Article

Multidrug-Resistant *Pseudomonas aeruginosa* Triggers Differential Inflammatory Response in Patients With Endophthalmitis

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Purpose: Infections with multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA) lead to poor clinical outcomes in endophthalmitis patients, and its interactions with the host immune system remain largely unknown. The current study aimed to determine the association of MDR-PA infection with the cytokine expression profile in patients with endophthalmitis.

Methods: Vitreous of 12 patients with culture-proven MDR-PA along with 12 samples from antibiotic-susceptible *P. aeruginosa* (S-PA) and 20 non-infectious controls were included in the study. Expression patterns of IL-6, IL-10, IL-1α, IL-1β, IFN-γ, TNF-α, IL-8, and GM-CSF in the vitreous were analyzed by multiplex immunoassay and correlated with the clinical severity. We also assessed the phosphorylation level of different immune pathway molecules.

Results: In the MDR-PA group, significantly ($P < 0.05$) increased expression of IL-6, IL-8, IL-10, IL-1β, and TNF-α was observed in comparison with the S-PA group. The increased inflammatory mediators in MDR-PA correlated with the disease severity. Additionally, the increased expression of inflammatory mediators was positively correlated to the activation levels of Akt, STAT3, JNK, p70 S6 kinase, and NF-κB ($P < 0.05$) in the MDR-PA group.

Conclusions: The current study shows the differential host immune response and phosphorylation levels of signaling molecules in MDR-PA endophthalmitis, thereby linking antibiotic resistance with distinct immune regulation.

Translational Relevance: This study provides evidence for the use of inflammatory mediator levels of IL-6, IL-8, IL-10, IL-1β, and TNF-α as potential diagnostic biomarkers of MDR endophthalmitis warranting prompt administration of immune modulators to avoid irreversible damage to the retina and vision loss.

Introduction

The rise in multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA) endophthalmitis across the globe, especially in India and Southeast Asia is a major concern among ophthalmologists and threatens our ability to treat and manage the fulminant clinical course associated with this organism.[1,2] An earlier study at our institute revealed *P. aeruginosa* to be the main etiology in nearly 72% of the multidrug-resistant bacterial endophthalmitis cases diagnosed over 12 years.[2] The poor outcome in these patients can be attributed not only to the production of PA virulence factors but also to the interaction between the bacteria and the host's innate immune system.[3] Considerable studies have shown that the severity of endophthalmitis is strongly correlated with

intraocular inflammation, characterized by the infiltration of neutrophils and the production of inflammatory cytokines.[4–8] An earlier investigation by our group on retinal cells highlighted an exacerbated response of interleukin (IL)-6, IL-1α, IL-8, IL-10, IL-1β, tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) in retinal and microglial cells exposed to MDR strains,[9] suggesting that MDR-PA infections have a more virulent behavior, and this excessive inflammatory response may hamper resolution of the infection.

Activation of the responses of several cytokines and chemokines is accompanied by the activation of multiple intracellular signaling cascades.[10] Innate recognition activation of associated pathways is a complex event requiring coordinate regulation of multiple cellular signaling components.[11] To date, several studies on severe pneumonia[12–14] and sepsis[15,16] have evaluated the inflammatory response; however, data regarding the inflammatory response in PA endophthalmitis are limited.[9] In fact, the issue has not been examined in human vitreous fluids, nor has the possible correlation of multidrug resistance with the ability of pathogens to elicit an immune response and subsequent induced inflammatory sequelae been assessed. Targeting the inflammatory pathways could be a viable immune-modulatory approach that might help in preserving the retinal function. The purpose of this study was to validate the differential response of previously reported immune mediators and to correlate this expression with the phosphorylated signaling intermediates in MDR-PA endophthalmitis in human vitreous samples. We also correlated these expressions with *P. aeruginosa* clinical outcomes and attempted to predict MDR infection. This study may help us refine the use of therapeutics or it may lead to new strategies for controlling cytokine-mediated damage in endophthalmitis.

Methods

Study Design and Collection of Samples

Patients were selected prospectively with a clinical diagnosis consistent with presumed infectious endophthalmitis seen at the Retina Clinic of the L V Prasad Eye Institute between September 2018 and October 2020. This study was conducted according to the tenets of the Declaration of Helsinki, and prior consent was obtained. The study design and protocol were approved by the Institutional Review Board of the L V Prasad Eye Institute. Patients were excluded if the vitreous sample was inadequate after routine microbiology workup. The study population included 24 patients diagnosed clinically with microbiologi-

cally proven *P. aeruginosa* endophthalmitis; of these patients, 12 cases were found to be multidrug resistant to almost all antibiotics tested. In addition, 20 non-infectious controls were patients undergoing posterior vitrectomy for idiopathic macular holes. All diagnosed patients underwent complete ophthalmological examinations, including B-scans, slit-lamp biomicroscopy, and visual acuity (VA) measurements. The best-corrected visual acuity measurements were converted to the logarithm of the minimum angle of resolution (logMAR)[17] for comparative analysis. The initial VA was recorded on the same day of the endophthalmitis diagnosis and the final VA after 6 months of follow-up. The vitreous samples from both patients and controls obtained during vitrectomy were collected aseptically and sent to the laboratory for microbiological workup; 50 μL of each sample was transferred into a presterilized microcentrifuge tube and stored at –80°C for further analysis.

Microbiological Work-Up and Antibacterial Susceptibility Testing

Vitreous samples from patients were investigated using an institutional protocol as described earlier.[18] Briefly, the sample was inoculated onto an array of bacterial and fungal media, which were incubated at 37°C and 25°C, respectively, for 7 days. Following growth on culture media, *P. aeruginosa* strains were identified using a VITEK 2 (bioMérieux, Craponne, France) after confirmation by biochemical tests. For antibiotic susceptibility testing, minimum inhibitory concentration was determined using E-test strips (HiMedia, Mumbai, India) or VITEK 2 antibiotic susceptibility testing cards according to the manufacturer's protocol.[19] Antibiotics tested included ciprofloxacin, moxifloxacin, gatifloxacin, ofloaxacin, ceftazidime, gentamicin, tetracycline, amikacin, tobramycin, piperacillin, norfloxacin, colistin, and imipenem. All results were compared to the Clinical and Laboratory Standards Institute interpretative guidelines, and the isolates were classified as susceptible or multidrug-resistant.[34] Multiple drug-resistant phenotypes were assigned for strains that were resistant to three or more classes of antibiotics.

Multiplex Immunoassay

Multiplex proteomic analysis was used to measure the levels of inflammatory cytokines, chemokines, and growth factors in the vitreous samples. The MILLIPLEX Human Cytokine/Chemokine/Growth Factor Panel A–Immunology Multiplex Assay

(HCYTA-60K-08; MilliporeSigma, Burlington, MA) was used for the MILLIPLEX enzyme-linked immunosorbent assay (ELISA). The concentrations of eight human mediators (IL-1β, IL-1α, IL-6, IL-8, IL-10, IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor [GM-CSF]) were measured using a MAGPIX multiplex assay instrument (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Standard curves of known concentrations of recombinant human cytokines (R&D Systems, Minneapolis, MN) were used to convert fluorescence units to cytokine concentration (pg/mL). The protein concentration of each sample was determined using the bicinchoninic acid (BCA) method (G-Biosciences, St. Louis, MO).

Cell Signaling Mediators

Each sample was processed using cell lysis buffer with phosphatase and protease inhibitors (Millipore-Sigma) and analyzed according to the assay protocol. The protein concentration of the cell lysate was measured using a BCA assay (G-Biosciences) and the MILLIPLEX MAP Multi-Pathway Magnetic Bead 9-Plex–Cell Signaling Multiplex Assay (48-681MAG; MilliporeSigma). The following probes were included in the kit: extracellular signal-regulated kinase/mitogen-activated protein kinase 1/2 (ERK/MAPK 1/2; Thr185/Tyr187), protein kinase B (Akt; Ser473), signal transducer and activator of transcription 3 (STAT3; Ser727), c-Jun N-terminal kinase (JNK; Thr183/Tyr185), ribosomal protein S6 kinase beta (p70S6K; Thr412), nuclear factor-kappa B (NF-κB; Ser536), signal transducer and activator of transcription 5 (STAT5A/B; Tyr694/699), cAMP-response element binding protein (CREB; Ser133), and p38 mitogen-activated protein kinases (p38; Thr180/Tyr182). The assay was carried out following the manufacturer's protocol, and fluorescence was assessed using xMAP technology on the MAGPIX platform (Luminex, Austin, TX). Mean fluorescent intensities were obtained in triplicate for each sample and normalized to total protein content.

PCA and Heat-Map Cluster Analysis

The PCA plots were generated using R Script (R Foundation for Statistical Computing, Vienna, Austria), and heat maps were generated using Prism 9.0.0 (GraphPad, San Diego, CA) based on differentially expressed inflammatory mediators (IL-6, IL-8, IL-10, IL-1β, and TNF-α) and cell signaling mediators (Akt, STAT3, JNK, p70 S6 kinase, and NF-κB).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 9. Results were analyzed statistically using the Kruskal–Wallis test with appropriate post hoc analysis because of the skewed distribution of the data. The cytokine and phosphorylated levels in each group were compared using the Mann–Whitney U test. To analyze correlations among variables, Pearson's correlation test was used. The quantitative variables were expressed by the mean and standard error of the mean. Categorical variables were compared between groups using Pearson's χ^2 test or Fisher's exact test. A two-tailed $P < 0.05$ was considered statistically significant. R Script was used to construct box-and-whisker plots. Data regarding cytokine and pathway mediators are presented as individual points with a bar representing the median value.

Results

Clinical Characteristics of the Study Population

We recruited a total of 44 patients, including 24 patients with culture-proven *P. aeruginosa* endophthalmitis and 20 non-infectious controls. The mean age of all patients was 49.54 years (range, 8–81); 26 were male and 18 were female. The study group included 12 MDR-PA strains and 12 *P. aeruginosa* susceptible (S-PA) strains. Out of the 12 MDR-PA strains, eight were extensively drug resistant to all antibiotics tested, and the rest were resistant to at least five classes of antibiotics (ceftazidime, cefepime, tigecycline, ticarcillin, and piperacillin/tazobactam). The clinical and other demographic details are included in Table 1. Although final VA ranged from no light perception to 20/50 in the study group, only one patient from the MDR-PA group (8%) and two patients from the S-PA group (16.6%) had favorable outcomes (>20/200). Two patients in both groups did not respond to treatment and underwent evisceration. The number of surgical interventions for the resolution of infection was higher in MDR-PA infections, although the difference was not statistically significant.

Elevated Levels of Inflammatory Mediators in MDR-PA Endophthalmitis

Previously, we have shown that in vitro *P. aeruginosa* infection in human microglia and retinal pigment epithelial cells elicits a robust proinflammatory immune response. Here, we tested the ability of MDR-PA and

Table 1. Clinical and Demographic Details of All Patients Included in the Study Group

Characteristic	Controls (n = 20)	P. aeruginosa (n = 24)		P
		MDR-PA (n = 12)	S-PA (n = 12)	
Age (yr), median ± SD (range)	46.14 ± 19.43 (9–71)	60.41 ± 9.01 (39–73)	52.33 ± 19.31 (8–81)	0.12[a]
Gender (male:female), n	12:8	6:6	8:4	0.70[b]
Diagnosis, n				
Postoperative (cataract surgery)	—	12	6	—
Traumatic	—	—	4	
Endogenous	—	—	2	
Macular hole/macular edema	18	—	—	
Rhegmatogenous retinal detachment	2	—	—	
Initial visual acuity (logMar)				
0.6–2	13	—	—	—
2.6	—	4	5	
3.2	7	8	7	
Final visual acuity (logMar)				
0.4–1.3	11	2	1	—
2–2.9	2	1	1	
3–3.2	7	7	8	
Evisceration, n	—	2	2	
Inflammation/polymorphs (vitreous), n				
0–3	—	2	4	—
0–5	—	2	3	
0–10/0–plenty	—	8	5	
Surgical interventions, n				
<2	19	3	7	—
≥2	1	9	5	

[a]Student's *t*-test.
[b]Pearson's χ^2 test.

S-PA to induce the immune response in the vitreous fluid of endophthalmitis patients. We used a multiplex approach to capture the broader range of inflammatory mediators. All of the assays were performed in duplicate, and a mean value was obtained for each data point. The duplicate values for all the inflammatory mediators are provided in Supplementary Table S1. Multi-group statistical analysis using the Kruskal–Wallis test with appropriate post hoc analysis revealed significant differences in the levels of IL-1β, IL-10 IL-6, IL-8, and TNF-α inflammatory mediators between the MDR-PA and S-PA groups ($P < 0.05$) in the vitreous fluids of patients with endophthalmitis. However, IL-1β and IL-10 were below the detection limit in the case of the non-infectious control group, and the Mann–Whitney U test was used to calculate the difference. The R^2 value of all of the standard curves was 0.98 to 1.

Most notably, there was a significant increase in levels of IL-10 (3539.48 ± 1232.94 vs. 293.50 ± 89.11 pg/mL; $P = 0.0005$), IL-6 (19,744.05 ± 4001.07 vs. 6319.85 ± 1444.64 pg/mL; $P = 0.004$), IL-8 (10,633.41 ± 72391.85 vs. 2915.3 ± 661.32 pg/mL; $P = 0.0005$), and IL-1β (24,219.04 ± 7600.23 vs. 4966.86 ± 901.28 pg/mL; $P = 0.01$) in vitreous fluids of patients with MDR-PA endophthalmitis compared with patients with S-PA endophthalmitis. Similarly, a significant increase in the concentration of TNF-α was observed in the samples in the MDR-PA group compared with the S-PA group (2199.22 ± 643.92 vs. 193.77 ± 54.12 pg/mL; $P = 0.0005$) (Fig. 1). Correlating with concentrations in the S-PA group, the levels of IL-10, IL-6, IL-8, IL-1β, and TNF-α in MDR-PA were, respectively, about 12-fold, 5-fold, 3.6-fold, 4.8-fold, and 11.3-fold higher, respectively, as shown in Figure 1.

Changes in IFN-γ, GM-CSF, and IL-1α concentrations were not significant in either group following post hoc analysis; however, the mean concentrations of IFN-γ (729.21 ± 333.08 vs. 83.81 ± 27.08 pg/mL) and IL-1α (96,890.19 ± 60,407.81 vs. 21,620.28 ± 6598.78 pg/mL) were higher for the MDR-PA group
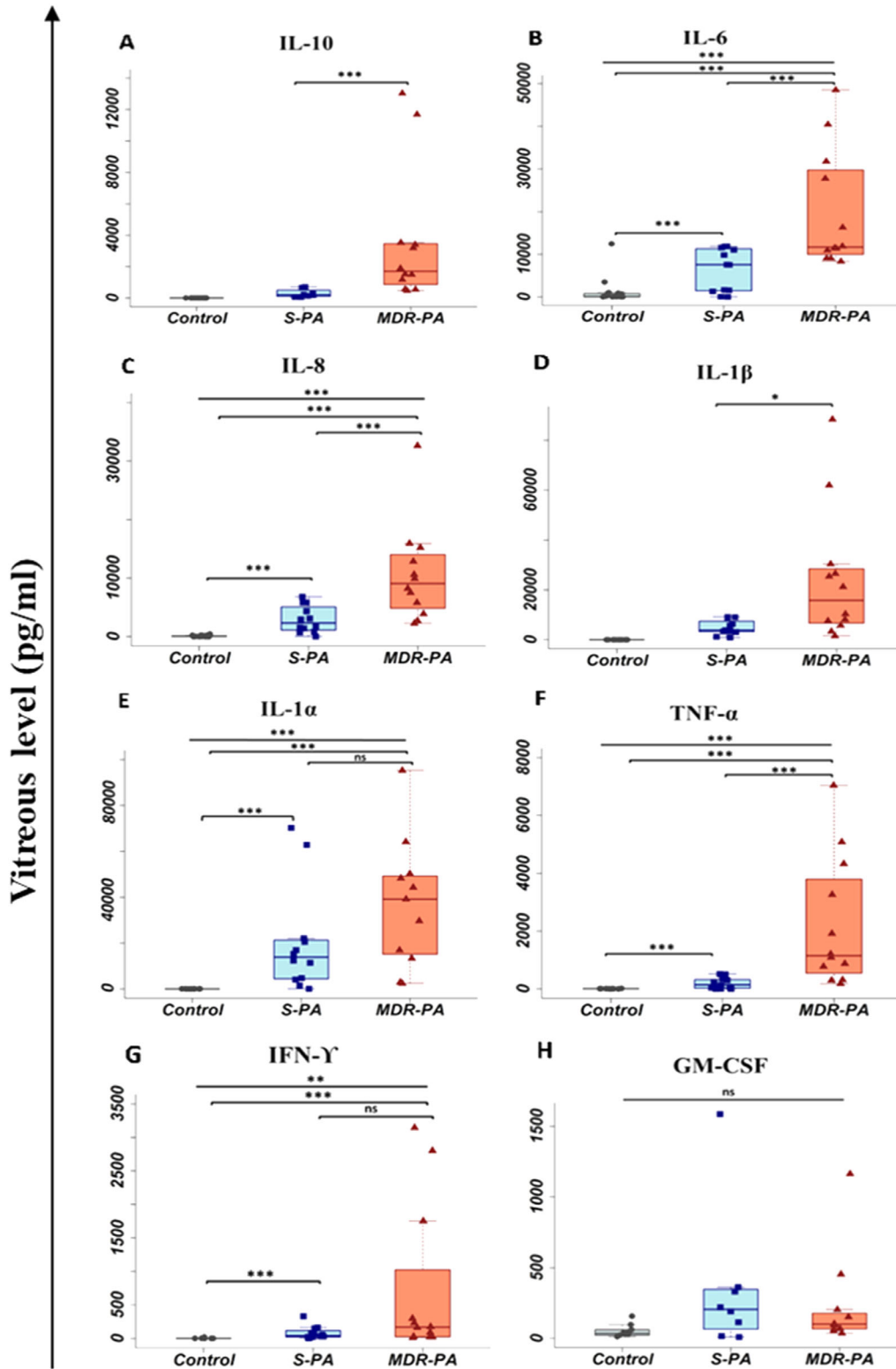
Figure 1. Comparison of the level of immune mediators in human vitreous infected with MDR-PA and S-PA. Box-and-whisker jitter plots show the expression levels of IL-10 (A), IL-6 (B), IL-8 (C), IL-1β(D), TNF-α (E), IL-1α (F), IFN- γ (G), and GM-CSF (H) in the vitreous of patients with macular hole (controls) and patients with *P. aeruginosa* endophthalmitis caused by S-PA and MDR-PA strains. The concentrations of inflammatory mediators were compared among the above-mentioned three groups by Kruskal–Wallis tests with appropriate post hoc analysis. In the case of IL-1β (D) and IL-10 (A), the Mann–Whitney *U* test was used. Significant *P* values are as shown in each graph. $P < 0.05$ was considered significant. All of the assays were performed in duplicate, and a mean value was taken for each data point. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

Table 2. Comparison of Various Inflammatory Mediators Expressed in the MDR-PA and S-PA Endophthalmitis Groups by Multiplex ELISA

Markers	Study Group	Concentration Range (pg/mL)	Detection, n (%)	Group Comparison	Dunn–Bonferroni P
IL-1α	Controls (A)	4.33–53.84	13 (65)	A vs. B	**<0.001**
	S-PA (B)	62.1–70219.7	12 (100)	A vs. C	**<0.001**
	MDR-PA (C)	2420.3–755928.6	12 (100)	B vs. C	0.27
IL-1β	Controls (A)	—	—	A vs. B	—
	S-PA (B)	907.8–9127.3	11 (91.6)	A vs. C	—
	MDR-PA (C)	1521–88434	12 (100)	B vs. C	**0.02**
IL-10	Controls (A)	—	—	A vs. B	—
	S-PA (B)	56.1–700.6	8 (66.6)	A vs. C	—
	MDR-PA (C)	467.4–13034	12 (100)	B vs. C	**0.002**
IL-8	Controls (A)	0.5–417.6	20 (100)	A vs. B	**0.002**
	S-PA (B)	18.4–6809.8	12 (100)	A vs. C	**<0.001**
	MDR-PA (C)	2270.5–32635.2	12 (100)	B vs. C	**0.05**
IL-6	Controls (A)	1.3–12457.3	20 (100)	A vs. B	**0.02**
	S-PA (B)	12.6–11877.3	12 (100)	A vs. C	**<0.001**
	MDR-PA (C)	8319.3–48507.2	12 (100)	B vs. C	**0.04**
TNF-α	Controls (A)	1–16	10 (50)	A vs. B	**0.02**
	S-PA (B)	6–517	12 (100)	A vs. C	**<0.001**
	MDR-PA (C)	180–7039	12 (100)	B vs. C	**0.01**
IFN-γ	Controls (A)	1.3–19.4	6 (30)	A vs. B	**0.02**
	S-PA (B)	4.1–331.6	12 (100)	A vs. C	**0.001**
	MDR-PA (C)	12.2–3144.6	12 (100)	B vs. C	0.22
GM-CSF	Controls (A)	13.4–157.1	10 (50)	A vs. B	0.08
	S-PA (B)	9.9–1585.7	8 (66.6)	A vs. C	0.08
	MDR-PA (C)	37.5–1163.4	11 (91.6)	B vs. C	0.75

Data represent the average values of duplicate experiments.

when compared with the S-PA group. Only GM-CSF levels (227.06 ± 100.01 vs. 354.35 ± 181.825 pg/mL) were found to be higher in the S-PA group, and there was no statistical difference found for comparisons between the controls and the MDR-PA group. Pro-inflammatory mediators, as well as GM-CSF and chemokine, were significantly elevated as compared with all controls. Table 2 summarizes the concentrations of all seven inflammatory mediators and one stimulating factor of all 44 patients recruited in this study. The difference in vitreous inflammatory mediator concentrations in the MDR-PA group did not, however, correlate with initial VA or overall prognosis.

MDR-PA Induces Higher Phosphorylation of the Pathway Mediators

We further wanted to determine if there was an association between these inflammatory mediators and the phosphorylation level of the downstream signaling cascades in vitreous lysates. After comparison, we found that the mean fluorescence intensities of phosphorylated Akt (8184 ± 5155 vs. 267.1 ± 51.45; $P = 0.007$), STAT3 (8139 ± 5428 vs. 323.8 ± 87.26; $P = 0.02$), JNK (7105 ± 4173 vs. 209.4 ± 35.94; $P = 0.006$), p70 S6 kinase (10749 ± 6209 vs. 359.0 ± 87.82, $P = 0.04$), and NF-κB (5331 ± 3862 vs. 2972 ± 1980, $P = 0.05$) in vitreous lysates were significantly higher in the MDR-PA group (Fig. 2). These experimental data suggest that MDR-PA induces Akt, STAT3, JNK, NF-κB, and p70S6 kinase activation in human vitreous to a significantly greater extent than S-PA after infection. Although the exact mechanisms remain unresolved, future experiments will aim to uncover the intricacies involved in this response.

Principal Component and Heat-Map Analysis Identified a Distinct Group of Cytokine and Pathway Mediators Associated with MDR-PA–Induced Endophthalmitis

Next, we generated a principal component analysis (PCA) using the concentrations of vitreous cytokines
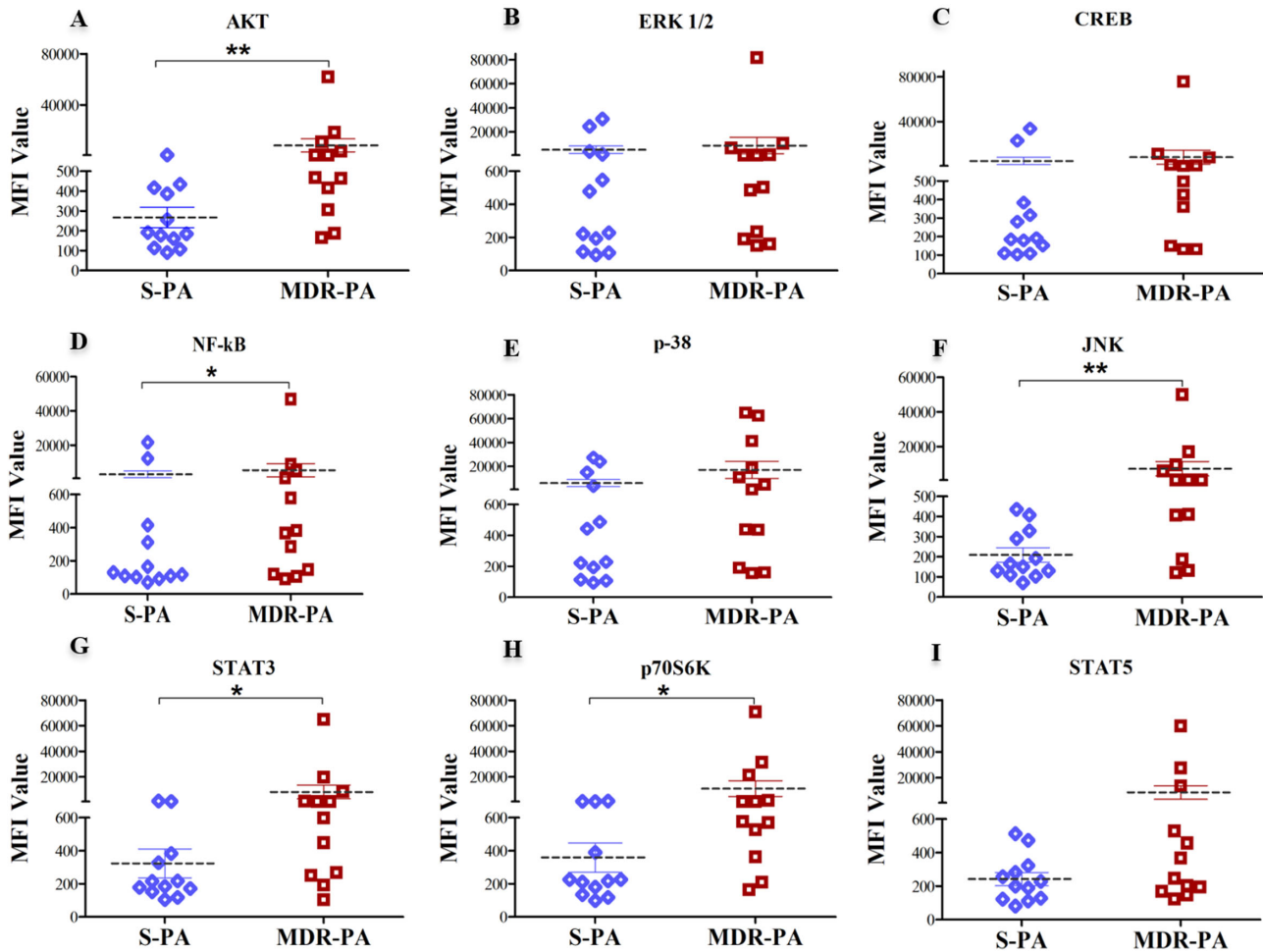
Figure 2. Quantitative analysis of phosphorylation of pathway molecules infected with MDR-PA and S-PA. Shown is multiplex ELISA quantification of phosphorylated multi-pathway molecules in MDR-PA– and S-PA–infected vitreous lysates of patients ($n = 12$ in each group). The lysates were prepared using a suitable lysis buffer with protease and phosphatase inhibitors. Stimulated cell lysates were used as a positive control, whereas unstimulated cell lysates were used as negative controls for the experiment. The mean fluorescence intensities of phosphorylated pathway molecules were compared between the MDR-PA and S-PA groups by the Mann–Whitney *U* test. Significant *P* values are noted in the upper part of each graph. $P < 0.05$ was considered significant. All of the assays were performed in duplicate, and a mean value was taken for each data point. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

(IL-10, IL-6, IL-8, IL-1β, and TNF-α) and pathway molecules (Akt, STAT3, JNK, p70 S6 kinase, and NF-κB). As shown in Figures 3A and 3B, we did observe distinct clustering of the aforementioned cytokines and the pathways in comparisons between the two study groups. PCA illustrated that 75% of the total variance in response to five inflammatory mediators and five phosphorylated pathway molecules was expressed by two principal components. For the plot of the inflammatory mediators, the first component accounted for a total of 65.66%, and the second accounted for 20.99% of the total variance (Fig. 3A). PCA plots of cytokine concentrations revealed that a total of eight patients out of 12 patients infected with MDR-PA were clustered separately. The S-PA–infected

patients were clustered together and showed a positive correlation; they could easily be differentiated from the group infected with MDR-PA. In the case of pathway molecules, the first component accounted for a total of 51.09%, and the second accounted for 46.86% of the total variance (Fig. 3B); two of the S-PA–infected patients were clustered along with the MDR-PA group. Altogether, the cytokines were better able to discriminate MDR-PA and S-PA distinctively, whereas the pathway mediators showed minimal overlap between the clusters. The heat maps summarize the concentrations of the five significantly different inflammatory mediators and pathway molecules identified in all 24 patients in this study. The concentration levels of the five cytokines in the vitreous were able
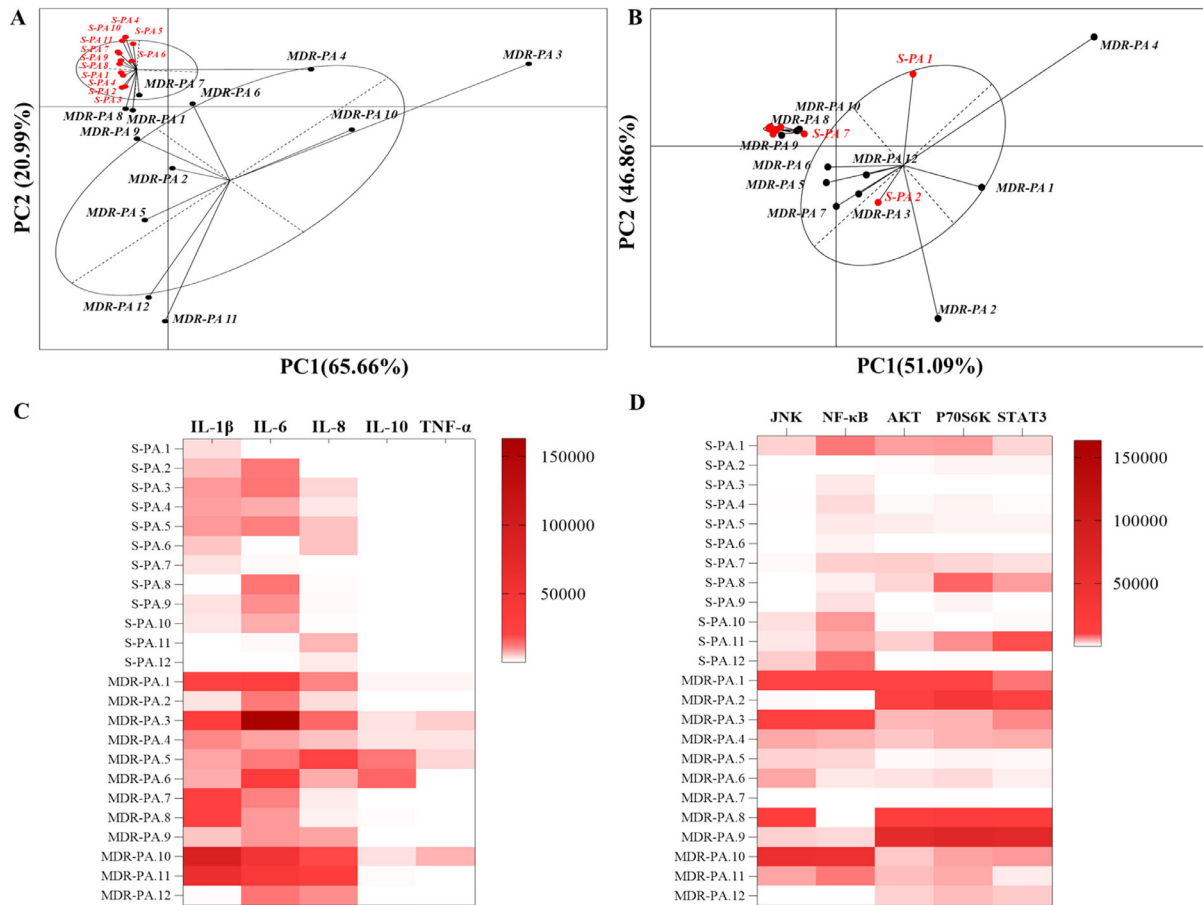
Figure 3. PCA and heat map depicting significant differentially expressed cytokines and pathway molecules. Shown are PCA plots of the concentrations of cytokine and pathway molecule concentration of cytokines and pathway molecules in MDR-PA– and S-PA–infected individuals, where the *x*-axis and *y*-axis represent the first and second principal components, respectively. The PCA represents the two principal components of variation. A two-dimensional representation is given by the two first principal components, with 65.66% and 20.99% (**A**) and with 51.09% and 46.86% variation explained (**B**). The differently clustered MDR-PA infected samples are labeled. Schematic heat maps represent the cytokine (**C**) and pathway molecule (**D**) concentrations in MDR-PA–infected and S-PA–infected individuals. All of the cytokines and pathway molecules presented in the heat maps differed significantly ($P < 0.05$). The rows represent patients, and the columns represent cytokines. The *red* and *white color gradients* represent the upregulation and downregulation of genes. *Red* indicates increased and *white* represents reduced levels.

to discriminate between S-PA–infected patients and MDR-PA–infected patients.

Discussion

The rapid and dangerous emergence of MDR bacteria is a major concern and has pushed the research community to invest greater effort in studying these bacteria and novel therapeutics. Recent reports show that pathogenicity and virulence can be remarkably different between MDR and non-MDR strains in systemic infections.[20] There have been reports of vancomycin-resistant enterococci, which are associated with serious MDR infections and persistent colonization due to evasion from the host's immune response and reduced inflammation.[21] Here, in the vitreous of unique clinical patients, we show for the first time, to our knowledge, that there is an exacerbated inflammatory response of IL-10, IL-6, IL-8, IL-1β, and TNF-α in MDR-PA endophthalmitis compared with infection by S-PA, suggesting a possible relation between non-response to antibiotic treatment and inflammation-causing irreversible damage to the retina.[22] Our results are in concordance with findings in conditions such as MDR tuberculosis (MDR-TB), acute organ dysfunction, and bacteremia, in which IL-1β concentrations were found to be significantly higher in MDR-TB[23] and in MDR *Escherichia coli* and *Klebsiella* infections.[24] Additionally, Basingnaa et al.[25] reported that the mean levels of IL-10 and TNF-α in MDR-TB cases

were relatively higher compared with levels in drug-susceptible tuberculosis cases.

Studies have shown an elevated amount of IL-10 in MDR *P. aeruginosa*–induced sepsis as compared with non-MDR *P. aeruginosa* infection in sepsis patients, as well as in a mouse model of *P. aeruginosa* pneumonia.[3,26] It has also been suggested that IL-10 in response to *Mycobacterium tuberculosis* infection, may downregulate the immune response and limit tissue injury, but overexpression of these cytokines may have a negative impact on the capacity to control infection.[27] Some studies have also shown that, in patients with human immunodeficiency virus infection[28,29] and other experimental infections,[30–32] high levels of IL-6 correlated with susceptibility to the pathogen and the disease severity of associated diseases. In agreement with our results, Gómez-Zorrilla et al.[3] observed higher IL-6 levels in MDR-PA bacteremia. Another important inflammatory mediator is IL-8, which is known to promote the recruitment of polymorphonuclear leukocytes[33] and neutrophil infiltration in endophthalmitis; it was significantly higher in the vitreous of MDR-PA–infected patients, which may, in part, explain the poor outcome in patients with MDR-PA, despite prompt treatment with intravitreal antibiotics.

Along with inflammatory mediators, we also observed differences in the phosphorylation levels of Akt, STAT3, JNK, p70 S6 kinase, and NF-κB between the MDR-PA and S-PA groups. In cultured human chondrocytes, TNF-α activates JNK1/2 enzymatically,[34] which might be a possible reason behind the higher phosphorylation levels of JNK1/2 in the MDR-PA group. NF-κB inhibition has been shown to decrease the viability of intracellular *M. tuberculosis* in human macrophages via induction of apoptosis and autophagy. NF-κB inhibition also increased the formation of autophagosomes,[35] a finding that correlates with our previous in vitro study where the MDR-PA burden was found to be higher than S-PA strain.[9] Interferon-stimulated genes and their association with STAT1[36] were investigated, and STAT1 overexpression was reported in cases of a drug-resistant TB strain. In *Bacillus*[11] and *S. aureus*[37] endophthalmitis, it has been shown that phosphorylation of STAT3 influences NF-κB activation and promotes the inflammatory response. The roles of IL-6 in activating STAT3 and GM-CSF in activating STAT5 are well proven.[38,39] In our study, we did observe differential expression of IL-6 and STAT3, whereas no statistical significance was found for either GM-CSF or STAT5. Nevertheless, this study highlights the roles of cytokines and immune signaling pathways in the initiation, magnitude, and duration of MDR-PA endophthalmitis. Additionally, macrophages have long been appreciated as potent

host antimicrobial immune cells equipped with several receptors that allow for rapid recognition, phagocytosis, and killing of pathogenic microbes. The secretion of immunostimulatory cytokines further orchestrates a robust multifaceted antibacterial immune response.[40] Therefore, understanding how these cytokines and immune pathways are modulated in MDR-PA infections might aid in resolving the inflammation and hence the tissue damage.

The color-coded heat maps of the concentrations of the five inflammatory mediators and pathway molecules provided a good overview of their profiles in MDR endophthalmitis. We hypothesized that a decrease in the threshold for induction of the proinflammatory reaction in response to resistant strains of bacteria can be considered to be an immune system compensatory response to the changed fitness cost of the bacterial population compared with the population of the susceptible strain. Although we could not clarify the reasons for the larger variability in MDR samples, as the number of subjects used in the present study was limited, we determined that local variability in the burden of multidrug-resistant *P. aeruginosa* bacilli is an important factor that in turn impacts the reporting of clinical signs and collection of samples at the hospital. Although the sample size was small, eight of 12 patients clustered distinctively in the PCA biplot based on vitreous levels of the cytokine and pathway mediators. Although these results are preliminary and should be confirmed and validated in mouse models, they are of some importance, as significant differences were found in our patients' vitreous fluids in spite of the large clinical and demographic variations. Moreover, these samples were collected over a period of 2 years at a tertiary eye care referral institute in a country that has the largest number of Gram-negative endophthalmitis cases in the world; obtaining vitreous samples for research, especially, MDR-infected vitreous fluids, is a challenge. Nevertheless, studies on larger clinical cohorts are needed to validate the findings of the current study and verify the ability of inflammatory mediators to discriminate between MDR-PA and S-PA infections. Because of the small sample size of this study, we were not able to relate disease severity with multidrug resistance. Given the breadth of data implicating cytokines in hyper-inflammation, it is not surprising that these inflammatory mediators could serve as potential diagnostic markers and help in identifying MDR-PA infection.

This study did conclusively prove our earlier hypothesis under in vitro conditions. Overall, however, our approach allowed us to identify a group of inflammatory mediators that might be potential diagnostic markers for MDR-PA endophthalmitis. In the

future, pharmacological inhibition or immunomodulation of validated inflammatory mediators could be used to study their impact on endophthalmitis severity.

In conclusion, the comprehensive comparative analysis demonstrated increased production of predominantly proinflammatory cytokine, chemokine, and pathway mediators in the vitreous humor of patients with MDR-PA–induced endophthalmitis. Our data from clinical patients provide evidence that the higher levels of inflammatory mediators and pathway molecules in MDR-PA endophthalmitis might be a reflection of more severe inflammation. Future validation studies and targeting of inflammatory molecules are warranted to assess the translational role of immunomodulatory therapies in patients with drug-resistant endophthalmitis.

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