2	No growth		+	+	1.04	1.56	yes	yes
14	72	M	4 days	2.3	No growth	+	+	–	5.04	9.74	yes	yes
15	45	M	3 days	2.3	No growth	+	–	+	0.88	1.39	yes	yes
16	62	F	4 days	2	No growth	+	–	+	1.07	1.01	no	yes

Table 3 Comparison of TLR2 and TLR4 expression in blood between cases and controls was comparable

Parameter	Cases (N = 16)	Controls (N = 10)	Mann–Whitney U test	Independent sample median test
TLR2 MFI	1.26(1.02–6.15)	1.38(1.01 to 2.84)	0.856	1.00
TLR4 MFI	1.58(0.65 to 17.14)	2.54(0.94 to 7.98)	0.897	0.688

Table 4 Correlation between delay in presentation to vitreous TLR expression

Parameter	Correlation	P value
TLR2 MFI	0.577	**0.024**
TLR4 MFI	0.086	0.7611

Bold value indicates to signify the level of significance ($P < 0.05$)

Discussion

Postoperative endophthalmitis leads to the development of an inflammatory reaction by the host. This inflammatory response generated by the host is a double-edged sword. On the one hand, it is necessary to remove the infection, on the other, it is responsible for causing irreversible damage to the retina [8]. The inflammatory reaction is directly related to the amount of innate and adaptive immune responses generated. The most important step in the initiation of this immune response is the recognition of pathogen by TLRs present on cells associated with innate immunity like neutrophils and monocytes [9]. TLR recognize microbes by highly conserved biochemical structures called pathogen-associated molecular patterns (PAMP). TLR provides considerable specificity for a different class of pathogens. TLR 1,2,4,5 and 6 are present on the surface of cell and are involved in

recognition of PAMPs derived from bacteria, fungi and protozoa while TLR3, 7, 8 and 9 are intracellular and are involved in the recognition of viruses. The TLR-mediated signaling is a complex and less understood area. The cytokines and other mediators released in the blood stream after any localized inflammation may lead to upregulation of systemic TLR expression [10].

TLR2 has been linked with the recognition of gram-positive bacteria and TLR4 is recognized as a receptor for gram-negative bacteria [11].

The current treatment protocol for endophthalmitis involves the intravitreal injection of broad-spectrum antibiotics and pars plana vitrectomy (PPV). The only available anti-inflammatory treatment option is corticosteroids. There are conflicting reports on the role of corticosteroids in the management of fungal endophthalmitis which is not so rare in tropical countries [12, 13]. Main concerns are emerging resistance to antibiotics and lack of definitive treatment for inflammation-induced damage [14].

It is a well-known fact that few cells are present in the normal vitreous gel, predominantly in the cortex. These cells consist of hyalocytes, astrocytes and glial cells. Coupland et al. studied vitreous cytology and noted that abundant neutrophils in vitreous fluid cytology suggest bacterial (suppurative) endophthalmitis [15]. Thus, the cytological evaluation of vitreous is a valuable modality in diagnosing intraocular disease in adjunct to microbiological evaluation [16]. In the present study also, cytological evaluation of vitreous in endophthalmitis cases showed the presence of neutrophils (87%%, $n = 14$), monocytes (75%, $n = 12$) and lymphocytes (68.8%, $n = 11$).

In our study, the level of expression of TLR2 and TLR4 on CD14 + cells was evaluated by calculating MFI from fluorescence histogram. In vitreous TLR2 evaluation of endophthalmitis patients, MFI of more than 1 was seen in 93.75% of cases and MFI of TLR4 was observed to be more than 1 in 100% cases ($n = 16$). This result suggests that upregulation of TLR2 and TLR4 can be detected from the vitreous samples of endophthalmitis patients. To the best of our knowledge, this is the first study on the detection of TLRs from the human vitreous of endophthalmitis patients. Since the level of detection of TLRs in endophthalmitis cases is high, it may be possible that the neutrophils and monocytes were attracted to the site of infection. The negative culture results in the majority of the cases do not rule out endophthalmitis as there was a delay in inoculation of samples due to logistic reasons. In various studies, the culture positivity results in endophthalmitis seem to vary between 30–63% [17, 18]. The possibility of other microorganisms like fungi or slow growing micro-organisms cannot be excluded. Pan bacterial and pan fungal PCR could be complementary to cultures from the vitreous biopsy in clinical endophthalmitis cases.

With the added knowledge, more weapons can be added to our armor to control the damage caused by the induced inflammatory response. Every effort needs to be done to save the precious vision of the patients. More studies with larger sample size are needed to validate the above result.

Studies conducted by Kumar et al. in experimental mice models of *Staphylococcus aureus* endophthalmitis have shown that microglial cells (macrophages of central nervous system) have surface expression of TLR and with pre-endophthalmitis injection of TLR2 ligand, Pam3cys, and there was decreased inflammation and increased phagocytic response [2]. In experimental *Bacillus cereus*, endophthalmitis that retinal function was preserved to a greater degree in TLR2 and TLR4 deficient mice proving that TLRs are an important component of intraocular inflammatory response in endophthalmitis [16, 19]. The above studies suggest that mechanism of TLR2-mediated immune response in different organisms may be different and level of expression varies with timing of sampling. The role of TLRs and polymorphonuclear neutrophil infiltrations has also been established in fungal endophthalmitis [20].

To identify the systemic influence of endophthalmitis, the blood level of TLRs in CD14 + monocytes was evaluated. In blood, MFI of TLR2 was more than 1 in 100% patients and MFI of TLR4 was more than 1 in 87.5% of patients suggesting upregulation of receptors in blood. However, the blood levels of TLR in cases and controls were comparable. Chang et al. evaluated TLR2 and TLR4 polymorphism in blood neutrophils and monocytes of 9 patients with acute anterior uveitis (AAU) by flow cytometry [5]. They found significant reduction in the level of TLR2 expression in patients of AAU when compared with healthy controls. The 5 of 9 patients with AAU were having a systemic disease, and 7 patients were positive for HLA-B27. So, the possibility of specific alteration could be due to associated systemic disease.

The level of expression of TLR2 in vitreous showed a statistically significant correlation with an increase in the time interval of cataract surgery and intervention for endophthalmitis ($p < 0.05$), but the same was not observed for TLR4. The correlation of TLR with the delay in presentation is suggestive of the increasing inflammatory load and uncontrolled multiplication of invading pathogen with late presentation. This highlights the importance of early intervention in endophthalmitis cases. Further studies are needed to understand the basis of the differential response of TLR2 and TLR4.

To compare if there is any selective perturbation in the level of expression of TLRs, ten age and gender-matched control patients were taken who were undergoing vitrectomy for causes other than an infection. TLR status in vitreous of control patients was not evaluated due to acellularity of the sample. In the blood of control patients, MFI of TLR2 was more than 1 in all patients and MFI of LTR4 was more than 1 in 90% of patients. The elevation of TLR in peripheral blood has been proposed in many non-infective diseases like neurological diseases, glial damage, diabetes mellitus and heart diseases, etc.[21, 22].The understanding of the TLR upregulation in non-infective diseases is still in its infancy. We did not specifically exclude systemic co-morbidities in our control group, and this could explain the presence of TLR in the control group.

The varying level of TLR expression in vitreous may depend upon the precise timing of vitreous sampling, and there may be a dynamic change in the level of TLR expression with the evolution of the disease. The findings of the present study implicate TLR activation in the pathogenesis of endophthalmitis. Further studies involving a greater number of patients are needed to confirm and expand the present findings.

Study limitations

1. The study was conducted on small sample size.
2. The poor culture positivity in our study can be due to non-availability of immediate inoculation of vitreous sample for microbiological evaluation.
3. The timing of vitreous sampling after acquiring the infection was variable. In the control group, the patients with any systemic disease were not excluded which can influence the level of expression of TLRs in blood.

Funding This study was not funded by any other agency.

Compliance with ethical standards

Conflict of interest None of the authors has any conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. (cleared by institutional review board, IEC Regd No. ECR/658/Inst/PB/2014/RR-2017).

Informed consent
Informed consent was obtained from all individual participants included in the study.

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